Detection of *Mycobacterium leprae* using real-time PCR in paucibacillary leprosy patients with negative acid-fast bacilli smears

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pISSN: 0853-1773 • eISSN: 2252-8083
https://doi.org/10.13181/mji.v28i4.2643
Med J Indones. 2019;28:351–7

Received: March 16, 2018
Accepted: May 16, 2019

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ABSTRACT

BACKGROUND Leprosy is an infectious disease that is still a health problem worldwide, including in Indonesia. The clinical symptoms are similar to other skin diseases and it is difficult to establish a diagnosis for paucibacillary (PB) leprosy. Current serological and histopathological tests have limitations, especially in patients with negative acid-fast bacilli (AFB). Serological tests often give false-negative results, while histopathological results often consist of non-specific inflammation. Probe-based real-time polymerase chain reaction (RT-PCR) assays is an alternative test that may be more sensitive and more specific to detect *Mycobacterium leprae*.

METHODS This study was done in June 2015 until March 2016; detected *M. leprae* in PB patients with negative AFB smears using TaqMan® probe-based RT-PCR assay on slit skin scrapings and skin biopsy specimens from 24 patients. The skin scrapings were obtained from skin tissue on ear lobes, skin lesions, as well as those from biopsy. Samples were tested with RT-PCR while histopathological examinations were only performed on skin from biopsy.

RESULTS The RT-PCR assay showed positive results of 21%, 25%, and 96% for specimens obtained from skin scrapings of the ear lobe, skin lesions, and skin biopsy, respectively. On the other hand, the positive rate for the histopathological test from skin biopsy was 79%. It indicated that the TaqMan® RT-PCR assay could increase the diagnostic capacity of histopathological examination by as much as 17%.

CONCLUSIONS TaqMan® PCR assay can improve the diagnostic capacity of histopathological examinations, which could be used as the new gold standard for the diagnosis of leprosy.

KEYWORDS *Mycobacterium leprae*, paucibacillary leprosy, real-time PCR

Leprosy is a chronic granulomatous infectious disease. The causal organism is *Mycobacterium leprae.* The bacterium is an acid-fast bacilli (AFB) that cannot be cultured *in vitro.* In countries with a high prevalence of leprosy, diagnosis is generally made based on clinical symptoms as well as microscopic examinations.[1] Clinically, leprosy is called "the great imitator" because it has similar symptoms as well as histopathological examination results to other skin diseases, such as pityriasis alba, pityriasis versicolor, vitiligo, tinea circinata, psoriasis, and others.[2] Sometimes, it is difficult to establish the diagnosis based only on the clinical signs and symptoms, such as for PB-type leprosy with negative AFB results.[1] It occurs because of the small number of AFB which was not detected by microscopy, which has a relatively low detection limit of 10,000 bacilli.[3] The negative result does not exclude the possibility that someone may be infected by leprosy. Other laboratory tests that can be used to diagnose leprosy are serological, histopathological, and molecular. The most commonly used serological test detects the presence of the phenolic glycolipid-1

protein.[4] However, this serological test often gives false-negative results due to a low number of bacilli or low antibody levels.[4] The sensitivity of the serological test on PB-type leprosy ranges only between 15% and 40%.[5] Therefore, serological testing is not recommended for diagnosing PB-type leprosy due to its low sensitivity.[5]

Histopathological testing, which is carried out by performing skin biopsy, is currently the gold standard diagnostic test for leprosy.[5] However, the test has several limitations because it is invasive and impractical.[6] Results of histopathological tests also do not always detect the presence of AFB in samples; even though patients show the characteristic symptoms of leprosy.[7] The examination can only confirm the diagnosis of PB-type leprosy in as much as 35–45% of cases.[6]

With the advancements in molecular medicine based on the amplification of nucleic acids, polymerase chain reaction (PCR) has become a sensitive, specific, and reliable diagnostic test to identify the pathogens of numerous infectious diseases. PCR may help establish the diagnosis for PB leprosy in patients with non-specific clinical symptoms and histopathological features.[8] It may improve the diagnosis of leprosy compared to microscopic examinations by acid-fast staining and histopathological examinations. This method can detect the bacilli even where they were present in small numbers, or even in subjects with false-negative AFB results.[7,9]

One of the PCR methods used for detecting *M. leprae* is the TaqMan® probe-based real-time PCR (RT-PCR) assay. This probe is commonly used in RT-PCR because it is relatively cheap compared to other probe-based RT-PCR methods such as beacon, etc. The advantage of probe-based RT-PCR is more specific than other types with the same level of sensitivity. The threshold of DNA detection for RT-PCR is more sensitive than conventional PCR.[3,6] The clinical sensitivity of RT-PCR in PB leprosy is also better than conventional PCR with a similar specificity.[10]

The capacity of RT-PCR assay to detect *M. leprae* in specimens with negative AFB smear results may enable us to differentiate PB-type leprosy from other diseases with similar symptoms. Other advantages include more rapid examination time and the use of a closed system to minimize the risk of contamination.[3,11] Therefore, this molecular-based assay can be used to confirm diagnosis.[4,12]

Based on the data, this study was aimed to identify the proportion of positive results from TaqMan® probe-based RT-PCR assay compared to histopathological examinations in PB leprosy patients with negative AFB smears. Moreover, this study would also identify the proportion of positive results from TaqMan® probe-based RT-PCR assay using skin scraping and skin biopsy specimens. To our knowledge, this is the first study testing the efficacy of TaqMan® probe-based RT-PCR in detecting leprosy from both sample types in Indonesia. It is expected that the TaqMan® probe-based RT-PCR assay may be utilized as a diagnostic tool to assist doctors to establish the diagnosis in patients with vague clinical symptoms and/or inconclusive results from laboratory tests, including microscopic or histopathological examinations.

METHODS

This study is a descriptive study with a cross-sectional method. The study was conducted between June 2015 and March 2016. Sample collection and histopathological examinations were performed at the Outpatient Clinic of the Department of Dermatology and Venereology, Cipto Mangunkusumo Hospital. The RT-PCR assay was carried out at the Laboratory of Clinical Microbiology, Faculty of Medicine, Universitas Indonesia. The patients with PB leprosy who were over 18 years of age, with or without reversal reaction were included. The diagnosis was made by history taking and physical examination performed by a dermato-venereologists. Also, the patients with negative results from AFB smears across all specimen tissues were included (bacterial index was zero). All patients have consented to participate in the study by filling the informed consent form. The patients with coagulation disorder and anemia were excluded, as they are contraindicated for surgical procedures such as skin biopsy. This study had obtained approval from the Medical Research Ethics Committee at the Faculty of Medicine, Universitas Indonesia with a letter of ethical clearance (No: 393/UN2.F1/ETIK/2015).

Eligible patients underwent procedures for the collection of specimens, including slit skin scraping from both ear lobes and two different skin lesions. Then, the specimens were sent for AFB smear testing. The scalpel used for the slit skin scraping was put into a falcon tube containing 1.5 ml 70% alcohol. Patients with negative AFB results were then subjected to

skin biopsy procedure, and specimens were obtained from the same site as the AFB smear specimen collection. The biopsy was performed by making an elliptical incision (1 cm diameter and subcutaneous deep excision). The excision of biopsy tissues was subsequently divided into two equal parts. One part was transferred into an Eppendorf tube containing 500 µl 70% alcohol. The tube was then sealed and brought to the laboratory along with the falcon tube containing the slit skin scrapings for RT-PCR assay. The remaining part was transferred into a plastic tube containing 10% formalin for histopathological examination. A representative picture of the skin biopsy conducted for RT-PCR and histopathological examinations are presented in Figure 1.

Tissues obtained from the slit skin scraping and the biopsy was extracted separately using the QIAamp® DNA Mini Kit (Qiagen®) as per the manufacturer's instructions. The products of the DNA extraction were stored at –30°C until they were used. The primers and probe used in this study was based on the previous study by Truman et al.[13] The primers for TaqMan® probe-based RT-PCR assay were selected from a common region of *M. leprae*-specific repetitive element (RLEP) gene which was 70 bp amplicons in size. The sequence selected was 5'-AGTATCGTGTTAGTGAACAGTGCATCGATGATCCGGCC GTCGGCGGCACATACGGCAACCTTCTAGCG-3'; where the capital letters in bold represent the sequence on which the forward and reverse primers were built. The sequence in lower case italics was selected for building the fluorescent TaqMan® probe.[13] PCR and data analysis were performed using a PCR-iQ™5, iCycler iQ™ Multicolor RT-PCR detection system. The 20 µl total reaction volume contained 5 µl of the template DNA, primers at a final concentration of 0.2 µM (forward and reverse) and 0.2 µM probes in DNase-free water. The PCR amplifications were performed under the following conditions: initial activation at 95°C for 3 min, 45 cycles of amplification of 15 sec at 95°C and 55°C for 1 min. DNA purified from *M. leprae* and blank distilled water were included as positive and negative controls, respectively. Positive controls were taken from clinical samples which was previously diagnosed with lepromatous leprosy. The RT-PCR assay is qualitatively assessed based on the cycle threshold (Ct). The Ct cut-off value used for the RT-PCR results was 40. Ct values higher than or equal to the cut-off were considered as negative. The RT-PCR results were considered positive if Ct values were below the defined cut-off.[14]

The frequency distribution of demographic characteristics, as well as proportion of positive results from RT-PCR was compared to the results from the histopathological examinations. All data were processed using Microsoft Excel 2013.

RESULTS

Of the 24 studied subjects, 13 were male and the others were female with an age range of 19–67 years and a median age of 37 years. There were 8 new patients who never had received any treatment. The other subjects had received treatment for leprosy. Subject characteristics are summarized in Table 1.

The RT-PCR assay on slit skin scraping obtained from ear lobes demonstrated positive results in 21% of patients (5 out of 24); while the positives for slit skin scraping from skin lesions was 25% (6 out of 24). One subject had positive RT-PCR results from slit skin scraping specimens obtained from both ear lobes and skin lesions.

Table 1. Characteristics of the subjects

Characteristics	n (%) (N = 24)
Age (year), median (min–max)	37 (19–67)
Male sex	13 (54)
Education background	
Primary–Junior High School	9 (37)
Senior High School	9 (37)
College	6 (25)
Occupation	
Housewive, n/total	5/11 (46)
Employee	5 (21)
Laborer	4 (17)
Entrepreneur	3 (13)
Unemployed	2 (8)
College student	1 (4)
Housemaid	1 (4)
Teacher	1 (4)
Retired	1 (4)
Merchant	1 (4)
Ongoing leprosy treatment	8 (33)
Diagnosis (Ridley & Jopling classification)	
Borderline tuberculoid (BT)	20 (83)
Borderline tuberculoid with reversal reaction	4 (17)

Table 2. Comparison of RT-PCR assay results from slit skin scraping and skin biopsy tissues

TaqMan® based RT-PCR assay	Slit skin scraping, n (%) (N = 24)	Skin biopsy, n (%) (N = 24)
Positive*	10 (42)†	23 (96)
Negative	14 (58)	1 (4)
Total	24 (100)	24 (100)

RT-PCR=real time-polymerase chain reaction
*Cycle threshold was below the defined cut-off of 40 cycles; †The RT-PCR results of slit skin scraping were considered positive if either specimens obtained from ear lobes and/or skin lesions was positive

Comparison of RT-PCR results on slit skin scraping and skin biopsy tissue are presented in Table 2. This data indicated that tissue specimens obtained from skin biopsy yielded a two-fold higher positive result compared to specimens from slit skin scraping. Pictures of the RT-PCR results from representative samples of these different types of samples are presented in Figure 1.

Of the 23 skin biopsy specimens (96%) that yielded positive RT-PCR results, 19 specimens among these (79%) were also positive for leprosy from histopathological examinations. There was one case (4%) where the patient had the clinical symptoms and signs but showed a negative result from both the RT-PCR assay and histopathological examination. The comparison of the RT-PCR result and histopathological examination on skin biopsy tissue is presented in Table 3.

DISCUSSION

Leprosy affects the skin and the peripheral nervous system and can cause nerve damage and deformity. Thus, it is important to diagnose the disease rapidly and accurately at its early stages. This bacterium cannot be cultured *in vitro*, unlike other bacteria species. Diagnosis is made based on clinical symptoms and the slit skin smear test. Other laboratory tests that can be used to diagnose leprosy are serological, histopathological, and molecular. Sometimes, it is difficult to establish the diagnosis based only on the clinical signs and symptoms because of its clinical similarity to other skin diseases. There are two main types of leprosy, that is paucibacillary (PB) and multibacillary (MB) leprosy that classified based on the number of bacteria present. It is difficult

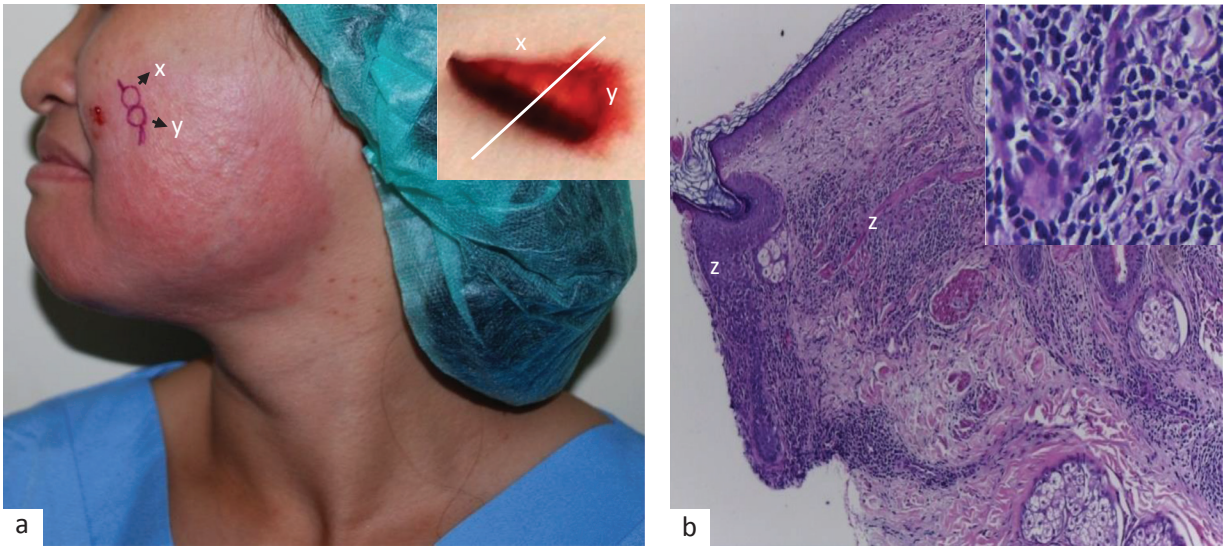

Figure 1. (a) Representative picture of skin biopsy for RT-PCR and histopathological examination. Photo of skin lesions of PB-type leprosy in the form of erythematous lesion. Skin biopsy was performed on skin lesions marked a and b. Half of the skin biopsy (x symbol) was used for the RT-PCR assay, the remaining half (y symbol) was used for histopathological examination; (b) Borderline tuberculoid leprosy (PB-type leprosy): in the dermis to the subcutis layer was elongated granulomatous inflammatory infiltrates (z symbol) on the perineural and periadnexal (H&E stain, magnification 100x); Datia Langhan cells and lymphocytes inside the granuloma (H&E stain, magnification 400×). RT-PCR=real time-polymerase chain reaction; PB=paucibacillary; H&E=hematoxylin and eosin

Table 3. Comparison of RT-PCR assay and histopathological examination results on skin biopsy tissue

TaqMan® based RT-PCR assay	Histopathology, n (%)		Total
	Positive (Leprosy)	Negative (Non-Leprosy)	
Positive	19 (79)	4 (17)	23 (96)
Negative	0 (0)	1 (4)	1 (4)
Total	19 (79)	5 (21)	24 (100)

RT-PCR=real time-polymerase chain reaction

to establish a diagnosis by microscopic examination using slit skin smear particularly for PB leprosy with negative AFB results. Serological and histopathological examination of PB leprosy also has low sensitivity.[5,6] Molecular tests such as RT-PCR had been reported to be useful for rapid and accurate detection of *M. leprae*. According to theory and previous studies, PCR is a sensitive assay that can detect microorganisms even in low numbers.[3,7,9] The threshold of DNA detection for RT-PCR is as low as 8 femtograms or equal to 240 bacterial cells,[3] which is more sensitive than conventional PCR.[6] The clinical sensitivity of RT-PCR in PB leprosy is also better than conventional PCR (79.2% *versus* 62.5%), with similar specificity.[10] It is believed that PCR able to overcome the low sensitivity problems of prior diagnostic methods.[12] The authors chose to test negative AFB smear results to minimize the possibility of obtaining positive results from the RT-PCR to evaluate the role of PCR to detect *M. leprae* in the diagnosis of leprosy.

The RT-PCR assay based on slit skin scraping tissues obtained from ear lobes only gave positive results in 21% of cases; while those obtained from skin lesions were at 25%. The low proportions in positive results might be caused by the relatively superficial specimen collection skin scraping procedure, which only reached 2 mm deep.[1] Usually, *M. leprae* are found in the eccrine glands, inside the nerves, sebaceous gland secretions, arrector pili muscle, and around the hair follicle.[5,15] The results of this study are similar to the results of the study conducted by Turankar et al[16] that used conventional PCR on leprosy patients with zero bacterial index. The positive result of PCR assay on slit skin scraping specimens using RLEP target genes was 30%. A study conducted by Wichitwechkarn et al[17] on PB leprosy patients with negative AFB smears also demonstrated a similar proportion of positive results (i.e., 20%) using a conventional method.

The selection of specimens may also affect the PCR results. Positive RT-PCR results from slit skin scraping specimens of ear lobes and skin lesions were 42%; while for skin biopsy tissues, it was 96%. Therefore, the sensitivity of the PCR assay in PB leprosy detection on negative AFB skin biopsy specimens is higher than those of skin scraping specimens. This result was two-fold higher for skin biopsy specimens. This probably occurred because the biopsy could obtain all skin layers and parts. Therefore, the amount of tissue available for DNA extraction was more than that of the skin scraping specimens, which could only reach 2 mm deep.[17,18] With a larger amount of tissue, the amount of extracted DNA was also higher and therefore, it may result in a greater proportion of positive results. Similar differences in the proportion of positive results (around two-folds) were also found in previous studies conducted by Kampirapap et al[18] and Wichitwechkarn et al[17] Both studies also compared the RT-PCR results in both types of specimens using conventional PCR with target genes (531 bp fragment of the *pra* gene) encoding the species-specific 36-kDa antigen of *M. leprae*. A study conducted by Kampirapap et al[18] showed that the sensitivity of PCR for PB leprosy in negative AFB skin biopsy specimens was 44.2%; while in skin scraping specimens, the sensitivity was 22%. Similar results were also obtained in a study by Wichitwechkarn et al[17] with a sensitivity of 36.4% for skin biopsy specimens and 18.2% for skin scraping specimens. The RT-PCR assay on skin biopsy tissue demonstrated positive results in 96%; while the remaining 4% (only 1 out of 24 samples) showed a negative result. Non-leprosy results had also been found in histopathological examinations; that is, 1 negative sample where the patient had clinical symptoms consistent with leprosy (i.e., a hypopigmentation lesion with slight anesthesia). These results support the data of previous studies, which indicated that the TaqMan® probe-based RT-PCR has good specificity and it can be used to exclude other skin disorders that are not caused by *M. leprae*.

A portion of samples (17%) gave positive results in the RT-PCR assay but had negative results in the

histopathological examination. This may be caused by the similarity of skin lesions of PB leprosy to other skin disorders, which can result in similar and non-specific histopathological features in the form of chronic granulomatous inflammation. These results provided evidence that the TaqMan® probe-based RT-PCR assay is more sensitive and more specific than histopathological examinations. The histopathological examination on skin biopsy yielded positive leprosy results in 79%, while for RT-PCR it was 96%. The different positive results between RT-PCR assay and histopathological examination found in this study were similar to the results of a study conducted by Dayal et al.[19] The study compared the proportion of positive identification between the PCR assay and histopathological examination on skin biopsy tissue. They conducted a study on both MB and PB leprosy patients with non-specified histopathological examination results. The study results indicate that PCR can improve diagnosis by as much as 15% compared to traditional histopathological examination. Our study showed different positive results in as much as 17% for both tests, which suggest that the TaqMan® probe-based RT-PCR can improve diagnostic capacity by as much as 17% compared to histopathological examinations. This result showed that TaqMan® probe-based RT-PCR assay can be used as a diagnostic tool to establish the diagnosis in patients with vague clinical symptoms and/or inconclusive results from laboratory tests, including microscopic or histopathological examination.

There are some limitations in this study. The treatment status of the subjects, which may have a great influence on the bacteria number and affect the RT-PCR results was not evaluated. Also, this study was only conducted on 24 subjects. Further studies with a larger sample size are necessary to identify whether the RT-PCR assay method would yield positive results up to the standard found in this study. Moreover, studies using quantitative RT-PCR should be conducted for monitoring therapy and to identify drug resistance by genotyping.

In conclusion, TaqMan® probe-based RT-PCR can improve diagnostic capacity as much as 17% compared to histopathological examinations. Therefore, TaqMan® probe-based RT-PCR assay should be considered for use as a diagnostic tool in patients with vague clinical symptoms and/or inconclusive results from laboratory testing.

Conflicts of Interest

The authors affirm no conflict of interest in this study.

Acknowledgment

The authors would like to thank the colleagues from Department of Dermatology and Venereology, Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital, Jakarta, Indonesia, that greatly accomodated and assisted in sampling. The authors would also express gratitude for the molecular staff of Laboratory of Clinical Microbiology, Faculty of Medicine, Universitas Indonesia, Jakarta, that assisted in specimens processing.

Funding Sources

This research is funded by research grant Universitas Indonesia 2015 and Cipto Mangunkusumo Hospital.

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