The Journal of International Medical Sciences Academy
2021; (April - June); Vol 34; No. 2

ORIGINAL ARTICLE

Rapid Diagnosis of Tuberculous lymphadenitis using Polymerase chain reaction with IS6110. A 5 year Meta-analytic study with special emphasis on predicted impact of COVID-19 associated social stigma and guidance for the implementation of the End TB Strategies.

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Abstract

Background: Tuberculosis remains one of the major cause of morbidity and mortality in developing countries like India despite intense health campaigning and Government Programmes. Tuberculous lymphadenitis being the most common extra pulmonary manifestation of tuberculosis the incidence still on higher margin especially in resource poor areas which often goes unnoticed and remains as a diagnostic challenge to General Pathologists and sometimes even to Cytopathologists. Moreover the sequelae of COVID-19 pandemic had drastically impacted the management of TB, World Health Organisation had insisted to maintain continuity of essential services for people affected with TB during the COVID-19 outbreak worldwide.

Aims & Objectives: To document the prevalence of tuberculous lymphadenitis among various patient groups and also to advocate the best method in early and prompt diagnosis of tuberculous lymphadenitis with an novel objective of proposing operational guidelines in the strategy of eradicating Tuberculosis.

Materials & Methodology: In the prospective study conducted in SBV University Hospital, Puducherry a total number of 151 cases clinically suspected of tuberculous LN were included and patients with neoplastic lesion were excluded from this study. The lymph node aspirate collected were analysed for tuberculous cytomorphological changes by using routine cytological stains and observations were correlated with results of various ancillary diagnostic procedures.

Results & Observations: Among 151 cases studied, 120 cases showed tuberculosis indicating high prevalence. 31 aspirates showed classical tuberculous pattern on cytomorphology, correlating well with histopathology (100%), Polymerase chain reaction (PCR) (100%) and culture (92%) respectively. In rest of the 119 cases carrying non tuberculous pattern on cytology, further exploration by ancillary diagnostic procedures revealed up tuberculosis in 79 cases with confirmation by culture, Histopathology and PCR.

Conclusion: Our study showed prevalence of tuberculous lymphadenitis is on higher margin especially among low sociodemographic group and cytological procedure in adjunction with ancillary procedures proved to be reliable and helpful to Cytopathologists. PCR procedure could be widely employed by the Government especially in endemic regions with resource poor diagnostic facilities with scope for creating platform of multiplexing with additional resources so that PCR instruments can serve multipurpose testing tool

Keywords: Tuberculosis Lymphadenitis, PCR, COVID-19, Diagnostic tests

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Received: May 2020
Accepted: August 2020

Introduction

Tuberculosis (TB) being a chronic granulomatous infection still remains a major global health problem affecting millions of populations annually worldwide [1]. It is ranks sixth among the top ten causes of death worldwide and tops the table in cases of infectious condition [2]. It had been estimated that by the year 2017, TB infection would present

in all known manifestations 10.4 million with average risk of 1.7 million deaths annually [1,3].

Geographically, Tropical Countries like India still continues to be one among the nation to have highest tuberculosis (TB) burden globally, with an reported incidence of 2.81 million laboratory proved TB cases and among that 147000 MDR/RR-TB cases in 2017 [1,4]. The case load had increased exponentially from 1.2 million to 2 million during short period of 2012 to 2018 [3,4]. World Health Organization [WHO] has recognized India as a vulnerable hotspot zone for TB Epidemic and as a leading cause of mortality accounting to 27% on global rate [1,4,5]. In addition due to various factors and evolvement of new strains, surge in incidence and prevalence of drug resistance of TB (MDR-TB) had also been reported [6].

The high level of inconsistency concerning clinical diagnosis and post mortem outcomes is disturbing among several nations. Although younger children are highly susceptible to the disease condition. TB strains have unique features making it complicated in the diagnosis of the disease.

Regardless of several awareness and management programmes, there is steady rise in rate concerning a number of 7 million new cases being reported and the expected range of 9 to 11 million cases by the end of 2018, worldwide [3,4,6]. Whereas the present global pandemic scenario, all the wherewithal are being focussed on COVID-19 management, TB eradication programmes and management had encountered a transient static which may lead to severe consequences as documented by Research organizations [3,4].

Tuberculosis infection being a T-cell mediated immune infection, it is known to occur in many forms and are broadly categorized into pulmonary and extrapulmonary manifestations of TB [1,7]. Extrapulmonary TB accounts for three-fifth of tuberculous incidence in India with peripheral immune organs especially lymph nodes being the commonest site if manifestation and it is identified in the form of Tuberculous Lymphadenitis [LNTB] [8]. Cervical group of lymph nodes are commonly affected followed by axillary and inguinal group of nodes [9]. An Indian paediatric group study demonstrated prevalence of superficial lymphadenitis as 27.5/1000 children and among the incidence LNTB accounts for 4.61/1000 cases [1,10].

Mycobacterium Tuberculosis [MTB] being the causative organism of LNTB, its diagnosis remains crucial challenge for clinicians and diagnostic laboratory departments [11]. Revised National Tuberculosis Control Programme [RNTCP] proposed Fine needle aspiration cytology [FNAC] along with demonstration for Acid fast bacilli [AFB] from clinical isolated as an essential screening procedure for any suspected LNTB or superficial lymphadenopathy [1,12]. The diagnosis can be made on cytomorphology by demonstrating presence of epithelioid cell granuloma with langhans giant cells and caseating type of necrosis [13,14]. Since past decade of time, FNAC with demonstration of AFB were the validated diagnostic procedure for LNTB owing to its minimal invasive and rapid report in spite of its known limitations [15, 16].

Many instances cytomorphology had many mimickers with differentials, a multisystem approach is warranted to diagnose LNTB which includes culture studies and recently Genotyping expression of MTB by Polymerase chain reactions [PCR] [17, 18]. Mycobacterium culture being highly sensitive method in terms of sensitivity and specificity, it has its own limitations in terms of duration to show positive growth (approximately 6 to 8 weeks). PCR came into existence which is based on targeting sequences to detect MTB from clinical isolates at the level of gene complexes and even species [19]. Various targeting sequences namely Insertion sequences [IS6110] ,H37RV strain TRC4, hsp60,etc are applied on various specimens like FNAC material, tissue biopsy, peripheral blood mononuclear cells, etc. [20,21].

The various diagnostic techniques which are utilized in detecting LNTB had only added to the already prevailing dilemma concerning to the diagnosis [1,22]. While majority of studies conducted in India focusses on Pulmonary TB & its diagnosis, more light needs to be thrown upon Extrapulmonary manifestations of TB especially LNTB and its diagnostic modalities in terms of sensitivity and specificity [22, 23].

COVID-19 Pandemic: Considerations for tuberculosis (TB) care

Globally in the present scenario of COVID-19 pandemic, it is mandatory to ensure that all essential services and programmes dealing with chronic long standing health diseases should keep perusing to protect lives of patients suffering from TB. In India health programmes had been commissioned to combat TB being monitored under 'NIKSHAY portal', still TB remain rampant leading to 0.5 million death every year with 7 million peoples getting affected by the disease annually which is grossly higher that the COVID-19 associated mortality rate. While strategies are framed to eradicate TB by the end 2030, the recent COVID-19 lockdown sequelae had predicted to be retard the testing rate of TB by 75% as per WHO data which is feared to end up with additional of 0.5 million deaths due to TB with India being highly vulnerable. Modelling work had proposed that if the sequelae of COVID-19 pandemic had led to a global reduction of 25% in expected ratio of detection of TB for past 3 months – a realistic possibility of

disruption in TB services being observed in several countries with an anticipation of 13% increase in TB deaths, bringing us back to the incidence of TB mortality noted a decade back.

While the laboratory diagnostic methods for TB and COVID-19 are quite distinct in respect to specimen's nature, molecular technique [PCR is the currently advocated diagnostic method for both the diseases. and individuals being evaluated for both conditions require specimens which are commonly different. The pipeline for effective diagnosis of COVID-19 has flourished impressively within a time span of few months, potential for multiplexing with additional resources is being proposed by widely Stop TB Partnership's Global Drug Facility

Hence the present study was commissioned in our hospital located in resource poor domain with a novel aim to identify the prevalence of LNTB and to analyse the comparative metric of efficacy of various diagnostic tools including histopathological evaluation of lymph node biopsies adding uniqueness to the study analysis.

MATERIALS & METHODOLOGY

Study Design

The prospective cross-sectional study was conducted in the Department of Pathology, Sri Balaji Vidyapeeth (Deemed to be University) located in South-Eastern Coastal part of India mostly covering low sociodemographic population. The study was conducted for a period of 5 years from 2014 to 2019 after obtaining Institute Ethics committee clearance and informed written consent from the patients.

Patient Inclusion criteria

(i) Patients referred to Cytology laboratory with superficial lymphadenopathy with strong clinical acumen and suspicion of LNTB including patients of anti-tuberculous treatment.

(ii) Significant Lymphadenopathy.

Exclusion criteria

Aspirates which showed features of neoplastic lesions including lymphoma, metastatic deposits on cytomorphology were excluded from the study but directed for further work up for malignancies.

Sample Size:

151 cases with lymphadenitis.

Data & Sample collection

The study was categorized into 2 sections.

First Phase included registration of patient details, sample collection by FNAC, culture study for isolated and Zeil-Neelsen studies (ZN stain).

Sample collection

The lymph nodes from the enrolled patients were palpated and aspiration was performed with 23-23 gauge needle as the standard operating procedure (SOP) of our laboratory. A total of five parallel smears were made of the aspirates and subjected to routine cytochemical stains (i). May Grunewald stain (ii)? Papanicalou stain (iii). Two smears for ZN stain for evaluating cytomorphology under light microscope. The staining were performed as per approved guidelines and SOP. A positive case of LNTB on cytomorphology was rendered by presence of epithelioid granuloma, longhand giant cells and caseating necrosis. A positive case on ZN stain is documented by presence of AFB as eosinophilic slender bacilli and confirmed it presence in two consecutive slides.

Under strict aseptic precautions a portion of the aspirated material (0.1-0.2ml) was subjected for culture study and the sample was inoculated on Lowenstein Jensen slant [LJ] medium and incubated 37°C and the slant was screened for growth.

Polymerase chain reaction (PCR)

A portion of the remaining samples left out in the needle was directed to PCR study-conventional type (Qiagen, Hilden, Germany) for DNA genotyping with an insertion element IS6110 oligonucleotide. Totally 3 primers were used one forward and two reverse set for detection of MTB with species. MTB reference strain (H37RV) grown on LJ agar was used as reference strain to document positive cases. An assay mixture with composition 25µl was formulated with the following (i). Forward primer 0.6µM, reverse primer N-tb & BMB–R each. With a composition of 0.325µl with addition of PCR buffer reagents as provided by the manufacturer kit. The primer composition sequences applied are IS-F 5' –CCTGCGAGCGTAGGCGTCGG-3' and IS-R 5' – CTCGTCCAGCGCCGCTTCGG-3'. The positive controls for every reaction was composed of 2 tubes of DNA of MTB (H37RV strain-Ladder pattern). The PCR procedure was run at the temperature of 95°C with amplification of the 123bp fragment of IS6110. First cycle was run at temperature of 95°C for 10 minutes duration and other 45 cycles at same 95°C & 60°C each for a duration of 30 seconds and 72°C for 40 seconds and at last step brought down to room temperature for cooling down. Various plots were generated by picking up positive and negative controls, appropriate melting points etc.

Histopathology Evaluation of Biopsy samples

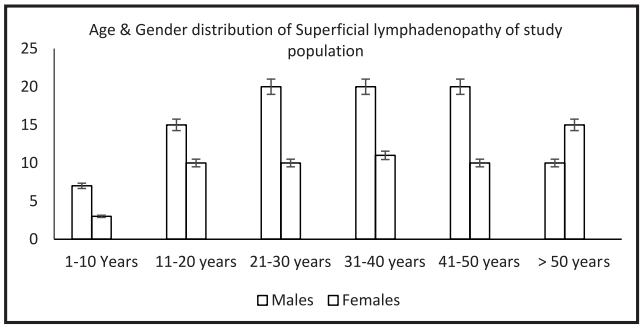
Excision biopsy of lymph nodes were advised in cased

where a definite diagnosis could not be arrived on cytology and culture studies and in patients where there was no response to treatment at clinician's discretion. The biopsy specimens obtained were fixed in 10% formalin and processed by automated tissue processor adopting the 24 hours scheduling. Microscopical sections of 3 to 4 microns were cut for conventional histopathological evaluation with haematoxylin and eosin stain. The histopathological diagnosis of tuberculous lymphadenitis was made based on the morphological presence of typical caseating necrosis with epithelioid granuloma, langhans multinucleated giant cells and ZN stain was performed to look for the presence of bacilli.

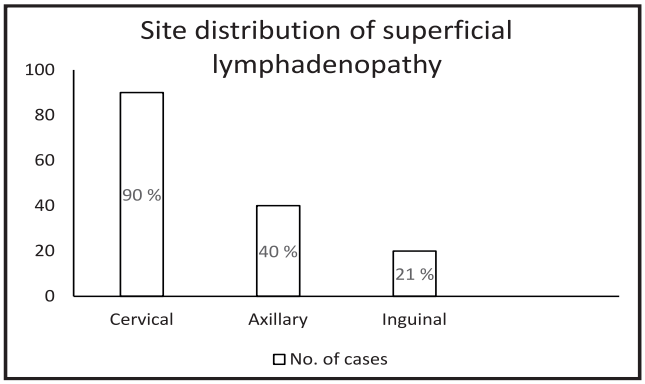
Results & Observations

The study analysed various diagnostic modalities LNTB on 151 suspected patients enrolled in the study. The age incidence ranged between 2 years to 60 years with high incidence from 3rd decade to 5th decade as shown in Graph-1. With regard to gender, male preponderance was observed up to fourth decade with average of Male: Female ration of 2:1 and the ratio started to flatten in equal proportion after 4th decade as shown in Graph-1.

With context to site distribution, cervical group of nodes topped the table (60%) followed by axillary (27%) and Inguinal group (13%) as depicted in Graph-2. The duration of lymphadenopathy ranged between 20 days to 24 months



Graph 1: Age & Gender distribution of superficial lymphadenopathy of study population.



Graph 2: Site Distribution of superficial lymphadenopathy of enrolled patients.

indicating the chronicity of the infection.

The cytomorphological patterns on the smears were categorized into two broad spectrums

(i) Classic granulomatous tubercular pattern constituting 31 cases

(ii) Non-tubercular pattern-which included non-necrotizing (20 cases), suppurative (35 cases), reactive (55 cases) and non-diagnostic (09 cases). Patients with non-tubercular pattern who were on antibiotics started empirically showed poor response to treatment thereby warranting subsequent biopsy and further explorations. The cytomorphological patterns correlated and varied in equal proportion with subsequent Histopathology report of lymph node biopsy and PCR reports from isolated which were elaborated in Table-1. Substantial variations were observed in case of reactive lymphadenitis where 55 cases showing reactive picture on cytology turned out to be LNTB on subsequent biopsy (N=15) and PCR revealed LNTB in 29 cases (n=55). Similar variations were observed in case of acute suppurative and non-necrotizing granulomatous lymphadenitis thereby directing cytopathologists to be judicious while dealing with non-tuberculous pattern on cytology smears.

Classic LNTB on cytomorphology correlated well with subsequent reports whereas non-classical pattern varied significantly reiterating the need for additional diagnostic studies to rule out possibility of LNTB. Interestingly, 8 out of 9 cases which were reported as non-diagnostic on cytology proved to be LNTB on PCR and confirmed on biopsy thereby emphasizing the limitations of cytology.

The data from culture, ZN stain were compared individually and also comparative metric keeping PCR results as reference standard as tabulated in Table-2, from which it is evident that ZN stain has low pick up rate whereas culture study though significant also proved to vary in few cases thereby emphasizing the demerits of standalone procedures.

Statistical Analysis

The data obtained by various diagnostic tests were analysed in terms of sensitivity, specificity, positive predictive value and the diagnostic odds ratio for prevalence.

Keeping histopathological evaluation of lymph node excision biopsy as gold standard methods (n=80/150) and PCR as standard reference methods, the parameters were calculated for TB lymphadenitis =120; True positive (TP) = 108; True negative (TN) = 30; False positive (FP) = 3; False negative (FN) =25

Prevalence of the disease= $T^{disease}$ /Total population X 100(133/151X100= 89%)

Table 1: Comparison of FNAC, histopathology and PCR in patients with superficial lymphadenopathy

Disease group on FNAC Impression	LNTB on Histo-pathology (n=80)	TB positive on PCR (n=120)
Reactive lymphadenitis (N=55)	15 (N=15)	29
Acute suppurative lymphadenitis (N=35)	25 (N=25)	32
Classical tuberculous lymphadenitis (N=31)	10 (N=10)	31
Non-necrotizing Granulomatouslymphadenitis (N=20)	20 (N=20)	20
Others/NDA (N=09)	09	08

Table 2: Comparative metric of conventional procedures with PCR

Diagnostic method (Conventional Procedures)	Total number of clinical sample from isolates	PCR Positive cases	PCR negative cases
TB related cases(n=120)	(N=151)	(N=151)	(N=151)
ZN stain Positive	55	54	05
ZN stain negative	65	66	31
LJ positive for MTB	97	90	10
LJ Negative for MTB	23	25	05
ZN positive	30	30	--
LJ positive	29	30	--
ZN negative	25	25	05
LJ positive	25	22	05
ZN negative	65	25	05
LJ negative	23	05	05

Prevalence of Disease = 9.9%

Positive predictive value (PPV) of the disease: A/ (A+B) X100

PPV= 97%

Negative predictive value of disease: (NPV): D/ (D+C) X100

NPV= 56%

Table 3: Statistical parameters analysis

A TP= 108	**B** FP=03
C FN=25	**D** TN=30
T disease= 133	**T Non disease=33**

Sensitivity and specificity

The parameters sensitivity and specificity being characteristics of tests and calculated by,

Sensitivity: A/ (A+C) X100; Specificity: D/ (D+B) X100.

All the diagnostic test had significant p value with sensitivity and specificity as depicted in Table-4.

When combined diagnostic tests are performed it proved to be significant and reject 'null hypothesis' (with 95% confidence interval) than standalone procedure in diagnosis for LNTB as evident from Table-5 which shows the statistical analysis in quanta. The observations showed the 'power value of the study rejected Type-2 error which is more among studies in tropical countries

Table 4: Statistical Analysis of data (keeping Histopathology as Gold Standard)

Diagnostic procedures	Sensitivity (%)	Specificity (%)	P value
PCR	89	96	0.002
LJ Culture study for MTB	80	86	0.004
ZN staining	57	86	0.006

Table- 5: Summary of statistical analysis and comparative metric values of various diagnostic tools in detection of LNTB (N=120 LNTB)

Diagnostic Procedure			Conventional method to be compared with		Conventional method to be compared with	
			Culture	Culture	ZN stain	ZN stain
			++	--	++	--
PCR IS 6110 H37RV	**Result**	Total	97	23	55	65
¤	(+)	120	115	5	53	65
	(-)	05	5	-	02	05
				95% of confidence level (with 5% error)		95% of confidence level (with 5% error)
Sensitivity			88.4%	90.5 to 96%	55.9%	70.5 to 79%
Specificity			90.5%	91.5 to 96%	84.9%	91.5 to 96%
Positive predictive value			90.9%	82.5 to 66%	86%	82.5 to 92.5%
Negative predictive value			97.9%	80.5 to 86%	65%	60.5 to 70.2%
Disease prevalence			78%	60.5 to 76%	52%	70.5 to 76%
AFB smear	(+)	55	54	-		
	(-)	65	01	05		
				95% of confidence level (with 5% error)		
Sensitivity			65%	54.5 to 75%		
Specificity			96%	80.4 to 92%		
Positive predictive value			90.5%	89.2 to 95%		
Negative predictive value			52.5%	45 to 55.5%		
Disease prevalence			70.2%	70.1 to 81%		

Discussion

The incidence of extra pulmonary manifestation of tuberculosis is highly on increasing margin as proposed by WHO with high morbidity and mortality with emergence of Multi drug resistance [MDR] especially among rural population [1-5]. FNAC procedure is useful rapid diagnostic method for detection of etiology of lymphadenitis [6-8]. Inflammation and swelling of lymph node is one of the common clinical presentation and an indicator of various pathological conditions and it is often treated as a systemic disease [9-11]. In India till date, the diagnosis of LNTB is based primarily on clinical evaluation and lab diagnosis with culture study with many pros and cons [1,2,12]. The present study was also aimed to analyse the efficacy of various diagnostic methods in diagnosing classic as well as non-classical cases with underlying evidence of tuberculous lymphadenitis in high prevalence areas.

Researchers have proposed that effective diagnosis of LNTB could be achieved with a multi-diagnostic approach rather than standalone procedures. Lymphadenitis of granulomatous inflammation is of chronic nature known to yield good amount material on aspirates due to high proliferation of lymphoid population of cells as immune response [14-17].

Cytomorphology detection of LNTB is based on demonstration of epithelioid granuloma with caseating necrosis (Fig-1), but it has its own limitation with a sensitivity and specificity of 65 % and 89% respectively as evident from the present study concords with prior studies done. [22-24]. The reasons attributed to the varied efficacy are multiple factors like foci of aspiration, nature of aspirate, technical expertise, mimickers of several other granulomatous lesions on microscopy thus owning its limitations in clinical situations [25]. Lymphadenitis due to non-MTB are usually resistant to anti-tubercular drugs and very often categorized as MDR-TB. Nevertheless, this technique paves way for collecting materials for further additional examinations [26,27].

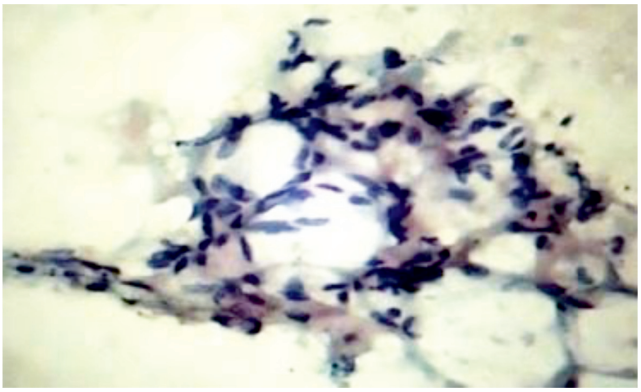


Figure 1: Cytomorphology of lymph node showing epithelioid cell granuloma, Pap-40X

Zeihl Neelsen (ZN) staining is a technique to demonstrate Acid-Fast bacilli (MTB) which has high specificity (96%) despite very low pick-up rate (sensitivity-65%) as observed in our study concurring with previous studies done (Fig-2) [28]. The overall positivity of AFB in aspirates ranges from 36 % to 60 % as seen in the present study as well. The reason for the low sensitivity and low negative predictive value is the bacterial load, foci of aspiration and treatment history [27,28]. The concentration of bacterial load is directly proportional to sensitivity of ZN stain and it has been explored that that a minimum of 10000 bacilli/UL is required for demonstration for ZN stain which is highly uncommon in majority of cases as observed in the study [29].

Figure 2: Zeihl-Neelsen stain showing AFB, ZN-Oil Immersion, 100X

Culture studies is which being more sensitive and specific is considered as gold standard method to demonstrate MTB along with biochemical test. In the present study culture study has a sensitivity of 88.4% and specificity of 90.5%. Prior studies proposes that sensitivity and specificity of culture varies between 40% to 90% in clinically suspected cases of LNTB and our observation falls into the reported range [2,7,27]. Although being highly efficient, it requires 6 to 8 weeks before a positive visual growth is noted. Another major shortcoming in culture study is that it cannot differentiate and categorize speciation of the genus of the mycobacterium thus unhelpful to the Clinicians to initiate treatment since the course of treatment varies according to species [28,29]. In 5 cases culture study was negative where PCR and biopsy was positive for LNTB due to the reason that the patient was already on ATT. All these shortcomings are inherent and Bac T system could be advocated in resource poor areas to avoid longer duration [21,29].

In past decade, various studies had been carried out to emphasize PCR from FNAC aspirates or subsequent excision biopsy of lymph node could improve the sensitivity of diagnosis of LNTB to 99% when compared to conventional techniques alone [21,27]. PCR being highly

sensitive method for detection of MTB in Extra pulmonary LNTB, it has a sensitivity and specificity of 95% and 96% as evident from our study. (Fig -3) The reason for high efficacy is that it could detect MTB even in very low bacterial load concentration of 100/µL whereas other techniques require at least 1 million bacilli/µl to show positive results. The efficacy of PCR was found to be at par with Histopathological evaluation of LNTB (Fig-4). Few cases (5 cases) in the present study were found be negative in PCR with positive on culture studies. The reason was unlike culture, PCR does not distinguish between live and dead MTB, which is also a major limitation as it could not be applied for screening for viable MTB in samples like dairy products, exposure to antibiotics which is known to inhibit MTB. Another major limitation of PCR is challenge to get expertise and financial support as it is relatively expensive procedure [29]. Single cell RNA seq can precisely resolve cellular states for low-cost RNA sequencing of single cells at high throughput.

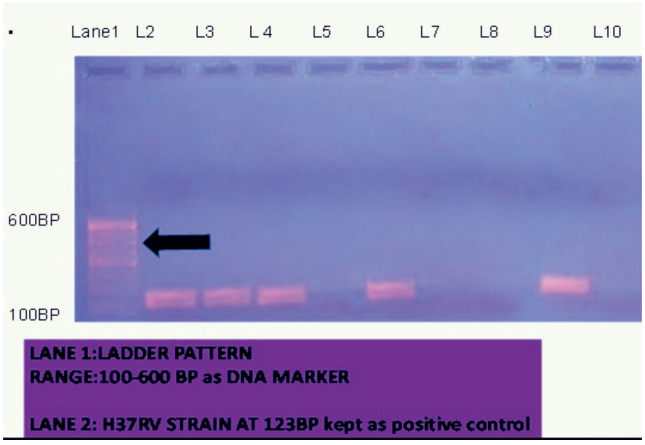


Figure 3: Conventional PCR with IS6110M sequence and H37RV strain with Ladder pattern

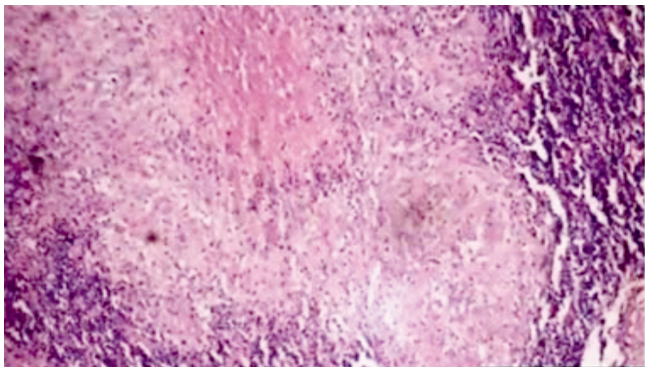


Figure 4: Histopathology of LNTB showing caseating necrosis, H&E-40X-High Power

Critical Appraisal of the study

The present study is cross sectional type with meta-analysis of observations in reference to recent researches on LNTB worldwide including Tropical countries. The incidence of LNTB among South-East Coastal population is substantially higher with diagnostic interpretations showing varied efficacy. While biopsy being an invasive procedure, PCR test serving as a non-invasive highly efficient robust tool in diagnosis of LNTB, situation still remains as a contemplatory vision to get it established in outlying backwoods areas. Hence Government and state health care authorities must step up initiatives on an emergency to establish diagnostic testing instruments with optimal resources at rural areas as per the respective population ratio apart from nodal testing centres. New Resources rolled-out for COVID-19 testing should be mobilised during this episode with scope for multiplexing for testing TB as well, rather than solely relying only on the existing available resources that are used for diagnosing TB, to ensure that the diagnostic coverage for TB is maintained as a mandate.[30-3] While several PCR kits being purchased for testing COVID-19 pandemic at all regions, manufacturer can be instructed to install inbuilt provisions for TB testing with primer and procure critical supply with collaborative partners thereby by making a platform for testing TB as proposed by WHO and Stop TB Partnership's Global Drug Facility [34]. This upgradation in diagnostic equipment will also justify by substantiating judicious and simultaneous use of wherewithal's [36].

Conclusion

In the Modern era of Medicine with several innovative, the incidence of tuberculous lymphadenitis is on higher margin than assumed and expected among the study population and diagnosis still remains a challenging task to clinicians and Pathologists. Combination of diagnostic procedures could serve as a novel method in detection of LNTB especially in patients with poor response to treatment in resource poor areas. Application of molecular procedures for routine diagnosis in country like India depends on varied factors. We conclude with a novelty that even conventional PCR with IS6110 is a robust rapid, economical, sensitive method, it be could be advocated as routine diagnostic tool in primary care centres which warrants minimal infrastructure to complete assays in areas with minimal resources. Health Care Programmes need to be continued that the diagnostic needs of TB patients are not neglected while COVID-19 testing is rolled out with scope for incorporating multiplexing of PCR instruments.

Conflict of interest:	All authors declare no COI
Ethics:	There is no ethical violation as it is based on voluntary anonymous interviews
Funding:	No external funding
Guarantor:	Dr. Anandraj Vaithy K. will act as guarantor of this article on behalf of all co-authors.

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