

# Comparative Analysis of Various Diagnostic Techniques for Tubercular Lymphadenitis: A Pilot Study from a Resource Poor Country

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**Abstract:** Background: Tuberculosis lymphadenitis (LNTB) is the most common presentation of extra pulmonary tuberculosis. The main causative agents reported were predominantly *Mycobacterium tuberculosis* (*M.tb*), closely related *Mycobacterium bovis* (*M.bovis*) and non-tuberculous mycobacteria (NTM). Over the past decade, a set of tedious cytological and microbiological diagnostic tests i.e. fine needle aspiration cytology (FNAC), microscopic smear examination & culture were used. Although FNAC & smear examination were rapid, but none of these techniques were able to differentiate between *M.tb* and other members of *Mycobacterium* spp., which is highly essential for planning anti-microbial therapy programme. Methodology In the present study, smear, FNAC, culture and *hupB* gene (Rv2986c) based PCR, were applied and each method was analyzed in terms of sensitivity, specificity, along with reliability and cost effectiveness. Results Considering culture as a gold standard, all other diagnostic methods were compared. Direct PCR, showed the sensitivity & specificity of 47% & 75% whereas when performed on culture isolates, the sensitivity rose to 76%. The sensitivity & specificity of FNAC were 60% & 49% respectively whereas of direct smear examination was 50% and 70% respectively. Conclusion: we conclude that smear and FNAC are rapid, cost effective, easily available, but has lower specificity and may not be able to differentiate tubercular lymphadenitis from non tubercular lymphadenitis. PCR(*hupB* gene based) being a singular target for *M.tb* showed reliability and potential to rapidly detect & identify causative agent of LNTB, can help clinician to initiate correct and timely treatment.

**Keywords:** Tuberculosis lymphadenitis, culture, *hupB* gene, fine needle aspiration cytology (FNAC)

## INTRODUCTION

Tuberculous lymphadenitis (LNTB) being one of the most frequent cause of lymphadenopathy<sup>1</sup>, accounted for about a half of 2,19,945 of total extra-pulmonary TB cases reported in the year 2008 in India<sup>2</sup>. An Indian pediatric study showed prevalence of peripheral lymphadenopathy as 27.2/1000 children and that of LNTB as 4.43/1000<sup>3</sup>. Although *Mycobacterium tuberculosis* complex (MTC) organisms i.e. *Mycobacterium tuberculosis* (*M.tb*), *Mycobacterium bovis* (*M.bovis*), *Mycobacterium africanum* and *Mycobacterium microti* were the main cause of mycobacterial lymphadenitis cases, nontuberculous mycobacterial (NTM) lymphadenitis (NTM-LN) with high frequency in human immunodeficiency virus type 1 (HIV-1)-infected individuals<sup>4</sup> reported to be an emerging causative agent. Cervicofacial lymphadenitis, the most frequent head and neck manifestation of NTM infection, often presents as chronic, unilateral lymphadenopathy with characteristic violaceous overlying skin changes. Lymphadenitis due to infection with the MTC is more chronic in nature, while NTM-LN often has a more rapid course<sup>5</sup> and their treatment follow-up were also different as tuberculous adenitis is best treated as a systemic disease with anti-tuberculosis medication whereas NTM infections can be addressed as local infections and are amenable to surgical therapy. Therefore, species identification is also of paramount importance.

Over the past decade, The efficacy of fine needle aspiration cytology (FNAC) and direct microscopical screening of stained slides for AFB were validated as a diagnostic tool for LNTB because of their simplicity, rapidity, and performance friendly nature, but have their own limitations<sup>6,7</sup>. Mycobacterial culture technique although being more sensitive & specific, requires 6-8 weeks before a positive visual result is obtained<sup>8</sup>. The main shortcoming of these parameters was that none of these were able to perform speciation of the genus mycobacteria thus causing misery to the clinician to initiate therapy.

After considering these limitations of existing diagnostic tools, molecular techniques like polymerase chain reaction (PCR) based on the amplification of target sequences were introduced to rapidly detect and identify mycobacterial agent in clinical samples at the genus, complex, and species levels<sup>9</sup>. Various targets like IS6110 insertion element<sup>10</sup>, *hupB*<sup>11-13</sup>, *katG*<sup>14</sup>,

*pncA*<sup>15</sup> genes and even peripheral blood mononuclear cells<sup>16</sup> etc were used to differentiate *M. tb* from other members of mycobacterium group. The various diagnostic tools are available to diagnose LNTB, but it has only added to already existing confusion regarding diagnosis. In this study, we have tried to compare already available diagnostic tools and suggest in terms of sensitivity, specificity, reliability and cost effectiveness and tried to suggest the better method to help clinician initiate timely and correct therapy to tubercular lymphadenitis patients. We have used *hupB* gene (Rv2986c) encoding a histone-like protein of *M. tb*, based PCR on clinical aspirates to identify the causative agent in LNTB as a target for detection and identification of *M. tb* and closely related *M. bovis* from other members of the MTC and NTM. Sequence analysis of *M. tb* and *M.bovis* has shown that *M.bovis* lacks the 12.7 kb fragment containing the *mce3* operon<sup>17</sup>, whereas all *M. tb* isolates examined showed the presence of the 12.7 kb fragment, while all the *M.bovis* strains lacked this fragment. We exploited the differences in the organization of the *mce3* operon in the two species.

## METHODS

A collaborated pilot study was undertaken at Department of Histopathology and Microbiology, at Vardhman Mahavir Medical College & Safdarjung Hospital (VMMC & SJH), New Delhi, and Department of Biotechnology, at All India Institute of Medical Sciences (AIIMS), New Delhi, India during Jan.2009 to August 2009. The study was divided in two parts. First phase involved patient registration, sample (aspirates) collection, smear preparation and staining and culture by both solid Lowenstein Jensen (L J) & Liquid broth based BacT/ALERT 3D automation and was performed at Safdarjung Hospital. The second phase involved DNA extraction & PCR from clinical aspirates and was performed in AIIMS.

In this study, 89 clinical suspected patients (52 male and 37 female) of tubercular lymphadenitis were included, after taking ethical clearance and informed written consent from patients or from parents (in case of children). There were 23 (25.8%) cases of children <15 yrs. The duration of lymph adenopathy varied from 10 days to 20 months.

Fine needle aspirations (approx. volume 0.5-2 ml) from all enrolled patients were performed in the Department of Histopathology under sterile aseptic conditions on the suspected lymph nodes. Two smears were prepared and

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stained by Giemsa stain and acid fast staining by Ziehl Neelsen (ZN) method as per the approved guidelines<sup>18</sup> and the findings were recorded.

0.1-0.2 ml aspirate was inoculated on Lowenstein-Jensen (LJ) slant & 0.2-0.5 ml (depending upon total volume of sample aspirated) was put into BacT/ALERT enriched bottles for cultivation of bacilli. The growth on LJ slant was checked everyday in first week to look for rapid growers bacteria and thereafter on weekly basis till sixth week. The growth was finally confirmed by ZN stain for the presence of AFB. In the same way, when BacT/ALERT system flashes positive signal, the bottle was taken out and smear was made to confirm TB bacilli.

**DNA Extraction and PCR:** DNA extraction and PCR were performed as per in-house protocol developed in the Department of biotechnology, AIIMS, New Delhi<sup>11</sup>.

Three primers were used: Forward primer CMB-F common to *M. tb.* and *M.bovis*, and 2 reverse primers N-tb & BMB-R specific for *M. tb.* and *M.bovis* respectively. The assay mixture (25µl reaction) contained: forward primer FP (0.625µM), the reverse primers N-tb & BMB-R (0.325µM each), 1x PCR buffer (100mM Tris/HCl, pH 8.8, 500mM KCl, 0.8% nonidet-P40), 2.5mM MgCl<sub>2</sub>, 0.3mM dNTPs and 1.25U Taq DNA polymerase. The thermal cycle parameters were 95°C for 10 min and 40 cycle of each, of 45 sec at 95°C, 45 sec at 58°C and 45 sec 72°C and final extension at 72°C for 10 min. The Forward primer i.e CMB-F used was common to both *M. tb* & *M.bovis*; whereas reverse primers, N-tb has been derived from the 12.7 kb fragment and BMB-R from the region adjacent to the 12.7 kb fragment (Fig.1). N-tb primer was specific for *M. tb*; while the sequence of the BMB-R reverse primer was present in both *M. tb* and *M.bovis*. However amplification occurs in case of *M.bovis* with the CMB-F & BMB-R exclusively and not in case of *M. tb*, as the 12.7 kb insert prevents Taq polymerase mediated amplification.

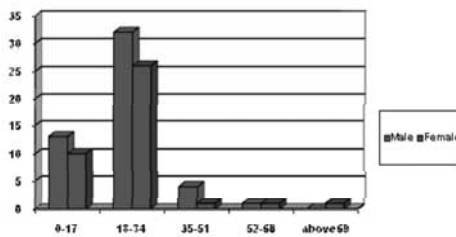


Table 1: Age & gender of patients studied

The standardized assay was used for detection of *M. tb.* as well as *M.bovis* in clinical samples. The amplified product obtained in CM-PCR assay is 162 & 127bp for *M. tb.* & *M.bovis* respectively (Table-2).

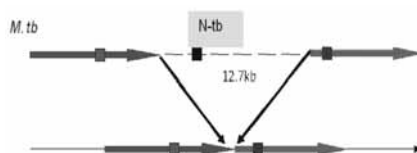


Table-2: Primers used

## RESULTS

The aspirated lymph nodes included cervical (67%), supraclavicular (11%), submandibular (6%), auricular (11%), submental (5%) of the total cases. The final diagnosis was made if FNAC showed epithelial cell granuloma with necrosis and/or smear positivity for AFB and/or growth on LJ or bactec, were seen<sup>7</sup>. Based on this, 54 cases were termed as positive. Considering culture as a gold standard, this assay when applied directly on clinical aspirates, showed the sensitivity & specificity of 47% & 75% whereas when performed on culture isolates, the sensitivity rises to 76%. The sensitivity & specificity

of FNAC were 60% & 49% respectively. The overall acid-fast bacilli positivity in fine needle aspiration smears was 38.4% of the total cases and in 50% of all culture-positive aspirates whereas of direct smear examination was 50% and 70% respectively (Table 3). LN-PCR was positive in 47% of the aspirates from patients, while PCR on culture isolates, showed sensitivity

**Table 3: Comparison of sensitivity, specificity of PCR & other tests with culture**

Test	Sensitivity	Specificity
PCR(Clinical samples)	47%	75%
PCR(Culture isolates)	76%	N.A *
FNAC	60%	49%
Smear	50%	70%

\* Can't calculate as it is performed only on positive culture isolates

to 76%.

## DISCUSSION

In endemic areas like India, the detection of LNTB with traditional diagnostic tools is always a major challenge. In the past decade, various studies describing lymph node PCRs from fine needle aspirates or biopsy specimens have consistently shown improved sensitivity (61-78%) when compared with conventional microbiologic methods<sup>10,12,13,19-21</sup>. The PCR assay described in the study is based on the *hupB* gene of *M. bovis* and *M. tb*. The specificity of the *hupB*-based PCR assay to detect and identify *M. bovis* and *M. tb* has been established and the sensitivity were reported to detect as low as 10 - 20 picogram DNA of the tubercle bacilli<sup>11-13</sup>.

In the present study, the LN-PCR was positive in 47% of the aspirates from patients. This lower sensitivity may be attributed either to the small volume of aspirate remaining after distributing the sample for the microbiological and cytological assays or due to presence of PCR inhibitors<sup>12</sup>. To confirm the cause, we again performed PCR on culture isolates, the PCR sensitivity then jumps to 76%. This was expected as inadequate sampling predominantly influences the assay. We performed PCR from the leftover aspirate after performing conventional parameters and identified *M.tb* in the vast majority of positive cases whereas *M.bovis* was not found. There were three pediatric cases in which only culture & PCR came positive whereas both FNAC & smear microscopic were negative. These results indicate that culture-enhanced PCR is a highly sensitive and specific method for the detection of *M. tb* in extrapulmonary specimens especially in children and would diminish the chance of open biopsy.

The only limitation found was unlike culture, the PCR technique does not distinguish between live and dead mycobacteria, a feature that is of the utmost importance when screening for viable mycobacteria in samples such as dairy products following pasteurization or pre-exposure of broad spectrum antibiotics such as amoxicillin-clavulanic acid & fluoroquinolones known to inhibit *M.tb*<sup>23</sup>.

Revised National Tuberculosis Control Programme (RNTCP) mainly recommends cytology & ZN smear microscopy for the diagnosis of LNTB<sup>24</sup>. In FNAC, diagnosis is based on the presence of granulomas, central necrosis and if possible, demonstration of acid-fast bacilli by staining. Cytology has a sensitivity of approximately 32-59%<sup>6,25</sup>. In our study, it was 60%. But when compared with culture, FNAC showed low specificity (49%). However, absence of specific cytologic findings of granulomatous lymphadenitis or negative acid-fast bacilli (AFB) smears requires additional open biopsy or repeated FNAC, thus this method has limitations in clinical situations. Another shortcoming of FNAC lies in the difficulty of differentiating tuberculosis from other granulomatous diseases or nontuberculous mycobacterial. Lymphadenitis caused by nontuberculous mycobacterial species usually resistant to anti-tubercular drugs and they would be misdiagnosed as multi-drug resistant tuberculosis (MDR-TB)<sup>26</sup>. However, this technique

provides an easy way for collecting materials for bacteriological examination. The concentration of organisms in the clinical specimen has a direct relationship with the sensitivity of the ZN stain and a concentration of  $\geq 10^4$  organisms/ml would normally guarantee a positive smear. The overall acid-fast bacilli positivity in fine needle aspiration smears can vary from 37.4% to 59.4%<sup>6,7,25</sup>. In the present study, it was 38.4% of the total cases and in 50% of all culture-positive aspirates. The low sensitivity was probably due to the low concentration of mycobacteria in the aspirate.

Culture reports from different studies<sup>1,8</sup> detect fine needle aspirates between 39 to 80% positive in the clinically suspected TB-L cases. Our observation also falls in between the reported range (43.1%). However, low sensitivity and extensive time requirements of culture studies limit its usual application. Traditionally, culture followed by a panel of biochemical tests has been used for speciation of mycobacteria but has inherent shortcomings<sup>9</sup>. In the present study, the time consumed for primary isolation on L J media ranges from 4 to 6 weeks and 2-3 weeks by liquid broth based automated BacT/ALERT system. Although BacT Alert 3D system recovers mycobacteria rapidly even this is too long as it is necessary to commence treatment as soon as possible.

These results confirm that PCR from the remainder of fine-needle aspirate could be a good initial diagnostic tool. Given the availability of a thermal cycler, the rest of the procedure has a cost similar to other routine assays for LNTB diagnosis. Therefore, this PCR assay could be of immense utility in redefining research priorities and public health strategies for control and prevention of both human and bovine tuberculosis and it can reduce the need for more invasive diagnostic approaches.

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**LITERATURE REVIEW**

**Maternal Obesity and Pregnancy Outcome: A Prospective Analysis**

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**Objective:** To analyze whether the obese women have an increased risk of pregnancy complications and adverse fetal outcome.

**Methods:** The longitudinal prospective study was carried out in the Obst and Gynae department, IPGME and R, Kolkata. The study enrolled 422 pre-pregnant obese women with pregnancy as study population and equal number of non obese pregnant mothers as controls. Body mass index (BMI) was  $e^{\circ} 30.0\text{kg/m}^2$  and  $20\text{-}22\text{ kg/m}^2$  in obese and control group respectively. Results: In comparison to average weight pregnant women, obese pregnant women were at increased risk of gestational diabetes mellitus (19.43 vs 3.79%;  $p < 0.001$ ), pregnancy induced hypertension (12.32 vs 2.36%;  $p < 0.001$ ), pre-eclampsia (8.76 vs 3.31%;  $p < 0.001$ ), preterm labor in less than 34 week gestation (7.58 vs 3.55%;  $p < 0.001$ ), cesarean section (36.72 vs 17.53%;  $p < 0.001$ ), instrumental deliveries (12.32 vs 5.21%;  $p < 0.001$ ) and postpartum infection morbidities (9.95 vs 3.79%;  $p < 0.001$ ). These women were more prone to develop overt diabetes (2.36% vs 0) and chronic hypertension (5.21 vs .47% ) in future as well. Neonates of obese women were mostly large for gestational age, macrosomic and they had high incidences of birth injuries, shoulder dystocia, premature deliveries, late fetal deaths and congenital malformations particularly spina bifida, cleft lip, cleft palate and heart defect. Conclusion: As obesity is considered to be a modifiable risk factor, preconception counseling and creating awareness regarding health risks associated with over weight and obesity should be encouraged.