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Molecular Diagnosis of Tubercular Lymphadenopathy from Fine-Needle Aspirates in Pediatric Patients

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Keywords
Pediatric patients · Real-time PCR · Fine-needle aspirates · Tubercular lymphadenopathy

Abstract
Objectives: The diagnosis of peripheral tubercular lymphadenopathy (TBLN) in pediatric patients is often a challenge because features evident on fine-needle aspiration cytology (FNAC) or tissue biopsy can be deceptive for the reason that they result from an immunological response. This study aimed to evaluate polymerase chain reaction (PCR) for *Mycobacterium tuberculosis* complex (MTBC) in pediatric patients under clinical suspicion for TBLN and to assess its role in the evaluation of cases cytodiagnosed as reactive lymphoid hyperplasia. ***Methods:*** This was a cross-sectional study conducted on 45 pediatric patients clinically suspected and unsuspected for TBLN. FNAC, culture on Löwenstein-Jensen medium, and real-time PCR were performed. Comparative values with reference to the culture were calculated. ***Results:*** Cytology had a sensitivity and specificity of 38.5 and 87.5%, respectively. Real-time PCR had a sensitivity and specificity of 84.6 and 81.3%, respectively. Of the 32 cases with a cytodiagnosis of reactive lymphoid hyperplasia, 53% were positive both on PCR and culture for *M. tuberculosis*; the φ value of 0.93 demonstrated a strong association between these 2 methods. ***Conclusion:*** Real-time PCR is useful in detecting MTBC in pediatric patients, and it also helps in the diagnosis of cases missed on FNAC.

Introduction

Peripheral tubercular lymphadenopathy (TBLN) in the pediatric age group is often associated with nonspecific symptoms and deceptive clinical signs [1, 2]. The diagnosis is done on fine-needle aspiration cytology (FNAC) or tissue biopsy, which is interpreted by the presence of necrosis, i.e., a granular-appearing, necrotic background, together with mature lymphocytes, tangles of epithelioid histiocytes, and giant multinucleated Langerhans-type histiocytes [3]. These features are the result of an immunological response triggered by the *Mycobacterium tuberculosis* antigen, which relies on the development of cell-mediated immunity (CMI).

Bacteriological evidence of *M. tuberculosis* complex (MTBC) is obtained from aspirates or tissue processed with Ziehl-Neelsen (ZN) stain and culture. ZN staining lacks sensitivity and specificity and the culture requires time [4]. Molecular techniques like PCR are rapid, sensitive, and specific, and can be used on lymph node aspirates for the diagnosis of tubercular lymphadenitis.

This study aimed to evaluate the diagnostic intervention of real-time PCR in pediatric tuberculosis (TB) patients clinically suspected or unsuspected for TBLN in relation to cytology and microbiological methods. It also assessed the role of real-time PCR for detecting MTBC upon evaluation of cytodiagnosed cases of reactive lymphoid hyperplasia in pediatric patients.

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Materials and Methods

Study Design and Participants

A cross-sectional study was undertaken by the Department of Pathology, Jawaharlal Nehru Medical College, Datta Meghe Institute of Medical Sciences, Sawangi, Wardha, India, between August 1, 2015, and July 31, 2016.

Pediatric patients presenting with peripheral lymphadenopathy, clinically suspected and unsuspected to be of tubercular origin, at any superficial body site (>2 cm) and not relieved by 2 weeks of antibiotic treatment as per the existing protocol [5] of the Central TB Division, India, were included in the study. All patients previously diagnosed with TB on antitubercular treatment and those with laboratory evidence of HIV infection were excluded from the study.

A complete blood count, measurement of the erythrocyte sedimentation rate, the Mantoux test, and chest X-ray were done as advised by the clinician. The study protocol was explained to the guardians/parents of the study participants and their written consent was obtained. The study protocol was approved by the institutional review board and permission was obtained for ethics clearance (approval letter ref. No. DMIMS(DU)/IEC/2014–15/863).

Fine-Needle Aspiration Cytology

FNAC of the lymph node was done under clinical guidance with a 23-gauge needle under aseptic conditions [6, 7]. The aspirate was divided into 5 parts. One part was smeared onto a slide and fixed immediately with 95% alcohol for Papanicolaou staining [8]. Another two smeared slides were prepared and air-dried for ZN [9] and May-Grünwald Giemsa [8] staining, respectively. One portion of the material was collected in an Eppendorf tube containing sterile PBS and processed for PCR, and the last portion was stored at 4°C for culturing in Löwenstein-Jensen medium.

Culture

For the culture [10, 11], all specimens were fully processed by digestion, decontamination, and concentration. The N-acetyl-L-cysteine and sodium hydroxide method was used for digestion and decontamination. Thereafter, specimens were concentrated by centrifugation at 3,500 *g* for 15 min and resuspended in 1 mL of sterile phosphate buffer (pH 6.8). The processed specimen was used to inoculate Löwenstein-Jensen slants (HiMedia Laboratories).

DNA Extraction and Nucleic Acid Precipitation

Procedure for DNA Extraction [12]

Sample preparation reagent (100 µL; Mylab Lifesolutions Pvt. Ltd., Pune, India) was put into the Eppendorf tubes, 100 µL of processed sample was added, and 1 µL of internal control (Mylab Lifesolutions) was also added to each tube. The tubes were vortexed vigorously for 10–30 s and then placed in a water bath at 100°C for at least 30 min. They were removed from the water bath and allowed to cool to room temperature for 2 min. They were spun in the refrigerated microcentrifuge at 10,000 *g* for 2 min; 50 µL of the clean supernatant was then transferred into a second set of labeled Eppendorf tubes and the remaining supernatant was discarded. The Eppendorf tubes were stored at –20°C. Before use, they were thawed, vortexed, and centrifuged.

Precipitation of Nucleic Acids [12]

The sample extract (50 µL) was transferred into a fresh 1.5-mL Eppendorf tube; 400 µL of 1X TE buffer and 50 µL of sodium acetate (3 M) were added. The tube was vortexed, 500 µL of absolute isopropanol was added, and then it was vortexed again. The sample was allowed to stand at room temperature for at least 15 min, and pelleted by spinning in a microcentrifuge at 13,000 *g* for 10 min at room temperature; 500 µL of 70% chilled ethanol was added and it was centrifuged at 13,000 *g* for 10 min at 4°C. The supernatant was decanted without disturbing the pellet, and the pellet was allowed to air dry. The pellet was resuspended in 50 µL of sterile distilled water; 5 µL of the sample was used for PCR analysis.

M. tuberculosis *Identification by Real-Time PCR [13–16]*

We used the PikoReal™ real-time PCR system by Thermo Fisher Scientific; reagents and kit were obtained from Mylab Lifesolutions. A multiplex real-time PCR based on TaqMan florigenic probes was designed for the detection of the MTBC-specific insertion sequence, IS*6110*, with 71 bp as the final amplified product. An internal positive control composed of synthetic DNA (105 bp) was also used, along with oligonucleotide primers and a dual-labeled hydrolysis probe for the in vitro qualitative detection of *M. tuberculosis*.

Master Mix was prepared using 10 µL of PCR mix, 1 µL *of M. tuberculosis* detection mix, 1 µL of internal positive control detection mix, and 3 µL of nuclease-free water. To this, 5 µL of extracted sample was added. A separate positive control using a positive template and a negative control using nuclease-free water were prepared and run with each reaction setup. The reaction profile included uracil-N-glycosylase incubation at 50°C for 2 min followed by denaturation at 95°C for 10 min. Annealing and extension for 45 cycles was done at 95°C for 15 s and at 58°C for 1 min (for each cycle). FAM was selected for positive signals of MTBC, HEX for the internal positive control, and ROX to minimize the noise of unwanted signals. A signal detected in the fluorescence channels FAM and HEX before 40 cycles was considered as having MTBC DNA. The colony growth on culture was stained with ZN stain for acid-fast bacilli and was also processed for real-time PCR.

Statistical Analysis

Data entry and statistical analysis were performed using SPSS v16.0 (SPSS Inc., Chicago, IL, USA). The results of the cytological diagnosis, the ZN stain, the culture, and PCR were analyzed. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the accuracy of cytology and PCR were calculated against culture (taken as a gold standard). The χ^2 test was done for the association of agreement. $p < 0.05$ was considered statistically significant. The φ value was calculated to assess the relationship between cytology, PCR, and culture. All the results were correlated with findings in the literature, and random results are discussed separately.

Results

Our study included 45 pediatric patients (age range 1–14 years) who presented to the Cytology Department with enlarged peripheral lymph nodes. A total of 35.7% of patients presented only with enlarged lymph node and

no other symptoms. Out of the remaining 64.3%, fever was the most common symptom, i.e., in 52.4% of the total number of cases. 03/15 (20%) of the patients presenting as only having enlarged lymph nodes and no other symptoms were detected as having TBLN, and 18/27 (67%) of patients with clinical symptoms were diagnosed as having reactive lymphoid hyperplasia on cytology. Erythrocyte sedimentation rate was elevated in 25% of the patients with TBLN. The Mantoux test was reported as positive in 40% of the TBLN cases. Chest X-ray was suggestive of TB in 10% of the patients with TBLN.

Cervical lymph nodes were involved in 81% of cases. A cytodiagnosis of TBLN was offered in 12 cases (28.6%), reactive lymphoid hyperplasia in 30 (71.4%), 2 cases had a diagnosis of a cyst, and 1 of suppurative lymphadenitis. Among the 12 cases diagnosed as TBLN, 5 (42%) had features of necrotizing granuloma, i.e., mature lymphocytes with epithelioid cells and necrosis, 3 (25%) showed the presence of granuloma only, and the remaining 4 (33%) had necrosis only. The cases with cyst or suppurative lymphadenitis were negative on ZN staining, in culture, and on PCR, and were not included in the analysis. It was also observed that only 3/12 cases were positive on ZN staining in the TBLN group and none was positive in the reactive group. ZN positivity was 25% in the TBLN cases, and overall positivity was 7%. A histopathological diagnosis was available only in 6/42 cases; TBLN was diagnosed in 1 and the other 5 had reactive lymphoid hyperplasia as a final diagnosis.

Sensitivity, specificity, PPV, NPV, and accuracy of cytology for *M. tuberculosis* detection compared to culture are depicted in Table 1. It was observed that sensitivity of cytology was 38.5% and specificity was 88.2%. No significant relationship could be detected between the cytology and culture diagnoses.

Sensitivity, specificity, PPV, NPV, and accuracy of PCR for *M. tuberculosis* detection compared to culture as a gold standard in the pediatric group are depicted in Table 2. It was observed that sensitivity of PCR was 84.6%. PCR yielded 7% false-positive cases. PCR missed 9% of cases which were culture-positive, possibly because the growth on culture could be due to nontubercular mycobacteria or due to mutation of the nucleic acid sequence of the strain of MTBC which could not be detected on PCR. Accuracy was 79%. A highly significant relationship was observed between PCR and culture diagnosis.

Sensitivity, specificity, PPV, NPV, and accuracy of PCR for *M. tuberculosis* detection compared to culture as a gold standard in cases diagnosed as reactive lymphoid hyperplasia on cytology are depicted in Table 3. Out of 30 cases of reactive lymphoid hyperplasia, 16 (53%) were positive on both PCR and culture. PCR was positive in 17 (56%) cases and culture was positive in 16 (53%) cases. Diagnostic accuracy was 97%. Real-time PCR graphs with Ct values in pediatric patients with a cytodiagnosis of reactive lymphoid hyperplasia are shown in Figure 1.

A histopathological diagnosis of reactive lymphoid hyperplasia was also correlated in 5 cases. On both PCR and culture, 3 were positive for *M. tuberculosis* and 2 were negative.

A highly significant relationship was established between PCR and culture in cases diagnosed as reactive lymphoid hyperplasia. The implication of this is that all lymph node aspirates should undergo PCR and culture, as part of the diagnostic algorithm, to be evaluated for the diagnosis of TBLN.

A strong positive association was observed between PCR and culture results ($\varphi = 0.94$), indicating that both these methods measured the percentage of patients tested that had TBLN even when there was a cytodiagnosis of reactive lymphoid hyperplasia.

Table 1. Comparative results of cytology and culture

Cytology	Culture		Total
	positive	negative	
TBLN	10 (24%)	2 (04%)	12 (28%)
RLH	16 (38%)	14 (34%)	30 (72%)
Total	26 (62%)	17 (38%)	42 (100%)

TBLN, tubercular lymphadenopathy; RLH, reactive lymphoid hyperplasia. Sensitivity 38.5%; specificity 87.5%; positive predictive value 83.3%; negative predictive value 46.7%; accuracy 58.1%.

Table 2. Comparative results of real-time PCR MTBC detection and culture

PCR	Culture		Total
	positive	negative	
Positive	22 (52%)	3 (7%)	25 (59%)
Negative	4 (10%)	13 (31%)	17 (41%)
Total	26 (62%)	16 (38%)	42 (100%)

Sensitivity 84.6%; specificity 81.3%; positive predictive value 88%; negative predictive value 76.5%; accuracy 79%. $p \leq 0.0005$ shows significance (Pearson χ^2 test).

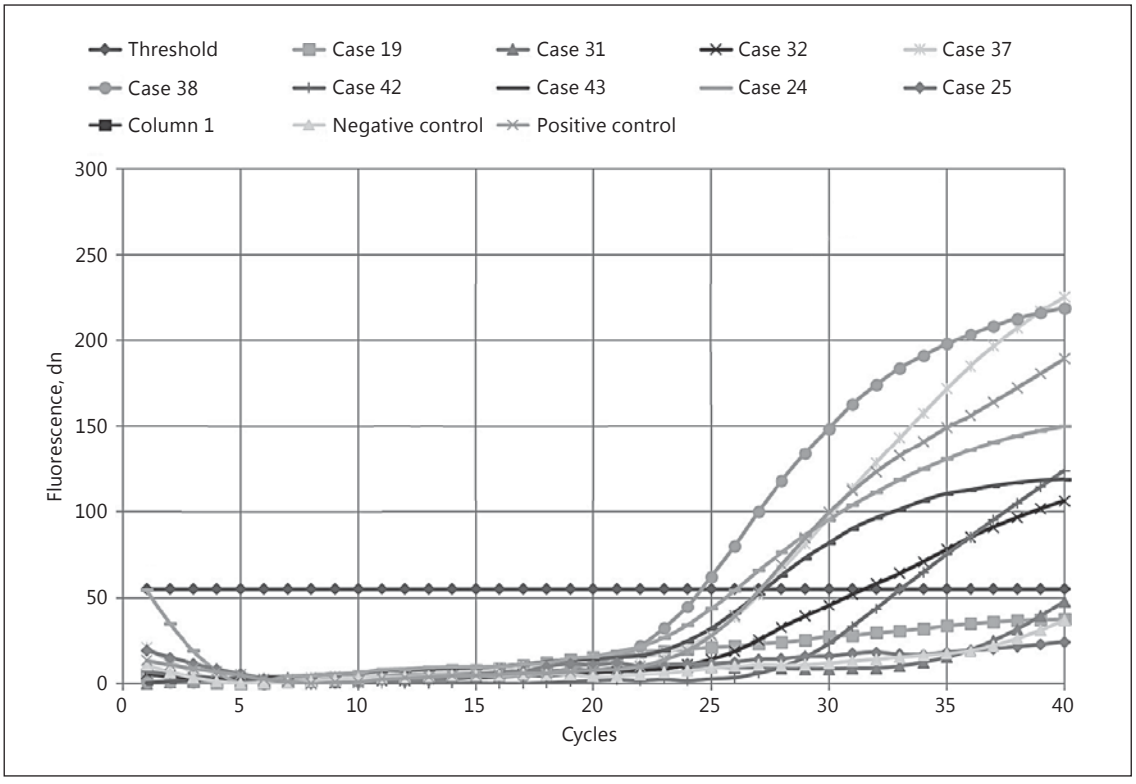

Fig. 1. Real-time PCR graph for different cases (Ct <35) with cytodiagnosis of reactive lymphoid hyperplasia.

Table 3. Comparative results of PCR MTBC detection and culture in reactive lymphoid hyperplasia

PCR	Culture		Total
	positive	negative	
Positive	16 (53%)	1 (3%)	17 (56%)
Negative	0 (0%)	13 (54%)	13 (54%)
Total	16 (53%)	14 (57%)	30 (100%)

Sensitivity 94.1%; specificity 100%; positive predictive value 100%; negative predictive value 92.9%; accuracy 97%. $p \leq 0.0005$ shows significance (Pearson χ^2 test).

Discussion

The group of nodes affected in peripheral lymphadenitis depends on the location of the initial focus of infection, as it is considered part of the primary complex and a manifestation of early postprimary TB. In this study, the most common site of involvement among the peripheral lymph nodes was the cervical lymph nodes, also seen in other studies [6, 17, 18]. Reactive lymph nodes were present in 71.4% of the patients. This is similar to the report by Aljafari et al [6]; in Sharma et al. [17], this group comprised only 30% of the overall study population.

ZN staining for acid-fast bacilli is generally regarded as insensitive for identifying organisms, as it may be dependent on the bacterial load [19]. In this study, ZN staining showed a positivity of 25% in TBLN cases and an overall positivity of 7%. This is in agreement with other studies that reported 22.9% positivity in TBLN cases [18] and 10% overall positivity [19]. Low positivity of ZN staining on smears could be because, in order to demonstrate the bacilli in smears, they should appear in numbers of 10,000–100,000/mL of material [20].

The appearance of cytological patterns is a response to CMI in TBLN, and it ranges from well-defined granuloma with necrosis, to fewer epithelioid and multinucleated histiocytes, and to necrosis only. In the pediatric age group, CMI is not fully developed until 15 years of age, so even though live bacilli are present and grow on culture, the cytological response does not develop. It is likely that such patients may not show any cytological features of TBLN [21]. Difficulty in obtaining bacteriological specimens and

the paucibacillary nature of pediatric disease further contribute to the challenges faced. In this study, 38% cases were positive on culture but did not show any cytological features suggestive of TBLN. . The sensitivity and specificity of cytology in the pediatric population were 38.5 and 87.5%, respectively. Low sensitivity could be associated with immune system development in pediatric patients. In the study conducted by Wright et al. [22] in South Africa, sensitivity and specificity were 84.9 and 50.9%, respectively.

If cytology and ZN staining are negative prior to culture becoming positive, a definitive diagnosis may be delayed for up to 8 weeks, which is unacceptable in regions with a high prevalence of MTBC, as it results in the continued presence of infected and infectious patients in communities and a further spread is likely. To alleviate this, we performed PCR utilizing IS*6110* as a target for MTBC on FNAC samples. The sensitivity of PCR was 84.6% and the specificity was 81.3%, which is in contrast to the study conducted by Wright et al. [23] in South Africa with a sensitivity and specificity of 51.9 and 94%, respectively. Selecting a different target sequence, i.e., one not common in a particular population, could have accounted for such a difference. However, there is a scarcity of studies conducted in Indian pediatric populations to offer further comparison of the results.

PCR could additionally detect 21 cases (12 in the reactive lymphoid hyperplasia group and 9 in the TBLN group) which were missed on ZN staining, proving it to be better for investigation than ZN staining. Similar findings were reported by Raoot and Geeta [24].

In the cases of reactive lymphoid hyperplasia, PCR was positive for MTBC in 17 (56%) cases and culture was found to be positive in 16 (53%) cases. A highly significant relationship was established between PCR and culture in the cases diagnosed as reactive lymphoid hyperplasia. Three out of six cases (50%) with a histopathological diagnosis of reactive lymphoid hyperplasia were also positive on PCR and culture. Our study is the first on pediatric patients to report PCR and culture positivity for TB in reactive lymphoid hyperplasia.

Culture for *M. tuberculosis* infection is the most accurate means of diagnosis available, but, it too, is beset by an irreducible false-negative rate. It is thus possible that the 3 patients who were positive on cytology and/or PCR in this study but negative in subsequent culture did, in fact, have TB. Only a long-term prospective study (clinicoradiological and cytological) incorporating a review of patient records and response to therapy over time could answer this dilemma. An analysis of this nature is outside the capabilities of this laboratory-based study.

The φ value suggested a strong positive association between PCR and culture results, suggesting that both of these methods measure the percentage of patients tested as having TBLN. Ours are the first reference data that have used the φ value at significance to measure the positive association between PCR and culture in a pediatric population.

The insertion sequence IS*6110* shows polymorphic patterns in MTBC as it is present in multiple copies in the genome. There are low-copy-number and high-copy-number strains of MTBC; the former contain <6 copies of IS*6110* and the latter >6 copies. The molecular epidemiology of MTBC isolates in India shows only 17% to be low-copy-number strains [25]. However, out of 6 major lineages of global phylogeny of MTBC, 3 are present in India, and members of these lineages contain the IS*6110* as an insertion sequence [26].

The target insertion sequence adopted and used for TB PCR in this study for the detection of clinically suspected and unsuspected TBLN was for commonly infecting mycobacterium, with reference to the molecular epidemiology of MTBC in India. Nontubercular mycobacteria do not share the targeted insertion sequence IS*6110* and hence could not be detected by the primer and probes used in this PCR. The drug resistance of MTBC cannot be predicted from this study.

Conclusion

PCR is a useful molecular method for the detection of MTBC in pediatric patients, with a high sensitivity, specificity, and accuracy on aspirates of clinically suspected and unsuspected TBLN cases. TBLN in the pediatric population is frequently missed by FNAC of lymph nodes, due to the lack of a cell-mediated immune response. PCR has proved to be highly effective in the diagnosis of such cases.

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Disclosure Statement

We declare no competing interests.

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Author Contributions

V.G. and A.B. contributed to concept and design of study. V.G. carried out the study, did acquisition of data along with analysis and interpretation. V.G. drafted the manuscript and reviewed it and also gave final approval for submitting and publication. V.G. and A.B. helped in drafting and revised the manuscript and also gave approval for the submitting and publication.

References

1 Eamranond P, Jamarillo E: Tuberculosis in children: reassessing the need for improved diagnosis in global control strategies. Int J Tuberc Lung Dis 2001;5:594–603.
2 Starke JR: Pediatric tuberculosis: time for a new approach. Tuberculosis 2003;83:208–212.
3 Mittal P, Handa U, Mohan H, Gupta V: Comparative evaluation of fine needle aspiration cytology, culture and PCR in diagnosis of tuberculous lymphadenitis. Diagn Cytopathol 2011;39:822–826.
4 de Waard JH, Robledo J: Conventional diagnostic methods; in Palomino JC, Leão SC, Ritacco V (eds): Tuberculosis 2007: From Basic Science to Patient Care, ed 1, chapt 12. Antwerp, Kamps and Bourcillier, 2007, pp 401–424.
5 http://tbcindia.nic.in/. New Delhi, Government of India Central Tuberculosis Division Directorate General Health Services. http://www.tbcindia.nic.in/ showfile.php?lid=2904 (updated October 2015; cited 15 June, 2016).
6 Aljafari AS, Khalil EAG, Elsiddig KE, Hag AE, Ibrahim ME, Elsaif MEMO, et al: Diagnosis of tuberculous lymphadenitis by FNAC, microbiological methods and PCR: a comparative study. Cytopathology 2004;15:44–48.
7 Mittal P, Handa U, Mohan H, Gupta V: Comparative evaluation of fine needle aspiration cytology, culture and PCR in diagnosis of tubercular lymphadenitis. Diagn Cytopathol 2010;39:822–826.
8 Bales CE: Laboratory techniques; in Koss LG, Melamed MR (eds): Koss Diagnostic Cytology and Its Histological Bases, ed 5. New York, Lippincott Williams & Wilkins, 2006, pp 1570–1634.
9 Churukian CJ: Pigments and minerals; in Bancroft JD, Gamble M (eds): Theory and Practice of Histological Techniques, ed 6. Philadelphia, Churchill Livingstone Elsevier, 2008, pp 247.
10 Chien HP, Yu MC, Wu MH, Lin TP, Luh KT: Comparison of the BACTEC 960 with Löwenstein-Jensen medium for recovery of mycobacteria from clinical specimens. Int J Tuberc Lung Dis 2000;4:866–870.
11 Central TB Division: Revised National TB Control Programme Training Manual for Mycobacterium Tuberculosis Culture & Drug Susceptibility Testing, April 2009, pp 13–16.
12 Green MR, Sambrook J (eds): Isolation and quantification of DNA; in: Molecular Cloning: A Laboratory Manual, ed 4. New York, Cold Spring Harbor Laboratory Press, 2012, pp 64–65.
13 Van Soolingen D, Hermans PW, de Haas PE, Soll DR, van Embden JD: Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J Clin Microbiol 1991;29:2578–2586.
14 Alonso H, Samper S, Martín C: Otall; Mapping IS6110 in high copy number *Mycobacterium tuberculosis* strains shows specific insertion points in the Beijing genotype. BMC Genomics 2013;14:422.
15 Pierce KE, Wangh LJ: Effectiveness and limitations of uracil-DNA glycosylases in sensitive real-time PCR assays. Biotechniques 2004;36:44–48.
16 Herrera EC, Sperhacke RD, Becker D, Kritski A, Zaha A, Rossetti MLR: Internal control in PCR for *Mycobacterium tuberculosis*: usefulness and improvement of the diagnosis. Braz Arch Biol Technol 2008;51:685–691.
17 Sharma M, Sethi S, Mishra AK, Chatterjee SS, Wanchu A, Nijhawan R: Efficacy of an in-house polymerase chain reaction assay for rapid diagnosis of *Mycobacterium tuberculosis* in patients with tubercular lymphadenitis: comparison with fine needle aspiration cytology and conventional techniques. Indian J Pathol Microbiol 2010;53:714–717.
18 Patwardhan SA, Bhargava P, Bhide VM, Kelkar DS: A study of tubercular lymphadenitis: a comparison of various laboratory diagnostic modalities with special reference to tubercular polymerase chain reaction. Indian J Med Microbiol 2011;29:389–394.
19 Gupta AK, Nayar M, Chandra M: Critical appraisal of fine needle aspiration cytology in tuberculous lymphadenopathy. Acta Cytol 1992;36:391–364.
20 Tiwari RP, Hattikudar NS, Bharmal RN, Kartikeyan S, Deshmukh NM, Bisen PS: Modern approaches to a rapid diagnosis of tuberculosis: promises and challenges ahead. Tuberculosis 2007;87:193–201.
21 Simon AK, Hollander GA, McMichael A: Evolution of the immune system in humans from infancy to old age. Proc Biol Sci 2015;282:20143085.
22 Wright CA, Zyl YV, Burgess SM, Blumberg L, Lieman G: Mycobacterial autofluorescence in Papanicolaou-stained lymph node aspirates: a glimmer in the dark. Diagn Cytopathol 2003;30:257–260.
23 Wright CA, Hoek KG, Marais BJ, van Helden P, Warren RM: Combining fine needle aspiration biopsy (FNAB) and high resolution melt analysis to reduce diagnostic delay in mycobacterial lymphadenitis. Diagn Cytopathol 2010;38:482–488.
24 Raoot A, Geeta D: Detection of mycobacterial DNA directly from FNAC samples of tuberculous lymphadenopathy using real time PCR: a preliminary study. Indian J Tuberc 2013;60:124–127.
25 Purwar S, Chaudhari S, Katoch VM, Sampath A, Sharma P, Upadhyay P, Chauhan DS: Determination of drug susceptibility patterns and genotypes of *Mycobacterium tuberculosis* isolates from Kanpur District, North India. Infect Genet Evol 2011;11:469–475.
26 Brites D, Gagneux S: Old and new selective pressures on *Mycobacterium tuberculosis*. Infect Genet Evol 2011;12:678–685.

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Reactive Lymphoid Hyperplasia or Tubercular Lymphadenitis: Can Real-Time PCR on Fine-Needle Aspirates Help Physicians in Concluding the Diagnosis?

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Keywords
Reactive lymphoid hyperplasia · Tubercular lymphadenitis · Real-time PCR · Fine-needle aspirates · Clinical diagnosis

Abstract
Background: Enlarged lymph nodes in adult patients often present a diagnostic challenge. In the absence of granuloma or necrosis, the cytology/tissue findings are misleading and relate the enlarged lymph nodes to reactive lymphoid hyperplasia (RLH), because granuloma formation is an immunological response that usually takes 14–100 days to develop. This study assesses the role of real-time (RT)-PCR in the diagnosis of the *Mycobacterium* complex (MTBC) in lymph node aspirates compared with culture in cases of RLH. ***Methods:*** A cross-sectional study was conducted on 112 patients, aged 15–74 years, with a diagnosis of RLH on cytology. RT-PCR for MTBC detection and culture on Löwenstein-Jensen medium for tubercular bacilli was done on lymph node aspirates. Comparative values with reference to culture were calculated. The χ^2 value, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratios (LR) were calculated. ***Results:*** Out of 112 RLH cases, 35 (31%) were positive on both RT-PCR and culture. RT-PCR was positive in 43 cases and culture was positive in 44 cases. The χ^2 test was found to be highly significant. PPV, NPV, positive LR, and negative LR were 81.4%, 87%, 6.76, and 0.23, respectively. ***Conclusion:*** RT-PCR for MTBC proves to be useful in arriving at a conclusive diagnosis in patients with a cytological diagnosis of RLH.

Introduction

Enlarged lymph nodes in patients are often a challenge to the physician, especially when patients are asymptomatic. The available investigations for diagnosis are fine-needle aspiration cytology (FNAC) and tissue biopsy. Tubercular lymphadenitis (TBLN) [1] is concluded as the diagnosis if epithelioid cell granuloma is present, with or without multinucleate giant cells and necrosis, on FNAC and/or on tissue biopsy. The epithelioid granuloma formation in tuberculosis is a result of the *Mycobacterium tuberculosis* antigen triggering a cell-mediated immune response, which usually takes 14–100 days to develop [2]. However, the changes that precede the granuloma formation are paracortical hyperplasia (a T cell-mediated im-

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mune response) and/or the accumulation of activated macrophages, which are both considered as evidence of reactive lymphoid hyperplasia (RLH). Patients with such lymph nodes are treated for RLH rather than being given antitubercular treatment.

Diagnostic methods such as Ziehl-Neelsen (ZN) staining provide evidence of tuberculosis by detecting *Mycobacterium* bacilli, but lack sensitivity and specificity [3]. Culture is considered the ideal method for detecting bacilli but is time-consuming [3]. Molecular techniques, such as real-time (RT)-PCR, are sensitive, specific, and rapid, but their role in the diagnosis of *M. tuberculosis* complex (MTBC) has not yet been determined. RT-PCR utilizes an insertion sequence, IS6110, that is specific for MTBC. IS6110 generally occurs in 1–20 copies per cell, dispersed in the *M. tuberculosis* genome, and is an ideal target for amplification. This multicopy insertion sequence IS6110 differentiates MTBC from other mycobacteria [4].

This study assesses the role of RT-PCR in the diagnosis of MTBC in lymph node aspirates in comparison to diagnosis with culture in patients cytodiagnosed as having RLH.

Methods

Study Design and Participants

A cross-sectional study was undertaken in Department of Pathology, Jawaharlal Nehru Medical College, Datta Meghe Institute of Medical Sciences (DU), Sawangi, Wardha, India, between 1 December 2014 and 1 December 2016. The patients presenting with lymphadenopathy in the outpatients department or admitted to hospital underwent FNAC, irrespective of sex and site. Only patients aged ≥15 years were included, as cell-mediated immunity (CMI), that mounts the immune response (when triggered by *M. tuberculosis* antigens), is not fully developed until the age of 15 years [5]. Patients aged ≤14 years were studied separately [6]. All of the patients had peripheral lymphadenopathy, at any superficial body site, and had been diagnosed as having RLH on the basis of cytology or histopathology. All patients on antitubercular treatment and with evidence of HIV were excluded from the study. The study protocol was explained to the enrolled patients and their informed consent was obtained. Institutional review board permission was obtained for ethics clearance (approval letter ref. No. DMIMS(DU)/IEC/2014–15/863). The study is registered under the clinical trial registry of India as No. CTRI/2017/01/007720.

FNA Analysis

FNAC was performed on the lymph nodes using a 23-gauge needle under aseptic conditions, and the obtained material was divided into 5 parts [1, 6, 7]. One part was smeared onto a slide and processed for Papanicolaou staining [7]. From another 2 parts, air-dried smears were prepared, and stained with ZN [8] and May-Grünwald-Giemsa [9] stains, respectively. One portion of the material was collected in an Eppendorf tube containing sterile PBS (pH 6.8) and processed for PCR. Remaining material was stored at 4 °C for culturing in Löwenstein-Jensen (LJ) medium [10].

Culture

All specimens were fully processed by digestion, decontamination (using the N-acetyl-L-cysteine and sodium hydroxide method), and concentration (by centrifugation at 3,500 *g* for 15 min and resuspension in 1 mL of sterile phosphate buffer, pH = 6.8), and finally inoculated on LJ slants obtained from Hi Media (cat. No. SL001, Mumbai, Maharashtra, India) [10, 11].

DNA Extraction and Nucleic Acid Precipitation

To extract DNA, 100 µL of sample preparation reagent (MyLab Lifesolutions Pvt. Ltd., cat. No. PMTBS100, Pune, Maharashtra, India) was put into the Eppendorf tubes, 100 µL of processed sample was added, and 1 µL of internal control (provided in the Pathodetect *M. tuberculosis* detection kit (MyLab Lifesolutions). The tubes were vortexed vigorously for 10–30 s, placed in a water bath at 100 °C for at least 30 min, removed, and then allowed to cool down to room temperature for 2 min. They were spun in the refrigerated microcentrifuge at 10,000 *g* for 2 min; 50 µL of the clean supernatant was then transferred into a second set of labeled Eppendorf tubes and the remaining supernatant was discarded. The Eppendorf tubes were stored at –20 °C. Before use, they were thawed, vortexed, and centrifuged.

To precipitate the nucleic acids, 50 µL of sample extract was transferred into a fresh 1.5-mL Eppendorf tube; 400 µL of 1X TE buffer (Promega, cat. No. V6231, Madison, WI, USA) and 50 µL of sodium acetate (3M) were added. The tube was vortexed, 500 µL of absolute isopropanol (Loba Chemie, cat. No. 271, Mumbai, Maharashtra, India) was added, and then it was vortexed again. The sample was allowed to stand at room temperature for at least 15 min, and then pelleted by spinning in a microcentrifuge at 13,000 *g* for 10 min at room temperature; 500 µL of 70% chilled ethanol was added and it was centrifuged at 13,000 *g* for 10 min at 4 °C. The supernatant was decanted without disturbing the pellet, and the pellet was allowed to air-dry. The pellet was resuspended in 50 µL of sterile distilled water; 5 µL of the sample was used for PCR analysis [12].

M. tuberculosis *Identification by RT-PCR*

The PikoReal™ RT-PCR system (Thermo Fisher Scientific, TCR0096, Foster City, CA, USA) was used. Reagents and kit were obtained from MyLab Lifesolutions. Multiplex RT-PCR based on a TaqMan fluorescence-labeled probe with an FAM fluorophore at the 5-end and a HEX quencher molecule at the 3-end was designed for the detection of *M. tuberculosis*. The MTBC-specific primers were used for insertion sequence IS6110. The final amplified product was 71 bp. An internal positive control (IPC), composed of synthetic DNA (105 bp) was also used, along with oligonucleotide primers and a dual-labeled hydrolysis probe for the in vitro qualitative detection of *M. tuberculosis*.

Master Mix was prepared using 10 µL of PCR mix, 1 µL *of M. tuberculosis* detection mix, 1 µL of IPC detection mix, and 3 µL of nuclease-free water. To this, 5 µL of extracted sample was added. A separate positive control using a positive template and a negative control using nuclease-free water were prepared and run with each reaction setup. The reaction profile included UNG incubation at 50 °C for 2 min and polymerase activation at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s. Annealing and extension

for 45 cycles was done at 60 °C for 15 s and 58 °C for 1 min for each cycle. FAM (6-carboxyfluorescein [6-FAM]) for positive signals of MTBC, HEX (hexachloro-fluorescein) for the IPC, and ROX (6-carboxyl-X-rhodamine) to minimize the noise of unwanted signals, were selected for the reaction profile. A signal detected in the fluorescence channels of FAM and HEX before 40 cycles was considered as having MTBC DNA [13–16].

RT-PCR on all culture isolates was performed by processing using the same protocol. The colony growth on culture was stained with ZN stain for acid-fast bacilli. The physicians concluded that lymph nodes positive on both culture and PCR as TBLN were positive for MTBC.

Data Collection

All data were collected on a standardized form, kept securely and entered online on a password-protected database. Data extracted for research purposes were used for the statistical analysis.

Statistical Analysis

Statistical analysis was performed using the software package SPSS v21.0 (SPSS Inc., Chicago, IL, USA). The results of cytological diagnosis, ZN stain, culture, and PCR were analyzed. The $\chi 2$ test was done to see the association of agreement. $p < 0.05$ was considered statistically significant. The PCR and culture results were cross-tabulated in a 2 × 2 contingency table. PCR- and culture-positive cases were considered true-positive (TP), PCR- and culture-negative cases true-negative (TN), PCR-positive and culture-negative cases false-positive (FP), and PCR-negative and culture-positive cases as false-negative (FN). Sensitivity was calculated as TP/(TP + FN), Specificity was calculated as TN/(TN + FP). Positive predictive value (PPV) was calculated as TP/(TP + FP), negative predictive value (NPV) as TN/(TN + FN), positive likelihood ratio (LR) as sensitivity/(1 – specificity), and negative LR as (1 – sensitivity)/specificity, respectively.

Results

The age of the patients ranged from 15 to 74 years. Gender distribution and lymph node site of 112 patients diagnosed as having RLH on cytology are depicted in Table 1.

The cytology features of 112 cases did not reveal any granuloma, necrosis, or multi nucleate giant cells in the stained smears. The smears showed lymphocytes, macrophages, and immunoblasts. Histopathological diagnosis was available only in 15 of the 112 cases. All 15 cases had RLH as the final diagnosis on histopathology. None of the cases was positive on ZN stain.

Comparative values of PCR for *M. tuberculosis* detection against culture in cases diagnosed as RLH on cytology are depicted in Table 2. Out of the 112 cases of RLH, 35 (31%) were positive on both PCR and culture. PCR was positive in 43 cases and culture was positive in 44 cases. Histopathological diagnosis of 15 cases as RLH was also correlated; 6 (40%) were positive and 8 were negative for mycobacteria on PCR and culture. Reports of PCR and culture in cases diagnosed as RLH were diagnostically complementary.

Table 1. Demographics and lymph node site in 112 patients

	Age range, years	Median age, years	*N*	Percent
Male	15–74	28	54	48.2
Female	17–62	32	58	51.8
Lymph node site				
Cervical			85	75.9
Submandibular			15	13.4
Supraclavicular			10	8.9
Inguinal			2	1.8

Table 2. Comparative results of *M. tuberculosis* detection by PCR and culture in reactive lymphoid hyperplasia

PCR	Culture		Total
	positive	negative	
Positive	35 (31%)	8 (7%)	43 (38%)
Negative	9 (08%)	60 (54%)	69 (62%)
Total	44 (39%)	68 (61%)	112 (100%)

Sensitivity, 79.5%; specificity, 88.2%; positive predictive value, 81.4%; negative predictive value, 87%; positive likelihood ratio 6.76; negative likelihood ratio 0.23; accuracy, 84.8%. The Pearson χ^2 statistic is significant at $p \leq 0.0005$.

Nine (8%) cases were positive on culture but negative on PCR, due to either the growth on culture of nontubercular mycobacteria or mutations in the nucleic acid sequence of the strain of MTBC, which failed to be detected by PCR.

Additionally, 8 (7%) cases were positive on PCR but negative on culture. A highly significant relationship ($p < 0.0005$) was established between culture and PCR in the cytology/histopathology-diagnosed cases of RLH: PPV 81.4%, NPV 87%, positive LR 6.76, and negative LR 0.23, respectively.

PCR could detect 43 (38%) cases of RLH that were missed on ZN staining.

A repeat FNAC could be performed only in 18/52 patients positive by culture and/or PCR after 100 days. These patients were initially cytodiagnosed as having RLH and did not undergo antitubercular treatment. Five of the 18 cases (28%) showed features of TBLN (granuloma formation and necrosis) on cytology. Distribution of culture, PCR, and cytology findings in the 18 cases is depicted in Table 3.

Table 3. Distribution of culture, PCR, and cytology findings in patients with repeat FNA

Cases	Repeat FNA positive for TBLN	Repeat FNA negative for TBLN	Total
PCR only positive	4	4	8
Culture only positive	0	9	9
PCR and culture positive	1	0	1
Total	5	13	18

FNA, fine-needle aspiration; TBLN, tubercular lymphadenitis.

Discussion

The cytological appearance of features ranging from well-defined granuloma with necrosis, to fewer epithelioid cells and/or multinucleated histiocytes, and to only the presence of necrosis is dependent on the immune status of patients which is influenced by socioeconomic, nutritional, and hormonal factors [17].

PCR and culture was positive for MTBC in 35 (31%) cases of the cytology-diagnosed RLH. Six (40%) of 15 cases with a histopathological diagnosis of RLH were also positive on PCR and culture. Our study comprises the first reference data, on patients aged ≥15 years, to report PCR and culture positivity for tuberculosis in cases of RLH. The factors affecting granuloma formation in pediatric patients have been studied previously [6]; granuloma formation is attributed to CMI which is not fully developed in children. However, by the age of 15 years, the CMI is well developed, and it usually takes 14–100 days for the formation of granuloma after exposure to an *M. tuberculosis* antigen [2]. Before the formation of granuloma, there is a presence of paracortical hyperplasia, and smears show lymphocytes, macrophages, and immunoblasts. This implies that the lymph node aspirates should undergo PCR and culture as a part of diagnostic algorithm for evaluating the diagnosis of TBLN at an early stage. The appearance of granuloma after 100 days in 5 of 18 patients subjected to repeat FNAC supports this fact.

PCR alone was positive in 43 (38%) cases. Aljafari et al. [7] reported 10 cases which were PCR-positive in a group of reactive lymph nodes that also responded to antitubercular treatment. Another study [18] demonstrated that 20 of 63 cases of RLH were positive on culture for tubercular bacilli.

There were 9 PCR-negative but culture-positive cases (8%), possibly due to the growth on culture of nontubercular mycobacteria or to mutations in the nucleic acid sequence of the strain of MTBC. A few studies [19, 20] do report such instances, with 10.5% PCR-negative and 48% culture-positive cases, but these particular studies considered only cytology-diagnosed TBLN cases, whereas our study was conducted on cytology-diagnosed RLH cases.

There were 8 PCR-positive but culture-negative cases (7%). This is possibly because the limit of detection (LOD) for culture and PCR are different. Culture needs a minimum of 10 genomic copies of MTB DNA to be positive (LOD 10–100 cfu/mL), compared to RT-PCR using IS6110 that needs only 5 genomic copies of MTB DNA (LOD 5 cfu/mL) [21].

FNAC assumes an important role in the evaluation of peripheral lymphadenopathy as a possible noninvasive alternative to excisional biopsy [1]. RT-PCR performed on aspirates of lymph nodes not only provides early diagnostic evidence of tuberculosis but also helps in arriving at a definitive diagnosis so that the patient can be referred for antitubercular treatment.

Quantitative estimation of genomic copies of *M. tuberculosis* can also be done by RT-PCR but our study aimed only at qualitative detection of *M. tuberculosis* in lymph node aspirates.

The target insertion sequence adopted and used for tuberculosis PCR in the study for the detection of TBLN was for the commonly infecting mycobacteria, in reference to the molecular epidemiology of *M. tuberculosis* in India. Target insertion sequences of atypical mycobacteria, i.e., nontubercular mycobacteria, were not utilized for PCR in this study. This would probably limit the detection of nontubercular mycobacteria, which could be associated with the lymphadenopathy.

Conclusion

Asymptomatic patients with lymphadenopathy on FNAC frequently reveals cytomorphology of RLH. Investigation of such cases by RT-PCR for MTBC on FNA offers a definitive and comparable diagnosis of TBLN. This helps in arriving at a diagnostic conclusion. Policy-makers all over the world, especially in developing and third-world countries, could achieve tuberculosis control if RT-PCR for MTBC were included as a primary investigation by the simple technique of FNA.

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Disclosure Statement

There are no conflicts of interest to declare. The funder had no role in study design, data collection, data analysis, data interpretation, or writing of the report. All the authors had full access to the data and vouch for data integrity and accuracy. The authors were solely responsible for final review and approval of the report. The corresponding author had final responsibility for the decision to submit for publication.

References

1 Mittal P, Handa U, Mohan H, Gupta V: Comparative evaluation of fine needle aspiration cytology, culture and PCR in diagnosis of tuberculous lymphadenitis. Diagn Cytopathol 2011;39:822–826.
2 Capuano SV, Croix DA, Pawar S, Zinovik A, Myers A, Lin PL, Bissel S, Fuhram C, Klein E, Flynn JL: Experimental *Mycobacterium tuberculosis* infection of cynomolgus macaques closely resembles the various manifestations of human *M. tuberculosis* infection. Infect Immun 2003;71:5831–5844.
3 Waard JH, Robledo J: Conventional diagnostic methods; in Palomino JC, Leao SC, Ritacco V (eds): Tuberculosis, ed 1, chapt 12. Antwerp, Kamps and Bourcillier, 2007, pp 401–424.
4 Makeshkumar V, Madhavan R, Narayanan S: Polymerase chain reaction targeting insertion sequence for diagnosis of extrapulmonary tuberculosis. Indian J Med Res 2014;139:161–166.
5 Simon AK, Hollander GA, McMichael A: Evolution of the immune system in humans from infancy to old age. Proc R Soc B 2015; 282:2014–3085.
6 Gupta V, Bhake A: Molecular diagnosis of tubercular lymphadenopathy from fine needle aspirates in pediatric patients. Acta Cytol 2017;61:173–178.
7 Aljafari AS, Khalil EAG, Elsiddig KE, Hag AE, Ibrahim ME, Elsaif MEMO, et al: Diagnosis of tuberculous lymphadenitis by FNAC, microbiological methods and PCR: a comparative study. Cytopathology 2004;15:44–48.
8 Churukian CJ: Pigments and minerals; in Bancroft JD, Gamble M (eds): Theory and Practice of Histological Techniques, ed 6. Philadelphia, Churchill-Livingstone, 2008, p 247.
9 Bales CE: Laboratory techniques; in: Koss LG, Melamed MR (eds): Koss Diagnostic Cytology and Its Histological Bases, ed 5. New York, Lippincott Williams & Wilkins, 2006, pp 1570–1634.
10 Chien HP, Yu MC, Wu MH, Lin TP, Luh KT: Comparison of the BACTEC 960 with Löwenstein-Jensen medium for recovery of *Mycobacteria* from clinical specimens. Int J Tuberc Lung Dis 2000;4:866–870.
11 Central TB Division: Revised National TB Control Programme Training Manual for *Mycobacterium tuberculosis* culture and drug susceptibility testing. New Delhi, Ministry of Health and Family Welfare, 2009, pp 13–16.
12 Green MR, Sambrook J (eds): Isolation and quantification of DNA; in: Molecular Cloning: A Laboratory Manual, ed 4. New York, Cold Spring Harbor Laboratory Press, 2012, pp 64–65.
13 Van Soolingen D, Hermans PW, de Haas PE, Soll DR, van Embden JD: Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J Clin Microbiol 1991;29:2578–2586.
14 Alonso H, Samper S, Martín C: Otal l: Mapping IS6110 in high-copy number *Mycobacterium tuberculosis* strains shows specific insertion points in the Beijing genotype. BMC Genomics 2013;14:422.
15 Pierce KE, Wangh LJ: Effectiveness and limitations of uracil-DNA glycosylases in sensitive real-time PCR assays. Biotechniques 2004;36:44–48.
16 Cortez-Herrera E, Sperhacke RD, Becker D, Kritski A, Zaha A, Rossetti MLR: Internal control in PCR for *Mycobacterium tuberculosis*: usefulness and improvement of the diagnosis. Braz Arch Biol Tech 2008;51:685–691.
17 Purohit MR, Mustafa T, Mørkve O, Sviland L: Gender difference in clinical diagnosis of tuberculous lymphadenitis – a hospital based study from central India. Int J Infect Dis 2009; 13:600–605.
18 Gupta V, Bhake A: Diagnosis of clinically suspected and unsuspected tubercular lymphadenopathy by cytology, culture and smear microscopy. Indian J Tuberc 2017;64:314–317.
19 Pahwa R, Hedau S, Jain S, Jain N, Arora VM, Kumar N, et al: Assessment of possible tuberculous lymphadenopathy by PCR compared to non-molecular methods. J Med Microbiol 2005;54:873–878.
20 Derese Y, Hailu E, Assefa T, Bekele Y, Mihret A, Aseffa A, et al: Comparison of PCR with standard culture of fine needle aspiration samples in the diagnosis of tubercular lymphadenitis. J Infect Dev Ctries 2012;6:53–57.
21 Reed JL, Basu D, Butzler MA, McFall SM: XtracTB assay, a *Mycobacterium tuberculosis* molecular screening test with sensitivity approaching culture. Sci Rep 2017;7:3653.