Role of Polymerase Chain Reaction (PCR) in the Diagnosis of Paucibacillary Leprosy

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Abstract

Leprosy caused by Mycobacterium leprae, is still considered a major health problem in many developing countries. The routine bacteriological test on the demonstration of acid-fast bacilli in skin smears is not sufficiently sensitive or specific in the paucibacillary leprosy patients. To detect DNA of Mycobacterium leprae by Polymerase Chain Reaction (PCR) from skin biopsy specimen as a means of diagnosis of paucibacillary leprosy. It was a cross-sectional study of clinically diagnosed 33 cases of Paucibacillary leprosy patients. Laboratory works were performed in the Department of Microbiology and Immunology and Department of pathology; Bangabandhu Sheikh Mujib Medical University(BSMMU) and Leprosy Control Institute and Hospital, Mohakhali, Dhaka from November 2013 to June 2015. It was observed that the mean age of patients was found 30.9±13.7 years, 19(57.6%) patients had two to five skin lesions, all (100%) patients had tuberculoid type of leprosy, all (100%) patients had loss of sensation and 18(54.5%) patients had nerve thickening. Regarding slit skin smear test, it was observed that all (100%) patients were slit skin smear negative for leprosy bacilli, 8(24.2%) patients had positive skin biopsy for histopathology and 15(45.5%) patients had positive skin biopsy for PCR. The validity of PCR for identification of paucibacillary leprosy correlated with sensitivity 100%, specificity 72%, accuracy 78.8%, positive predictive value 53.3% and negative predictive value 100%. Polymerase chain reaction (PCR) has definite value in the diagnosis of paucibacillary leprosy and can be regarded as a sensitive and specific diagnostic modality.

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Introduction

Leprosy, also known as Hansen's disease, caused by Mycobacterium leprae (M. leprae), is a chronic infectious disease affecting the skin and peripheral nerves resulting in disabling deformities and disfiguration.[1-3] The prevalence rate of leprosy in Bangladesh on 2012 is 0.223 per 10,000 people.[4] According to WHO classification, leprosy is two types, a paucibacillary and a multibacillary leprosy. A different way of classification of leprosy by Ridley-Jopling, are tuberculoid (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL) and lepromatous (LL) forms.[5] If the results of skin smears are negative (there are five or fewer lesions), the disease is called paucibacillary.[6]

M. leprae is an acid fast intracellular, rod shaped aerobic organism. Leprosy is thought

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to be transmitted mainly acrogenically, and it is generally recognized that the nasal cavity is involved in the carriage and shedding of M. leprae.[7,8] The major reservoir and dominant source of infection of the disease are the untreated leprosy patients. The delay in presentation of leprosy symptoms results in long transmission period. [9] In adult cases in men outnumber those in women 1.5:1, although Hansen's disease occurs in all ages. The latency period between exposure and overt signs of disease is usually 5 years for paucibacillary cases.[10,11] Although making a clinical diagnosis is frequently straightforward, however no ideal method is available for confirming it. Delay in diagnosis can have important negative outcomes, such as increased risk of nerve damage. [12]

Microscopic examination is not enough for detection of M. leprae because at least 10^4 organisms per gram of tissue are needed for the detection of M. leprae by AFB staining procedure.[13] Visualizing the organism in skin-biopsy specimens or slit-skin smears is not difficult in multibacillary disease, with the slit smear giving positive results in nearly 100% of lepromatous and 75% of borderline leprosy cases. [14] In contrast, the organism is often problematic to identify in paucibacillary disease, with only 5% of slit smears demonstrating organisms in tuberculoid leprosy cases. [15,16] However recent techniques have proven useful in (1) evaluating specimens in which bacilli are sparse. (2) Differentiating leprosy from other mycobacterial infections on specimens in which bacilli are numeruos, and (3) evaluating response to therapy. Sensitiveness of molecular assays have ranged from 34% to 80% in patients with paucibacillary disease to 100% in patients with multibacillary disease, with an overall specificity of 100%.[17,18]

In the past two decades or more, several reports on the use of the PCR for detection of M. leprae have been published. The strength of PCR is its extreme sensitivity and high specificity. Various clinical samples can be used for PCR mediated detection of M leprae including skin, lymph node, blood, nerve biopsy, hair bulbs and nasal secretion.[13] Most investigators showed that PCR is very sensitive, only 1-100 organisms are enough to detect M. leprae.[19] Thus PCR is a useful tool for the diagnosis of leprosy, observed by one study.[20]

Methods

It was a cross-sectional study and conducted with clinically diagnosed 33 cases of Paucibacillary leprosy patients. The study was carried out in the Department of Dermatology and Venereology and Outpatient Department of Leprosy Control Institute and Hospital, Mohakhali, Dhaka. Laboratory works were performed in the Department of Microbiology and Immunology and Department of pathology Bangabandhu Sheikh Mujib Medical University(BSMMU) and Leprosy control Institute & Hospital, Mohakhali, Dhaka. The duration of the study was from November 2013 to June 2015. Consecutive type was the sampling technique. Inclusion Criterias were patients of all ages and both sexes and one to five asymmetrical hypopigmented or erythematous anaesthetic macule or patch or plaque, patient who will give informed consent and willing to comply the study procedure. Exclusion criterias were patients under leprosy treatment and clinically diagnosed leprosy (other than tuberculoid and borderline tuberculoid).

Data regarding age, sex, socioeconomic condition were recorded in a predesigned data collection sheet questionare. The Preliminary screening panel for each patients were included the complete history, physical examination and the necessary laboratory tests. Laboratory works were performed in the Department of Microbiology and Immunology, and Department of pathology; BSMMU. Data including age, slit skin smear, skin biopsy for histopathology and PCR of Lepra bacilli were recorded.

Ethical consideration

Before starting this study, the research protocol was approved by the Institutional

Review Board (IRB) of BSMMU, Dhaka. All participants were thoroughly informed about the nature, purpose and implication of the study in easy understandable way as well as the entire spectrum of benefits and risk of the study. All study subjects were assured of adequate treatment of any risk developed in relation to study purpose. Subjects were assured about their confidentiality and freedom to withdraw themselves from the study any time. Data obtained from the study was used only for the research purpose and the confidentiality of all study information was maintained strictly. Finally informed written consent was taken free of duress and without exploiting any weakness of the subject.

Study Procedure

Laboratory procedure

Skin biopsy for histopathology and PCR for lepra Bacilli and slit skin smear were done on all clinically suspected Paucibacillary leprosy patients

Microscopic examination:

Slit skin smear

Slit skin smear was taken from skin lesions as well as from left forehead and ear lobules for Ziehl-Neelsen staining for microscopic examination to detect M. leprae. A sterile scalpel with a round tip, a glass slide, a cotton swab-dipped in 70% alcohol, a swab-stick soaked in benzoin and a diamond pencil for labeling the slide will be taken. The skin was cleaned with 70% alcohol. The site was pinched between thumb and the index finger, a small cut of 0.5 cm deep was made with a sterile scalpel. The tissue was exposed in the cut was scraped and a little bit of the tissue along with the tissue fluid was removed. The material obtained was evenly spread on the glass slide in a circular area, about 0.8 cm in diameter. The smear was dried on air for 15-20 minutes and was fixed by passing slide over flame several times for 2-3 seconds each time and was stained by modified Ziehl-Neelsen staining method.

The stained smear was examined under light microscope using 100x oil-immersion objective. Red, straight or slightly curved rods occurring singly or in groups (M. leprae) was seen against a blue background.

Grading of ZN staining:

The grading was done according to WHO classification based on National guideline, 2005: [4]

No AFB (acid fast bacilli)	Negative
1-10 bacilli/100 fields	1+
I-10 bacilli/10fields	2+
1-10 bacilli/field	3+
10-100 bacilli/field	4+
100-1000 bacilli/field	5+
Over 1000 bacilli/field	6+

Punch Skin Biopsy

Using aseptic procedure 2 ml of 2% Lidocaine HCl was administered subcutaneously from where the skin sample was taken. Punch skin biopsy samples (two) were taken from the skin lesions (up to dermis) of leprosy patients. Each biopsy specimen was 4 mm in size. One sample was merged in 10% formalin and sent to Department of Pathology for histopathology. Other sample was merged in normal saline solution and sent to Department of Microbiology and Immunology for PCR of lepra bacilli. A section from biopsy material was stained by hematoxylin and eosin stain for histopathological examination.

Histopathological examination

Biopsy tissues fixed in formalin was left for 12-24 hours in the Pathology Laboratory of BSMMU. Tissues was processed, embedded in paraffin wax and cut into thin section by microtome. The sections were stained with haematoxylin and eosin. The slides were examined by a histopathologist of BSMMU and the reports were interpreted as tuberculoid, borderline tuberculoid, borderline borderline, borderline lepromatous and lepromatous leprosy.

Polymerase chain reaction

Extraction of M. leprae DNA

Skin biopsy specimens stored at - 20°C was cut into small pieces with sterile scissors. These samples were homogenized in a manual homogenizer (mortar and pestle) with

300 µl sterile distilled water. Suspension was then taken into eppendorf tube and suspension was mixed with 100 microliter of lysis buffer (100 mM Tris-HCl, pH 8.5, 0.05% Tween 20 and 60 µg of proteinase K per ml). The content of the tube was vortexed and was kept at 60°C for 18 hrs. Paraffin oil (40 µl) was layered on top to prevent evaporation and was vortexed again. Then the samples were kept in heating block at 97°C for 15 min. The tube was removed from the heating block and was immediately placed on ice bag. Lysed cells were removed by centrifugation at 11000 rpm for 3 minutes and the supernatant was taken in another eppendrof tube. The supernatant was stored at - 20°C for PCR (Srisungngam et al. 2007).

PCR amplifications

The isolated DNA was amplified using the insertion sequence S_{13} and S_{62} primers {(synthesized by Oswell DNA Service, Edinburg, UK in a thermal cycler (Eppendrof AG, Master Cycler gradient, Hamburg, Germany)}. Oligoneucleotide primers were used for PCR amplification of M leprae DNA is as follows:

Primer	Sequence	Concentra-tion	Product size
S13	5'- CTC CAC CTG GAC CGG CGA T-3'	196 µg/34.1 nmol	
			530 bp
S62	5'-GAC TAG CCT GCC AAG TCG-3'	155 µg/28.3 nmol	

PCR was performed in a final reaction volume of 25 µL, containing 1.5 µL of each primer, 3 µL of extracted DNA, 12.5 µL mastermix and 6.5 µL of nuclease free water (Promega Corporation, USA). After initial denaturation at 95°C for 10 minutes, the reaction was subjected to 36 cycles (each cycle consisting of denaturation at 95°C for 1 minute, annealing at 60°C for 45 seconds and elongation at 72°C for one and a half minute) followed by a final extension at 72°C for 10 minutes. Then the product was hold at 4°C. After amplification, the products was processed for gel documentation or kept at - 20°C till tested.

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PCR products were detected by electrophoresis on 1.5% agarose gel (Appendix-iii). Gel was prepared with IxTBE buffer (Tris Borate EDTA). For 1.5% agarose gel preparation, 0.18 gm of agarose powder (LE, Analytical grade, Promega, Madison, USA) were mixed with 12.5 ml of TBE buffer. After mixing thoroughly it was boiled using microwave oven for few minutes to dissolve the agarose completely. Gel was cooled to 60-70° C before pouring it into the casting tray and was allowed to stand for 30 minutes for solidifications before removing the analytic comb.

The gel with the tray was placed in the electrophoresis tank and IxTBE buffer was poured in it. One micro liter of Gel loading dye was taken on a piece of parafilm, 4 µl product was taken and mixed well with the dye. The whole volume aliquot of amplified sample with dye was loaded into a well of agarose gel submerged in TBE buffer in tank and was

subjected to electrophoresis for 30 minutes at 100 volts. In each experiment a negative control, replacing the sample DNA with 'Escherichia coll ATCC 25922' during amplification was included.

Visualization and Interpretation of results

The agarose gel was stained with ethidium bromide. For staining, the gel was submerged in distilled water mixed with ethidium bromide (5 µl ethidium bromide in 100 ml distilled water) for 30 minutes. It was then distained with distilled water for 15 minutes. The gel will be observed under UV Transilluminator (Gel Doc, Major science, Taiwan) for DNA bands and was photographed. The DNA bands were identified according to their molecular size by comparing with the molecular weight maker (100 bp DNA ladder) loaded in a separate lane. Samples showing the presence of s13, s62 specific DNA band corresponding to 530 bp band were considered positive for the presence of leprae bacilli.

Considering histopathology report as gold standard sensitivity, specificity, positive predictive value, negative predictive value and accuracy were calculated using following formulas:

Sensitivity = (TP×100) / (TP+FN)
Specificity = (TN×100) / (FP+TN)
Positive predictive value = (TP×100)/ (TP+FP)
Negative predictive value = (TN×100) / (FN+TN)

Here TP = True positive
TN = True negative
FP = False positive
FN = False negative

Accuracy = (TP+TN) × 100/Total population

Data analysis

All data were compiled and edited meticulously by thorough checking and rechecking. All omissions and inconsistencies were corrected and were removed methodically. After collection, data was checked for inadequacy and inconsistency. Irrelevant and inconsistent data was discarded. Statistical analyses were carried out by using the Statistical Package for Social Sciences version 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA). The mean values were calculated for continuous variables. For the validity of study outcome, sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the PCR evaluation for skin biopsy was calculated.

Results

Table I shows age of the study patients, it was observed that 18(54.5%) patients belonged to age 21-30 years. The mean age was found 30.9±13.7 years with range from 9 to 64 years.

Table 1: Distribution of the study patients by age (n=33)

Age (in year)	Number of patients	Percentage
< 20	5	15.2
21-30	18	54.5
31-40	4	12.1
41-50	3	9.1
>60	3	9.1
Mean±SD	30.9 ±13.7	
Range (min, max)	(9, 64)	

Table 2 shows number of skin lesion of the study patients, it was observed that 19(57.6%) patients had two to five skin lesions and 14(42.4%) had single skin lesion. Regarding clinical type of the study patients, it was observed that all (100%) patients had tuberculoid type of leprosy.

Table 2: Distribution of the study patients by number of skin lesion and by clinical type (n=33)

Number of skin lesion	Number of patients	Percentage
Single	14	42.4
Two to five	19	57.6
Clinical type	**Number of patients**	**Percentage**
Tuberculoid	33	100.0
Borderline tuberculoid	0	0.0

Table 3 shows that all (100%) patients had loss of sensation and 18(54.5%) patients had nerve thickening. Regarding number of nerve thickening of the study patients, it was observed that 18(54.5%) of the patients had one nerve thickening.

Table 3: Distribution of the study patients by number of skin lesion (n=33)

Loss of sensation	Number of patients	Percentage
Present	33	100.0
Absent	0	0.0

Nerve thickening	Number of patients	Percentage
Present	18	54.5
Absent	15	45.5

Number of nerve thickening	Number of patients	Percentage
One	18	54.5
More than one	0	0.0
No nerve thickening	15	45.5

Table 4 shows slit skin smear of the study patients, it was observed that all (100%) patients were slit skin smear negative for leprosy bacilli. Regarding skin biopsy for histopathology of the study patients, it was observed that 8(24.2%) patients had positive skin biopsy for histopathology and regarding skin biopsy for PCR of the study patients, it was observed that 15(45.5%) patients had positive skin biopsy for PCR.

Table 4: Distribution of the study patients by slit skin smear, skin biopsy for histopathology and skin biopsy for PCR (n=33)

Slit skin smear	Number of patients	Percentage
Positive	0	0.0
Negative	33	100.0

Skin biopsy for histopathology	Number of patients	Percentage
Positive	8	24.2
Negative	25	75.8

Skin biopsy for PCR	Number of patients	Percentage
Positive	15	45.5
Negative	18	54.5

Table 5 shows the validity of PCR evaluation of diagnosis of paucibacillary leprosy by calculating sensitivity, specificity, accuracy, positive and negative predictive values. It was observed that sensitivity 100%, specificity 72%, accuracy 78.8%, positive predictive value 53.3% and negative predictive value 100%

Table 5: Sensitivity, specificity, accuracy, positive and negative predictive values of PCR evaluation for skin biopsy (n=33)

Test of validity	Percentage
Sensitivity	100.0
Specificity	72.0
Accuracy	78.8
Positive predictive value	53.3
Negative predictive value	100.0

Discussion

This cross sectional study was carried out with an aim to detect DNA of M. leprae by PCR from skin biopsy specimen. A total of 33 patients of Department of Dermatology and Venereology, Bangabandhu Sheikh Mujib Medical University and outpatient Department of Leprosy control Institute & Hospital Mohakhali, Dhaka, during November 2013 to June 2015 were included in this study. In this study it was observed that 18(54.5%) of the patients with paucibacillary leprosy were in 3rd decade and the mean age was found 30.9±13.7 years varied from 9 to 64 years. In this study it was observed that 54.5% patients had nerve thickening. However, James et al. mentioned that enlarge nerves are found in 75-85% patients with pacibacillary disease.[6]

All (100%) patients were slit skin smear negative for leprosy bacilli. In this study it was observed that 24.2% patients had positive skin biopsy for histopathology. In this study it was observed that 45.5% patients had positive skin biopsy for PCR. Whatever the relationship between positivity of PCR and development of the disease, PCR is much more sensitive than microscopic examination for direct detection of the bacilli, observed by Santos et al.[21]

In this series it was observed that PCR evaluation for skin biopsy, true positive 8 cases, false positive 7 cases and true negative 18 cases evaluated by histopathological diagnosis. Sensitiveness of these molecular assays varied from 34% to 80% in patients with paucibacillary disease to 100% in patients with multibacillary disease, with an overall specificity of 100% observed by Swick. [14] In this study it was observed that the validity of PCR for identification of paucibacillary leprosy correlated with sensitivity 100%, specificity 72%, accuracy 78.8%, positive predictive values 53.3% and negative predictive values 100%.

Yan et al. mentioned that the real-time PCR test is especially valuable for detection of low numbers of bacilli with BI negative samples, with the positive rate reaching 50% for such patients in this study. Yan et al.we obtained in their study that 70.8% with the real-time PCR test in paraffin-embedded skin biopsy for AFB-negative specimens. The overall positive rate of AFB was 31.4% (16 of 51), compared with positive real-time PCR and histopathological results of 74.5% and 52.9% ($P < 0.05$), respectively. It is of interest to note that for negative AFB samples belonging to BT and TT the real-time PCR positive rates were 70.8% and 55.6%, respectively, indicating the real time PCR test is more sensitive in detecting AFB-negative leprosy cases. Among 51 cases with clinical symptoms, BI was positive in 16, through pathological examination the other 11 with leprosy pathological changes were confirmed to be leprosy, a total of 27 patients were diagnosed with leprosy, the remaining 24 were increased by 11, 10 positive cases, respectively, by real-time PCR and nested PCR, the total diagnosed cases went up to 38 by real-time PCR. The greater sensitivity and specificity of the RLEP TaqMan PCR can be an especially useful tool for the rapid detection of M. lepare DNA in clinical specimens in which no AFB are detectable microscopically and should be used in difficult to diagnose cases such as the specimens with no leprosy pathological changes. The ability of real-time PCR to detect M. leprae DNA on regular bacteriological negative samples would be helpful in differentiating leprosy from diseases that cause similar symptoms ensuring a correct diagnosis. [22]

Conclusion

It can be concluded that the skin biopsy for PCR is useful diagnostic method in the diagnosis of paucibacillary leprosy and it should be worthy to note that PCR can help the physician in the rational approach for patient management.

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