

Comparison of PCR with standard culture of fine needle aspiration samples in the diagnosis of tuberculosis lymphadenitis

Yohannes Derese^{1, 2}, Elena Hailu¹, Tekalign Assefa², Yonas Bekele¹, Adane Mihret¹, Abraham Aseffa¹, Jemal Hussien¹, Ibrahim Ali^{1, 2}, Markos Abebe¹

¹Armauer Hansen Research Institute, Addis Ababa, Ethiopia

²Addis Ababa University, School of laboratory Science, Addis Ababa, Ethiopia

Abstract

Introduction: Lymphadenopathy is the commonest form of extrapulmonary tuberculosis (TB) Clinical diagnosis of TB in lymph nodes requires aspiration of the material and isolation of mycobacteria. Bacterial culture is the gold standard for detection of tubercle bacilli, but it is time-consuming and requires specialized safety procedures and a BSL3 laboratory. However, PCR is a rapid method which requires small volumes of samples and can also be performed on killed bacilli to ensure safety. This project was designed to compare direct fine needle aspirate (FNA) PCR with culture in the diagnosis of tuberculosis lymphadenitis.

Methodology: Direct examination of samples with EZN staining, culture, cytology and PCR was performed on previously collected FNA from the patients with suspected tuberculosis lymphadenitis

Results: In total, 38% of the samples were positive for TB by culture, 11.8% by EZN staining, 23.4% by PCR, and 59.8% by cytology. Cytology had the highest sensitivity (81%) and EZN stain the least (22.9%). The specificity of EZN stain was the highest (92.4%) while cytology was the lowest (50%). In this study, out of 50 culture-positive samples, 21 (42%) were positive by PCR while 8 (10.8%) out of 74 culture-negative samples were positive by PCR.

Conclusions: Although PCR is a sensitive diagnostic method, its sensitivity was shown to be low in this study. Therefore, we recommend that further studies should be conducted on fresh aspirate samples to investigate for possible PCR inhibitors which may limit the sensitivity of PCR diagnosis.

Key words: tuberculosis; lymphadenitis; FNA; PCR

J Infect Dev Ctries 2012; 6(1):53-57.

(Received 28 April 2011 – Accepted 27 July 2011)

Copyright © 2012 Derese *et al.* This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction

Lymphadenitis is the commonest form of extrapulmonary tuberculosis (EPTB) [1] caused by *Mycobacterium tuberculosis* complex or NTM (none tuberculosis mycobacterium) although *S. aureus* or *S. pyogenes* could also be related to this finding [2].

Its pathogenesis is not fully understood and it therefore remains a diagnostic and therapeutic challenge because it mimics other pathologic processes and yields inconsistent physical and laboratory findings. A high index of suspicion is needed for the diagnosis of mycobacterial cervical lymphadenitis. A unilateral single or multiple painless lump can occur usually in the posterior cervical or supraclavicular region. Diagnosis could be made by a thorough history and physical examination, tuberculin testing, staining for acid-fast bacilli, radiologic examination, fine-needle aspiration (FNA) cytology, histology (on biopsy specimen), and PCR. It is also important to

differentiate tuberculous from nontuberculous mycobacterial lymphadenitis to treat the disease because their treatment protocols are different [3]. Definitive diagnosis is often difficult as most of the available techniques are either lower in sensitivity or specificity and others are expensive and require highly skilled manpower.

In developing countries the detection of TBLN is a major challenge [1]. The clinical parameters for the diagnosis of TB in lymph nodes are not specific for some cases and the absence of lymphadenitis does not rule out TB involvement. Clinical features, though indicative of tuberculous etiology, are not adequate for making a definitive diagnosis [4]. Fine needle aspiration cytology (FNAC) is a simple and rapid diagnostic technique but faces several limitations [5]. The conventional Erlich-Ziehl-Neelsen (EZN) method on direct smears for acid fast bacteria AFB is widely used and plays a key role in the diagnosis and also for

the monitoring of treatment, but it has low sensitivity, ranging from 20% to 43% [6] due to the few number of mycobacterial cells in the FNA specimens [3]. Mycobacterial culture is the gold standard method for detection of tubercle bacilli (70% to 80%), but it is time-consuming and requires specialized safety procedures and must be performed in a biosafety level 3 facility [6]. The diagnosis of TBLN performed by either lymph node cytology or biopsy histology lacks specificity due to the difficulty of distinguishing other granulomatous pathologies in the absence of acid-fast bacilli [2]. Serological techniques lack sensitivity and specificity [6]. In developed countries PCR is routinely used to diagnose pulmonary TB, tuberculous meningitis, and lymph node TB [2]. Unlike culture, PCR is a rapid and sensitive method which requires a small volume of sample, and can be performed on killed bacilli; however, it needs highly skilled technologists and the cost could be high. In this study, we compared PCR, EZN staining, cytology and the standard culture methods in the diagnosis of tuberculous lymphadenitis.

Methodology

We completed a retrospective study on previously collected FNA specimens from Woldia, Butajira, and Gonder hospitals as well as from the Gimbi health center in Ethiopia following ethical clearance obtained from the National Research Ethics Review Committee. Specimens were stored at -80°C until used. After being thawed, they were subjected to EZN staining for AFB, TB culture, PCR, and cytology to diagnose lymphadenitis tuberculosis.

EZN staining for the detection of AFB bacilli

Smears were stained with EZN and examined for the presence of AFB under oil-immersion (1000x) using a light microscope [7].

Lowenstein–Jensen medium culture

Four Lowenstein–Jensen (LJ) medium (two with glycerol and two with pyruvate) were used for inoculation and samples were incubated at $35 \pm 2^{\circ}\text{C}$. Slanted bottled media were incubated in a horizontal plane until the inoculum was absorbed (one week), then examined weekly for eight consecutive weeks, during which time the presence or absence of growth was noted. Smears from the LJ media were examined for AFB and the results were recorded [8]. *M. gordonea*, which grows quickly on LJ, was used for culture quality control; *i.e.*, if LJ quality is good, growth is seen after an overnight incubation. Known

positive strains for *M. tuberculosis* and *M. bovis* were used as positive controls.

FNA PCR

DNA extraction was performed as previously described [9]. Briefly, 200 μl of the FNA material was incubated in a water bath at 80°C for 20 minutes to inactivate the bacteria and then diluted with 500 μl of Tris-EDTA buffer (Sigma-Aldrich, Steinheim, Germany). The bacteria were then lysed with 50 μl lysozyme (10 mg/ml) and vortexed before incubation for one hour at 37°C . The lysozyme-treated samples were incubated at 65°C for 10 minutes in the presence of 10 mg/ml of proteinase K and 10% sodium dodecyl sulphate (SDS). A 5 M solution of sodium chloride-cetyltrimethylammonium bromide (NaCl-CETAB) was added to the sample, and phenol/chloroform/isoamyl alcohol (25:24:1) extraction was performed. The DNA precipitate was obtained by adding isopropanol after storage for 30 minutes to 1 hour at -20°C and the DNA was collected by centrifugation at 12,000 RPM for 15 minutes, washed with 70% ethanol, and resuspended in 30 μl of distilled water or Tris-EDTA. Finally, the DNA was stored at 2°C to 8°C until PCR was performed.

PCR was performed using IS 1081 primers, HotStar Master Mix (Qiagen, Germantown, MD, USA), and Qiagen RNase-Free Water (Germantown, MD, USA). A standard PCR series was run (*i.e.*, denaturation at 95°C for 1 minute, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds) for a total of 35 cycles using a thermocycler (Eppendorf, Hamburg, Germany). Next 6 μl of 100 bp of the DNA ladder was mixed with 4 μl of loading dye and loaded into the first well as a reference. Then 5 μl of PCR product from the respective samples was mixed and loaded into the remaining wells. Next 10 μl of the mixture was loaded into the first well and run on agarose gel electrophoresis (prepared 1.5% agarose in 1 x TAE buffer with 5 μl ethidium bromide added to make a 100 ml preparation) subjected to electric power of 110 voltage for 40 to 50 minutes. Finally the presence or absence of mycobacterium complex was detected by exposing the gel and looking for a visible band of 136 bp in reference to the 100 bp DNA ladder on UV light. Known positive strains for *M. tuberculosis* and *M. bovis* were used as positive controls (136 bp), and water was used as negative control.

FNAC

For cytological examination, smears were prepared directly on slides, air-dried, and stained with Wright stain [7]. Slides were examined by a pathologist for characteristics suggestive of TB. Known positive and negative control slides were also used for comparison. Two laboratory technicians blindly rechecked the stained slides.

Statistical analysis

Double data entry and statistical analysis were performed using software packages SPSS version 17 (SPSS Inc, Chicago, Illinois, USA). Negative predictive value, positive predictive value, sensitivity and specificity were calculated. Chi-square test was done along with P-value to see the presence of associations or agreement. P-values less than 0.05 were considered statistically significant.

Results

A total of 134 FNA samples were analyzed in the study; among these, 80 were from females and 54 were from males with the mean age of 28.61 ± 12.73

Table 1. Comparison of PCR with culture

PCR	Culture		Total
	Negative	Positive	
Negative	66	29	95
Positive	8 (10.8%)	21 (42%)	29
Total	74	50	124

years (range two to 60 years). The highest suspicion for TBLN was in the age group 18 to 25 years.

In this study, out of 50 culture positives 21 (42%) were positive by PCR, and out of 74 culture negative samples 8 (10.8%) were positive by PCR (Table 1).

Among 35 culture-positive samples, 8 (22.9%) were AFB positive by ZN stain, and among 66 culture negative samples 5 (7.8%) were positive by EZN stain (Table 2).

As shown in Table 3, among 48 culture-positive samples, 39 (81.3%) were positive by cytology and among 66 culture negative samples, 33 (50%) were diagnosed as TBLN by cytology. Statistically significant correlation was also found between culture

Table 2. Comparison of EZN stain with culture

EZN stain	Culture		Total
	Negative	Positive	
Negative	61	27	88
Positive	5 (7.8%)	8 (22.9%)	13
Total	66	35	101

and direct PCR (p = 0.002), culture and direct ZN stain (p = 0.04) and culture and cytology (p = 0.006).

Table 3. Comparison of cytology with culture

Cytology	Culture		Total
	Negative	Positive	
Negative	33	9	42
Positive	33 (50%)	39 (81.3%)	72
Total	66	48	114

Among the three methods tested, cytology had the highest sensitivity (81%) while EZN stain had the lowest (22.9%) sensitivity. The specificity of ZN was the highest (92.4%) while cytology was the least specific (50%). Positive predictive value (PPV) of PCR (72.4%) was the highest and that of cytology (54.2%) was the lowest. Negative predictive value (NPV) of cytology was the highest (78.6%) but not significantly different from those of PCR and ZN stain, which were 69.5% and 69.3% respectively. PCR had good test efficiency (70.2%) while cytology (63.2%) had the least test efficiency (Table 4).

Table 4. Sensitivity, specificity, positive predictive value and negative predictive value of PCR, ZN stain, and cytological result against the culture

Methods	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Direct PCR	42	89.2	72.4	69.5
ZN stain	22.9	92.4	61.5	69.3
Cytology	81	50	54.2	78.6

Generally, the detection rate of culture was 38.8%, EZN stain was 11.8%, cytology was 59.8% and PCR was 23.4%.

Discussion

TB continues to be a major public health problem especially in developing countries. It has formed a lethal partnership with HIV/AIDS. It is therefore essential to have a reliable diagnostic method that is rapid and cost-effective for early detection and management of TB patients. TBLN is the commonest presentation of EPTB. It is most frequently found in children and young adults [9], which is reflected in our study with most of the TBLN cases falling in the age group 18 to 25 years.

In this study we observed a culture-positivity rate of 38.8% which is in line with the results of other

Figure 1. IS 1081 PCR from DNA extraction of direct FNA sample. A representative gel showing the positive (136 bp) and negative FNA samples



similar studies conducted on FNA samples from TBLN where culture positivity rate was between 17% and 55.5% [9]. The detection rate of PCR was lower (23.4%). The possible reason for the lower detection rate of PCR in this study, which leads to false negative results especially on samples from tissue specimens, is usually the presence of PCR inhibitory substances [10]. Other reasons could be fewer DNA copies available in the specimen or DNA degradation by lytic enzymes. On the other hand, 8 (10.8%) PCR positive samples were negative by culture, which is an indicator of the presence of mycobacterial genomic DNA although live organisms were not detected by culture. This may be due to the presence of dead bacilli, scanty bacilli in the sample, and/or the means of transportation and storage of the sample, as reported by Pahwa R *et al.* [9].

The sensitivity of PCR against the gold standard culture was 42%. In the past decade, experimental studies showed that PCR done on FNA or biopsy specimens from lymph nodes have consistently shown improved sensitivity (61% to 78% depending on the study) when compared with conventional microbiologic methods [11].

In the study by Osoro *et al.*, PCR was positive in 33% of the aspirates from patients. This is a lower

sensitivity compared with other reports for TBLN patients, perhaps due to the small amount of aspirate remaining after splitting the specimen for the additional microbiologic and cytologic assays. In one study, comparative analysis showed biopsies had higher sensitivity than aspirates, presumably due to their larger size. However, in the routine clinical setting, aspirates are preferred because taking FNA is less invasive and easier [11].

PCR positivity of several cases that were culture negative indicates that PCR is more sensitive but it also picks up dead bacilli. PCR is a powerful and reliable technique for rapid diagnosis of *M. tuberculosis*, with a reported sensitivity of 55% to 95% in culture positive samples [9].

A study in Ethiopia by Iwnetu *et al.* has shown the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of histopathology FNAC against culture to be 76%, 88%, 100% and 55%, respectively [12]. In our study, the sensitivity, specificity, PPV, and NPV of FNAC was 81%, 50%, 54.2%, and 78.6% respectively. These results show that cytology had the highest sensitivity (81%), which may lead to over-diagnosis of TBLN. Furthermore, cytology also had low specificity (50%), as TBLN were diagnosed among 66 culture negative samples. In

general, cytopathological morphology in FNAC lacks specificity but has higher sensitivity because non-tuberculous granulomatous patients could also be diagnosed as TBLN. Inexperienced pathologists who read the slides may also add to the low specificity and higher false positivity rate of FNA cytology. Therefore, relying on cytology alone could lead to false case reporting to policy makers as well as wrong patient management and improper use of anti-TB drugs.

In this study, EZN stain for AFB had sensitivity and specificity of 22.9% and 92.4% respectively. This low degree of sensitivity is in line with the findings of other studies with sensitivity ranging from 20% to 43% [6]. In another study, AFB smear positivity was 27.1% [4]. The quality of the smear as well as the scanty bacilli found in the FNA could be the main factor for decreased sensitivity and, as expected, the specificity was the highest of the three methods. However, 7.8% (5/66) of the FNA culture negative smear samples were positive by EZN stain. This result may be due to the presence of dead bacilli that failed to grow on culture.

PCR and EZN detected 10% and 7.8% of the culture negative samples respectively showed that bacilli could be dead during sample transportation and processing, which may also lead to a lower sensitivity of the culture. Although the sensitivity of FNA PCR in this study was lower than expected, its higher specificity as well as higher PPV and NPV are promising.

We recommend further prospective studies on fresh aspirate samples to investigate for possible PCR inhibition. Since FNAC has the highest sensitivity and AFB the highest specificity, we recommend FNAC to be used as a screening tool and when there is high suspicion of false positivity to use AFB or PCR for confirmation of the results. This method would help to provide better management of the disease.

Acknowledgements

The authors would like to acknowledge the assistance of Dr Nebiat Aragie in taking biopsy samples. The cost of this project was covered by NORAD and SIDA through the AHRI core budget.

References

1. Mirza S, Restrepo B, McCormick J, Fisher-hoch SP (2003) Diagnosis of tuberculosis lymphadenitis using a polymerase

- chain reaction on peripheral blood mononuclear cells. *Am J Trop Med Hyg* 69: 461-465.
2. Peters TR, Edwards KM (2000) Cervical Lymphadenopathy and Adenitis. *Pediatr Rev* 21: 399-405.
3. Bayazit YA, Bayazit N, Namiduru M (2004) Mycobacterial Cervical Lymphadenitis. *ORL* 66 :275-280.
4. Dua T, Ahmad P, Vasenwala S, Beg F, Malik A (1996) Correlation of cytomorphology with AFB positivity by smear and culture in tuberculous lymphadenitis. *Ind J* 43: 81-84.
5. Aljafari A, Khalil E, Elsiddig K (2004) Diagnosis of tuberculous lymphadenitis by FNAC, microbiological methods and PCR: a comparative study. *Cytopathology* 15: 44-48.
6. Annam V, Karigoudar MH, Yelikar BR (2009) Improved microscopical detection of acid-fast bacilli by the modified bleach method in lymphnode aspirates. *Indian J Pathol Microbiol* 52: 349-352.
7. Reddy VC, Aparna S, Prasad CE (2008) Mycobacterial culture FNA a useful tool in diagnostic tuberculosis lymphadenitis. *Indian Journal of medical microbiology* 26: 259-261.
8. WHO (2006) "Guidelines on Standard Operating Procedures for Laboratory Diagnosis of HIV-Opportunistic Infections" WHO/SEARO BCT Unit. Available: http://www.searo.who.int/en/Section10/Section17/Section53/Section367_1135.htm. Accessed 17 March 2011
9. Pahwa R, Hedau S, Jain S, Jain N, Arora VM, Kumar N, Das BC (2005) Assessment of possible tuberculous lymphadenopathy by PCR compared to non-molecular methods. *J Med Microbiol* 54: 873-878.
10. Cardoso MA, Cardoso RF, Hirata RD, Hirata MH, Leite CQ, Santos AC, Siqueira VL, Okano W, Rocha NS, Lonardoni MV (2009) Direct detection of *Mycobacterium bovis* in bovine lymph nodes by PCR. *Zoonoses Public Health*. 56: 465-70.
11. Osore F, Oscar NO, Verdonck K, Are'valo J (2006) Clinical Evaluation of a 16S Ribosomal RNA Polymerase Chain Reaction Test for the Diagnosis of Lymph Node Tuberculosis. *Clin Infect Dis* 43: 855-859.
12. Iwnetu R, Hombergh J, Woldeamanuel Y (2009) Is tuberculous lymphadenitis over-diagnosed in Ethiopia? Comparative performance of diagnostic tests for mycobacterial lymphadenitis in a high-burden country. *Scandinavian Journal of Infectious Diseases* 41: 462-468.

Corresponding author

Markos Abebe Alemayehu, PhD
Armauer Hansen Research Institute
PO Box 1005
Addis Ababa, Ethiopia
Telephone: +251911744583
Fax: +251113211563
Email: markosabebe@yahoo.com

Conflict of interests: No conflict of interests is declared.