Demography, clinical presentation and laboratory diagnosis of leprosy by microscopy, histopathology and PCR from Dhaka city in Bangladesh

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Summary
Background: Recent data regarding demography, clinical presentation and molecular diagnosis of leprosy patients are lacking in Bangladesh.
Methods: A cross sectional study on 55 leprosy cases of 16–70 years old, both sexes was done. Ziehl-Neelsen (Z-N) stain, histopathology and PCR of skin specimens were done.
Results: 40% patients were between 21–30 years, mean age 34.5 years, male to female ratio was 2.9:1. Fourteen (25%) patients were destitute, 11 (20%) were housewives. 62% patients had more than 5 skin lesions. 58% had two and 36% had single nerve involvement. Altogether 9 (16%) were smear positive for AFB, 3 (33%) were graded as 2+, 2 (22%) as 3+ and 4 (44%) as 4+. Histologically, 52 (95%) showed features of leprosy, 14 (25%) were diagnosed as tuberculoid and 5 (9%) were lepromatous leprosy. In total, 40 (73%) of 55 patients were positive by PCR. Thirty (88%) of 34 multibacillary leprosy and 10 (48%) of 21 paucibacillary leprosy patients were PCR positive. One of 3 histopathologically negative leprosy patients was PCR positive.
Conclusion: Multiple skin lesions and multiple nerve involvement are the predominant features. For diagnosis of multibacillary leprosy, Z-N stain and for paucibacillary leprosy, PCR are suitable methods. In smear negative cases, results of histopathology may be suggestive, and PCR can give confirmatory results.

Keywords: Bangladesh, Diagnosis, Histopathology, Leprosy, PCR, Ziehl-Neelsen Stain

Place of work: The research was carried out in the department of Microbiology of Dhaka Medical College of Bangladesh. Leprosy patients were recruited from the Leprosy Control Institute and Hospital, Mohakhali, Dhaka of Bangladesh.

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Introduction

Leprosy remains a major problem in several countries despite significant progress in reducing the prevalence of leprosy over the past 50 years. Transmission of leprosy continues and the total number of leprosy patients was 175,554 at the end of 2014, while the number of new cases globally in 2014 was 213,899. Leprosy is endemic in India, Nepal and Bangladesh. In 2012 the prevalence of leprosy was 3,668 in Bangladesh, 134,752 in India, 3,492 in Nepal, 2,191 in Sri Lanka, 1,206 in China and 3,013 in Myanmar, all being neighbouring countries of Bangladesh. The major reservoir and dominant source of infection of the disease is the untreated leprosy patient. The multibacillary type of leprosy spreads silently before it is clinically detected.

Currently the main focus is on promoting early detection of leprosy cases to reduce the disease burden and especially the disabilities it causes. Although the clinical diagnosis of leprosy is straightforward, no ideal method is available for confirming it. Delay in diagnosis can lead to increased risk of nerve damage. As M. leprae cannot grow in vitro, at present, the diagnosis of leprosy in Bangladesh is based on microscopic detection of acid-fast bacilli in tissue smears, in combination with histopathological and clinical evaluation.

The skin test and serological assays for the detection of M. leprae infection have less sensitivity and specificity. Serological tests reflect past infection, giving no information about current bacteriological status. 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 the AFB staining procedure. Only in 35% of early cases histopathological examination shows definitive features of leprosy and in the remaining cases histology shows only chronic inflammatory signs which are common to many dermatoses. DNA amplification through polymerase chain reaction (PCR) is used to detect M. leprae particularly when low numbers of bacteria are present.

In Bangladesh, the diagnostic accuracy of PCR has not yet been tested, which seems essential before advocating its widespread use for the diagnosis of leprosy. The present study was undertaken to know the present demography of leprosy patients, their clinical presentation and to test the diagnostic accuracy of PCR in detecting M. leprae in skin biopsies from clinically suspected cases of leprosy, compared with Z-N staining and histopathology.

Materials and Methods

STUDY DESIGN

The cross sectional study was carried out in the department of Microbiology, Dhaka Medical College of Bangladesh. Patients attending the Leprosy Control Institute and Hospital, Mohakhali, in Dhaka were the study population. Clinically suspected leprosy patients who gave informed consent were included. Already treated leprosy patients, who were receiving anti leprosy drugs were excluded. Data were collected by face to face interview in a semi-structured questionnaire. A total of 55 cases were recruited.

ETHICAL ISSUES

In compliance with the Helsinki declaration for medical research involving human subjects (1964), all the patients were informed about the nature, purpose and procedure of the study.
Written consent was obtained from each study patients. Ethical approval was obtained from the ethical review committee of Dhaka Medical College, Dhaka.

**CLINICAL DIAGNOSIS**

The diagnosis of leprosy was based on different clinical parameters which involved detailed examination of skin lesions and peripheral nerves. Persons with the presence of one or more of the following cardinal signs were diagnosed as leprosy, provided such signs were considered active and definitive, including a hypo-pigmented or erythematous patch or patches on the skin with diminished or loss of sensation, and enlarged and/or tender and thickened peripheral nerves or nerve trunks.

**CLASSIFICATION OF LEPROSY**

This was according to the National guideline and technical manual on leprosy, 2005.\(^{14}\)

(a) Multi-Bacillary (MB) type: Bacillary load is high. It includes lepromatous (LL), borderline lepromatous (BL) and mid-borderline (BB) forms, in which the skin smear is positive and/or the number of skin lesions is more than five.

(b) Pauci-Bacillary (PB) type: Bacillary load is low. It includes tuberculoid (TT), borderline tuberculoid (BT), indeterminate (I) and pure neuritic (PN) forms, in which the skin smear is negative and/or the number of skin lesions is 1–5.

**LABORATORY PROCEDURE**

Skin biopsy and slit skin smear were done on all clinically suspected leprosy patients.

**MICROSCOPIC EXAMINATION OF SLIT SKIN SMEARS**

A slit skin smear was taken from skin lesions as well as from the left forehead and ear lobes for Ziehl-Neelsen (Z-N) staining and microscopic examination to detect *M. leprae*. A sterile scalpel with a round tip, a glass slide, a cotton swab dipped in methylated alcohol, a swab-stick soaked in benzoin and a diamond pencil for labeling the slide were used. The skin was cleaned with methylated spirit. The site was pinched between thumb and index finger, a small cut of 0.5 cm deep was made with a sterile scalpel. The tissue exposed in 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 the slide over a flame several times for 2–3 seconds each time and was stained by the Ziehl-Neelsen 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*) were seen against a blue background.

**GRADING OF ZN STAINING**

The grading was done according to the WHO classification.\(^{14}\)
PUNCH SKIN BIOPSY

Punch skin biopsy samples were taken from the skin lesions (up to dermis) of leprosy patients. Using an aseptic procedure, 2 ml of 2% Lidocaine HCl was administered subcutaneously from where the skin sample was taken. Each biopsy specimen was 6 mm in size and was cut in two halves; one half was immersed in 10% formalin for histopathology and the other half was stored at −20°C until used for DNA extraction for PCR.

HISTOPATHOLOGICAL EXAMINATION

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

EXTRACTION OF M. LEPRAE DNA FOR PCR

Skin biopsy specimens stored at −20°C were cut into small pieces with sterile scissors. These samples were homogenised in a manual homogeniser (mortar and pestle) with 300 μl sterile distilled water. The suspension was then put into a micro-centrifuge tube and 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 a heating block at 97°C for 15 min. The tube was removed from the heating block and was immediately placed on an ice bag. Lysed cells were removed by centrifugation at 11,000 × g for 3 minutes and the supernatant was put into another micro-centrifuge tube. The supernatant was stored at −20°C for PCR.

PCR AMPLIFICATIONS

A 530 bp fragment of DNA of M. leprae was amplified by PCR using oligonucleotide primers forward, S13 5′- CTC CAC CTG GAC CGG CGA T-3′ reverse S62 5′- GAC TAG CCT GCC AAG TCG -3. 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 master mix 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. PCR products were detected by electrophoresis on 1.5% agarose gel for 30 minutes at 100 volts, stained with ethidium bromide, destained and visualised by UV transilluminator.

DATA ANALYSIS

Data analysis used Microsoft Excel programme (version 2007). Sensitivity and specificity were calculated using the standard formula.

Results

The mean age of the patients was 34.5 years and youngest and the oldest patients were 16 and 70 years respectively (Table 1).

Out of the total of 55 clinically diagnosed leprosy cases, 41 (75%) were male and 14 (25%) were female with a male to female ratio of 2.9:1. One-quarter (25%) of the patients were destitute followed by 11 (20%) housewives (Figure 1).

Thirty four (62%) patients had more than five skin lesions and 21 (38%) had up to five lesions. Three (6%) patients had no nerve involvement, 20 (36%) had single nerve involvement and 32 (58%) had two or more nerves involvement.

Among the multibacillary cases, seven (13%) were lepromatous (LL) and 27 (49%) were borderline lepromatous (BL). Of the 21 paucibacillary cases, 10 (18%) were tuberculoid leprosy (TT) and 11 (20%) were borderline tuberculoid leprosy (BT). All paucibacillary cases were negative by Z-N stain. Out of 27 BL, two (7%) were Z-N stain positive and all the seven (100%) LL cases were Z-N stain positive. Altogether nine cases (16%) were positive for acid-fast bacilli. Of the nine smear positive cases, 3 (33%) were graded as 2+, 2 (22%) as 3+ and 4 (44%) as 4+.

A total of 52 cases (95%) had definite features of leprosy, while three (5%) were negative on histopathological examination. Of the histopathologically positive leprosy patients, one-quarter (25%) were diagnosed as TT (Figure 2), 11 (20%) as BT, 22 (40%) as BL and five (9%) as LL leprosy (Figure 3). Three patients were not diagnosed as leprosy by histopathology; two of those had normal findings, one had autolysis. Of the 52 histopathologically diagnosed cases, 9 (17%) were also positive by Ziehl Neelsen (Z-N) stain. See Table 2 – note that the clinical classification and the histological classification may not be the same in every case.

Table 1. Age distribution of the patients (n = 55)

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Number (%)</th>
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<tbody>
<tr>
<td>&lt;20</td>
<td>3 (5)</td>
</tr>
<tr>
<td>21–30</td>
<td>22 (40)</td>
</tr>
<tr>
<td>31–40</td>
<td>11 (20)</td>
</tr>
<tr>
<td>41–50</td>
<td>13 (24)</td>
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<tr>
<td>&gt;50</td>
<td>6 (11)</td>
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</tbody>
</table>
Of the 55 patients, four (40%) of the 10 TT, six (55%) of the 11 BT, 23 (85%) of 27 BL and all the seven (100%) LL cases were positive for *M. leprae* DNA by PCR (Table 2). Overall 40 (73%) were positive by PCR (Figure 4).

PCR was positive in 30 (88%) of the clinically diagnosed 34 multibacillary leprosy cases, while it was positive in only 10 (48%) of the 21 paucibacillary cases. All nine (100%) Z-N stain positive and 31 (67%) of the 46 Z-N stain negative cases were PCR positive. Of the 52 histopathology positive cases, 39 (75%) were PCR positive and one (33%) of the three histopathology negative cases was PCR positive.

**Discussion**

To achieve the goal set by WHO for 2020, early diagnosis and treatment of leprosy is required to interrupt transmission of the disease. However, no single test has shown sufficient
sensitivity and specificity to serve as diagnostic tool for leprosy. In this study, PCR has been tested to see its usefulness in the diagnosis of leprosy.

In our study, the mean age of the patients was $34.5 \pm 12.08$ years. Many (25%) patients were found among the destitute, followed by 20% among housewives from very poor families. It is a disease of poor and marginalised populations. The age distribution and the classification of cases is similar to that reported by others.\textsuperscript{15–18}

Sixteen percent were positive by Z-N stain which is in agreement with some other reports.\textsuperscript{19} In contrast, 30% ZN stain positive cases were reported in other studies.\textsuperscript{15,20} Z-N stain positivity depends on smear collection, preparation, quality of staining and microscopic observation. Higher positivity indicates presence of multibacillary leprosy because at least $10^4$ organisms per gram of tissue are needed for the detection of \textit{M. leprae} by AFB staining methods.\textsuperscript{9} Moreover, many patients might have started anti-leprosy treatment but did not disclose that during the current visit. This might be a cause of less positivity by Z-N stain in the present study.

On the basis of histopathology, 95% cases were positive. Overall concordance in clinical and histological classification was observed in 75% cases.\textsuperscript{15} In another study, clinico-histopathological correlation was found to be 53% overall, with maximum parity in LL cases

\begin{table}[h]
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\begin{tabular}{lllll}
\hline
\textbf{Clinical types} & \textbf{Clinical diagnosis} & \textbf{Z.N. Stain positive} & \textbf{Histopathology diagnosis} & \textbf{PCR positive} \\
& \textit{n} (\%) & \textit{n} (\%) & \textit{n} (\%) & \textit{n} (\%) \\
\hline
Paucibacillary (\textit{n} = 21) & & & & \\
TT & 10 (18) & 0 & 14 & 4 \\
BT & 11 (20) & 0 & 11 & 6 \\
Multibacillary (\textit{n} = 34) & & & & \\
BL & 27 (49) & 2 & 22 & 23 \\
LL & 7 (13) & 7 & 5 & 7 \\
\textbf{Total} & \textbf{55 (100)} & \textbf{9 (16)} & \textbf{52 (95)} & \textbf{40 (73)} \\
\hline
\end{tabular}
\caption*{Table 2. Results of Z.N. stain, histopathology and PCR among 55 patients, by classification}
\end{table}

TT, Tuberculoid leprosy; BT, Borderline tuberculoid leprosy; BL, Borderline lepromatous leprosy; LL, Lepromatous leprosy.
(76%) followed by BL (59%), BT (53%), TT (47%) and least in mid-borderline cases (37%).

PCR diagnosed 73% of cases of clinical leprosy. PCR confirmed the diagnosis in 72%–82% cases in some studies. In this study, 40% of TT, 55% of BT, 85% of BL and 100% of LL cases were PCR positive, which is also similar to other studies.

Identification of \(M. leprae\) in stained slides is a confirmatory test. The disadvantage of Z-N staining is at least 10\(^4\) organisms per gram of tissue are required to become positive. Thus, many paucibacillary leprosy patients and most tuberculoid leprosy patients will be negative on Z-N staining.

In the absence of an absolute diagnostic test to diagnose leprosy, it is advocated that the final diagnosis should be reached by a combination of multiple criteria considering clinical examination, Z-N staining, histopathology and PCR.

**Conclusion**

Microscopy may be recommended for detecting early lepromatous leprosy cases and PCR may be used for difficult to diagnose leprosy cases who are skin smear negative. In smear negative cases, the results of the clinical examination, histopathology and PCR may be used in combination to give a final diagnosis.

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References


