Research Article

Comparison of conventional and molecular methods in diagnosis of extrapulmonary (cutaneous) tuberculosis in a tertiary care hospital in Delhi

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Abstract

Background: Tuberculosis (TB) is a potentially fatal contagious disease caused by *Mycobacterium tuberculosis*. The one third of the world's population is infected with tubercle bacilli. According to World Health Organisation (WHO), global incidence of TB in 2014 was 9 million cases out of which 2.2 million cases were of India only. India has the highest burden of TB. Extrapulmonary Tuberculosis (EPTB) constitutes about 15–20% of TB cases. Cutaneous TB constitutes about 1.5% of all EPTB cases. The incidence of cutaneous TB amongst total dermatology patients varies between 0.1% and 2% in different studies.

Objective: To compare the conventional and molecular methods in diagnosis of extrapulmonary (cutaneous) tuberculosis.

Materials and methods: A cross-sectional study was conducted on consecutive patients of cutaneous tuberculosis in the departments of Microbiology and Dermatology at UCMS & Guru Teg Bahadur Hospital Delhi, from all sample received during the time period between Nov 2010 and March 2012. Conventional method of diagnosis of tuberculosis was performed on biopsy samples i.e. staining and culture methods. The conventional methods of diagnosis are then compared with molecular methods PCR targeting 16S rRNA. The two set of primers were used. The outer (16 SOL,16 SOR) and inner pairs (16 SIL,16 SIR) of primers are expected to be the genus specific and species specific primers for 16S rRNA gene amplification, respectively.

Result: 31 samples received during the time period between November 2010 and March 2012. Female predominance was found in present study 54.8% (17). The clinical types of the received samples from cutaneous tuberculosis patients includes ,TBVC (6.4%), SFD (25.8%) and LV (67.7%). Fifty eight percent of patients were found to be in age group 11–20 years. Ziehl- Neelsen (ZN) staining was performed on smears of all biopsy specimens. The 6.4% (2) were ZN stain positive and 12% (4) were Auramine stain positive and 6 (19.3) were culture positive. Nested PCR was performed on 31 biopsy specimens. Eight (25.8%) specimens were found to be positive for common *Mycobacterium* species. Out of 8, DNA from 6 biopsy specimens were amplified by both genus specific and species specific primers based on 16S rRNA gene amplification. They were diagnosed as M. *tuberculosis* infection.

Conclusion: PCR tests offer alternative robust approach to detect M. *tuberculosis* in paucibacillary EPTB specimens that show rapid results with good diagnostic accuracy. Although, these tests cannot replace the conventional AFB smear, culture identification or histopathological observations but they contribute significantly for an early diagnosis of EPTB and exert an acceptable impact on the clinical management of disease.

KEYWORDS: Cutaneous tuberculosis, Ziehl-Neelsen (ZN) stain, Lowenstein Jensen (LJ), nested PCR.

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Introduction

Tuberculosis (TB) is a potentially fatal contagious disease caused by *Mycobacterium tuberculosis.* The one third of the world's population is infected with tubercle bacilli. The WHO declared TB as a global emergency in 1993.[1] The incidence of TB reported in India was 2.2 million in 2014.[2] TB is still progressing endemically in developing countries despite many prevention

programs but rare in developed countries. Ten percent of all cases of TB are extrapulmonary and cutaneous tuberculosis represents a small proportion 4.8% of all cases of extra pulmonary TB.[3] Extrapulmonary tuberculosis (EPTB) constitutes about 15–20% of TB cases and can constitute up to 50% of TB cases in HIV-infected individuals.[32] As India has high burden of TB cases, thus proportionately higher number of EPTB cases are also observed in this country.[37] Cutaneous TB constitutes about 1.5% of all EPTB case.[16] The incidence of cutaneous TB amongst total dermatology patients varies between 0.1% and 2% in different studies which is increasing proportionately with the overall increase in TB.[4,5] *Mycobacterium tuberculosis* was related to cutaneous lesions with great diversity of clinical and histopathological aspects. This diversity has a direct relation with the individual's immunological status, the mycobacterial virulence, the infection circumstances and the presence or not of mycobacteria in the lesion. The cutaneous tuberculosis can occur by direct penetration of M. *tuberculosis* in the dermis (primary infection) or by dissemination of the same, beginning with a pulmonary focus (secondary infections).[6] Cutaneous tuberculosis and atypical mycobacteria skin infection (AMI) present a wide range of clinical manifestations, varying from warty, nodules and papulonecrotic lesions, to ulcerations and abscesses.[7] In a study from north India Lupus vulgaris (LV) was the most frequent manifestation (55%), followed by scrofuloderma (SFD) (27%), TB verrucosa cutis (TBVC) (6%), tuberculous gumma (5%), and tuberculids (7%). The disease continues to present in various morphological forms that elude clinicians. With the arrival of improved culture methods and the availability of polymerase chain reaction (PCR), the diagnostic capabilities have increased. Cutaneous tuberculosis responds satisfactorily to the recommended antituberculous treatment, but increasing reports of multidrug resistance is a matter of concern. Although the impact of drug-resistant strains causing cutaneous tuberculosis is not yet a significant issue as in pulmonary tuberculosis, the situation that is emerging may change in the coming years.[3]

The diagnosis of TB depends on acid fast bacilli demonstration on smears and culture of the microorganism on Lowenstein Jensen media. This encourages the use of more rapid culture methods. The laboratory confirmation of cutaneous tuberculosis is often difficult using conventional methods[8] as cutaneous tuberculosis is often paucibacillary. Thus PCR has emerged as a promising tool in the diagnosis of various forms of cutaneous tuberculosis, most commonly targeting IS6110 gene specific for M. *tuberculosis* complex.[9] Keeping this in mind this study was planned to detect, isolate, and identify mycobacterium in patients of cutaneous tuberculosis and pattern of anti mycobacterial drug susceptibility in these isolates. The study will also evaluate the usefulness of PCR for detection of cutaneous tuberculosis in comparison with the conventional methods.

Materials and Methods

A cross-sectional study was conducted on 31 consecutive patients of cutaneous tuberculosis in the departments of Microbiology and Dermatology and STD UCMS and Guru Teg Bahadur Hospital Delhi, from November 2010 to March 2012.

Selection of patient

Inclusion criteria include clinically diagnosed and histopathologically documented new cases of cutaneous tuberculosis of any sex and age group or untreated cutaneous tuberculosis patients with antitubercular therapy in past 3 months or volunteers were enrolled for this study.

Exclusion criteria include patients with antitubercular therapy or its constituents drugs (aminoglycosides and quinolones) in the past 3 months.

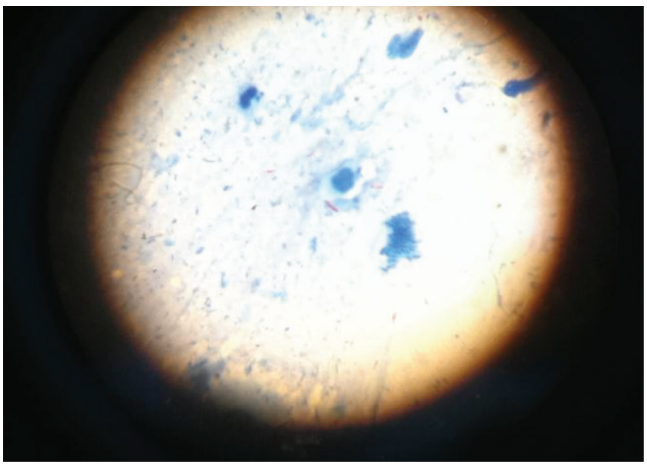
The control group was also studied from the patients who had diagnosis other than cutaneous tuberculosis.

Sample processing

Biopsy specimen were transported from the Dermatology department in sterile universal container in 0.85% saline, the samples were homogenized. Smear was prepared and stained with ZN and auramine.[10] Homogenized specimen were decontaminated with 4% H_2SO_4 for 10 min. The mixture was neutralized with 4% NaOH (pH 6.8–7.0), centrifuged at 3000 x g for 15 min, and the pellet was resuspended in 4 mL of sterile distilled water. Aliquots (200 μL) were then inoculated onto Lowenstein Jensen (LJ) slopes. LJ slopes were incubated in slanted position with screw lightly loosen for atleast 1 week for even distribution of inoculums at 37ºC incubator. The growth was checked weekly till 8 weeks before declaring it as negative growth. A positive culture was confirmed microscopically for acid fast bacilli after staining with Ziehl Neelsen stain Rough, tough, buff irregular colonies were seen (Picture 2). The absence of growth after 8 weeks on LJ medium was taken as negative. Bacterial colonies were identified as M. *tuberculosis* by conventional identification methods.[36]

DNA extraction

The biopsy tissue was used for DNA extraction Mini Kit (Qiagen) and was processed according to the manufacture's



Picture 1: Ziehl Neelsan staining.

protocol. Extracted DNA was amplified by 16S rRNA primers. The two set of primers were used. The outer and inner pairs of primers are expected to be the genus specific and species specific primers for 16S rRNA gene amplification, respectively.[10]

The outer primers:

Forward	16 SOL	(5'TGCACTTCGGGATAAGCCTG 3')
Backward	16 SOR	(5'ATTCCAGTCTCCCCTGCAGT 3')

The inner primers:

Forward	16 SIL	(5'GGATAGGACCACGGGAT 3')
Backward	16 SIR	(5'TACCGTCAATCCGAGAG 3')

PCR reaction mixture contains (25 µl) 10 X Taq Buffer (Genetix), 10 mM (Genetix), 3 units of Taq polymerase (Genetix), 100 pmoles of each set of primers and 20 µg of extracted DNA followed by addition of nuclease free water to make up to final volume (25 µl). Amplification was performed for 25 cycles with annealing temperature of 57° C.

These PCR product obtained after first round of PCR was used as template for second round of PCR using inner primers. The amplification was performed for 25 cycles with annealing temperature of 57° C.

The reaction was then incubated for an additional 10 min at 72°C and maintained at 4°C till resolved by electrophoresis. The PCR product was analyzed on 1% agarose gels and amplicon size compared with 100bp DNA ladder (Genetix).

Amplified product was identified on the basis of its molecular weight and migration in ethidium bromide gel and comparing the band of the amplified PCR product with appropriate markers.

The H_{37}RV strain was used as a positive strain (TB Reference Centre, Delhi). The PCR mix without DNA was used as negative control. PCR amplified DNA detection by agarose gel electrophoresis. A 10 µl of aliquot of PCR product was checked for DNA amplification by electrophoresis in 1.5% agarose gel (Himedia). The gel was analyzed using the UV gel documentation system (G.Box, Sygene) at 300 nm.

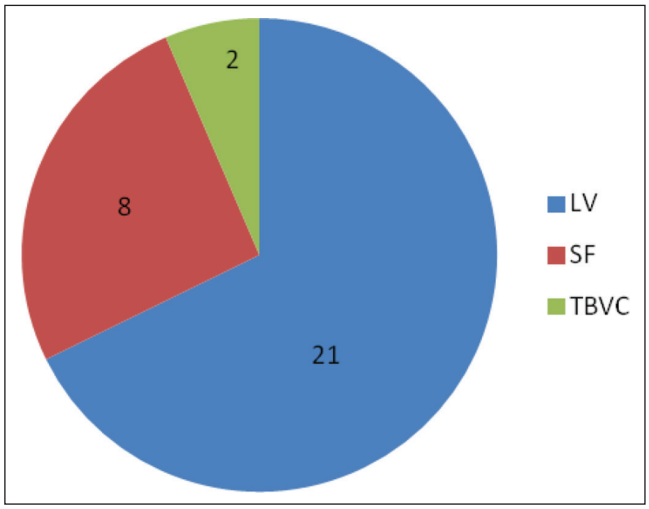


Figure 1: Distribution of clinical types of cutaneous tuberculosis.

Picture 2: Culture showing *Mycobacterium tuberculosis*.

Result

In present study, out of 31 cases, 14 (45.1%) were male patients and 17 (54.8%) were female patients showing marginal female preponderance. In this study, 2 were cases of TBVC, 8 were of SFD and 21 were of LV (Figure 1). The 58% of patients were in age group 11–20 years. Ziehl-Neelsen staining was performed on smears of 31 biopsy specimens. Thin red rod shaped, slightly curved and isolated colonies were in pairs, or in clumps which stand out clearly against the blue background (Picture 1).

Only 2 (6.4%) were AFB positive out of 31 specimens. Auramine phenol staining was performed on smears of 31 biopsy specimens. Bright yellow fluorescent rods were seen against a dark background. Out of 31 only 4 (12.9%) were smear positive. Biopsy specimens of 31 patients of cutaneous tuberculosis were homogenized and decontaminated by 4% NaOH method and were inoculated on 2 bottles of LJ media each for isolation. M. *tuberculosis* showed eugenic growth. All of them grew as dry, rough, and irregular with wrinkled surface (Picture 2). The colonies were not easily emulsifiable. The growth was detected at 37°C at 4–6 weeks. Out of 31 specimens, only 6 (19.3) were culture positives out of those, only 4 isolates were positive for niacin and nitrate (Figure 2).

Amplification of nuleic acid was done by 2 steps, the first step was nested method using 16S rRNA sequence as the target to detect a 555 bp DNA fragment for *Mycobacterium* species and in second step, inner primers were used to detect 306bp DNA fragment for M. *tuberculosis* complex. The outer primers were 16 SOL and 16 SOR and inner primers were

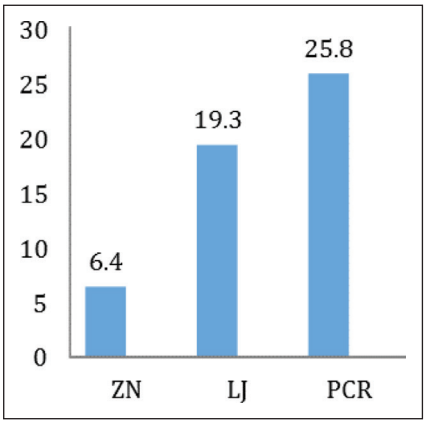
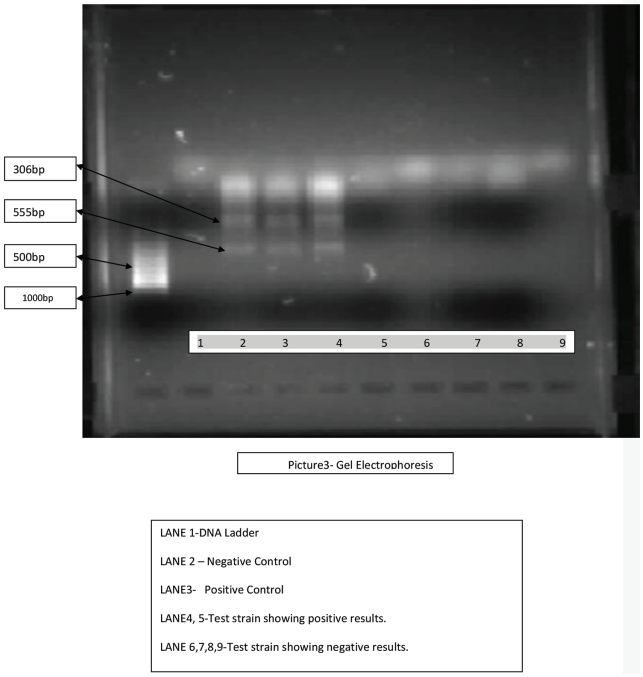


Figure 2: Positivity in ZN smear, LJ culture and PCR.



Picture 3: Gel electrophoresis.

16 SIL and 16 SIR which were expected to be genus specific and species specific, respectively. The specimen that had both 555bp and 306bp fragment or had only 306bp fragment was diagnosed as M. *tuberculosis* infection (Picture 3). The negative reagent blank and positive control reaction were processed parallel in each sets of specimens. The results which gave the correct negative and positive control ($H_{37}RV$) in each sets were used for analysis. Nested PCR was done on 31 biopsy specimens. Eight (25.8%) specimens were positive for common *Mycobacterium* species. Out of 8, DNA from 6 biopsy specimens were amplified by both genus specific and species specific primers based on 16Sr RNA gene amplification. They were diagnosed as M. *tuberculosis* infection. Out of 8, 6 cases were both PCR and culture positive and 2 were culture and smear negative. There were only 2 cases which were found to be positive by smear, culture and PCR. These were cases of lupus vulgaris and scrofuloderma, respectively. Out of 6 nested PCR positive cases, 3 were cases of scrofuloderma and 3 were cases of lupus vulgaris, respectively. The remaining 2 which were amplified by outer primers gave only 555bp were cases of lupus vulgaris.

Discussion

In present study, out of 31 cases, 14 (45.1%) were male patients and 17 (54.8%) were female patients showing a marginal female preponderance. The same trend observed in an Indian study by Punia et al.[13] with female predominance (52% females and 48% males). The same trend was observed in study from karachi by Beyt et al.[14] on the pattern of cutaneous tuberculosis, with 38.14% males and 61.85% females. It indicates that females were more susceptible to cutaneous tuberculosis and they develop the illness at an earlier stage than males. In contrast to these studies the earlier study from same hospital on cutaneous tuberculosis by Aggarwal et al.[15] observed more of male (57.1%) patients than female (48.8%).

In the present study, the cases of cutaneous tuberculosis were mainly seen in younger age group i.e <30 years. The 58% of patients were in age group 11–20 yrs which is in concordance with Singal et al.[16] who found 67.1% cases in age group 10–29 years and more recent Indian study from Chakrabarty et al.[17] who found 58% cases in similar age group. In the present study, 21 (67.7%) cases were of lupus vulgaris, 8 (25.8%) cases were of scrofuloderma (SFD) and 2 (6.4%) were of tuberculosis verrocusa cutis (TBVC) (Figure 3). Lupus vulgaris was the most common clinical variant in this study which was in concordance with 3 studies of Chong et al., [18] Aggarwal et al.[15] and Singal et al.[16] Where as in one of Indian study, scrufuloderma (12.5%) was commonest clinical variant followed by Lupus vulgharis.[39] In present study, out of 31 biopsy specimens only 2 cases (6.4%), one lupus vulgaris and another one of SFD (Figure 1), were positive by ZN staining which was similar as compared to some of the other studies. Gopinathan et al.[20] had reported smear positivity of 9.8% which is in concordance to the present study results. Sehgal et al.[5] also reported similar smear positivity of 9.5% in skin biopsy specimens in their study of cutaneous tuberculosis. In their study Gopinathan et al.[20] also concluded that tissue exudates were found to be better clinical specimens for detection of acid fast bacilli in SFD cases. Since there was no tissue exudates in the present study, that can be one of the reason for low smear positivity. However, it is a rapid procedure but it lacks senstitivity. only 4 came out to be positive (12.9%) by auramine and phenol. Since data on fluorescent microscopy in context of cutaneous tuberculosis is very rare, the results can not be compared with the present study results. The culture techniques have been estimated to detect 10–100 viable AFB per ml of sample.[11] For detection of mycobacteria in clinical specimens, the current gold standard

consists of a combination of solid and liquid media. Out of 31 biopsy specimens, only 6 isolates (19.3%) grew on LJ media after a period of 4–6 weeks. Out of 6, 2 (2/8;25%) were cases of SFD and 4 (4/21;19%) were of lupus vulgaris.

The culture positivity of 56.86% has been reported in some indian studies whereas it was 43.24% in Brazilian study in biopsy specimens. while performing biochemical tests only 4 isolates were niacin and nitrate reduction test positive. Only 2 of them were cases of lupus vulgaris and 2 of them were of scrofuloderma. Only one isolate was heat stable and catalse positive i.e. vulgaris.

Amplification of nuleic acid was done by 2 steps, the first step was nested method using 16S rRNA sequence as the target to detect a 555bp DNA fragment for *Mycobacterium* species and in second step, inner primers were used to detect 306bp DNA fragment for M. *tuberculosis* complex. The outer primers were 16 SOL and 16 SOR and inner primers were 16 SIL and 16 SIR which were expected to be genus specific and species specific, respectively. The specimen that had both 555bp and 306bp fragment or had only 306bp fragment was diagnosed as M. *tuberculosis* infection. The negative reagent blank and positive control reaction were processed parallel in each sets of specimens. The results which gave the correct negative and positive control (H_{37}RV) in each sets were used for analysis.

Nested PCR was performed on all biopsy specimens. 8 (25.8%) specimens were positive for common *Mycobacterium* species (Table 1). Out of 8, DNA from 6 biopsy specimens were amplified by both genus specific and species specific primers based on 16Sr RNA gene amplification. They were diagnosed as M. *tuberculosis* infection (Picture 3). Out of 8, 6 cases were both PCR and culture positive and 2 were culture and smear negative. There were only 2 cases which were found to be positive by smear, culture, and PCR. These were cases of lupus vulgaris and scrofuloderma, respectively. Out of 6 nested PCR positive cases, 3 were cases of scrofuloderma and 3 were cases of lupus vulgaris, respectively. The remaining 2 which were amplified by outer primers gave only 555bp were cases of lupus vulgaris. The sensitivity of direct detection of M. *tuberculosis* by PCR depends on the efficiency of DNA isolation from mycobacerial cells, amplification of target DNA and detection of the amplified product.

In the present study, the PCR positivity was found out to be 25.8% which was slightly less than that reported by Chaiprasert et al.[22], who reported PCR positivity in 47 (35.8%) tissue specimens out of 131. In one of the Brazilian study, the mycobacterial DNA was detected in 24.32% of the biopsies[6] similar to the present study's nested PCR results (25.8%). But, their primers were different from this study. Another Iranian study by Meghdadi et al.[38] showed 20% of PCR positivity. Hijidin et al.[31] had reported PCR positivity of 5.1% which is very lower as compared to this study. Although they had used 16S rRNA based primers for detection of M. *tuberculosis* in joint biopsy specimens The low detection rate was still a problem in the present study. Moreover, sample size was small. The false negative result of PCR may also be due to the presence of inhibitors detected in the tissues itself.[30] Chan et al.[21] have reported that inhibitors were detected more frequently in extrapulmonary than in pulmonary tissue, whereas the authors of other studies have reported the opposite.[27,28,29,32] Solid specimens such as skin and lymph nodes also cause difficulties in DNA extraction and discordant results in different methods may be caused by non-homogenous distribution of AFB in the specimen.[30] Many genes and sequences are used as target DNA for amplification. They are repetitive and non-repetitive DNAs. IS 6110 is one of the frequently used repetitive sequences[22] while 16S rRNA is a nonrepetitive sequence.[12,22] IS 6110 is a good target for amplification because it presents in high copy number but some strains of M. *tuberculosis* do not contain IS 6110 sequence in their genomes.[32] In general, the high sensitivity of the IS 6110 PCR is due to the presence of multiple copies of IS 6110 in the M. *tuberculosis* complex and the versatility of 16S rRNA gene.

PCR, which can detect mycobacteria, by use of universal primers, might be considered useful.[31] 16S rRNA can detect some exceptional strains of *M. tuberculosis* which lack the *IS 6110* insertion sequence, or mycobacteria other than tuberculosis (MOTT). These mycobacteria are usually present among immunocompromised patients.[34] The nested PCR positivity for 16S rDNA in their study was 24.3% which was similar to the present study's nested PCR results (25.8%). But, their primers were different from the present study. Although, the rate of PCR positivity in the present study was less but PCR test was able to detect *Mycobacterium* species, it has advantages over the traditional diagnostic tests, the procedure is quick, and through the additional PCR tests, it is possible to typify the strain of *Mycobacterium* that is isolated.[35]

Table 1: Comparison of nested PCR results with culture results for M. *tuberculosis*

	Culture (+)	Culture (-)
PCR (+)(8)	6	2
PCR(-)(23)	0	23
Total	6	25

Conclusion

PCR can be used for early diagnosis of cutaneous tuberculosis in skin biopsy specimens that can help to initiate timely anti-tubercular treatment and prevent progression to irreversible changes.

The PCR appears to be a promising diagnostic tool as it is quick and easy to perform in cutaneous tuberculosis which is a limitation of cultural procedures, still its role in the routine diagnosis of cutaneous tuberculosis and atypical mycobacterial infection is uncertain as the technique is lesser in positivity prone to artifacts and amplification dead *Mycobacterium* can give false positive results. PCR assay can be used for rapid detection of M. *tuberculosis* from cutaneous tuberculosis cases, particularly when the staining for acid fast bacilli is negative and there is a lack of growth on culture or when fresh material has not been collected for culture.

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