692/OA/15
Q3/25/6

ORIGINAL ARTICLE

Rapid *Mycobacterium tuberculosis* DNA Detection on Fine Needle Aspirates from Extra Pulmonary Lymph Nodes

Aamer Ikram[1], Syed Azhar Ahmed[3], Farooq Ahmad Khan[1], Muhammad Tahir Khadim[2] and Luqman Satti[3]

Abstract

Objective: To evaluate the diagnostic efficacy of two rapid methods i.e. *Mycobacterium tuberculosis* (MTB) Polymerase Chain Reaction (PCR) on Fine Needle Aspiration (FNA) samples by comparing with cytology of respective site sample.
Study Design: Cross-sectional comparative study.
Place and Duration of Study: Department of Microbiology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan, from July 2010 through November 2013.
Methodology: A total of 105 extra pulmonary lymph nodes aspirates obtained through fine needle aspiration were processed. Cytology and PCR were done on each specimen. Cytology was taken as gold standard.
Results: Out of the total 105 samples, 71 (67.6%) were positive for the MTB PCR while 34 (32.4%) showed negative status. According to FNA cytology (FNAC) results, 72 (68.6%) cases were positive for the disease while 33 (31.4%) were negative. Sensitivity of PCR was 90.3%, specificity 81.8%, positive predictive value (PPV) 91.5%, negative predictive value (NPV) 79.4%, with diagnostic accuracy of 87.6%. Area under the curve was 0.860 ($p < 0.001$).
Conclusion: PCR is a sensitive tool for detection of MTB on FNA samples from EPTB cases. The results are available within few hours which is helpful for the clinicians to initiate therapy.

Key Words: *Fine needle aspirate. Fine needle aspiration cytology. Lymphadenopathy. Mycobacterium tuberculosis. Polymerase chain reaction. Tuberculosis. Tuberculous lymphadenitis.*

INTRODUCTION

Tuberculosis (TB), though preventable infectious disease, continues to haunt the masses across the world and more so in the developing countries. According to the World Health Organization (WHO) estimates, one-third of the world's population stays infected with the silent killer, MTB.[1] Twenty two high TB burden countries account for 81% of all estimated incident cases.[2] Around 95% of the TB cases occur in developing countries with sparse diagnostic and treatment facilities. During the year 2012, 8.6 million new TB cases have been reported with 1.3 million deaths; Asia (58%) and Africa (27%) continuing to lead.[2] The threat of Multidrug Resistant (MDR) and Extensively Drug Resistant (XDR) TB is not only adding to the existing problem but also hampering the control measures further taxing the developing nations.[3] Worldwide, 450,000 new cases of MDR TB were estimated in 2012 with high mortality rates; 9.6% of MDR were XDR.[2]

Presently, Pakistan stands at fifth position among the 22 high TB burden countries.[2] The incidence of TB cases in Pakistan during 2012 was 231/100,000 population with a prevalence of 376/100,000.[2] Poverty, overcrowding, dislodgment and lack of appreciation add exceedingly to the burden as three-fourths of sufferers stay undiagnosed. A multifaceted approach is mandatory for correct and timely diagnosis of TB including correct clinical judgment supported well by diagnostic facilities. WHO recommends molecular-based assays for diagnosis of TB.[4] Sensitive molecular methods can also be employed for contact tracing along with targeted approach.[5,6]

Extra pulmonary TB (EPTB) is an important clinical entity which may pose diagnostic difficulty.[7] According to WHO report 2013, EPTB accounts for 16% of TB cases in Europe and Americas, 17% in South-East Asia, 18% in African region, and 22% in Eastern Mediterranean region.[2] The frequency of extra pulmonary involvement is much higher among HIV positive cases (> 50%).[8] EPTB is most commonly manifested in the form of lymphadenitis.[9] Exact prevalence in Pakistan is not known, however, Butt *et al*. and Ahmed and Aziz reported EPTB frequencies of 25.2% and 33% respectively.[10,11] Extra pulmonary samples reported by the Aga Khan Laboratory, Karachi, Pakistan, amounted to 58% with lymph nodes as the main site.[12] An international study from Hong Kong reported EPTB frequency of 22.3%.[13]

The diagnosis of EPTB is conventionally established through smear microscopy, histopathology and myco-bacterial culture of biopsy material. For the last one and

[1] *Department of Microbiology / Histopathology[2], Armed Forces Institute of Pathology, Rawalpindi.*
[3] *Department of Microbiology, Baqai Medical University, Karachi.*

Correspondence: Dr. Aamer Ikram, 50-A, Lane 4, Harley Street, Rawalpindi.
E-mail: maahin1@yahoo.com

Received: February 13, 2015; Accepted: May 19, 2015.

a half decade, FNA has evolved as an important minimally invasive tool in evaluation of peripheral lymphadenopathy. The modality has been revolutionized by rapidity of testing through molecular methods. On the other hand, performing PCR on FNA sample is not easy because of minute quantity of the sample.

The objective of this study was to study the diagnostic efficacy of two rapid methods i.e. real time MTB PCR on FNA compared with cytological examination of the same site sample.

METHODOLOGY

This cross-sectional comparative study was carried out at Department of Microbiology in collaboration with Department of Histopathology, AFIP, Rawalpindi, Pakistan, from July 2010 to November 2013. The project was formally approved by Institutional Review Board and Institutional Ethical Committee of AFIP. All the data used in this study were anonymized; each case was given an identity number. Written informed consent was sought from all the patients for participating in this study along with permission for their data to be used for research purposes and publication later in any form and journal. In case of minors (less than 18 years), similar written informed consent was taken from the parents of respective patients.

Non-probability convenience sampling was adopted; all patients with clinical suspicion of tuberculous lymphadenitis were included in the study. Patients with diagnosed pulmonary TB and open TB, those already on anti-TB drugs or with previously completed treatment were excluded. Cases of lymphadenopathy that turned out to be negative for tuberculous cytology were taken as control group.

FNA was performed by a competent histopathologist; two samples were taken, one sent to histopathology and other to microbiology department. For histopathology smears were stained with haematoxylin and eosin, Diff-Quick, May Grunwald Giemsa and Papanicolaou Staining.[14] Cytological examination criteria of presence of multiple epithelioid cells with caseation necrosis in the background was taken as hallmark of tuberculous granulomatous inflammation.

For MTB PCR, specimens were transferred to sterile vials for extraction of DNA or storage at -20°C for extraction later. DNA extraction was performed by using Bacterial Xpress nucleic acid extraction reagents (Millipore Merck, USA). IS6110 with 123 bp fragment was the target point in the MTB DNA.

200 µl of bacterial express was transferred to a nuclease free microfuge tube (1.5 ml); 50 µl of sample was added, vortexed for 10 seconds and incubated for 5 minutes at room temperature; 250 µl of isopropyl alcohol was then added, vortexed for 10 seconds and centrifuged at 16000xg for 10 minutes; supernatant was discarded and pellet was washed by adding 400 µl of 70% ethanol, vortexed for 10 seconds and centrifuged (16000xg) for 10 minutes at room temperature; supernatant was removed; short spun for 3 seconds, ethanol was removed; air dried for 10 minutes; 50 µl of diluent was added to dried pellet; and kept at 55°C for 10 minutes. The re-suspended pellet was used for PCR amplification on real time smart cycler using Sacace Biotechnologies MTB Real TM Kit (Italy).[15]

DNA amplification was performed according to the manufacturer's instructions.[15] Briefly, required quantity of reaction tubes (or PCR plate) was prepared for samples and controls; new sterile tubes were prepared for each sample 10 (N+1) µl of PCR-mix-1, 5 (N+1) µl of PCR Buffer Flu, 0.5 (N+1) µl of TaqF DNA polymerase and 0.5 µl of UDG-enzyme; vortexed and centrifuged briefly. To each tube, 15 µl of reaction mix was added; 10 µl of extracted DNA was added to appropriate tube. Each panel was prepared for 2 controls: 10 µl of DNA buffer was added to the tube labeled Amplification Negative Control and 10 µl of C+ MTB and IC was added to the tube labeled Amplification Positive Control. Tubes were inserted in the smart cycler (Cepheid SmartCycler™ Real-Time Thermal Cycler, Fisher Scientific, USA). Sequences of the primers used were: 5'CCTGCGAGCGTAGGCGTCGG3' and 5'CTCGTCCAGCGCCGCTTCGG3'. Results were verified by FAM channel. Cut off value for positive control was 36 and for sample it was 38.

The data was analyzed using Statistical Package for Social Sciences (SPSS) version 17.0 (Chicago, Illinois, US). Qualitative variables were presented as percentage along with 95% confidence interval. Sensitivity, specificity, PPV, NPV and accuracy of MTB PCR were calculated. Various associations were calculated; p-value < 0.05 was considered as significant.

Quality control was ensured at each stage. For cytology, opinions were randomly reconfirmed through another histopathologist; in five TB cytology positive cases, lypmh node biopsy confirmed definitive diagnosis of tuberculous granulomata on histopathology. For PCR, H37Ra ATCC 25177 MTB was used as control strain; positive and negative controls were used with each batch; repeat testing was done on all the doubtful results; and samples were also randomly checked to rule out cross-contamination.

RESULTS

A total of 105 patients were included in the study. Average age of the patients was 30.31 ± 16.18 years with minimum age of 2 years and maximum age of 75 years. Fifty seven (54.3%) patients were females while 48 (45.7%) males with female to male ratio of 1.2:1.

Seventy nine (75.2%) patients had duration of the lymphadenopathy of less than 6 months followed by 14 (13.3%) patients with 12 months or more while 12 (11.4%) patients had duration between 6 to 12 months. Cervical lymph nodes were involved in most of the cases 57 (54.3%); followed by supraclavicular 21 (20%), submandibular 15 (14.3%), axillary 5 (4.8%), supra-sternal 3 (2.9%), 1 (0.9%) each from anterior auricular, posterior auricular, mediastinal and anterior chest regions. Average palpable area (size of 2 sides) of the lesion was 7.516 ± 8.23 cm^2; minimum = 0.01 cm^2, maximum = 49 cm^2. Minimum one-sided size was 1 mm and maximum 80 mm. According to the PCR results, 71 (67.6%) patients were positive for MTB while 34 (32.4%) patients had negative status. According to FNA cytology, 72 (68.6%) patients were positive for the disease and 33 (31.4%) were negative. On cross tabulation of FNA PCR and cytology, true positive cases were 61.9%, true negative 25.7%, false positive 5.7% and false negative 6.7%. Sensitivity of PCR was 90.3%, with 81.8% specificity, 91.5% PPV and 79.4% NPV, with diagnostic accuracy of 87.6% (Table I). Area Under the Curve (AUC) was 0.860 ($p < 0.001$, Figure 1).

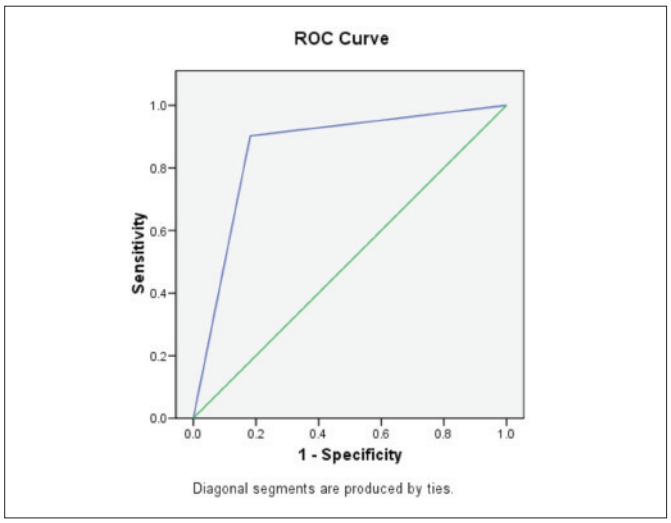

Figure 1: ROC curve for PCR diagnosis with FNAC diagnosis as gold standard.

Table I: Association of FNAC diagnosis with study variables.

Variables	FNAC Diagnosis		p-value
	Positive	Negative	
Age (years)			
≤ 10	7 (9.7%)	4 (12.1%)	0.043
11 - 20	12 (16.7%)	8 (24.2%)	
21 - 30	28 (38.9%)	3 (9.1%)	
31 - 40	13 (18.1%)	6 (18.2%)	
41 - 50	7 (9.7%)	7 (21.2%)	
≥ 51	5 (6.9%)	5 (15.2%)	
Gender			
Female	41 (56.9%)	16 (48.5%)	0.419
Male	31 (43.1%)	17 (51.5%)	
Duration of the lymphadenopathy			
< 6 months	53 (73.6%)	26 (78.8%)	0.687
6 - 12 months	8 (11.1%)	4 (12.1%)	
> 12 months	11 (15.3%)	3 (9.1%)	

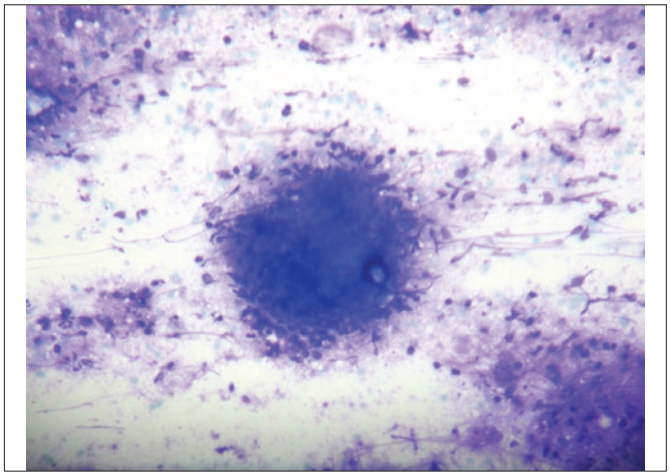

Figure 2: Photomicrograph of FNAC of lymph node showing epithelioid cell granuloma with caseous necrosis. Diff Quick stain (Magnification 40x).

Association of FNAC diagnosis with different variable is shown in Table I. Highest frequency of positive cases was in third decade (38.9%) followed by fourth decade (18.1%). Although in FNAC diagnosed positive cases frequency of females (56.9%) was higher than males (43.1%), the difference was statistically insignificant ($p = 0.419$). Association of FNAC diagnosis with duration of the symptoms of the disease was also statistically insignificant ($p = 0.687$) as frequency of positive and negative cases was higher in patients with duration of less than 6 months i.e. 73.6% vs. 78.8% respectively. Median (interquartile range) of palpable surface area of the lesion was 4.25 cm^2 (2.06 - 8.75) for positive cases and 6 cm^2 (4 - 10.5) for negative cases. The difference was statistically insignificant ($p = 0.184$).

Microscopy of FNA from lymph node of tuberculous pathology showed multiple epithelioid cell granulomata in a background of caseation necrosis; appearance with Diff Quick stain is depicted in Figure 2.

DISCUSSION

Tuberculous lymphadenitis is the most commonly occurring variety of EPTB which poses a diagnostic challenge to the developing world.[9,16] Presence of caseating granulomata in lymph node biopsy or epithelioid cell granuloma with caseation necrosis are important clues for the diagnosis of tuberculous lymphadenitis, however, their absence does not exclude this possibility. Culture of mycobacteria in lymph node tissue material, either obtained by FNA or excision biopsy, still remains the gold standard for definitive diagnosis of tuberculous lymphadenitis. Diagnosis of tuberculous lymphadenitis is cumbersome primarily due to several reasons: mycobacteria in the lymph node tissues are pauci-bacillary or sparse, and *Mycobacteria* in tissue have a propensity to group or clump unlike

respiratory specimens where dispersion is even.[16] PCR has been utilized for the exigent TB diagnosis; still FNA sample has been extremely challenging because of minute quantity, contrarily only two MTB bacilli are required to be amplified to yield a positive PCR.[17]

In this study, the average age of the patients was around 30 years with a wide range of 2 - 75 years. Maximum number of positive cases was in the third decade followed by fourth decade; FNAC diagnosis was significantly associated with age decades (p = 0.043). Although among diagnosed cases, frequency of females was higher than males, the difference was statistically insignificant. Association with duration of the lymphadenopathy was also statistically insignificant; however, the fact that majority of the patients had less than 6 months duration, highlighted early attention due to prominence of superficial lymph nodes. Cervical lymph nodes (54.3%) were most commonly involved site in this study; followed by supraclavicular (20%), submandibular (14.3%), axillary (4.8%) and suprasternal (2.9%) regions. In a study by Singh *et al*. most common site was supraclavicular followed by cervical and axillary regions.[18]

Out of the total 105 specimens, 67.6% were TB positive through PCR and 68.6% by FNAC, results of both the methods were comparable. Sensitivity of MTB PCR (90.3%) turned out to be excellent. Our results were comparable to a regional study by Purohit *et al*. in which the sensitivity and specificity for MTB PCR were 85% and 95% respectively.[19] In that study, DNA was eluted from dried smear of FNA. In another study of 22 cases by Singh *et al*., the sensitivity of PCR for FNA specimen was 55% though specificity was comparable to the present results.[18] Wright *et al*. reported sensitivity of 51.9% whereas specificity was 94%.[20] In a study by Goel *et al*. on 54 cases, sensitivity was 94.4% , however, specificity was very low (38.2%).[21] In the 17 cases studied by Baek *et al*. in 2000, 13 were positive for MTB PCR (76.4%).[22] Additionally, nested PCR on FNA from solitary pulmonary nodules has been reported for good sensitivity and specificity of 87.5% and 96% respectively.[23]

In a study by Coppenraet *et al*., sensitivity of PCR was 71.6% but this study mainly concentrated on atypical mycobacteria.[24] In the study conducted by Linasmita *et al*. on 73 patients, real time PCR sensitivity was 63.4% and specificity 96.9%.[16] Specificity of 81.8% in this study was fairly good. Results of this study were comparable to Pahwa *et al*. with specificity of 86.1% and sensitivity 89.5%.[25] However, in that study, PCR was compared with MTB culture. A specificity of 86% was detected by Singh *et al*., comparable to our results.[18] A higher specificity of 96% was established in a study comparing PCR with conventional culture and ZN staining.[19]

The present PPV and NPV were comparable to the regional study by Singh *et al*., 90% and 75% respectively.[18] Comparing it with Purohit *et al*., PPV (96%) was slightly lower in this study whereas NPV (59%) was much higher.[19] Wright *et al*. reported PPV of 90.3% similar to these results, whereas specificity was 90%.[20] The ROC curve (Figure 1) for the present study demonstrated an overall fine accuracy of the MTB PCR on FNA samples.

There were 6.7% specimens, which were positive by cytology but negative by PCR. This could be due to sampling error, inadequate sampling, undetectable number of bacilli, or incomplete extraction of DNA. 5.7% specimens were false positive; repeat PCR of these specimens were also positive. The reasons for false positive results might be due to cross-contamination which remains a concern, or DNA amplification of non-viable MTB. In this study, cytopathology diagnosed slightly more tuberculous lymphadenitis cases as compared to PCR but this difference was insignificant. Generally, cytopathology has higher sensitivity and lower specificity as compared to PCR. This study had certain limitations: firstly, we compared only FNAC and PCR results; presence of dead bacilli can give positive PCR results; secondly, FNA yields less material and may not be the true representative of area containing MTB.

The progression of molecular methods during the last decade has been tremendous and the diagnostic sensitivity on specimens like tissue biopsy and FNA samples has been encouraging. Recently published studies and especially the present study considering the large sample size augment this status. Presently, PCR is regarded as a powerful tool for MTB detection in specimens from clinically suspected TB cases. It is primarily helpful in providing rapid results especially in cytology negative cases.[18,19] With GeneXpert added to the diagnostic arsenal, rifampicin sensitivity is also readily available. Combination of conventional methods such as culture, Ziehl-Neelsen stain and FNAC along with molecular techniques will help in improving the overall TB diagnostic accuracy. The future plan of authors is to initiate a multicentre study on MTB PCR by GeneXpert and cytology of FNA samples along with other conventional methods to formulate comprehensive protocol for the diagnosis of tuberculous lymphadenitis at different hospital levels in our developing country.

CONCLUSION

PCR has rapidly gained importance for microbiological diagnostics and so has been its utilization for the challenging MTB. FNA is a simple and easy-to-perform minimally invasive procedure. A combination of FNAC and MTB PCR provide the rapidity requisite for the diagnosis for initiating prompt treatment. The results of this study conclude that PCR is a sensitive method for

detection of MTB DNA fragments on FNA samples with a good specificity. The results are available within few hours thus helpful to the clinicians for timely diagnosis.

REFERENCES

1. World Health Organization. Global tuberculosis control: surveillance, planning, financing: WHO Report 2008. Geneva, Switzerland: *World Health Organization;* 2008.
2. World Health Organization. Global tuberculosis control: WHO Report 2013. Geneva, Switzerland: *World Health Organization;* 2013.
3. Tanveer M, Hassan Z, Siddique AR, Ali A, Kanji A, Ghebremicheal S, *et al.* Genotyping and drug resistance patterns of *M. tuberculosis* strains in Pakistan. *BMC Infect Dis* 2008; **8**:171.
4. Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB): Policy Statement. Geneva, Switzerland: *World Health Organization;* 2008.
5. Sotgiu G, Migliori GB. Is tuberculosis elimination a reality? *Lancet Infect Dis* 2014; **14**:364-5.
6. Anderson LF, Tamne S, Brown T, Watson JP, Mullarkey C, Zenner D, *et al.* Transmission of multidrug-resistant tuberculosis in the UK: a cross-sectional molecular and epidemiological study of clustering and contact tracing. *Lancet Infect Dis* 2014; **14**:406-15.
7. Watts HG, Lifeso RM. Current concepts review: Tuberculosis of bones and joints. *J Bone Joint Surg Am* 1996; **78**:288-99.
8. Lee MP, Chan JWM, Ng KKP, Li PCK. Clinical manifestations of tuberculosis in HIV-infected patients. *Respirology* 2000; **5**: 423-6.
9. Mirza S, Restrepo B, Mccormick J, Fisher-Hoch SP. Diagnosis of tuberculosis lymphadenitis using polymerase chain reaction on peripheral blood mononuclear cells. *Am J Trp Med Hyg* 2003; **69**:461-5.
10. Butt T, Kazmi SY, Ahmad RN, Mahmood A, Karamat KA, Anwar M. Frequency and antibiotic susceptibility pattern of *Mycobacterial* isolates from extra-pulmonary tuberculosis cases. *J Pak Med Assoc* 2003; **53**:328-32.
11. Ahmed M, Aziz S. Pattern of tuberculosis in general practice. *J Pak Med Assoc* 1998; **48**:183-4.
12. Javaid A, Hasan R, Zafar A, Ghafoor A, Pathan AJ, Rab A, *et al*. Prevalence of primary multidrug resistance to anti-tuberculosis drugs in Pakistan. *Int J Tuberc Lung Dis* 2008; **12**:326-31.
13. Noertjojo K, Tam CM, Chan SL, Chan-Yeung MM. Extra-pulmonary and pulmonary tuberculosis in Hong Kong. *Int J Tuberc Lung Dis* 2002; **6**:879-86.
14. Bancroft JD, Gamble M, editors. Theory and practice of histological techniques. 5th ed. Edinburg; *Churchill Livingstone;* 2002.
15. MTB Real - TM (Sacace Manual). Sacace Biotechnologies Srl, Italy. REF TB15 - 50FRT: *VER* 28.02.11.
16. Linasmita P, Srisangkaew S, Wongsuk T, Bhongmakapat T, Watcharananan SP. Evaluation of real-time polymerase chain reaction for detection of the 16S ribosomal RNA gene of *Mycobacterium tuberculosis* and the diagnosis of cervical tuberculous lymphadenitis in a country with high incidence. *Clin Infect Dis* 2012; **55**:313-21.
17. Kim SS, Chung SM, Kim JN, Lee MA, Ha EH. Application of PCR from the fine needle aspirates for the diagnosis of cervical tuberculous lymphadenitis. *J Korean Med Sci* 1996; **11**:127-32.
18. Singh KK, Muralidhar M, Kumar A, Chattopadhyaya TK, Kapila K, Singh MK, *et al*. Comparison of in-house polymerase chain reaction with conventional techniques for the detection of *Mycobacterium tuberculosis* DNA in granulomatous lymphadenopathy. *J Clin Pathol* 2002; **53**:355-61.
19. Purohit MR, Mustafa T, Sviland L. Detection of *Mycobacterium tuberculosis* by polymerase chain reaction with DNA eluted from aspirate smears of tuberculous lymphadenitis. *Diagn Mol Pathol* 2008; **17**:174-8.
20. Wright CA, Hoek KGP, Marias BJ, van Helden P, Warren RM. Combining fine needle aspiration biopsy (FNAB) and high-resolution melt analysis to reduce diagnostic delay in mycobacterial lymphadenitis. *Diag Cytopathol* 2009; **38**: 482-8.
21. Goel MM, Ranjan V, Dhole TN, Srivastava AN, Mehrotra A, Kushwaha MR, *et al.* Polymerase chain reaction vs. conventional diagnosis in fine needle aspirates of tuberculous lymph nodes. *Acta Cytol* 2001; **45**:333-40.
22. Baek CH, Kim SI, Ko YH, Chu KC. Polymerase chain reaction detection of *Mycobacterium tuberculosis* from fine-needle aspirate for the diagnosis of cervical tuberculous lymphadenitis. *Laryngoscope* 2000; **110**:30-4.
23. Shim JJ, Cheong HJ, Kang EY, In KH, Yoo SH, Kang KH. Nested polymerase chain reaction for detection of *Mycobacterium tuberculosis* in solitary pulmonary nodules. *Chest* 1998; **113**:20-4.
24. Bruijnesteijn van Coppenraet ES, Lindeboom JA, Prins JM, Peeters MF, Claas ECJ, Kuijper EJ. Real-time PCR assay using fine-needle aspirates and tissue biopsy specimens for rapid diagnosis of *Mycobacterial lymphadenitis* in children. *J Clin Microbiol* 2004; **42**:2644-50.
25. 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**:873-8.