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Research Report

Mycobacterium leprae in Daily Water Resources of Inhabitants Who Live in Leprosy Endemic Area of East Java

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ABSTRACT

Leprosy still a health problem in Indonesia, where many leprosy pocket areas still persists, especially in the eastern part of the country. Although the program of WHO – Multidrug Therapy (MDT) regiment has been conducted elsewhere since 1980s, only the prevalence can be reduced but not the incidence of new leprosy cases. Theoretically after the source of leprosy (the infectious leprosy cases) has been treated, no more transmission of the disease and should be no more new leprosy cases will be found. To explain this phenomenon, the non-human resource of M.leprae became a new topic of debates, especially the existence of bacteria in the environment. A field study of the existence of M.leprae in the environment of leprosy endemic area had been conducted in a leprosy endemic area of the northern part of East Java. The aim of the study is to find any correlation of the existence of these bacteria in the environment with the presence of leprosy patients who live in that area, in order to study its role in the transmission of the disease. Ninety water samples from wells in the house of inhabitants who live in one endemic sub district were collected. The owner of the well was interviewed whether any leprosy patients who routinely use the water for their daily life activities. Water samples were examined by Polymerase Chain Reaction (PCR) method to detect M.leprae DNA, using the LpF-LpR and Lp3-Lp4 nested primers (99bp). The PCR results showed positive band for M.leprae in 22 out of 90 (24%) water samples. Water samples from wells that used by leprosy patients showed positive PCR in 11/48 (23%), while 11 out of 42 (26%) water samples from wells that never been used by leprosy cases showed positive result. Statistically there was no difference ($p>0.05$) in the positivity of M.leprae between the two groups. It was concluded that the existence of M.leprae in the daily water resource was not correlated with the present of leprosy cases in the area. Possible symbiosis between protozoan and mycobacterium in the environment were discussed.

Key words: *Leprosy, M.leprae, environment, water resources*

INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae,* which often cause disability of patients. The WHO-MDT Program has been introduced since 1980s and the majorities of leprosy endemic countries have achieved the elimination era, which means the prevalence rate is <1/10.000 population. But at the present time Indonesia still has a burden with around 17.000 new leprosy cases detected every year and become the 3rd highest number of the leprosy incidence in the world (Depkes RI, 2008; WHO, 2008). Theoretically, by treating all leprosy patients will eliminate the source of infection and no more transmission of the disease. But after more than 20 years of MDT Program, the new case detection rate has not been reduced and relatively stable. Many theories or opinions tried to explain this phenomenon, but the most possibility is the existence of non-human resource of *M.leprae* as mention in Noordeen (1994) also in Cree *and* Smith (1998). Up to present the bacteria could not be cultivated in artificial media and only growth by *in vivo* method using animal like mouse foot pad or armadillo.

Improvement of molecular biology techniques like Polymerase Chain Reaction (PCR) method makes it is possible now to detect a small amount of *M.leprae* in clinical or environmental specimens.

The aim of the study is to detect *M.leprae* in the daily water resources that used daily by inhabitants in leprosy endemic area using the PCR method, in order to find any correlation of the existence of *M.leprae* in the water environment with the present of leprosy cases in that area.

MATERIAL AND METHOD

Water samples collection

Ninety water samples from wells in the house of inhabitants who live in leprosy endemic area of one sub district in the northern part of East Java were collected. The prevalence of leprosy in this sub district was 8.02/10.000. Based on the information data regarding the user of each well, 48 wells are used by leprosy patients and another 42 wells have never been used by leprosy patients who live in that area. Using a sterile plastic bag, around 300 ml of water samples was collected from each well and kept in room temperature. Before PCR examination, 50 ml water samples was filtered using Millipore membrane filter 0.22 um. Membrane washed with PBST 1.5 ml and vortexes 10 minutes. The suspension then centrifuged at 13.000 rpm, 4° C for 20 minutes and pellet formed was used for making a smear for Ziehl Neelsen (ZN) staining and DNA extraction.

DNA extraction

The *Qiagen miniprep kit (Research Biolabs Co)* was used for DNA extraction from the pellet, following the manual book, to obtain PCR template.

PCR examination

PCR examination was performed using nested primers: *LpF-LpR* (*LpF*: 5'TATCGATGCAGGCGTGAG TGT3', *LpR*: 5'CTAACACGATACTGCTGCAC3') and *Lp3-Lp4* (*Lp3*: 5'TGAGGTGTCGGCGTGGTC3', *Lp4*: 5'CAGAAATGGTGCAAGGGA3'). PCR procedure modify from Donoghue *and* Spigelman (2001) to detect *M.leprae* from the specimen. The amplified products were electrophoreses on 3% Agarose gel and stained with ethidium bromide 0.1 ug/ml. Positive result was presented by a band at 99 bp, as indicated by positive control (*M.leprae* Thai 53 obtained from nude mouse culture in Leprosy Research Centre Japan).

Figure 1. Water samples collection

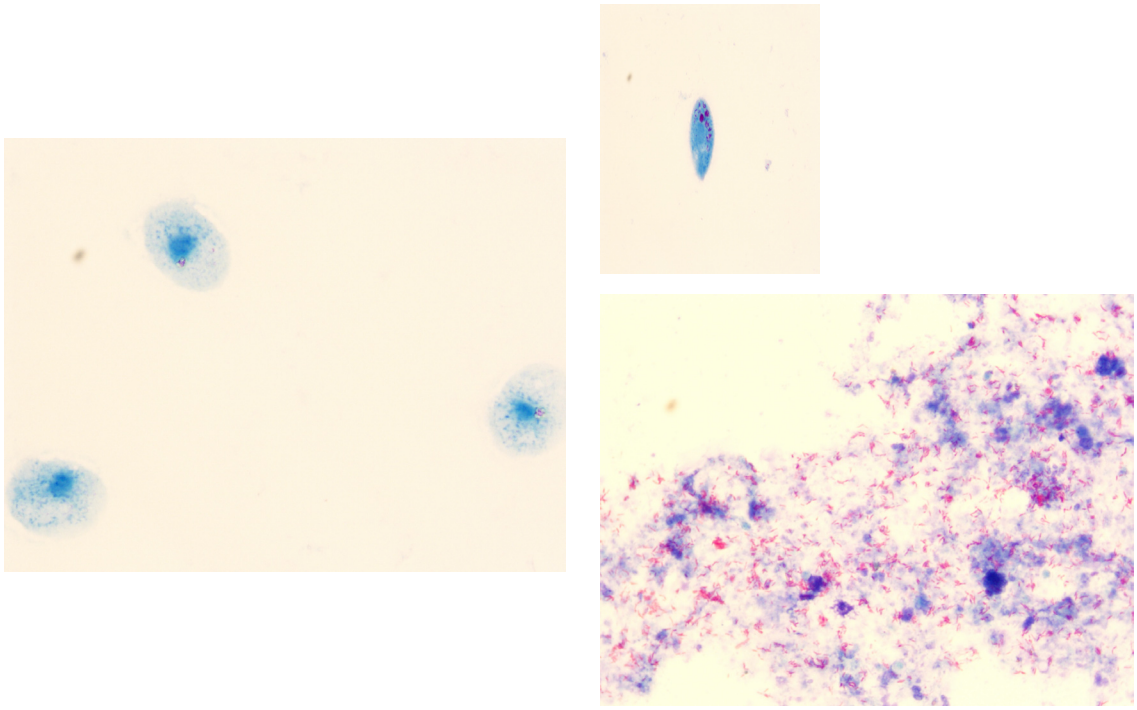

Figure 2. Ziehl Neelsen staining of water samples

RESULTS

All of the sediments from water samples showed a positive Acid Fast Bacilli (AFB) after staining with ZN method. Some of these bacilli were found inside "amoeba-like" protozoas, some of them were moving microorganisms.

Positive results were found in 22 out of the total 90 water samples (24%), consists of 11/48 (23%) water that often used by leprosy cases and 11/42 (26%) water from wells that never been used by leprosy patients.

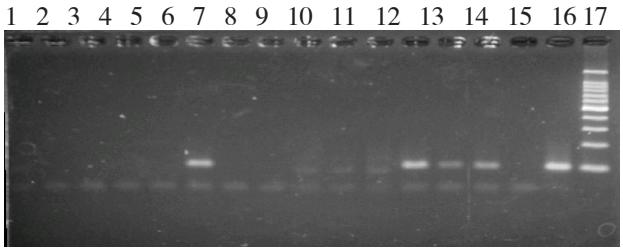

Figure 3. PCR of water samples (lane 1-14: samples, lane 15: negative control, lane 16: positive control, lane 17: 100 bp DNA ladder)

Table 1. PCR results for *M.leprae* detection and the status of wells

Well status	PCR *M.leprae* (+)	PCR M.leprae (–)	Total
leprosy cases (+)	11 (23%)	37 (77%)	48 (100%)
leprosy cases (–)	11 (26%)	31 (74%)	42 (100%)
Total	22 (24%)	68 (76%)	90 (100%)

Chi Square Test: $p = 0.909$, $df = 1$, $p > 0.05$

DISCUSSION

Leprosy is still endemic in three big countries i.e. India, Brazil and Indonesia. The last two countries have similar climate condition of tropical area like warm temperature and rainy season. This situation causes a typical tropical diversity, where millions of micro organisms are live symbiotically in the environment. It has been known that *M.leprae* as the causal agent of leprosy, can survive in the soil up to 40 days (Chakrabarty and Dastidar, 2002). Microbiological studies which indicate the existence of *M.leprae* in the water also has been reported by Kazda et al. (1990). This bacterium is an obligate intra-cellular microorganism, which is only growth if the agent lives inside the host cell.

Since there are many mycobacterium in the environment, special primers needed, because the *Lp1-Lp2* and *Lp3-Lp4* coding 18 kDa *M.leprae* antigen at region RLEP3 repetitive element (X17153) recommended by Donoghue *and* Spigelman (2001) are to short. We create a new nested primer called *LpF-LpR* for outer primer (260bp) and used together with *Lp3-Lp4* as inner primer (99bp) (Izumi *et al*., 2008 unpublished). These primers were sensitive and specific for *M.leprae*.

The first report on the existence of *M.leprae* in the public water resource in North Maluku, was reported by Matsuoka et al. (1999). Using the PCR method, he found 21/44 of public water resources were contaminated by *M.leprae*. Agusni et al (2004) reported the detection of *M.leprae* in some ponds that be used as water resource of inhabitants who live in leprosy endemic area in northern coast of East Java. Interestingly, positive results were found more in the root of water plants than in the water collected from the center site of the pond. This finding came to suspicion that the bacilli live in protozoan which are live in the root of many water plants.

Leprosy cases that often use the well for their daily activities could make the water contaminated with *M.leprae*. Therefore if the bacteria came to water, the PCR examination will be positive. Most of leprosy cases that live in this area have been treated by MDT regiment; some of them are already Release from Treatment (RFT). The results of this study showed that 11 out of 48 water samples (23%) from wells that being used by leprosy patients were positive after PCR test to detect *M.leprae*. But interestingly, *M.leprae* were also positive in 11 out of 42 (26%) water samples collected from wells that never been used by leprosy cases. Statistically, there was no significant difference on the PCR positive results for *M.leprae*.

The daily water supply in this area mostly from the well that is belonged to the family. Most of the owner said that the well only used by their family, so they know all the people who use their well. Since the list of registered leprosy patients is available in the health centre, the possibility of water contamination in these wells was minimal.

Mudatsir et al. (2006) reported the genomic study of *M.leprae* isolates collected from leprosy patients and their environment, using the TTC repeat method. The result concluded that that *M.leprae* found from leprosy patients, daily water resources and nasal swabs from healthy inhabitants were originated from one population. This means that *M.leprae* in the environment is one of the links belonged to leprosy problem and not a separate entity. Matsuoka (1999) also strongly suggests water as probable source of infection by showing significantly higher leprosy prevalence in water with *M.leprae* positive samples than in negative one.

Cirillo et al (1997) reported the survival of *M.avium* inside protozoa. While Jadin (1975) found *M.leprae* inside amoeba. These finding creates a speculation that *M.leprae* could also be survived inside the cell of environmental protozoa. If it is true, the following question is "how they can be transmitted to human?". Many reports on the nose swab studies found high prevalence of *M.leprae* positive in the nasal cavity among people who live in leprosy endemic area. Since they are healthy individuals, the bacteria were entered the nasal cavity from dust of the environment during breathing. This means that *M.leprae* was distributed generally in the environment of leprosy endemic area

and contaminates the dust or soil. Study by Lavania el al. (2006) showed the existence of *M.leprae* DNA in soil of endemic leprosy area in India, continuing study by Lavania et al (2008) found viable *M.leprae* (RNA *M.leprae*). These puzzles should be uncovered by more investigations.

CONCLUSION

The existence of *M.leprae* in the daily water resources of inhabitants in leprosy endemic area is not influenced by the presence of leprosy patients who live in the same area. More investigations are needed to find out the role of environmental *M.leprae* in the transmission of leprosy.

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