LIST OF PUBLICATIONS


Multiplex PCR technique could be an alternative approach for early detection of leprosy among close contacts - a pilot study from India. Communicated in Biomed Central Journal of Infectious Diseases, UK.

Rapid Identification of Mycobacterium species with the aid of Multiplex Polymerase Chain Reaction from clinical isolates. Open Microbiology Journal (Bentham Publications – Accepted 2009 December, in press)


Diagnosing leprosy: revisiting the role of slit skin smear with critical analysis of applicability of polymerase chain reaction (PCR) as diagnostic tool. (International journal of Dermatology – communicated)
India contributes about 80% of the global leprosy case load including case of fresh infection and reinfection. Due to lack of gold standard, diagnosis is done mainly based on routine clinical signs and symptoms, smear and histopathological evidences. There is a lot of lacunae in early confirmatory diagnosis in terms of sensitivity and specificity, especially in paucibacillary tuberculoid type. Moreover, the classification of different classes of leprosy is very important for selection of proper therapeutic schedule. Hence this study was undertaken to develop a multiplex polymerase chain reaction for the diagnosis and strain differentiation of M leprae. A multiplex polymerase chain reaction was developed using the primers R1 and R2 (a) amplifying 372bp DNA target from a repetitive sequence of M leprae and this repetitive sequence (372bp) that was used as a target DNA for amplification was reported to be specific for M leprae was not present in 20 mycobacterium species other than M leprae and primers TTCA and TTCB (b) amplifying (201 bp) DNA target of variable sizes from the regions flanking TTC repeats of M leprae genome. This multiplex polymerase chain reaction developed in our laboratory revealed that the number of repeats at each locus might be variable among Mleprae but they are found mostly in multibacillary (as the bacterial load is higher in multibacillary) type.

**Key words**: Polymerase chain reaction, leprosy, primers, bacterial index, histopathology.

Leprosy still remains today one of the major disabling diseases and imposes a considerable burden in terms of morbidity and social stigma not only on patients affected by the disease but also to members of immediate family. More than a century after its discovery, M leprae still remains uncultivable. Recent studies indicate that multidrug therapy (MDT), introduced by WHO in 1982, has not had a significant impact on reducing the incidence of leprosy in many parts of the world including India. The fall in the prevalence rate of leprosy has not due to a fall in the rate of detection of new cases. Shortening of the duration of treatment and removal from the registers of cured or defaulter patients, rather than a reduction in the transmission of M leprae infection could have largely caused the observed fall in the prevalence. The actual case detection rate provides the most helpful estimate of leprosy burden and it has increased during the past few years, which is also reflected in this study conducted among patients at the outpatients' clinic, department of dermatology, IPGME&R, Kolkata/Institute of PG Medical Education & Research, Kolkata. There remains today, hyperendemic areas in many countries eg, India, Brazil, Burma, Indonesia, Nepal which have shown no substantial decrease in the new case detection rates. New cases of leprosy continue at an incidence of 500,000 - 600,000 per year, and around 400,000 new cases occur in India. India alone contributes about 80% of the global leprosy case load. Little is known about the distribution and transmission of infections and the factors leading to the
DEVELOPMENT AND APPLICATION OF A NEW EFFICIENT AND SENSITIVE MULTIPLEX POLYMERASE CHAIN REACTION

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The epidemiology of leprosy is still poorly understood.

Apart from, Ridley and Jopling proposed classification in 1960 based on histology for the purpose of treatment. WHO in 1982 advocated, use of two different regimens of MDT for the treatment of leprosy which defined indeterminate (I), tuberculoid leprosy (TT), borderline tuberculoid leprosy (BT) cases of leprosy as being paucibacillary (PB) and borderline lepromatous (BL), lepromatous leprosy (LL) cases of leprosy as being multibacillary (MB). Under this classification bacterial index (BI) ≥ 2, indicates MB leprosy and BI value < 2 indicated for PB leprosy.

Currently, the diagnosis of leprosy is based on the clinical evaluation of characteristic lesions associated with neurological sequels and observations of acid-fast bacilli (AFB) in tissue smears or histological sections. Recently, a number of molecular based alternative methods for leprosy diagnosis are in practice in increasing numbers. Both serological tests and molecular probes have shown a certain potential for detection and identification of *M. leprae* in patients but these methods suffer from respectively limited specificity and sensitivity. Thus there is lack of a parameter which is highly sensitive and specific to diagnose leprosy.

Recently the polymerase chain reaction (PCR) based techniques for the detection of *M. leprae* DNA in environmental and clinical specimens has allowed investigators to begin the study of natural distribution as well. M. leprae DNA can be detected directly in various clinical specimens such as nasal swab, skin, etc. Most of the reports demonstrated that the PCR with or without DNA probes appeared very sensitive; so that even 1 to 100 organisms are detectable by this technique. Thus, this technique may be a useful diagnostic tool. However, PCR tools have not been fully evaluated for detecting *M. leprae* in detection of PB and MB form of clinical entities of leprosy.

In this study, we aimed to develop a multiplex PCR in this laboratory to diagnose cases of leprosy. Moreover, we have evaluated the sensitivity and specificity of this test and compared it with the traditional parameters to find out whether it can be considered as a better tool or not.

**Material and Method**

Classifications of patients were done clinically and histopathologically, according to the Ridley-Jopling scale. One hundred and eighty-five cases have been studied that comprised both the PB, having negative BI<2, but distinctive histopathological lesions diagnostic for leprosy and the MB having both histopathological lesions and a positive BI types. Disease types include I, TT, BT, mid-BL and LL. Skin biopsy and nasal swab have also been collected from each of 65 patients with skin diseases other than leprosy eg, post kala azar: leishmaniasis (PKDL), lupus vulgaris, sarcoidosis were used as control.

**Sample collection** — Untreated patients' (clinically MB/PB) samples were collected after obtaining their informed consent forms and ethical committee clearance. Patients with new skin patches or nodules with or without evidence of nerve damage were selected for the study. Samples which include (a) slit-skin smears and punch biopsy (n=185) and (b) nasal swab (n=30) have been obtained according to standard procedures for newly diagnosed untreated leprosy patients. Skin biopsy (4mm thick) / slit-skin smears from 6 sites depending on clinical type of leprosy for each patient have been prepared. A portion of biopsy samples/ slit-skin specimens from each patient had been used for paraffin embedding and the other portion had been stored at −20°C until used for PCR. Bacterial indices were determined microscopically from paraffin section of biopsy specimens.

**Collection of nasal swabs** — The surface of the nasal cavity of each patient had been swabbed with sterilised wet cotton swabs, which, were then frozen until further use. The cotton was washed in 0.5 ml of phosphate-buffered saline (PBS) containing 0.05% Tween80 to release the sample from cotton swabs and the aliquot was centrifuged at 10,000 X g for twenty minutes. The sediment was digested with lysis buffer (100mM Tris-Hcl, pH8.5 containing 0.05% Tween-20 and 60 µl of proteinase K per ml) for preparation of template DNA for PCR.

**Extraction of DNA from skin tissues** — Lysis buffer (300 µl) was added to frozen section of tissues and after mixing, were incubated for 18 hours at 60°C. Paraffin oil (40µl) was layered on top to prevent evaporation. Thereafter, the samples were incubated at 97°C for 15 minutes. The skin biopsy specimens were then incised to small pieces with sterile scissors. They were then homogenised by hand homogeniser with 1ml of sterile distilled water followed by heat inactivation of proteinase K as described above. After that to the lysed or homogenised products, equal volume of phenol-chloroform-isooamyl alcohol (25:24:1) was added. The tube was shaken vigorously for 1 minute. After centrifugation for 8 minutes at 8000 RPM in a microcentrifuge, the aqueous phase was collected and mixed with an equal volume of chloroform-isooamyl alcohol. Alcohol then precipitated with ethanol. The precipitated DNA was resuspended in 100µl of distilled water and used for PCR.

**Extraction of DNA from nasal swab** — In the same way, the lysed sediment obtained from nasal swab was
treated for DNA extraction. The newly developed multiplex PCR then tested all the samples.

**Primers and development of multiplex PCR** — A multiplex PCR have been developed in this laboratory based on primers amplifying the 372 bp of the repetitive sequence of *M. leprae*, reported to be specific to *M. leprae* and not present in 20 mycobacterial species other than *M. leprae* according to Yoon et al\(^4\).

A pair of primers designed to amplify 201 bp flanking entire 21 TCT repeats specific for MB and absent in PB and other mycobacterium species as per Shin et al\(^5\). The specificity and sensitivity of the primers: R1 and R2 and TTCA and TTCB had already been examined earlier\(^6,7\).

**Sequences for (a)**: 
- 5'-CGG CGG GAT CCT CGA TGC AC-3' (primer R1)
- 5'-CGT AAG CTT GTC GGT GG-3' ( primer R2)

**Sequences for (b)**: 
- 5'-GGT AAT CCC TCA TCT CGG GGA TG-3' (TTCA)
- 5'-CTC AGA GGC CGA AGC CTG-3' (TTCB)

Reaction mixtures and conditions of reactions and cycling conditions were optimised as follows. The reaction mixture in 50 μl of 10mMTris-HCl (pH 8.3), 50mMKCl, 1.5 MgCl\(_2\), 0.01% (weight/volume) gelatin, 200μM each dATP, dGTP, dCTP and dTTP, 1U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn) 0.5μm each primer and DNA purified DNA was added to the PCR mix, the tubes were kept for at least 10 minutes at room temperature. After amplification was finished, a 20μl portion of the reaction mixture was run in a 2% agarose gel. After electrophoresis, the gel was stained with ethidium bromide, and the 372bp and 201bp bands were examined under ultraviolet illumination.

Data were tabulated for sensitivity, specificity, positive predictive value and negative predictive value which were calculated according to the standard formula. Significance of the results were calculated by McNemar’s test\(^8\).

**OBSERVATIONS**

A simple method for the extraction of DNA from clinical samples was developed. By using this simple DNA extraction method, multiplex PCR was applied on frozen skin biopsy samples and nasal swabs from leprosy patients and controls.

It was observed, that primers (R1 and R2) amplifying 372bp of a repetitive sequence of *M. leprae*, was specific for *M. leprae* only and same was and is not present in 20 other mycobacterial species, (*M. avium, M. smegmatis, M. kansasii, M. tuberculosis, M. bovis and M. bovis BCG, etc*), and 10 other bacterial species such as *E. coli, P. aeruginosa, P. vulgaris, S. dysenteriae, E. cloacae, S. pyphi, S. marcescens, Staph aureus, Strept viridans and C. diphtheriae*\(^9\). While the primers TTCA and TTCB were found to amplify 201bp of TCT repeats in MB patients; the same was not found to amplify in PB patients (probably due to the lower bacterial load) and other mycobacterial species. The multiplex PCR results were compared with AFB count and it was observed that patients with high BI showed a strong 372bp and 201bp band and patients with low BI (even in one or two bacilli) showed a faint 372bp band (Fig1). Since the average BI obtained from slit-skin smears and histopathological slides were available, multiplex PCR results of biopsy and nasal swab specimens were compared with the BI and histopathological tests. We have studied a total of 185 cases among which 65 cases had skin diseases other than leprosy (control) and the remaining 120 were clinically diagnosed leprosy patients. Of these 120 cases studied, 50 were clinically diagnosed with PB and 38 with MB. Table 1 shows the distribution of study population according to different diagnostic parameters. Table 2 shows comparison of different diagnostic parameters. The significance of difference between two parameters was done by McNemar’s Chi-square test. Table 3 shows role of PCR in identifying MB and PB cases of leprosy.

**DISCUSSION**

This study was to initiate an original work in this laboratory to develop multiplex PCR as a better diagnostic tool for the detection of leprosy at an early stage.

The primer R1 and R2 is very specific for *M. leprae* and

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>pGEM (Molecular weight) marker</td>
</tr>
<tr>
<td>2</td>
<td>372bp amplicon using primers R1 and R2</td>
</tr>
<tr>
<td>3</td>
<td>201bp amplicon using primers TTCA and TTCB</td>
</tr>
<tr>
<td>4</td>
<td>Multibacillary</td>
</tr>
<tr>
<td>5</td>
<td>Paucibacillary</td>
</tr>
<tr>
<td>6</td>
<td>Paucibacillary</td>
</tr>
<tr>
<td>7</td>
<td>Paucibacillary</td>
</tr>
<tr>
<td>8</td>
<td>Multibacillary (positive control)</td>
</tr>
<tr>
<td>9</td>
<td>Negative control</td>
</tr>
</tbody>
</table>

*Fig 1 — Multiplex PCR Products of Different Clinical Samples Using Primer R1R2 (372bp Target) and TTCA and TTCB (201bp Target) for Detection of Leprosy*
was absent in 20 other mycobacterial species and the primers TTCA and TTCB are specific for MB patients only and were absent in PB and other mycobacterial species. Both the primers showed no amplification in the control subjects (who were suffering from skin diseases other than leprosy).

Detection of leprosy cases is difficult because the disease is usually asymptomatic in the early stage. So patients report late. Moreover, due to social stigma patients are reluctant to report the disease. In this backdrop the traditional parameters like BI and histopathological examination needs a lot of pursuance. Moreover, if these are negative the diagnosis is accurate (100% specificity and 100% positive productive value), but the sensitivity of the test is low (28% and 31% respectively). This may be due to technical difficulty. PCR on the other hand not only has a high sensitivity (80%), but also its sensitivity is high (80%). This difference in sensitivity between PCR, BI and PCR, histopathology have been found to be statistically significant (p< 0.001 in both the cases).

It should be noted that the multiplex PCR were positive in 89.5% cases of MB and 76.8% in case of PB. It should be expected, as the bacterial load is higher in MB. The PB type carries so few bacteria that these cases could not be detected microscopically (BI=0); 76.8% PCR positivity becomes meaningful and clearly reflects an advantage over microscopic examination. Even indeterminate cases studied, yielding positive results with multiplex PCR is very encouraging, since the indeterminate type is the earliest to appear in the clinical spectrum of leprosy.

Though the prevalence of leprosy is declining, new case detection rate is either stable or increasing. New case detection is a better indicator of the disease, as it is not affected by the changing case definition or duration of the treatment. According to WHO the elimination was based on the hypothesis that a prevalence of <1 case per 10,000 population is inconsistent. The International Leprosy Association's Technical Forum noted that there is little evidence to support this hypothesis as declination in the prevalence rate may not really indicate the elimination of the disease as: (i) Leprosy has long incubation period ranging from 2 to 20 years, (ii) patients newly diagnosed with leprosy may have transmitted the disease to others in the family. Moreover, the true incidence of leprosy is difficult to measure and the rate of infection in a community cannot be measured in contrary to tuberculosis. Using WHO's definition South Africa had attained elimination in 1924 but new cases continued to be detected in Northern Transvaal. The principal means of transmission of *M. lepra* is probably by aerosol spread of nasal secretion and uptake through nasal or respiratory mucosa. *M. lepra* DNA can also be detected in nasal swabs from up to 5% of healthy individuals in India and Indonesia, which suggest that subclinical infection occurs more frequently in these areas than previously thought.

In such circumstances this multiplex PCR can be a very useful diagnostic tool to detect the bacteria, where the bacterial load is very low and the chances of getting unnoticed can be decreased. Family members of newly detected patients can also undergo the test from the nasal swab to control the spread and transmission of the disease.

This technique can also be used even after release from treatment (RFT) to prevent relapse and resistant cases as, *M. lepra* DNA has been detected even eight years after completion of antileprosy therapy; and overlook of the cases can be reduced where leprosy is not endemic. Moreover, this multiplex PCR can detect both types as PB or MB at a very early stage (as the treatment varies in either case) to prevent acute nerve damage and disability.

Multiplex PCR because of its expenses and technical challenges may not completely replace conventional diagnostic methods such as smear, histopathological and clinical examination, rather, it can be positioned as a very useful complimentary tool in the diagnosis of doubtful cases and early detection of the bacteria. Further aid and fund is required to support this diagnostic tool in the endemic areas, as it can be a cost effective diagnostic tool for the detection, which can also help in control of the spread and transmission of the disease to support our motto of giving a "final push to elimination of leprosy", as a
public health problem.

Acknowledgment

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References

5. Smith WCS — We need to know what is happening to the incidence of leprosy. Lepr Rev 1997; 68: 165-72.
Multiplex PCR technique could be an alternative approach for early detection of leprosy among close contacts - a pilot study from India.

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Abstract:

Background: Implementation of Multi drug Therapy (MDT) regimen has resulted in the decline of the total number of leprosy cases in the world. Though the prevalence rate has been declining, the incidence rate remains more or less constant and high in South East Asian countries particularly in India, Nepal, Bangladesh, Pakistan and Srilanka. Leprosy, particularly that of multibacillary type spreads silently before it is clinically detected. An early detection and treatment would help to prevent transmission in the community. Multiplex PCR (M-PCR) technique appears to be promising towards early detection among contacts of leprosy cases.

Methods: A total of 234 paucibacillary (PB) and 205 multibacillary (MB) leprosy cases were studied in a community of an endemic area of Bankura district of West Bengal (Eastern India). They were assessed by smear examination for acid-fast bacilli (AFB) and M-PCR technique. These patients were treated with Multidrug Therapy (MDT) as prescribed by WHO following detection. A total of 110 MB and 72 PB contacts were studied by performing M-PCR in their nasal swab samples.
Results: 83.4% of MB patients were observed to be positive by smear examination for AFB and 89.2% by M-PCR. While 22.2% of PB patients were found to be positive by smear examination for AFB, 80.3% of these patients were positive by M-PCR. Among leprosy contacts (using M-PCR), 10.9% were found to be positive among MB contacts and 1.3% among PB contacts. Interestingly, two contacts of M-PCR positive MB cases developed leprosy during the period of two years follow up.

Conclusion: The M-PCR technique appears to be an efficient tool for early detection of leprosy cases in community based contact tracing amongst close associates of PB and MB cases. Early contact tracing using a molecular biology tool can be of great help in curbing the incidence of leprosy further.

Key words: Leprosy, Multiplex-PCR, Contact tracing, Early detection, and Leprosy Incidence, Paucibacillary (PB), Multibacillary (MB).

Background:

From time immemorial, leprosy is a grossly mutilating disease associated with social stigma and taboos, particularly in underdeveloped nations. The global caseload of leprosy has reduced by almost 90% over the last 20 years and 15 million cases have been detected and cured worldwide. Three hundred thousand (0.3 million) new cases were detected during the year 2005 (1). The principal factor contributing to this worldwide success is attributed to the introduction of standardized MDT regimens against the causative agent, *Mycobacterium leprae*. Further, leprosy
elimination campaigns for case detection in communities, training of physicians and leprosy
health care workers, community awareness towards prevention and control of leprosy have also
proven to be beneficial. **Elimination is defined as a reduction in the prevalence of leprosy
patients receiving antibacterial therapy to less than 1 per 10,000 populations.** (2), which
indicates that the disease is no longer considered a major public health problem. India has
achieved the prevalence rate of less than 1 per 10,000 populations in 2006. But the incidence rate
remains high in six countries of South East Asian region including India. India alone accounted
for 60% of the world’s newly detected cases (1, 3). This might be due to lack of consistent
information on the core elements of this infectious disease, e.g. source of infection, reservoir and
mode of transmission, host factors related to immunity of disease etc (4, 5, 6). It has been
observed that though prevalence has declined since initiation of MDT, the incidence has not
shown a similar decline during the same period i.e. after implementation of MDT (7, 8, 9, 10).

Leprosy, particularly MB type, is highly contagious and infectious may spread to several
contacts of patients even before clinical diagnosis. In case of MB leprosy, contacts staying in the
same house are at higher risk of getting infection compared to contacts staying at the neighboring
houses (10, 11, 12, 13, 14). Therefore, early detection of infections among close contacts
followed by effective chemotherapy is likely to bring down the spread of disease leading to a
decline in the overall incidence rate. Unfortunately, the conventional method of contact tracing
fails to detect fresh cases before it becomes transmissible to others (person to person
transmission) (15) Hence, it is necessary to have an alternative and more effective tool for an
early detection to prevent and control further transmission.
Previous work carried out by us, has led to the development of a Multiplex PCR (M-PCR) for early diagnosis of leprosy. The technique was standardized and was evaluated with high sensitivity and specificity (16).

The present study was carried out to evaluate whether the same technique could be used as a better diagnostic tool for early detection of leprosy cases and contacts for prediction of future cases of leprosy.

**Materials and Methods:**

**Patients:**

This study was conducted in an endemic population in the district of Bankura (prevalence rate greater than 2 per 10,000 populations) in West Bengal, India. After taking formal consent, a total no of 234 paucibacillary (PB) and 205 multibacillary cases (MB) attending the Public Health Centers were assessed by AFB (Acid Fast Bacilli) smear examination as well as multiplex-PCR (M-PCR) to assess the diagnostic efficiency of the later. Out of 234 PB cases 140 were tuberculoid (TT) and 94 were borderline tuberculoid (BT). Similarly, of 205 MB cases 53 were borderline lepromatous (BL) and 152 were lepromatous leprosy (LL).

Patients were grouped in the following categories:

(i) Patients without treatment, (ii) Patients on treatment not more than two months, (iii) Patients complaining of hypoesthesia but showing no clinical symptoms of leprosy – considered as
Indeterminate type, and (iv) Patients released from treatment (RFT) and later developed a new active lesion/ i.e. relapsed cases.

Slit skin smear (SSS) for acid -fast bacilli (AFB) staining were obtained from all patients for determination of Bacterial Index (BI). All diagnosed cases were given MDT as per the national leprosy control programme guidelines (17). Competent health care workers followed up household contacts of these patients. A total of 182 persons of which 110 were MB contacts and 72 were PB contacts, participated in this study voluntarily. Nasal swabs/slit skin smear specimens were obtained from all contacts after obtaining their necessary consent. The contacts were followed up for two years for observing the development of clinical leprosy.

**Ethical approval** was taken from the Ethical Committee of the Institute (Office of the Director, Inst of Post Graduate Medical Education & Research, Kolkata, Govt. Of West Bengal). Ref No. Inst/IEC/1835 dated 2.8.05 as a part of the project entitled “Development of Multiplex PCR for Early Diagnosis and Strain Differentiation of *M.leprae*” and since then reviewed periodically.

**Sample collection:**

**Slit Skin Smear:** SSS were obtained from each patient (from 3 to 6 sites, depending on the type of leprosy) for determination of bacterial index (BI). 4mm punch biopsy / SSS from three to six sites for each patient were obtained. Half of the biopsy samples from each patient was used for paraffin embedded sectioning and the other portions were stored at \(-20^\circ\text{C}\) until PCR was performed. BI (bacteriological index ) was also determined microscopically from paraffin section of biopsy specimens.
Collection of Nasal Swabs: The surface of the nasal septum either side of each patient were swabbed with sterilized wet cotton swabs, frozen in buffered saline containing 0.05% Tween80, which released the sample from cotton swabs. The aliquot was centrifuged at 10,000X g. The sediment was processed for DNA extraction as described bellow.

**DNA Extraction from clinical samples:**

**Extraction of DNA**

a) **From Skin Tissues:** The Frozen section of tissues /skin biopsy specimens were cut to small pieces with sterile scissors. These samples were homogenized in a manual homogenizer with 1ml sterile distilled water. It was then incubated in lyses buffer containing 300 μl of 100mM Tris-HCl, pH8.5 (containing 0.05% Tween 20 and 60 μg of proteinase K per ml) for 18hrs at 60°C. Paraffin oil (40 μl) was layered on top of the buffer to prevent evaporation. Thereafter, the samples were incubated at 97°C for 15 minutes (11), followed by heating for inactivation of proteinase K. Equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was later layered on the lysed homogenated product. The tube was shaken vigorously for 1 minute. After centrifugation of the material for 8 minutes, the aqueous phase was collected and again mixed with an equal volume of chloroform-isoamyl alcohol. This was followed up by another brief centrifugation. Then, the uppermost phase was collected and boiled for 10 minutes to destroy DNase, followed by precipitation of DNA with ethanol. The precipitated DNA was resuspended in 100 μl of distilled water and used for M-PCR.
b) Extraction of DNA from Nasal swab: Frozen samples were quickly thawed and centrifuged at 10,000X g for 20 minutes. The sediment was subjected to DNA extraction following the same procedure as mentioned above.

**Multiplex PCR**

A M-PCR was developed in our laboratory (16) based on:

(a) Primers amplifying the 372 bp of the repetitive sequence of *M. leprae*, known to be specific for *M. leprae* and is not present in twenty other mycobacterial species. (19)

(b) A pair of primers was designed to amplify 201 bp flanking entire 21 TTC repeats. (20)

Sequences for (a): 5’-CGG CCG GAT CCT CGA TGC AC-3’ (primerR1)

5’-GCA CGT AAG CTT GTC GGT GG-3’ (primerR2)

For (b): 5’-GGA CCT AAA CCA TCC CGT TT-3’ (TTC-A)

5’-CTA CAG GGG GCA CTT AGC TC-3’ (TTC-B)

Reaction mixtures, conditions of reactions and cycling conditions were optimized as follows:

The reaction mixture contains 50 ul of 10mM Tris-HCL (pH 8.3), 50mMKCL, 1.5 MgCl2, 0.01%(wt/Vol) gelatin, 200uM each dATP, dGTP, dCTP and dTTP, 1U of Taq polymerase (Perkin –Elmer Cetus, Norwalk, Conn) 0.5μm each primer and DNA extracted from biopsy samples.

PCR Condition: PCR is carried out in a thermocycler for 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 mins and primer extension at 72 °C for 3 mins. After purified DNA is added to the PCR mix, triple distilled water is used as negative control. The
tubes are kept for at least 10 mins at room temperature. After amplification is finished, a 20μl portion of the reaction mixture is run in an 2% agarose gel. After electrophoresis, the gel is stained with ethidium bromide, and the 372bp and 201bp bands examined under UV illumination.

**Statistical Analysis:** Sensitivity of smear examination for AFB and M-PCR of skin biopsy samples is calculated considering clinically diagnosed cases as true gold standard of positivity.

**Results:**

The findings of M-PCR and BI of SSS are presented in table I.

Table-I: Sensitivity of AFB and multiplex-PCR as diagnostic tools in detecting paucibacillary as well as multibacillary leprosy cases among clinically diagnosed patients:

<table>
<thead>
<tr>
<th>Type of disease</th>
<th>Clinical Forms</th>
<th>Cases confirmed by AFB In SSS (Slit skin smear)</th>
<th>Cases confirmed by Multiplex-PCR</th>
<th>Percent of positivity by AFB test</th>
<th>Percent of positivity by M-PCR test</th>
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<tr>
<td>Paucibacillary cases (n=234)</td>
<td>TT (n=140)</td>
<td>44 (BI&lt; 2)</td>
<td>111</td>
<td>31.4%</td>
<td>79.2%</td>
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<tr>
<td></td>
<td>BT (n=94)</td>
<td>18 (BI&lt; 2)</td>
<td>77</td>
<td>19.1%</td>
<td>81.9%</td>
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<tr>
<td>Total</td>
<td></td>
<td>52</td>
<td>188</td>
<td>22.2%</td>
<td>80.3%</td>
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<tr>
<td>Multibacillary cases (n=205)</td>
<td>BL (n=53)</td>
<td>38 (BI&gt; 2)</td>
<td>47</td>
<td>71.6%</td>
<td>88.6%</td>
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<tr>
<td></td>
<td>LL (n=152)</td>
<td>133 (BI&gt; 2)</td>
<td>136</td>
<td>87.5%</td>
<td>89.4%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>171</td>
<td>183</td>
<td>83.4%</td>
<td>89.2%</td>
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</tbody>
</table>
From the above table it is clear, that on the whole M-PCR can detect a higher number (79.2% to 89.4%) of leprosy cases compared to SSS test (19.1% to 87.5%). The sensitivity of M-PCR in case of tuberculoid type is higher than smear examination. It is noted that the positivity was considerably higher in tuberculoid cases (TT 79%, BT 81.9%) in comparison to SSS (TT 31.4%, BT 19.1%). Multiplex PCR of nasal swab was found to be positive 49.4% (42/85), 51.6% (32/62), 62.5% (25/40) and 78.1% (75/96) in the categories of TT, BT, BL and LL respectively. This indicates that nasal swab could be an alternative, non-invasive procedure for M-PCR for leprosy detection. PCR results were confirmed by sequencing.
Table-I a): Sensitivity of AFB and multiplex-PCR as diagnostic tools in detecting paucibacillary as well as multibacillary leprosy cases among different categories of paucibacillary patients:

<table>
<thead>
<tr>
<th>Clinical status of the disease</th>
<th>Cases confirmed by AFB</th>
<th>Cases confirmed by Multiplex-PCR</th>
<th>Percent of positivity by AFB test</th>
<th>Percent of positivity of M-PCR test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of case</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT (including 2 indeterminates) n=138 + 2 Indeterminate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients without treatment (New Cases)(n=128)</td>
<td>0</td>
<td>85</td>
<td>62 (72.9%)</td>
<td>50.58%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>43</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Patients on treatment not more than 2 months (n = 7)</td>
<td>0</td>
<td>6</td>
<td>3 (50%)</td>
<td>14.28%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Patients released from treatment (RFT)/ later developed a new active lesion / relapsed cases (n=3)</td>
<td>0</td>
<td>3</td>
<td>1* (33.3%)</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Patients complaining of hypoesthesia but showing no clinical symptoms of leprosy -- considered as Indeterminate type (n=2)</td>
<td>0</td>
<td>2</td>
<td>1 (50%)</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total n= 140</td>
<td></td>
<td></td>
<td></td>
<td>31.4% (44/140)</td>
</tr>
<tr>
<td>BT n=94</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical status of the disease</td>
<td>Cases confirmed by AFB</td>
<td>Cases confirmed by Multiplex-PCR</td>
<td>Percent of positivity by AFB test</td>
<td>Percent of positivity of M-PCR test</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------------</td>
<td>---------------------------------</td>
<td>----------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td></td>
<td>No of case</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients without treatment (New Cases)(n=85)</td>
<td>0</td>
<td>68</td>
<td>55 (80.8%)</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>17</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Patients on treatment not more than 2 months (n = 6)</td>
<td>0</td>
<td>5</td>
<td>2(40%)</td>
<td>16.6%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Patients released from treatment (RFT)/ later developed a new active lesion / relapsed cases (n=3)</td>
<td>0</td>
<td>2*,13</td>
<td>1* (33.33%)</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total n= 94</td>
<td></td>
<td></td>
<td></td>
<td>19.14% (18/94)</td>
</tr>
</tbody>
</table>
Table-I b): Sensitivity of AFB and multiplex-PCR as diagnostic tools in detecting multibacillary leprosy cases among different categories of multibacillary patients:

<table>
<thead>
<tr>
<th>BL n=53</th>
<th>Clinical status of the disease</th>
<th>Cases confirmed by AFB In SSS(Slit skin smear)</th>
<th>Cases confirmed by Multiplex-PCR</th>
<th>Percent of positivity by AFB test</th>
<th>Percent of positivity of M-PCR test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients without treatment (New Cases)(n=44)</td>
<td>BI</td>
<td>No of case</td>
<td>BI</td>
<td>No of case</td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>23</td>
<td>3</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients on treatment not more than 2 months (n = 5)</td>
<td>0</td>
<td>4</td>
<td>2(50%)</td>
<td>2(50%)</td>
<td>50%</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients released from treatment (RFT)(^{#}) / later developed a new active lesion / relapsed cases(^{#}) (n=4)</td>
<td>0</td>
<td>2*</td>
<td>2(0%)</td>
<td>2(0%)</td>
<td>71.6% (38/53)</td>
</tr>
<tr>
<td>2</td>
<td>1*</td>
<td>1*</td>
<td>3</td>
<td>2b</td>
<td>2b</td>
</tr>
<tr>
<td>4</td>
<td>1*</td>
<td>1*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total n= 53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>71.6% (38/53)</td>
</tr>
</tbody>
</table>

LL n=152

<table>
<thead>
<tr>
<th>Clinical status of the disease</th>
<th>Cases confirmed by AFB In SSS(Slit skin smear)</th>
<th>Cases confirmed by Multiplex-PCR</th>
<th>Percent of positivity by AFB test</th>
<th>Percent of positivity of M-PCR test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients without treatment (New Cases)(n=115)</td>
<td>BI</td>
<td>No of case</td>
<td>BI</td>
<td>No of case</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>37</td>
<td>5</td>
<td>31</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients on treatment not more than 2 months (n = 12)</td>
<td>0</td>
<td>4</td>
<td>1(25%)</td>
<td>2(25%)</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Patients released from treatment (RFT)(^{#}) / later developed a new active lesion / relapsed cases(^{#}) (n= 25)</td>
<td>0</td>
<td>14*;1b*</td>
<td>2(13.33%)</td>
<td>2(13.33%)</td>
</tr>
<tr>
<td>2</td>
<td>1*;2b*</td>
<td>3</td>
<td>3</td>
<td>2*</td>
</tr>
<tr>
<td>4</td>
<td>2*;1b*</td>
<td>3</td>
<td>5</td>
<td>1*</td>
</tr>
<tr>
<td>6</td>
<td>1*</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total n= 152</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The data obtained from M-PCR of nasal swab of 110 household contacts of MB patients and 72 of PB patients are presented in table II. The result shows that 11.7% adults and 9.5% children among MB contacts and 2% adult among PB contacts showed evidence of *M. leprae* DNA in their nasal swabs. These contacts were followed up prospectively for two years since the day of collection of nasal swabs. It was observed that 1 adult and 1 child developed leprosy from M-PCR positive MB contacts during the two-year observation period. None of the contacts from PB cases developed clinical leprosy during the follow up period (table-II)

Table-II: Showing M-PCR positivity and incidence of leprosy in household contacts of leprosy cases in nasal swabs:

<table>
<thead>
<tr>
<th>Multbacillary leprosy contact</th>
<th>Paucibacillary leprosy contact</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of persons tested with</strong></td>
<td><strong>Number positive (%)</strong></td>
</tr>
<tr>
<td>M-PCR (Nasal Swab)</td>
<td>(Nasal Swab)</td>
</tr>
<tr>
<td>Adult = 68</td>
<td>8 (11.7%)</td>
</tr>
<tr>
<td>Child = 42</td>
<td>4 (9.5%)</td>
</tr>
<tr>
<td>Total = 110</td>
<td>12 (10.9%)</td>
</tr>
</tbody>
</table>

| Adult =48 | 1 (2%) | 0 |
| Child=24 | 0 (0%) | 0 |
| Total=72 | 1 (1.3%) | 0 |
Discussion:

Leprosy is a disease with long incubation period and the symptoms are difficult to perceive at the early stage of infection. Self-healing does occur in a large number of infected cases. Moreover, many patients with early signs are not aware that they are suffering from leprosy and thus clinical diagnosis is often delayed. Clinical diagnosis is possible only when the patient is symptomatic, exhibiting lesions and satisfies the criteria of cardinal signs of leprosy (23). A sizable proportion of new cases are among children (WHO 2004), (21) as they often remain in close contact with infected family members sharing same dwelling units that facilitate infection in them (22) Similarly, an infected child could pass this infection to other children while they are in contact for longer duration, i.e., playing in a group or in school. This often goes un-noticed during initial phase because of its slow and silent nature of early transmission.

Considering the above, early detection of cases followed by effective chemotherapy appears to be the single most effective strategy for reducing incidence of leprosy cases as well as to prevent transmission. However, existing method of contact tracing and detection is not beyond criticism. The results of M-PCR that was developed and used in this pilot study as an alternative tool appears to be encouraging. In our study using M-PCR, we found that the molecular tool is much better in detecting high percentage of TT/BT cases (79.2% & 81.9% respectively), than cases detected by SSS (22.2%). The overall positivity of PB cases by PCR is 80.3%. As the disease progresses further, the positivity of both PCR (80.3% to 89.2%) and AFB smear examination are (22.2% to 83.4%) increased. This efficacy of M-PCR over SSS can be explained by the fact that, with the progression of disease there is increase of bacterial load resulting in availability of more genomic DNA of *M leprae* and thereby leading to PCR positivity. While specific PCR for
diagnosis of leprosy developed by previous scientists (27, 28) were successful in diagnosing only 50% of PB cases, the present M-PCR was able to diagnose 80.34% of PB cases. The reason for identifying more cases of early infection might be due to the use of combination of two specific primers in the same PCR reaction. However, unlike the previous workers, the M-PCR failed to detect 100% cases of MB leprosy. The reason for the failure may be due to the inclusion of large number of cases (205) compared to those of 37 (28) and 38 (27) cases of MB leprosy. From table I (a) and I(b) it is seen that MPCR is highly sensitive in new cases and the sensitivity gradually decreases in case of RFT/Relapse cases both in PB and MB patients. This result is along expected lines, as it is difficult to amplify mycobacterial DNA in patients after treatment due to genomic degradation of the bacteria. The specificity was not calculated in this study as the same was done in our earlier study while evaluating MPCR as a diagnostic tool and it was found to be very high. (16) From the present study, it can be suggested that M-PCR could be an alternative screening tool for detection of early leprosy cases or their close contacts with high sensitivity compared to any other available tools.

Another important and effective strategy for reduction of incidence of leprosy is contact tracing and early detection of cases among them. In our pilot study M-PCR technique was applied in the leprosy contacts for adopting a better way of community-based early case detection. Contacts of PB and MB cases both were followed up after testing their nasal swabs by M-PCR. It was noted that two of the twelve M-PCR positive cases developed as clinical cases and none of the M-PCR negative cases developed any form of leprosy during this period. It is interesting to note that a significantly larger numbers (12 nos) of MB contacts were positive by M-PCR than that of PB contacts (2 nos) who did not develop leprosy during the follow up period.
It has been estimated that 6-8% of household contacts develop clinical symptoms of leprosy within two years of follow up since the diagnosis of the index cases(24). In our study 10.9 % MB contacts were PCR positive and 1.8% developed the disease within two years of study period. This low number of detection in our study group could be due to the lower transmission dynamics in the community during the post elimination era. Keeping in mind the long incubation period of the disease it is imperative that the contacts need to be followed and observed for a longer period.

The trend that we find in our study can serve as important clue that the contacts of MB patients are at increased risk of developing leprosy in the future. This hypothesis needs to be tested on large scale of population over a long interval before coming to any conclusion, since the authors have no intention to put forth the MB patients into social ostracism under the fear of infection. Whatever the relationship between positivity of PCR and development of the disease, PCR is much more sensitive than microscopic examination for direct detection of bacilli (25, 26). Moreover, at present there is no other more sensitive alternative tool available for early detection of leprosy other than PCR and serological test (22). Present M-PCR test has been standardized based on the presence of *M. leprae* DNA which does not support the presence of viable organisms. Effort should be made to standardize a method based on mRNA detection so that viability of *M. leprae* bacilli could also be ascertained (27, 28, 29, 30).

It should be also noted that the PCR positivity in contact persons indicate the presence of *M. leprae* DNA only and not infection and therefore, a careful follow up of them should be done and treatment should be started immediately after the development of first sign and symptoms of leprosy.
Conclusion:

This study indicates that M-PCR can be used as an efficient tool for early detection of leprosy among contacts. However it needs further in-depth study with adequate population size and controls over a long period of time. As this M- PCR can only detect the presence of *M. leprae* DNA, hence a contact with positive PCR result must be followed up regularly for detection of any development of disease. As leprosy has a long and varied incubation period, a long term follow up/ observation is necessary to establish clearly the early case detection efficiency of M-PCR.

This M- PCR is a relatively expensive procedure compared to other methods of detection of infection caused by *M. leprae*. It has its limitations in the viability detection of the bacteria. In addition, it also requires a well-equipped laboratory which is difficult to replicate in the field. When tests are performed in bulk, the cost is expected to go down significantly. Hence, we recommend its application in a large number of samples to make it cost effective. The test needs to be evaluated further as it could serve as a better diagnostic tool for early case detection and their treatment to achieve faster control of leprosy.

Competing Interests :

The authors declare that they have no competing interests.

Author’s Contribution:

Surajita Banerjee: Developed and standardized the protocol, wrote the manuscript.

Prasanta Sinha Mahapatra & Samudra Guha: Collected the samples.

Soma Gupta, Siddhartha Gupta: Wrote and edited the manuscript,
Debasis Bandhopadhaya, Chaitry Ghosal, Suman Kalyan Paine, Rathindra Nath Dutta, Nibir Biswas: edited the manuscript.

Kamalesh Sarkar: Did the statistical analysis.

Basudev Bhattacharya: Guided the overall work and helped in writing and editing the manuscript.

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References:


**Corresponding Author:** Dr. Basudev Bhattacharya

**Department Of Biochemistry, IPGME&R, Kolkata, India**
Rapid Identification of Mycobacterium Species with the Aid of Multiplex Polymerase Chain Reaction (PCR) From Clinical Isolates

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3Department of Microbiology, R G Kar Medical College, Kolkata
4Department of Life Science & Biotechnology, Jadavpur University

Abstract: Mycobacteria are aerobic, nonspore forming, non-motile, single-cell bacteria. Of more than 40 currently recognized species of mycobacteria, Mycobacterium tuberculosis, the causative agent of human TB is the commonest pathogen for pulmonary and extra pulmonary tuberculosis cases. The other members of the Mycobacterium tuberculosis complex (MTC) or the nontubercular mycobacterium (NTM) produces similar diseases which cannot be differentiated from tuberculosis by clinical symptoms and signs. But this differentiation is important as the chemotherapy varies widely according to the strain of mycobacterium. The burden of morbidity and mortality of tuberculosis is rapidly growing worldwide, particularly with the HIV/AIDS epidemic. The strain identification of Mycobacterium remains a cumbersome, labor intensive and expensive procedure, which requires 3 to 12 weeks of time. The conventional methods of strain identification lack proper standardization and precise diagnosis. The prime objective of this study is to overcome these problems.

A multiplex PCR using 3 amplicons of 165, 365, and 541 base pair target sequences was done with a total number of 165 clinical isolates of suspected Koch’s patients. Strain identification was compared both by conventional methods and multiplex PCR. The results of the study show that this multiplex PCR is supposed to be less complicated, less time consuming, cost-effective and superior to the conventional methods. It is also applicable for culture negative samples where strain identification is not possible by conventional approach.

Key Words: M. tuberculosis, Non Tubercular Mycobacteriosis, culture, multiplex PCR, strain differention.

1. INTRODUCTION

Based on surveillance and survey data, WHO estimates that 9.27 million new cases of TB occurred in 2007. Of these 9.27 million new cases, an estimated 44% were new smear positive cases, 14.8% were HIV positive cases and 4.9% were MDR-TB (which includes 3.1% new cases and 19% previously treated cases). An estimated 1.32 million HIV-negative people died from TB in 2007 and there were an additional 4,56,000 TB deaths among HIV positive people [ICD-10]. India is ranking top among 27 high MDR-TB burden countries. Incidence of MDR-TB in INDIA is reported to vary from 0.1%-5.4%. MDR-TB among all new TB cases are 2.8%, whereas 17% among the previously treated cases [1-5]. Moreover, the HIV/AIDS has produced a devastating effect on TB control worldwide [6]. Among the mycobacterial diseases (both pulmonary and extrapulmonary) M. tuberculosis complex has got the lion’s share. But the infection with Nontubercular Mycobacterium (NTM) or Mycobacterium Other Than Tuberculosis (MOTT) is also increasing rapidly.

This MOTT complex can cause pulmonary infection as well as extrapulmonary lesions (Lymph node, skin & soft tissue, joint or systemic disseminated infection) in immunocompromised hosts. The majority of such infections are due to M. avium & M. intracellulare (MAI). Other responsible pathogens are M. scrofulaceum, M. kansasi, M. fortuitum, M. ulcerans etc. [7]. In 1959, Runyon classified NTM into four groups (I to IV) based on phenotypic characterization, particularly growth rate and pigmentation [8]. The treatment of diseases with M. tuberculosis complex and MOTT complex are different because many of the first and second line of antitubercular drugs are ineffective against many members of the MOTT organisms. The most effective drugs against these organisms are macrolide or aminoglycoside antibiotics. The treatment protocol heavily depends on the species of mycobacterium isolated, site and severity of infection [9]. The report of drug sensitivity testing is not always reliable and the duration of the treatment is protracted. On other side Pyrazinamide is ineffective in M. bovis infection [10]. So, strain identification from the clinical specimens is critical particularly in case of patients not responding to conven-

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tional therapy, relapse or in immunocompromised subjects. Moreover, an early diagnosis of species helps us to curtail huge burden of treatment with ineffective drugs and reduce patients' morbidity and mortality [11].

The conventional method of identification of species involves culture on LJ slants and liquid medium followed by observing the rate of growth and pigment production (where applicable). Moreover, the growth of cultures was studied in different temperature gradient to identify the mycobacterial species. Biochemical tests like niacin production, nitrate reduction, semi quantitative and heat resistant catalase production etc are also required [12].

These are time consuming, complicated and laborious procedures whereas a multiplex PCR involving three amplons (165bp, 365bp and 541bp fragments) can identify and differentiate between different species of mycobacterium [13, 14] and this method is also highly sensitive and specific [15].

2. MATERIALS & METHODS

2.1. Specimen Collection

This cross sectional study was done with the Institutional Biotechnical permission. Patients of all ages and both sexes recently diagnosed to be suffering from pulmonary or extrapulmonary tuberculosis were randomly chosen from in and out patients' departments of three tertiary care hospital in Kolkata, Eastern part of India. The diagnosis of tuberculosis is based on the clinical examination, microscopy for AFB, manteaux test, Chest Skiagram, ESR, Cytological examination and ADA estimation of aspirated fluid. Sputum samples were collected from pulmonary tuberculosis patients. Body fluids (like CSF, pleural fluid, peritoneal fluid); blood, tissue or lymph node aspirates from patients with extrapulmonary tuberculosis were collected aseptically using all precautions. Total numbers of samples were 165. The total span of the study was conducted from July 2006 to February 2008.

2.2. Specimen Processing

Sputum samples were liquefied and decontaminated using an equal volume of 4% NaOH, 2.9% Sodium citrate solution and N-Acetyl-L-Cysteine for 30 minutes incubation at room temperature. After decontamination, specimen neutralized with sterile PBS (pH6.8). After centrifuga 3000Xg for 30 minutes, supernatant was discarded and sediment was suspended in sterile water. The body were concentrated in usual methods. Tissue or lymph aspirates decontaminated and concentrated for AFB culture inoculation. PBMC layer were collected from blood samples of the patients by histopaque density gradient. All the samples were ready for isolating DNA.

2.3. Demonstration of Acid Fast Bacilli Smears

Smears were prepared using the dissolved sediment of all specimens, stained by Ziehl-Neelson (ZN) method examined for presence of AFB with a light microscope. Smears recorded as positive if at least 1 to 9 AFB were observed in high power fields were observed [16, 17].

2.4. Isolation of DNA and Multiplex PCR for Species Identification

After decontamination and concentration, samples moistened with adjusting amount of TE buffer (pH 8). After scrapping the cytological material, it was disrupted thoroughly in TE buffer. Mycobacterial DNA was extracted from clinical specimens using a modification of the method described by Sritharan and Barker (1991) [18]. Sediments were resuspended in 100 μl of TE-Triton buffer (10 Mm Tris, 1 mM EDTA, and 1% Triton X-1 8) with 200 μg/ml proteinase K, incubated at 56°C for 1 hour, and then incubated at 95°C for 30 min. After incubation, the specimens were placed on ice for 5 min. Protease-treated and heated specimens were next exposed to Phenol: Chloroform: Isoamyl Alcohol (25:24:1) aqueous phases of the organic extractions were precipitated with ethanol overnight. The DNA was dissolved in TE buffer [18]. Reference strains of M. tuberculosis (H37Rv), M. bovis, M. smegmatis, M. intracellula and M. avium were obtained from Tuberculosis Research Centre (TRC), Chennai, India. Extraction of genomic DNA done from reference strains cultured on LJ media was standard method. Multiplex PCR as earlier developed in our laboratory was done to amplify the following sequences [19, 20].

<table>
<thead>
<tr>
<th>Target Region</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>65 KDa (hsp proteins)</td>
<td>5’-CTA GGT CGG GAC GGT GAG GCC AGG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CAT TGC GAA GTG ATT CCT CCG GAT-3’</td>
</tr>
<tr>
<td>dna J gene</td>
<td>5’-AAG AGG AAG GAG AGA GGC-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GTC GTT GAG GTT GAA CTC-3’</td>
</tr>
<tr>
<td>IS 6110 insertion element</td>
<td>5’-GTG GGC ATG GTC GCA GAG AT-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CTC GAT GCC CTC ACG GTT CA-3’</td>
</tr>
</tbody>
</table>

The PCR amplification was done in a Biometra Thermal cycler with an initial cycle of denaturation (5min at 95°C), followed by 40 cycles each of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, with a final extension of 10 min at 72°C and a post incubation of at 4°C. Each DNA amplification experiment included a positive control (Reference strains like: M. tuberculosis DNA) and a negative control (distilled water). The am
Rapid Identification of Mycobacterium Species with the Aid of Multiplex

DNA products were visualized under UV light and photographed after 2.5% agarose gel electrophoresis by ethidium bromide staining (Fig. 1).

Fig. (1). Multiplex PCR amplification of 165bp, 365bp and 541bp regions of the clinical specimens of M. tuberculosis and Nontubercular mycobacteria on 2.5% agarose gel. Lane 1: 100bp Molecular Marker; Lane 2-12: Clinical specimens (2,4,6: Atypical); (3,5,7,9,10: M.Tuberculosis); (8: M.Bovis); (11,12: Inconclusive) (Lane 13: Positive Control; Lane 14: Negative Control.

2.5. Culture by Inoculation in LJ Medium and Liquid Medium

All the samples (N=165) were included in the study. The samples were inoculated on LJ media and liquid medium Middlebrook 7H9 with OADC supplement in our laboratory. The observations like rate of growth both at 30°C & 37°C, Colony character and pigment production (if any) in light and dark (on LJ slants) for colonies were noted.

2.6. Biochemical Tests

Then biochemical tests like Niacin Production, Nitrate Production, Catalase production (semi quantitative & heat resistant), TWEEN-80 Hydrolysis, Growth in presence of Paranitrobenzoic acid (PNB) were done to differentiate among different strains of Mycobacterium tuberculosis complex and NTM. To differentiate between Mycobacterium tuberculosis and Mycobacterium bovis, Growth on PZA containing media, Sensitivity to TCH, Niacin production, Nitrate Reduction were performed [12, 21].

3. RESULTS AND DISCUSSION

In this study we found that out of 165 samples, 126 (76.36%) were PCR positive whereas 95 (57.57%) were culture positive. The overall AFB positive samples was 85 (15.5%) (Table 1). Out of total samples 136(82.42%) were sputum. Rest were pleural fluid(n= 14),peritoneal fluid(n=2),CSF(n=6),Blood(n=4),Lymph node Aspirate(n=2) and tissues(n=1) (Table 2). Out of 126 PCR positive samples 102 (80.95%) showed all the three target amplifications and thus diagnosed as M.tuberculosis. Only 2(1.6%) cases were attributed to M.bovis which shows amplification only at 165bp region .18 (14.28%) cases were nontuberculous mycobacteria and the rest 4 (3.10%) were inconclusive showing nonspecific amplifications.

<table>
<thead>
<tr>
<th>Experiments/Tests</th>
<th>Positive Result in Percentage</th>
<th>Distribution in Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB</td>
<td>85 (51.5%)</td>
<td>PTB: 80 (58.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EPTB: 5 (12.6)</td>
</tr>
<tr>
<td>Culture</td>
<td>95 (57.57)</td>
<td>PTB: 57 (64.77)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EPTB: 7 (24.13)</td>
</tr>
<tr>
<td>Polymerase Chain Reaction</td>
<td>126 (76.36)</td>
<td>PTB: 110 (87.30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EPTB: 16 (55.17)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type of Tuberculosis</th>
<th>Sample</th>
<th>Number of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary (82.4%)</td>
<td>Sputum</td>
<td>136</td>
</tr>
<tr>
<td>Extrapulmonary (17.6%)</td>
<td>Pleural Fluid</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Peritoneal Fluid</td>
<td>02</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>06</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>04</td>
</tr>
<tr>
<td></td>
<td>Lymph node Aspirates</td>
<td>02</td>
</tr>
<tr>
<td></td>
<td>Tissue</td>
<td>01</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>165</td>
</tr>
</tbody>
</table>
The atypical mycobacteria (NTM) were identified either by amplification of both 165 and 365 bp target sequences or shorter than that of M.tuberculosis or M. bovis (target region 165 bp) due to small deletion. It can be used to differentiate this organism from the M.tuberculosis complex [19, 20].

Out of 95 culture positive samples 72(75.78%) were Mycobacterium tuberculosis whereas 3 (3.16%) were M.bovis and 6 (6.32%) were MOTT or NTM. 14 (14.74%) showed the isolations were 71% of in accordance with the study made by Schultz et al. [22], who demonstrated the isolations were 71% of M.tuberculosis and 34% of atypical mycobacterial strains from 191 archival specimens of tissues. In the study of Ergin et al. the respective isolations were 79.1% and 20.8% in 120 specimens [23].

Table 3. Types of Mycobacteria Identified By PCR (N=126) and Culture (N=98)

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR Positive</th>
<th>Culture Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>102(80.95%)</td>
<td>72(75.78%)</td>
</tr>
<tr>
<td>M. bovis</td>
<td>02(1.6%)</td>
<td>03(3.16%)</td>
</tr>
<tr>
<td>MOTT</td>
<td>18(14.28%)</td>
<td>06(6.32%)</td>
</tr>
<tr>
<td>Unidentified</td>
<td>04(3.1%)</td>
<td>14(14.74%)</td>
</tr>
<tr>
<td>Total</td>
<td>126(100%)</td>
<td>95 (100%)</td>
</tr>
</tbody>
</table>

Species identification by conventional cultural methods and biochemical tests are of limited value because of the comparatively low yield of the culture and long time to grow. Even the so-called rapid growers may require 1-3 weeks to grow. The differentiation of M.tuberculosis and M.bovis is not always conclusive. Niacin production and PZA sensitivity tests lengthen the reporting time span. Pigment production in light (photochromogens) and in both light and dark (scrochchromogens) are subjective observations that cannot be standardized. To achieve good pigment production colonies should be young, actively metabolizing, isolated and well aerated. [24] Some members require prolonged exposure to light. Some others (like M. szulgii) need incubation at 35 °C. In our study, we also observed the growth both at 30 °C and at 37°C. Moreover, many important members of MOTT complex e.g. M.avium-intracellulare (MAI) and M.ulcerans etc. are nonchromogenic i.e. do not produce any pigment at all. Complicated biochemical tests like Niacin production, Nitrate reduction, Tween80 hydrolysis, Catalase etc. are required to differentiate them from M.tuberculosis complex as already mentioned. [12, 25].

In the current study, even with this time consuming laborious effort the species identification by conventional methods is low. It was possible in 81 culture positive cases which are only 49.99% of total 165 cases. Schultz S et al. [22] also, in their study, found that only 41 out of 190 specimens (21.5%) were culture positive in which species identification could be made. On the other hand, species identification is much rapid and more accurate alternative where 122 cases out of 165(73.93%) could be categorized into different species. The overall isolation of M.tuberculosis Complex (M.tuberculosis and M. bovis) is 63% [M.tuberculosis 61.8% and M.bovis 1.2%] in comparison to MOTT which is 10.9%. 2.4% cases gave ambiguous results by showing non-specific bands on PCR followed by gel electrophoresis. This may be due to contamination. Ergin et al. [23] in their study, evaluated in share of M.tuberculosis as 79.1% and MOTT as 20.8% in 120 mycobacterial strains isolated from clinical specimens by PCR-RFLP. The result of our study is in accordance with the study made by Ergin et al. It is to be noted here that the failure in species identification by conventional methods is much higher (14 cases) which accounts for 8.48% of overall 165 cases. Over last decade, the rate of non-AIDS associated infection is also increasing and many of the newly identified NTM species have been identified using molecular biological tool (including DNA sequencing) rather than outdated phenotypic characterization. Thus PCR base studies along with some conventional tests are becoming indispensable for proper identification of NTM organisms [12].

The sensitivity and specificity of PCR depends on selection of the target sequence. Eisenaroth et al. used primer designed from IS6110 sequence to diagnose pulmonary tuberculosis [25]. Norle et al. demonstrated that IS 6110 based PCR assay is 91% sensitive & 100% specific in detecting Mycobacterium tuberculosis in sputum [26]. But Kent et al. detected 24 false positive cases out of 31 non tuberculosis cases. It was thought that mycobacterial species other than Mycobacterium tuberculosis might be responsible for this [27]. In this study we have included hsp 65 genes & dnaJ gone along with IS6110 and performed a multiplex PCR, so that it does not only diagnose Mycobacterium tuberculosis with greater accuracy but also help to identify the species of mycobacterium, as already mentioned. But one point should be kept in mind. Due to widespread and ubiquitous presence of MOTT organisms in nature, they can colonize in different human organs without causing infection. Hence, mere isolation of NTM organisms from clinical specimens is not tantamount to infection by itself. Therefore the American Thoracic Society recommends diagnostic criteria for NTM diseases to help physicians interpret laboratory results [12, 28].

4. LIMITATIONS OF THE STUDY

DNA sequencing, DNA micro array and line probe assay to identify the causative organisms are definitely more conclusive. If these procedures could be included, the study would get enriched. But belonging to third world country and having a huge burden of tuberculosis patients, these sophisticated tests could not be done as a routine practice.

5. CONCLUSION

To differentiate between M.tuberculosis complex and nontuberculous mycobacteria, multiplex PCR is a very good tool. If it is used in conjunction with culture and biochemical tests; it is likely to give best results. Moreover, our in house developed multiplex PCR will be much more cost effective than other molecular biological assay.

6. ACKNOWLEDGEMENT

Our team heartily acknowledges Department of Science and Technology (DST), Government of India for their financial assistance through the Project (SR/SO-HS-68/2006) to
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carry out the research work. Moreover we are grateful to RNTCP units of Kolkata Municipal Corporation for their regular support for detecting the tuberculosis patients.

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[14] Revised National Tuberculosis Control Programme Gaudatone, TBC India. (Directorate General of Health Services Ministry of Health Family Welfare.)


Adenosine deaminase estimation and multiplex polymerase chain reaction in diagnosis of extra-pulmonary tuberculosis

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Midnapore, ‡Department of Microbiology, R G Kar Medical College, Kolkata, India  

SETTING: Extra-pulmonary tuberculosis (EPTB), including mycobacteriosis, contributes 15–20% of all tuberculosis (TB) cases. The diagnosis of EPTB remains elusive because of the inadequate sensitivity of routine and conventional bacteriological methods for the detection of Mycobacterium tuberculosis and related organisms in clinical specimens such as cerebrospinal fluid (CSF), pleural fluid and peritoneal fluid.

OBJECTIVE: To develop a better diagnostic marker for EPTB.

DESIGN: In our study, 179 cases of EPTB were analysed for acid-fast bacilli (AFB) smear, adenosine deaminase activity (ADA) and multiplex polymerase chain reaction (PCR). Although estimation of ADA is helpful, its sensitivity and specificity varies widely. On the other hand, a multiplex PCR using amplicons such as IS6110, dnap and hsp65 genes has high sensitivity (60–88%) and specificity (81–100%).

RESULTS: On comparing AFB and ADA results with PCR, the PCR is clearly more effective than AFB (P < 0.001) and ADA estimation (P < 0.02) in CSF. The same result was observed with peritoneal fluid (P < 0.001 vs. P < 0.05) and pleural fluid (P < 0.001 and P < 0.05).

CONCLUSION: The study shows that multiplex PCR remains the best tool and is a much better marker for diagnosing EPTB.

KEY WORDS: tuberculosis; EPTB; AFB; ADA; multiplex PCR

IN THE LAST two decades, tuberculosis (TB) has re-emerged as one of the leading causes of death by a single infectious pathogen worldwide. The estimated 8.8 million new cases every year corresponds to 52,000 deaths per week, or more than 7,000 deaths per day, and accounts for 1000 new cases per hour each day.2,3

Thirty per cent of the world's TB-infected population lives in India.3 Of these TB cases, a sizeable percentage consists of extra-pulmonary TB (EPTB), such as tuberculous meningitis, pleural effusion, ascites, lymphadenopathy and synovial effusion.4

In spite of its high incidence and prevalence, the diagnosis of EPTB in its various clinical presentations, remains a true challenge. Clinicians underestimate these diseases, and the use of insensitive conventional analytical methods has contributed to the difficulties in managing patients with EPTB.5,6 The difficulty with the clinical specimens of EPTB is that they yield very few bacilli, and acid-fast bacilli (AFB) smear and culture therefore have low sensitivity. AFB staining is positive in less than 10% of patients with pleural effusion in most reports, whereas pleural fluid culture is positive in 12–70% cases with tuberculous pleural effusion.8 As it takes 4–6 weeks to obtain results on culture, treatment decisions have to be made before the laboratory diagnosis is available. Culture techniques also require viable organisms, and this is often a problem in partially treated patients.9

For tuberculous meningitis, identification of mycobacteria by the auramine or Ziehl-Neelsen (ZN) technique is seldom possible, except in the case of severe disease. Given the life-threatening nature of the condition, culture takes too long and requires skill. In a review by Molavi and Lefrock, cerebrospinal fluid (CSF) smears were positive in 10–40% of cases, while culture isolation was positive in 45–90% cases.10 In another study by Kennedy and Fallon, the authors found 37% smear positivity and 52% culture positivity.11 Other parameters, such as cell count and cell type, protein concentration and lactate dehydrogenase activity, are mostly non-specific and lack both sensitivity and specificity.1 At best, they can serve as supporting or indirect evidence.

Much attention has recently been given to the estimation of adenosine deaminase (ADA) enzyme activity.
in fluid samples of patients suffering from EPTB. ADA is an enzyme that catalyses the deamination of adenosine, forming inosine in the process. The physiological function of ADA is related to lymphocyte proliferation and differentiation; it is elevated in a number of diseases that evoke cell-mediated immune response and lymphocyte proliferation, such as the various chronic intracellular bacterial or viral infections, infectious mononucleosis, lymphomas and lymphatic leukaemia. Thus, although ADA increases in tuberculous meningitis or pleural effusion, it lacks sensitivity and specificity, as high values are obtained in other non-tuberculous conditions, even pyogenic meningitis. On the other hand, non-elevated values may be found in patients with TB but with poor lymphocytic response, such as concurrent human immunodeficiency virus/acquired immune-deficiency syndrome (HIV/AIDS).

Nucleic acid identification by polymerase chain reaction (PCR) is a rapid, sensitive and specific tool for the detection of Mycobacterium tuberculosis. It permits direct identification of the M. tuberculosis complex and is useful even in treated cases. It is applicable in the case of tuberculous meningitis and pleural effusion. In the case of tuberculous pleural effusions, PCR targets such as IS6110 and hsp65 kDa yield a sensitivity of 42–100% and a specificity of 85–100%. Results are available in a day or two. The few studies that have evaluated PCR for the diagnosis of EPTB have included a high percentage of HIV-positive patients. The sensitivity of PCR in these studies ranges from 42% to 93%. Using improved PCR techniques, the contribution to diagnosis of EPTB has been evaluated thoroughly.

MATERIALS AND METHODS

This was a cross-sectional study, with bioethical permission obtained from the respective departments. Here patients were randomly chosen from tertiary care centres in and around Kolkata, India, and divided into two groups. The first group (Group I) comprised patients with pleural effusion, meningitis or ascites who were diagnosed as cases of tuberculous origin on the basis of clinical examination, Mantoux test and diagnostic tests (chest X-ray, erythrocyte sedimentation rate [ESR], cytological examination of aspirated fluid) and on response to anti-tuberculosis drugs.

The second group (Group II) included in-patients with pleural effusion, meningitis and ascites, but in whom TB had been ruled out on the basis of clinical examination, diagnostic tests and treatment response. These patients served as the control group.

Patients of all ages and both sexes were included in the study population. In both groups pleural, peritoneal and cerebrospinal fluid was collected aseptically. The samples from all patients in the disease and control groups were subjected to the following tests: 1) demonstration of AFB by concentration and decontamination of the fluid, where applicable, using NaOH-NALC, sodium hydroxide and N-acetyl-L-cysteine mixture, followed by preparation of smear and ZN staining; 2) estimation of ADA activity, determined by a method based on the Berthlot reaction. ADA hydrolyses adenosine to ammonia and inosine; the ammonia thus formed further reacts with a phenol and hypochlorite in an alkaline medium to form a blue complex with sodium nitroprusside acting as a catalyst. The intensity of the blue-coloured iodophenol compound formed is directly proportional to the amount of ADA present in the sample. ADA was estimated by spectrophotometer at 600 nm and the assay temperature was 37°C.

One unit of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia/min from adenosine under standard assay conditions. Results were expressed as units per litre per minute (U/l/min).

The cut-off value of ADA was standardised at 10 U/l/min for CSF and 30 U/l/min for pleural and peritoneal fluid.

Isolation of DNA and multiplex PCR

After decontamination and concentration, the sample was moistened with an adjustable amount of Tris-EDTA (TE) buffer (pH 7.6). After scraping the cytological material, it was dissolved in TE buffer (pH 7.6) with thorough mixing. Mycobacterial DNA was extracted from clinical specimens using a modification of the lysis method described by Sritahan and Barker. The sediments were resuspended in 100 μl of TE-Triton X-100 buffer (10 mM Tris, 1 mM EDTA and 1% Triton X-100, pH 8) with 200 μg/ml proteinase K, incubated at 56°C for 3 h, and then at 95°C for 30 min. After each incubation, the specimens were placed on ice for 5 min. The protease-treated and heated specimens were next extracted with phenol; chloroform:isoamyl alcohol (25:24:1). The aqueous phases of the organic extractions were precipitated with ethanol overnight. The DNA was dissolved in 200 μl of TE buffer.

Reference strains of M. tuberculosis (H37Rv), M. bovis, M. smegmatis, M. intracellulare and M. avium were obtained from the Tuberculosis Research Centre (TRC), Chennai, India. Genomic DNA was extracted from reference strains cultured on Löwenstein-Jensen medium by the standard method. Multiplex PCR, as developed earlier in our laboratory, was performed to amplify the following target sequences:

1. a pair of oligonucleotide primers for gene coding 65 kDa hsp protein:
   5'-CTA GGT CCG GAC GGT GAG GCC AGG-3'
   5'-CAT TGC GAA GTG ATT CCT CCG GAT-3'
2. a pair of genus-specific oligonucleotide primers is designed based on the nucleotide sequence of the dnaJ gene:
5'-AAG AGG AAG GAG AGA GGC-3'  
5'-GTC GTG GAG GTT GAA CTC-3'

A pair of oligonucleotide primers is designed from IS6110:
5'-GTG GGC ATG GTC GCA GAG AT-3'  
5'-CTC GAT GCC CTC ACG GTT CA-3'

PCR amplification was performed in a Biometra Tpersonal DNA thermal cycler (Biometra, Gottingen, Germany) with an initial cycle of denaturation (5 min at 85°C), followed by 40 cycles each of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, with a final extension of 10 min at 72°C and a post-incubation period of 30 min at 4°C. Each DNA amplification experiment included a positive control (reference strains, e.g., M. tuberculosis H37Rv, DNA) and a negative control (distilled water). The amplified DNA products were visualised under ultra-violet light and GEL DOC (BIORAD, Hercules, CA, USA) was used for documenting picture after 2% agarose gel electrophoresis by ethidium bromide staining (Figure).

RESULTS

In this study, a total of 179 body fluid samples, CSF (n = 80), pleural fluid (n = 49) and peritoneal fluid (n = 50), were investigated for AFB staining, ADA activity and PCR. Table 1 shows the distribution of the study population. A total of 97 body fluid samples (CSF n = 44, pleural fluid n = 19, peritoneal fluid n = 34) were collected from EPTB suspects and 82 samples (CSF n = 36, pleural fluid n = 31, peritoneal fluid n = 15) from patients with diseases other than EPTB.

Table 1  Distribution of study population

<table>
<thead>
<tr>
<th>Body fluids</th>
<th>Patients n</th>
<th>Controls n</th>
<th>Total n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>44</td>
<td>36</td>
<td>80</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>19</td>
<td>31</td>
<td>50</td>
</tr>
<tr>
<td>Peritoneal fluid</td>
<td>34</td>
<td>15</td>
<td>49</td>
</tr>
<tr>
<td>Total</td>
<td>97</td>
<td>82</td>
<td>179</td>
</tr>
</tbody>
</table>

CSF = cerebrospinal fluid.

Table 2 shows the distribution of patients in the study population according to the tests performed on the samples. Based on this distribution, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated separately for AFB staining, ADA activity and PCR with CSF, pleural fluid and peritoneal fluid (Table 3).

Diagnosis of EPTB by AFB staining had 100% specificity and 100% PPV in all body fluid samples, but the sensitivity of the test was low (13.6%, 5.2% and 14.7% for CSF, pleural fluid and peritoneal fluid, respectively). A study by Nagesh et al. also reported 100% specificity and PPV for AFB staining, but they reported a sensitivity of 20% and an NPV of 71.2%. Their study was conducted exclusively on pleural fluid.

The low sensitivity of AFB staining is common, as it is very difficult to demonstrate bacteria even after

Table 2  Distribution of patients in study population according to the different tests performed on body fluid samples

<table>
<thead>
<tr>
<th>Body fluids</th>
<th>Tests</th>
<th>Patients n</th>
<th>Controls n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF (n = 80)</td>
<td>AFB-positive</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AFB-negative</td>
<td>38</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>ADA &gt; 10</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>ADA &lt; 10</td>
<td>23</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>PCR-positive</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>PCR-negative</td>
<td>12</td>
<td>33</td>
</tr>
<tr>
<td>Peritoneal fluid (n = 50)</td>
<td>AFB-positive</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AFB-negative</td>
<td>18</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>ADA &gt; 30</td>
<td>9</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>ADA &lt; 30</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>PCR-positive</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>PCR-negative</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>Pleural fluid (n = 49)</td>
<td>AFB-positive</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AFB-negative</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>ADA &gt; 30</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>ADA &lt; 30</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>PCR-positive</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>PCR-negative</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

CSF = cerebrospinal fluid; AFB = acid-fast bacilli; ADA = adenosine deaminase; PCR = polymerase chain reaction.
Table 3

<table>
<thead>
<tr>
<th>Body fluids</th>
<th>Parameters</th>
<th>AFB</th>
<th>ADA</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>Sensitivity</td>
<td>13.6</td>
<td>47.7</td>
<td>72.7</td>
</tr>
<tr>
<td></td>
<td>Specificity</td>
<td>100</td>
<td>69.4</td>
<td>91.7</td>
</tr>
<tr>
<td></td>
<td>NPV</td>
<td>48.6</td>
<td>52.0</td>
<td>73.3</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>Sensitivity</td>
<td>14.7</td>
<td>58.8</td>
<td>82.4</td>
</tr>
<tr>
<td></td>
<td>Specificity</td>
<td>100</td>
<td>60.0</td>
<td>80.0</td>
</tr>
<tr>
<td></td>
<td>NPV</td>
<td>34.0</td>
<td>39.1</td>
<td>66.6</td>
</tr>
<tr>
<td>Peritoneal fluid</td>
<td>Sensitivity</td>
<td>5.2</td>
<td>47.4</td>
<td>78.9</td>
</tr>
<tr>
<td></td>
<td>Specificity</td>
<td>100</td>
<td>25.8</td>
<td>87.1</td>
</tr>
<tr>
<td></td>
<td>NPV</td>
<td>63.3</td>
<td>44.4</td>
<td>87.1</td>
</tr>
</tbody>
</table>

AFB = acid-fast bacilli; ADA = adenosine deaminase; PCR = polymerase chain reaction; CSF = cerebrospinal fluid; PPV = positive predictive value; NPV = negative predictive value

Discussion

Confirming an EPTB diagnosis remains a challenge, as traditional methods such as AFB staining and M. tuberculosis culture have limitations. There is therefore a need for new tests to replace or supplement these traditional tests. ADA activity measurement and PCR assay are two new methods that have been introduced to diagnose EPTB. In this study, we evaluated the sensitivity, specificity, PPV and NPV of the two tests, and the probability of diagnosing EPTB using these methods compared with traditional AFB staining methods.

Although M. tuberculosis culture is considered the gold standard for diagnosing EPTB, we did not include it in our study, as culture results take 6–8 weeks and initiation of treatment is necessary before results are available. The management of patients with EPTB cannot therefore depend on this investigation.

ADA is superior to AFB staining in diagnosing positive EPTB. However, as demonstrated by its PPV and NPV, it cannot be used alone to confirm the diagnosis of EPTB. Another problem with this method is that studies have used different cut-off values to calculate its sensitivity and specificity. Nagesh et al. described a cut-off value of 50 U/l/min for pleural fluid, whereas in CSF, Kashyap et al. used a cut-off value of 11.39 U/l/min, while Gambhir et al. used 8 IU/l/min.

A high positive value for ADA activity cannot therefore confirm a diagnosis of EPTB, but it may be a useful supporting test.

The second new approach used to diagnose EPTB is the PCR assay, which is rapid, highly sensitive and specific. The greater sensitivity of PCR can be explained by the fact that even a low number of bacteria can be amplified and diagnosed using this procedure. However, PCR may also amplify the DNA of dead bacteria. A PCR assay may therefore remain positive until dead bacteria are cleared off. Many study groups have reported the high sensitivity of PCR assay, although it varies between studies. This may due to differences in the assay protocol, including how DNA is extracted.

The specificity of PCR depends on the selection of the target sequence. Eisenach et al. used an IS6110 primer to diagnose pulmonary TB. Norie et al. demonstrated that an IS6110-based PCR assay is 91% sensitive and 100% specific in detecting M. tuberculosis in sputum. Kent et al., however, detected 24 false-positive cases out of 31 non-TB cases. It was thought that mycobacterial species other than M. tuberculosis may be responsible for this. In the present study, we included the hsp65 and dnaJ genes along with IS6110 and performed a multiplex PCR, so that it not only diagnoses M. tuberculosis with greater accuracy but it also helps identify the mycobacterial species.

The uniqueness of this study is that different tests such as AFB staining, ADA activity and PCR assay...
have been performed separately and evaluated in different body fluids such as CSF, pleural fluid and peritoneal fluid. The comparison of new methods with traditional AFB staining and between each other is another unique calculation performed in this study. The use of multiplex PCR, which simultaneously diagnoses the mycobacterial species, can be considered an important parameter.

CONCLUSION

To overcome the limitations of traditional methods to diagnose EPTB, ADA activity can be measured as a supplementary parameter. The multiplex PCR assay is highly sensitive and specific and can be used for early detection of EPTB cases. Multiplex PCR coupled with ADA estimation is likely to yield optimal results.

References

CONTEXTE : La tuberculose extra-pulmonaire (TBEP), y compris les mycobactérioses, représente 15% à 20% de l'ensemble des cas de tuberculose (TB). Le diagnostic de l'EPTB reste insaisissable en raison de la sensibilité inadéquate des méthodes de routine et des méthodes bactériologiques conventionnelles pour la détection de Mycobacterium tuberculosis et des organismes voisins dans les échantillons cliniques tels le liquide céphalo-rachidien (LCR), le liquide pleural, le liquide péritonéal, etc.

OBJECTIF : Développer un meilleur marqueur pour TBEP.

SCHEMA : Dans la présente étude, on a analysé 179 cas de TBEP pour les frottis de bacilles acidorésistants (BAAR), les valeurs d'activité d'adénosine déaminase (ADA) et la réaction de polymerase en chaîne (PCR) multiple. Bien que l'estimation des valeurs d'ADA soit utile dans certains cas, il y a de larges variations de sensibilité et de spécificité. Au contraire, les réactions PCR multiples utilisant les amplicons comme IS6110, le gène dnaJ et les gènes hsp65 ont une sensibilité (60-88%) et une spécificité (81-100%) élevées.

RÉSULTATS : Dans le cas du LCR, lorsqu'on compare les résultats des frottis pour BAAR et des valeurs d'ADA avec ceux de la PCR, il est évident que la PCR a une supériorité définitive sur les BAAR (P < 0,001) et sur l'estimation de l'ADA (P < 0,02). On retrouve le même tableau dans le cas du liquide péritonéal (P < 0,001 et P < 0,05) ainsi que pour le liquide pleural (P < 0,001 et P < 0,05).

CONCLUSION : Dès lors, cette étude montre que la PCR multiple pour l'identification de l'ADN mycobactérienne reste le meilleur outil de diagnostic de l'EPTB et est un marqueur bien meilleur.

RESUMEN

MARCO DE REFERENCIA : La tuberculosis extra-pulmonar (TBEP), incluida la enfermedad por micobacterias atípicas, representa del 15% al 20% de los casos de tuberculosis (TB). El diagnóstico de este tipo de enfermedad continúa siendo difícil debido a una inadecuada sensibilidad de los métodos bacteriológicos convencionales utilizados sistemáticamente en la detección de Mycobacterium tuberculosis y de microorganismos similares, en muestras clínicas como el líquido cefalorraquideo (LCR), pleural y peritoneal.

OBJETIVO : Detectar un marcador superior para la TBEP.

MÉTODO : En el presente estudio, se analizaron 179 casos de TBEP mediante baciloscopía, determinación de la actividad de la adenosina desaminasa (ADA) y la reacción en cadena de la polimerasa (PCR) de amplificación múltiple. Si bien la determinación de ADA es útil en algunos casos, su sensibilidad y especificidad varían considerablemente. En contraste, la PCR múltiple con amplicones como la secuencia de inserción IS6110 o los genes de las proteínas dnaJ o hsp65 presenta alta sensibilidad (de 60% a 80%) y especificidad (de 81% a 100%).

RESULTADOS : Cuando se comparan los resultados de la baciloscopia y la determinación de ADA con los de la PCR, es clara la superioridad de la PCR con respecto a la baciloscopia (P < 0,001) y también a la medición de ADA (P < 0,02), cuando se trata de muestras de LCR. El resultado es equivalente en muestras de ascitis (P < 0,001 vs. P < 0,05) y en muestras de líquido pleural (P < 0,001 y P < 0,05).

CONCLUSIÓN : En consecuencia, los resultados del estudio ponen en evidencia que la PCR de amplificación múltiple para detección del ADN micobacteriano sigue siendo la mejor herramienta diagnóstica de la TBEP y es también un marcador muy superior.
Title of the paper: Diagnosing leprosy: revisiting the role of slit skin smear with critical analysis of applicability of polymerase chain reaction (PCR) as diagnostic tool

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Abstract

Background

Diagnosing leprosy is a challenge, especially in early cases, and need for a sensitive diagnostic tool is a much-felt need. PCR holds promise as simple and sensitive diagnostic tool but its usefulness in Indian perspective needs further evaluation. Slit skin smear (SSS) remains the conventional method of leprosy detection, hence the study was undertaken to evaluate and compare diagnostic efficacy of PCR versus SSS.

Methods

Punch biopsy of skin and SSS was obtained from active margins of lesions. Cases were clinically grouped into multibacillary and paucibacillary, and classified into TT, BT, BL, LL, histoid and indeterminate after clinico-pathological correlation. DNA was extracted from biopsy-specimens and multiplex-PCR was carried out incorporating primers meant for amplification of specific 372bp of repetitive sequence of M.leprae DNA.

Results

Among 164 patients, PCR was positive in 82.3%. The sensitivity of PCR was significantly more (p < 0.0001) than SSS in both multibacillary (85.9% vs. 59.8%) and paucibacillary (75.43% vs. 1.75%) subgroups; though difference in sensitivity is remarkable in paucibacillary subgroup. PCR and SSS positivity was found in 100% of LL and histoid leprosy; whereas PCR had significantly more (p<0.0001) positivity in BT and of definite increased value in indeterminate and TT leprosy.

Conclusion

PCR had higher sensitivity compared to SSS, especially in diagnostic challenging situations and paucibacillary cases. Thus use of this costly but sensitive tool should be restricted to this subgroup, since in LL and histoid leprosy SSS suffice in making the diagnosis.

Key words: Leprosy, Diagnosis
Introduction

Leprosy, an age-old chronic granulomatous infectious disease caused by *Mycobacterium leprae*, known to be ostracized by the society at large from the very early days of civilization, no longer remains an untreatable and incurable condition after the introduction of multi drug therapy (MDT) by World Health Organization (WHO) since 1982. Though, the ambitious elimination campaign that was launched in 1990s by WHO was not able to render out the World leprosy-free, in spite of the highly effective drug regime. The global prevalence of leprosy at the beginning of 2008 was estimated to be 2,12,802 (the major bulk of 1,20,967 being contributed by South-East Asia) and the new case detected in 2007 was 2,54,525 (India accounted for the majority, 1,37,685 new cases). The figures speak for themselves that the disease is yet to be tamed, and considering the long incubation period of leprosy, this too, may represent only the tip of ice-berg. The disease still persists in certain geographical pockets, where the prevalence is still higher than 1 per 10,000 population (e.g., 1.5 in Brazil, 1.8 in Nepal). The need of the hour is to detect cases early and to bring all cases under the cover of MDT. It is understandable that early case detection would require a sensitive diagnostic tool with point of care test.

The conventional diagnostic methods relies on clinical methods (needless to say, it demands a high degree of clinical suspicion and awareness among clinicians), microscopic evaluation of tissue smears for AFB and histopathology. However, its diagnosis still poses a challenge. For reliable detection, acid fast staining requires $10^4$ organisms per gram of tissue and its sensitivity is low at the tuberculoid end of the disease spectrum and early cases, where the bacilli are rare or absent Histopathological findings may too be non-specific at times. Serological tests for leprosy have shown certain potential for detection of host immune response against the bacilli. Even when satisfactory immunodetection is achieved, it may just be a reflection of a past infection, giving no information on the current bacteriological status. Thus detection of the bacilli is difficult and in India, where a huge burden of 87,228 cases were registered in 2008, diagnostic aid that can supplement the clinico-histological finding even in early cases, is a much-felt need. Polymerase chain reaction (PCR) holds promise as a simple and sensitive diagnostic tool, though it has some technical limitations.

A recently developed PCR-technique for the detection of *M. leprae* DNA in environmental and clinical specimens has allowed investigators to begin to study natural distribution as well the technique by which very small amount of *M. leprae* DNA can be detected directly in clinical specimens such as nasal swab, skin, etc.

The usefulness of PCR in the Indian perspective needs further evaluation. In this study, we applied PCR validated by previous study, for the diagnosis of leprosy patients and compared the results with the clinico-histological diagnosis and the findings of slit-skin smear. The study was undertaken with the objectives of evaluating the diagnostic efficacy of PCR in the diagnosis of leprosy, to determine the utility of PCR in the clinical setting and to compare the findings of PCR with slit skin smear, a conventional parameter for diagnosis of leprosy.

Materials and Methods

Cross sectional study was carried out in the Hansen's clinic of the dermatology OPD of Institute of Post Graduate Medical Education and Research, Kolkata and Medical College, Kolkata over a period of nine years. All procedures and protocol followed in the study was approved by the institutional ethical committee (IEC) of both the institutes and duly signed
informed consent was obtained before including the patient for the study. Leprosy was diagnosed clinically (WHO guideline)\(^7\) and further confirmed/corroborated by histopathology before including the study subjects for final analysis. The study population was classified into multibacillary (MB) and paucibacillary (PB) as per the clinical criteria of WHO.\(^7\)

**Skin biopsy, Histology and Slit skin smear**

Punch biopsy was taken from active margin of lesions or from the anaesthetic area in pure neural leprosy (where skin lesions were not present). For the purpose of biopsy, plaques/nodules were given preference over patches, in cases of concomitant presence of plaque/nodule and patch. The tissue samples were stained with Hematoxylin-eosin (H&E) stain for histology and Fite-faraco stain for demonstration of bacilli. The cases were classified after clinico-pathological correlation, into tuberculoid (TT), borderline-tuberculoid (BT), mid-borderline (BB), borderline-lepromatous (BL), lepromatous (LL), indeterminate, pure neuritic and histoid leprosy.\(^8\) For the diagnosis of indeterminate leprosy, any cases where the possibility of any diagnostic mimickers (e.g., pityriasis alba, patch stage of mycosis fungoides) was suspected, were excluded from the study. The slit skin smear (SSS) was taken from the lesion following the standard protocol and stained using modified Ziehl-Neelsen stain for the demonstration of acid-fast bacilli (AFB) (Figure 1).\(^9\) The biopsy samples were stored at -20°C in Tris buffer prior to DNA extraction.

**Extraction of Mycobacterium leprae DNA from clinical specimens:**

Genomic DNA was extracted from skin of study subjects with a modification of technique developed by Yoon KH \(et\ al.\)^\(10\) without using Zirconium beads.

The skin biopsy samples (both study samples and controls) were incised with no. 10 or 15 disposable scalpel blade in a petri dish into small pieces. They were then homogenized by hand homogenizer with 1 ml sterile distilled water. The homogenized material were then taken into separate 1.8 ml microcentrifuge tube and incubated with lysis buffer containing 300\(\mu\)l of 100mm Tris-HCl, pH 8.5 containing 0.05% Tween-20 and 60\(\mu\)g of Proteinase-K per ml for 18 hours at 60°C. Paraffin oil (40\(\mu\)l) was layered on the top of the sample to prevent evaporation. Thereafter, the samples were incubated at 97°C for 15 minutes to inactivate Proteinase K. After completion of incubation, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) were added to each of the tube containing the homogenized samples. Each of the tube was shaken gently for 1 minute. After centrifugation at 10,000 x g for 15 minutes, the supernatant was transferred to another microcentrifuge tube and again mixed with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifugation step was repeated. The supernatant was collected in a separate microcentrifuge tube and again equal volume of chloroform was added to each of the tube and centrifugation step was repeated. The supernatant fraction was collected in a microcentrifuge tube to which isopropanol (0.7% vol./vol.) was added. Then the samples were kept at -20°C overnight (12 hrs) and centrifugation was done at 12,000 x g for 30 minutes the next day. The pellet of DNA was collected, draining gently the upper supernatant portion from the tube without disturbing the pellet. 150\(\mu\)l of ethanol was added to each of the tube and mixed thoroughly. The DNA was then precipitated, lyophilized and re-suspended in 100\(\mu\)l of distilled water which was subsequently used as a template DNA for PCR.
**Polymerase Chain Reaction**

A multiplex PCR has been developed in our laboratory incorporating using two sets of primers. One component of the primer set of the multiplex PCR was designed according to Yoon KH, et al.\(^\text{[10]}\) The said pair of oligomers were meant for amplification of 372bp of the repetitive sequence of the *M. leprae* DNA. The basepair is reported to be very specific to *M. leprae* and was not found to be present in 20 other mycobacterial species. The specificity and sensitivity of the primers: Ri & R\(_2\) has been already established in earlier studies.\(^\text{[4]}\)

Enzyme amplification (PCR) was performed with following sequences of oligomer primers: 5' – CGG CCG GAT CCT CGA TGC AC- 3' (primer Ri) and 5' – GCA CGT AAG CTT GTC GGT GC- 3' (primer R\(_2\)). The reaction mixture consists of 50μl of 10 mM Tris HCl (pH- 8.5), 50 mM KCl, 1.5 mM MgCl\(_2\), 0.01% (wt/vol.) gelatine, 200μM of dATP, dGTP, dCTP and dTTP; 1U of taq DNA polymerase (Perkin – Elmer Cetus, Norwalk, Conn) 0.5μM of each primer and DNA prepared from the biopsy samples. Total 35 cycles of PCR was performed with thermocycler (PTC – 0150 Minicycler, Version: 4.0, M J research, INC, manufacturer Lincoln street, Wallthumb, Massachusetts-02451, USA) consisting of denaturation at 94°C for 1 minute followed by annealing at 60°C for 2 minutes with primer extension at 72°C for 3 minutes followed by final extension 72°C for 10 minutes (Figure 2).

**Statistical analysis**

Data were statistically described in terms of range, mean ± standard deviation (±SD), frequencies (number of cases), and relative frequencies (percentages) when appropriate. Z-test of proportion, chi-square test, kappa test and ANOVA tests were used as applicable. Medcalc statistical software ® version 9.6.4.0 (http://www.medcalc.be) was used for statistical analysis. P value <0.05 was considered statistically significant in this study.

**Results**

A total of 207 patients were screened, of which three patients did not consent for biopsy (all were females having only facial lesions due to cosmetic reasons) and in 39 cases the histopathology was not corroborative with leprosy. The study analyzed 164 patients (male:female ratio 5.07:1), with mean age at presentation 37.57±13.40 years (range 13 to 84, median 36 years), among whom 135 were not on any anti-leprotic medication (treatment naïve) and the rest 29 patients were undergoing treatment with MDT at the time of examination.

Clinico-pathologically, majority of the patients belonged to BT (n=85, 51.8%) subgroup of the disease spectrum, followed by TT (n=33, 20.1%), BL (n=22, 13.4%), LL (n=11, 6.7%), indeterminate (n=6, 3.7%), pure neuritic (n=5, 3%) and histoid leprosy (n=2, 1.2%). At presentation, Type I reaction was present in 25 (15.2%) and Type II in 3 (1.3%) patients of the total study population. Among 164 patients 107 (65.2%) patients were diagnosed to be multibacillary (MB) and the rest 57 (34.8%) patients were paucibacillary (PB).

PCR was positive in 82.3% (135 of 164 cases) and in treatment-naïve cases the positivity was 85.9% (116 of 135 cases). Among 107 MB cases, 92 (85.98%) patients yielded PCR positive, while 43 (75.44%) were positive among PB patients (Chi-square test, p=0.142). On the other hand, AFB could be demonstrated by SSS in 65 (39.6%) cases, of which positivity was significantly more (Chi-square test, p<0.0001) in MB (59.81%) than the PB cases (1.75%). The sensitivity of PCR was significantly more (Z-test of proportion, p < 0.0001) in both the
MB and PB subgroups; though the difference in sensitivity is remarkable in PB sub-group, where PCR yielded positivity in 75.43% compared to 1.75% cases in whom AFB was found in SSS (Table 1). PCR positivity was found in 72 (72.72%) among 99 patients who showed no AFB in SSS and in 63 (96.92%) of 65 cases where SSS showed AFB. In 2 patients PCR yielded negative results in spite of being positive by SSS. This difference is found to be significant (Chi-square test, \( p=0.0002 \)) with poor inter-rater agreement between the two tests \((\text{kappa}= 0.024)\).

Considering the disease spectrum PCR was maximally positive in the LL and histoid leprosy (Figure 3a, b, c) (100% positivity) followed by BL, TT, BT and indeterminate cases (Figure 4a, b). PCR was found to be negative in all the cases of pure neuritic variety. AFB positivity in SSS was found to be 100% in LL and histoid leprosy and was of absolute negative yield in TT, pure neuritic and indeterminate cases (Table 2)

PCR results did not significantly vary with the presence of lepra reaction (chi-square test, \( p=0.5 \)) or duration of illness (ANOVA test, \( p=0.364 \)) or number of lesion (ANOVA test, \( p=0.05 \)). Similarly AFB in SSS showed no significant association with presence of lepra reaction (chi-square test, \( p=0.06 \)) or duration of disease (ANOVA test, \( p=0.973 \)); though unlike PCR, its positivity significantly increased (ANOVA test, \( p<0.001 \)) with increase in the number of lesions.

Discussion

Leprosy is diagnosed clinically based on the presence of two or more cardinal clinical features\(^7\) and the clinical applicability of these signs are extensively reviewed. In spite of the high sensitivity of clinical methods, delay in diagnosis in more than 80% of new cases of leprosy was reported at the Hospital of Tropical disease in London, UK during 1995-99.\(^{111}\) This fact highlights the need for more sensitive tool, which is capable of diagnosing the early cases of leprosy, since early diagnosis is the only way to prevent the deformities and decrease subsequent morbidities.

Use of PCR in the diagnosis of leprosy has ushered in the hope that the long awaited search for a sensitive tool is going to end. Despite the enthusiasm, one has to test the reliability, utility and applicability of PCR rigorously, before it can be made available in clinical settings. Just like every good thing comes with a price, one has to remember that PCR too is expensive. In the resource poor countries of Africa and Asia, where unfortunately the load of leprosy is highest, this tool has to be used judiciously with the precise idea of where to use and where not to use. So the authors intended to explore the clinical situations where use of PCR would provide an edge over the other diagnostic methods for leprosy.

The present large scale study has shown a positive yield in 82.3% of all cases of clinico-pathologically diagnosed cases of leprosy by using amplification of 372bp of the repetitive sequence of the \(M.\ leprae\) DNA which is lower than the 93.1% yield reported with same set of primer.\(^{10}\) The difference may arise due to the difference in the profile of patients selected for analysis. The present study recruited even the pure neural variety (\(n=5\)), indeterminate cases (\(n=6\)) and even those who were on therapy with MDT (\(n=29\)), which are associated
with more negative yield. Other studies, which used 530 bp\textsuperscript{[12]} or M. leprae-specific 16S ribosomal RNA\textsuperscript{[13]} found PCR positivity of 92% and 100% in AFB positive cases, and 61% and 50% in AFB negative ones, respectively. The present study using 372 bp, found a better positivity than 530bp and comparable results with 16S ribosomal RNA, for both AFB positive (96.92%) and negative (72.72%) cases. PCR targeting 36 kDa gene of \textit{M. leprae} showed positivity in a modest 66.6% cases, and the same study found In situ hybridization showing positivity in 42.8% of early (Indeterminate/BT) and 46.7% in BB/BL group.\textsuperscript{[14]} In our study though, early (Indeterminate/TT/BT) and BL group showed 82.25% and 90.9% positivity, respectively. This highlights the superiority of the present method over In situ hybridization or PCR using 36Kda gene. One study by Goulart \textit{et al.} compared different methods of PCR and concluded 130 bp primer (positivity in 73.6% ) to be more sensitive than 372bp (positivity in 52.7%).\textsuperscript{[15]} The same study found 130 bp to be positive in 40% of TT, 55.5 % of BT, and 100% of BB, BL and LL. In contrast to Goulart \textit{et al.} we found better sensitivity profile in our study population using the 372 bp primer and the positivity was better than 130bp in both the TT and BT pole.

Several explanations can be put forward to explain PCR negativity. There may be no bacilli present in the sample at all, which is likely to be the case in some BI-negative cases, or bacilli may not be viable anymore, because of either a strong host immune response or killing by treatment.\textsuperscript{[16]} On the other hand, it cannot be ruled out that other factors, such as the age of the specimens, may also have an effect on the outcome of PCR. An explanation for negative results among PB patient samples might be their intrinsically higher ratios of human genomic DNA to \textit{M. leprae} DNA, which probably inhibit \textit{M. leprae} DNA amplification.\textsuperscript{[17]} In addition, there might be some technical limitations on the detection of \textit{M. leprae} DNA due to PCR inhibitors or other PCR procedures adopted in this study(reasons not clear to us).

The study has found that none of the pure-neuritic cases has shown positive result with PCR. This highlights that PCR from the skin biopsy of the anesthetic/hypoaesthetic skin area in pure neuritic leprosy is a waste of resource. Martinez AN \textit{et al} showed 83.3% positivity in pure neuritic patients after Triazol extraction of the samples.\textsuperscript{[17]} The difference might be due to difference in the extraction method which provides better yield of DNA. On the other hand PCR has shown 33.3% positivity in indeterminate cases, which is an optimistic sign, considering the fact that indeterminate cases are the one that are most difficult to diagnose. Clinicians would always be hesitant to start MDT (which would warrant at least 6 months of therapy, once started) only on the ground of perineural and/or peri-adnexal infiltrate from a hypopigmented macule (the clinico-pathological situation in indeterminate cases). Presence of positive PCR finding would provide them the much-needed laboratory back-up in making the diagnosis. If PCR can help in dispelling the diagnostic dilemma in 1/3rd of such clinically challenging situations, it will be welcome by the medical fraternity at large.

One need also to remember that in countries like India, leprosy is associated with stigma; hence patients (and the family members) are reluctant, if not non-receptive to accept the fact that they are being affected by leprosy. Often they seek objective evidence before coming to terms with the fact. The problem arises in paucibacillary cases, where the SSS is often negative (positive only in 1.7% in the present study). This is the situation where PCR (positivity in 75.4%) can provide the diagnostic confirmation to pacify the ailing sentiments
Thus in early leprosy (which poses diagnostically dilemma and often has to differentiated from pityriasis alba, seborrhoeic dermatitis, etc.) PCR is a better tool than SSS. Regarding clinical mimicker of leprosy, Post-kala-azar-dermal leishmaniasis (PKDL) deserves special mention, which is often confused with the LL pole. In our study population there was one case who was previously misdiagnosed as PKDL because of the similar clinical feature and the patient had a history of kala-azar in the past. The patient showed both AFB in SSS and a positive PCR result, though in such cases cost-benefit ratio would definitely favour the use of SSS over PCR. Here, it is worth mentioning that PCR is often criticized for the fact that it requires skilled personnel which is not always affordable to the developing countries, which bears the major brunt of the disease. Though PCR initially appears to be an expensive one but in the long run, PCR analysis in bulk is economically tolerable and cost effective.

From our study, it was evident that PCR always had a higher sensitivity compared to presence of AFB in SSS, which has been the conventional diagnostic tool for diagnosis of leprosy. However, 14.1% (in MB cases) and 24.57% (in PB cases), and overall 17.7% false negative results are of major concern. It is thus evident that almost 1 in 5 leprosy cases will be missed if we bank upon solely on PCR technique as followed in the study. Hence, PCR should be used as a supplementary tool for leprosy diagnosis and is never a replacement for clinical acumen.

It may be highlighted that PCR as diagnostic tool may not be useful in multibacillary cases (where SSS has a high yield), especially in lepromatous pole and histoid variety of leprosy, where SSS was found to be positive in all cases. Hence it is imperative that the use of this costly but sensitive tool be restricted to diagnostic challenging situations and paucibacillary cases (but not in pure-neural leprosy), where it can be a useful supplement to the existing diagnostic methods.
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