

ORIGINAL ARTICLE

Evaluating multiplex PCR, culture & histopathology in the diagnosis of Tubercular Lymphadenitis

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ABSTRACT

Background: Tubercular lymphadenitis (TBLN) is the most common manifestations of extra-pulmonary tuberculosis (EPTB) accounting for 30-40% of cases with multiple discrepancy in diagnosis. Rapid diagnosis and timely initiation of anti-tubercular therapy (ATT) is the key for successful clinical effects. This study was carried out to evaluate multiplex polymerase chain reaction (MPCR) using *MTP40*, *IS6110*, 32 kD-alpha antigen encoding gene, and mycobacterial specific genes (*rpoB*, *katG* & *inhA* promoter region), and compare with the conventional methods for rapid diagnosis of TBLN.

Materials and Methods: Lymph node aspirates samples were collected and analyzed from a total of 48 TB lymphadenitis cases and 20 non-TB controls. Each specimen was subjected to Ziehl-Neelsen (ZN) staining, culture on Lowenstein–Jensen (LJ) medium, cytological examination and multiplex PCR using *MTP40*, *IS6110*, 32 kD-alpha antigen encoding gene sequences and mycobacterial specific genes (*rpoB*, *katG* & *inhA* promoter region) as a primer.

Results: In total, 29.2% of the samples were positive for ZN staining, 31.2% of the samples were positive for TB by culture and 89.6% by MPCR assay. The sensitivity of ZN staining method was the lowest (29.2%) while sensitivity of MPCR assay was the highest (89.6%). In the control group, all the tests were found to be negative, thus giving a specificity of 100%.

Conclusion: Multiplex PCR assay is rapid, cost effective, highly sensitive and specific technique for the diagnosis of tubercular lymphadenitis.

Tuberculosis (TB), caused by *Mycobacterium tuberculosis complex* (MTBC), still remains the major killer disease worldwide, especially in developing countries in spite of considerable progress in diagnosis and treatment. It ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death worldwide^[1]. *Mycobacterium tuberculosis complex* includes *Mycobacterium tuberculosis* (MTB), *M. africanum*, *M. canettii*, *M. bovis*, *M. microti*, *M. orygis*, *M. caprae*, *M. pinnipedii*,

M. suricattae and recently recognized *M. mungi*^[2]. Globally, it infects one third of the world's population. The global tuberculosis burden in India is to be 26%. MTB mostly infects pulmonary sites but it can also manifest extra-pulmonary sites i.e. endometritis, lymphadenitis, pleuritis and tuberculous meningitis etc. Almost 20% of extra-pulmonary tuberculosis disease has been reported in India^[3]. Among the extra-pulmonary tuberculosis (EPTBs), tuberculous

lymphadenitis is the most common manifestation frequently caused by *Mycobacterium tuberculosis*, accounting 30-40% of all cases^[4,1].

The non-tuberculous mycobacterial (NTM) infections have also increased in many regions of the world along with MTBC infections and much of this increase in the burden of TB concurred with HIV infection in patients^[5,6]. The species of NTM associated with human disease are: *M. avium*, *M. intracellulare*, *M. kansasii*, *M. fortuitum*, *M. chelonae*, *M. szulgai*, *M. paratuberculosis*, *M. scrofulaceum* etc. Lymphadenitis due to infection with the MTBC is more chronic in nature, while NTM-LN (non-tubercular- lymphadenitis) often has a more rapid course^[7] and their treatment follow-up were also different as tuberculous adenitis is best treated as a systemic disease with anti-tuberculosis therapy whereas NTM infections can be addressed as local infections and are conformable to surgical therapy. Therefore, species identification is also most important.

LNTB diagnosis depends on the clinical history, symptoms and clinical findings of the patients. It depends mainly on the bacilli culture obtained after fine needle aspiration (FNAs) biopsy or excisional biopsy, with the microscopic demonstration of the acid-fast bacilli using Ziehl Neelsen stain (ZN)^[8]. Routine tests used for the microbiological confirmation of lymph node TB have important disadvantages: the sensitivity of AFB staining is <50% and could not distinguish between non-tuberculous mycobacterium (NTM) and MTBC, presumptive differentiation between MTBC and NTM. On the other hand, culturing takes 2–8 weeks to produce a definite result^[9,10].

Previously workers have dedicated attention to the role of PCR in the diagnosis of LNTB from FNA aspirates^[11,12,13]. The majority of the studies have used only a single target gene, that is, *IS6110*; however, it is missing or in low copy numbers in 10-40% of *M. tuberculosis* isolates from India^[14,15]. So, an alternative approach is to use multiplex PCR (MPCR) where more than two targets are amplified simultaneously. It is cost-effective and chances of contamination are also less and it can differentiate MTBC from NTM. Therefore, this study was carried out to evaluate MPCR using *MPT40*, *IS6110* and *32kD alpha antigen*. Further, mycobacterial specific genes (*rpoB*, *katG* & *inhA*) were also investigated by MPCR using specific primers and compare it with conventional methods such as microscopy, culture, and cytological examination for rapid diagnosis of LNTB.

MATERIALS AND METHODS

Study design and population

This study was conducted during the period March 2013-February 2014 in the Department of Microbiology and Pathology of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. The diagnoses of TBLN and non-tubercular lesions were based on the clinical assessment and cytological findings. Information was collected from medical files and compliance charts on demographic characters of case groups, personal characteristic. This prospective cross-sectional study included 48 successive patients of TBLN and 20 random patients with non-tubercular lymph node lesions (reactive hyperplasia and metastatic carcinoma).

Sample collection and processing

Fine needle aspiration was performed from palpable lymph nodes in both tubercular and non-tubercular groups by an experienced pathologist as an outpatient procedure using 21 Gauge needle attached to 10 ml syringe following standard procedure. One half of the fluid and aspirate sample was used for preparation of three smears; two was fixed for cytological examination by Papanicolaou and Giemsa staining and another was used for ZN staining, as described earlier^[16]. The other half flushed in 200µl phosphate buffered saline (PBS) at pH 6.8, concentrated by centrifugation at 3000 × g for 15 min and one loopful (approximately 0.1–0.15 ml) was inoculated on Lowenstein-Jensen (LJ) medium as well as used for PCR-based study.

On the basis of cytological examinations patients were grouped as follows: Group I is epithelioid cell granuloma with caseous necrosis, Group II is epithelioid cell granuloma only, Group III is caseous necrosis only and Group IV is necrosis and suppurative lesion^[17,18,19,20].

DNA extraction

Two hundred microliters of stored sample was used for DNA extraction according to the cetyltrimethyl ammonium bromide (CTAB)- chloroform extraction method, with minor modifications as described earlier^[21].

Multiplex polymerase chain reaction

MPCR was performed using primers (Eurofins, Bengaluru, India) for the *IS6110*, *MTP40* and *32kD alpha antigen*

encoding gene sequences^[22,23,24,25,26,27] (Table 1), *rpoB*, *katG* and *inhA* promoter region sequences. A 396-bp fragment of *MTP40*, 984-bp fragment of *IS6110* gene and 506-bp fragment of *32kD alpha antigen* encoding gene sequences, specific for *M. tuberculosis*, *M. tuberculosis* complex and NTM, were amplified by using primers as described in Table 1. Master mix (25 µl) for the MPCR was prepared by using 10x buffer (Merck, Bengaluru, India), 10mM dNTP mix (Merck, Bengaluru, India), 5U Taq polymerase (Merck, Bengaluru, India), 10 pmol of primer MTB F, MTB R, MTBC F and MTBC R (Eurofins, Bengaluru, India), 10 µl DNA template and deionized water (qs) to create a total volume of 25 µl. The amplification was carried out in a thermocycler (T-100™-Bio-Rad) under the following conditions: initial denaturation at 95°C for 3 min, 35 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 50 sec and a final extension at 72°C for 7 min.

DNA extracted from *M. tuberculosis* H37Rv and milliQ water was used as positive and negative control, respectively. 5µl of amplified products of both the cycles were analyzed by electrophoresis on 2% agarose gel and the amplified fragments were analyzed by ethidium bromide staining (0.5 µg/ml) under ultraviolet light.

Statistical analysis

Sensitivity, specificity, positive predictive value, and negative predictive value of the tests were calculated and compared by MedCalc software.

Ethical issues

For this study, the approval was obtained from the Institution Ethics Committee.

RESULTS

In this study, out of 48 clinically suspected patients of LNTB, number of male and female patients was 60.4% (29/48) and 39.6% (19/48), respectively with a male: female ratio of 1:0.5. The age group of patients ranged from 2 years to 65 years (Mean age 28.2 ±16.9). It was further observed that majority [64.6% (n=31)] of the patients were of 5 to 30 years age groups. Among these cases, cervical lymphadenopathy was observed among 81.2% (39/48) subjects whereas; axillary lymphadenopathy and inguinal lymphadenopathy were observed among 12.5% (6/48) and 6.2% (3/48), respectively. History of anti-tubercular treatment (ATT) was present in 39.6% (n=19) cases, for a variable period from 2 months to 9 years. Family history of TB was present in 12.5% (n=6) patients.

Out of 48 superficial lymph nodes aspirated, 13 cases showed ZN smear positivity while 31 cases were ZN smear negative with cytological pattern of LNTB. The suggestive cytological patterns of LNTB were observed in 66.7% (32/48) cases. The most common cytological finding was epithelioid cell granuloma with caseous necrosis (Group I; n=21), followed by necrosis and suppuration (Group IV; n=16), epithelioid cell granuloma (Group II; n=8) and

Table 1: Sequences of primers used for the detection of *Mycobacterium tuberculosis* in MPCR assay

Genes	Primers	Sequence (5'-3')	Size (bp)	Reference (Primers)
<i>MTP40</i>	MTBF	CGGCAACGCGCCGTCGGTGG	396	Portillo <i>et al.</i> 1991
	MTBR	CCCCCACGGCACCGCCGGG		
<i>IS6110</i>	MTBCF	CGGAGACGGTGCCTAAGTGG	984	Wojciech, 1992
	MTBCR	GATGGACCGCCAGGGCTTGC		
32 kD α-antigen	NTMF	TTCTGACCAGCGAGCTGCCG	506	Ohara <i>et al.</i> 1993
	NTMR	CCCCAGTACTCCCAGCTGTGC		
<i>rpoB</i>	rpoBF	GTCGCCGCGATC AAGGA	259	Mokrousov <i>et al.</i> 2003
	rpoBR	TGACCCGCGCGTACAC		
<i>katG</i>	katGF	GCAGATGGGGCTGATCTACG	435	Mokrousov <i>et al.</i> 2002
	katGR	AACGGGTCCGGGATGGTG		
<i>inhA</i> promoter region	mabAF	ACAAACGTCACGAGCGTAACC	451	Leung <i>et al.</i> 2006
	mabAR	GTGGCGTTGATGACCTTCTC		

caseous necrosis (Group III; n=3) (Table 2). Cytologically, 25% (12/48) cases in Group IV was interpreted as negative for LNTB based on non-specific findings of necrosis and suppuration, while 8.3% (4/48) cases could not be given any diagnosis due to paucity of cellularity (Table 2).

Z-N smear examination showed presence of acid-fast bacilli in 29.2% (14/48) cases and 31.2% (15/48) of the samples demonstrated growth [*M. tuberculosis* (n=14); NTM (n=1)] on LJ media. Cytological diagnosis of TB was supported by Z-N smear examination and culture in 38% (8/21) and 42.8% (9/21) cases of Group I and 25% (2/8) and 50% (4/8) cases of Group II, respectively. Although presence of caseous necrosis in Group III was interpreted as cytological evidence of TBLN, all three cases in this group were negative for Z-N smear while 66.7% (2/3) cases were culture positive. In contrast, cytological interpretation as negative for TBLN

in Group IV showed presence of AFB in 25% (4/16) cases but negative for culture (Table 2). Out of 19 cases, which were showing only necrosis and/or suppuration without granulomas (Group III and IV), only 21.0% (4/19) and 10.5% (2/19) cases showed Z-N smear and culture positive respectively whereas 78.9% (15/19) cases were confirmed as TBLN after multiplex PCR but remaining 21.0% (4/19) of the cases were negative by MPCR.

Further on subjecting all the cases for MPCR, 89.6% (n=43) cases were positive for either or both the target genes namely *IS6110* and *MTP40* (Table 3). In addition, with regard to PCR target detection in the multiplex PCR, 79.2% (n=38/48) samples were positive for both *IS6110* & *MTP40* while only 4.2% (n=2/48) samples were positive for *IS6110* and 2.1% (n=1/48) sample were positive for only *MTP40* (Fig. 1). Further 4.2% (n=2/48) samples were positive for 32

Table 2: Observation of Z-N smear, culture and MPCR positivity in different Cytological groups of TBLN cases (n=48)

Groups	Cytological findings	Z-N smear	Culture on LJ media	MPCR	<i>IS6110</i> & <i>MTP40</i> positive	Only <i>IS6110</i> positive	Only <i>MTP40</i> positive	Only 32 kD α -antigen
		No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Group I (n=21)	Epithelioid cell granuloma with caseous necrosis	8 (38.0)	9 (42.8)	21 (100)	20 (95.2)	1 (4.8)	1 (4.8)	1 (4.8)
Group II (n=8)	Epithelioid cell granuloma only	2 (25)	4 (50)	7 (87.5)	3 (37.5)	1 (12.5)	0	0
Group III (n=3)	Caseous necrosis only	0 (0)	2 (66.7)	2 (66.7)	2 (66.7)	0	0	0
Group IV (n=16)	Necrosis and suppuration	4 (25)	0	13 (81.2)	13 (81.2)	0	0	0
Total (N=48)		14 (29.2)	15 (31.2)	43 (89.6)	38 (79.2)	2 (4.2)	1 (2.1)	1 (2.1)
Control (n=20)	—	—	—	—	—	—	—	—

Table 3: Sensitivity & specificity of AFB smear, culture, single step and nested multiplex PCR methods for the diagnosis of TBLN

Test results	Final diagnosis		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	LNTB (N=48)	Control (N=20)				
AFB smear						
Positive	14	—	29.2	100	100	37.0
Negative	34	20				
LJ Culture						
Positive	15	—	33.3	100	100	42.3
Negative	33	20				
Multiplex PCR						
Positive	43	—	89.6	100	100	80
Negative	5	20				

kD a-antigen. In the control group of non-tubercular lesions (n=20), all the tests i.e. AFB smear, culture, and MPCR were found to be negative.

The sensitivities of Z-N smear, culture and MPCR assay were 29.2%, 33.3%, and 89.6% respectively. In the control group, all the tests were found to be negative, thus giving a specificity of 100% for all the tests in the study that is, Z-N smear, culture, cytological examination, and multiplex PCR (Table 3).

DISCUSSION

Tubercular lymphadenitis is often difficult to diagnose because of its diverse clinical presentation and low sample volume. The conventional techniques such as acid fast staining and bacterial culture have limited sensitivity due to low numbers and slow growth rate of bacilli in a significant proportion of clinical specimens^[13,15]. The ability to rapid detection of *M. tuberculosis* from clinical specimens has important implications in the treatment of TB disease. In recent decades, for the identification of microorganisms at the species level, molecular techniques are being increasingly used for the diagnosis and control of TB^[9,28,29,30]. The PCR assay provided a rapid, sensitive and specific diagnosis of TB. Various sets of primers have been used for the detection of MTB directly from clinical specimens^[31,32,33]. Although *IS6110* is a commonly used target in PCR, it had disadvantage of having lower or no copies in a large number of strains in different regions^[14,34,35]. The amplification of the species-specific *MTP40* genomic fragment, which is exclusively detected within the *M. tuberculosis* genome^[22,36] but not in the genomes of other mycobacterial species, are making it suitable for use in diagnosis.

The positivity rate of culture among LNTB cases in our study was 31.2% and is comparable with other studies which revealed 28% of LJ culture positivity of lymph node aspirates^[37]. But another study has reported a culture positivity of 38.8%, which was higher than this study^[38]. In this study, low positivity of culture could be explained by stage of the lymphadenitis, where high number of tubercle bacilli is present in central abscess/necrotic material or the use of only solid L-J media for cultivation not liquid media^[39].

Among LNTB cases, higher positivity of AFB, LJ culture and multiplex PCR was observed in group I (granuloma with necrosis) cytomorphological patterns and this finding was consistent with various studies showing higher positivity (75.6%) of AFB, culture (50%) among cytomorphological

pattern of granuloma with necrosis^[37,40]. But, slightly higher positivity (87.5%) was observed with multiplex PCR assay in non-necrotizing granulomatous cytomorphological pattern which is consistent with a previous study by Tadele *et al*^[37]. This means that multiplex PCR assay has a greater role in the diagnosis of most difficult cytological patterns.

The present study advocates that the use of multiplex PCR assay based on the simultaneous amplification of the species-specific *MTP40* gene, the *IS6110* gene fragment, and the *32kD-alpha antigen gene*, in a single step can identify and distinguished *M. tuberculosis* from *M. bovis* and from other, non-tuberculous mycobacteria. MPCR has many advantages such as less chances of contamination as all reaction takes place in same tube and cost-effective^[13].

In this study, cytological diagnosis supplemented with multiplex PCR assay as well as the microbiological examinations served in establishing a definitive diagnosis of tubercular lymphadenitis in 89.6% of the cases. The negative mycobacterial examination does not exclude the possibility of tuberculosis is apparent from our cases in which the conventional methods were negative for AFB but the MPCR was positive. Weather the cases with negative bacteriological examination should still regard as tuberculosis made easier by performing MPCR assay because of its high sensitivity. The limitation of the PCR assay is that it could not differentiate live from dead bacilli.

CONCLUSION

Multiplex PCR assay is a highly sensitive and specific technique, can play an important role in the rapid diagnosis of LNTB. It is necessary to use such a diagnostic technique especially in infections with paucibacillary conditions where low sample volume is obtained from invasive procedures. To the best of knowledge, we for the first time in India have evaluated MPCR using these three targets for rapid detection and differentiation of MTBC from NTM directly from clinical specimens of LNTB cases.

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