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THE AETIOLOGICAL CAUSES OF TUBERCULOUS LYMPHADENITIS IN BUTAJIRA, ETHIOPIA

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Table of contents

List of tables

List of figures

Abstract

The utility of MPB70 antigen in serodiagnosis of *M.tuberculosis* complex infection and the polymerase chain reaction (PCR) for rapid identification of the causative agent if cervical lymphadenitis were investigated. The PCR assay was based on detecting a 506-bp DNA segment belonging to the alpha 32 kDa antigen (85B) common in the genus *Mycobacterium,* a 984-bp DNA segment belonging to the insertion sequence IS 6110 specific for the *M. tuberculosis* complex, and a 185-bp *pnc*A gene segment at position 169 allele-specific for genetic differentiation of *M.tuberculosis* from *M. bovis*. For the ELISA purpose, sera from 25 tuberculous lymphadenitis (TBLN), 14 non-tuberculous lymphadenitis (NTBLN), 11 pulmonary tuberculosis (PTB) patients and 10 healthy control (HC) subjects were tested. For PCR, in 39 clinically diagnosed tuberculous lymphadenitis patients, fine needle aspirates (FNA) were processed and tested. Of these, which 14 were considered as non-tuberculous lymphadenitis by fine needle aspirate cytology (FNAC). Of the 39 clinically diagnosed TBLN, 27 (69%) were positive by ELISA. When this was compared with the assumed gold standard (FNAC), it showed 64% sensitivity, 21% specificity, 49% efficiency, 59% positive predictive value and 25% negative predictive value. With PCR, 29 (74%) of 39 were positive, showing 72% sensitivity, 21% specificity, 54% efficiency, 62% positive predictive value and 30% negative predictive value. When it was compared with FNAC.Of the 29 PCR positive for genus *Mycobacterium* and *M.tuberculosis* complex, 18(62%) of them were identified as *M. tuberculosis*, 3 (10%) of them were *M. bovis*

and 8 8%)of them were found co-amplifying *M.tuberculosis* and *M. bovis.* These data indicated that both serodiagnosis with MPB70 antigen of the *M.tuberculosis* complex and PCR assay are useful for rapid identification of tuberculous lymphadenitis and better management of patients.

I. Introduction

Tuberculosis is a leading public health problem worldwide. It affects all ages and both sexes within every socioeconomic group amongst the population .Due to the incompleteness of information system on TB in Ethiopia. The available figures are not reliable. However, in 1995 according to Ministry of Health, in our country TB is the leading cause of morbidity, hospital admission and the 1st cause of hospital death. Health institution reports show an increase in the total number of TB cases. The annual estimate of new cases amount 90.000, of which about 45% are pulmonary tuberculosis positive cases (National Tuberculosis Control Program of Ethiopia, 1997). Tuberculosis can affect any organ in the body other than respiratory organ. Of which lymph node is the one. Lymph node tuberculosis is, occurring mainly in the cervical region.

Lymphadenitis usually appears as an inflammation of a cervical lymph node. It can be caused by infection with *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. africanum* and *M. bovis*) and non-tuberculous mycobacteria (*M. avium intracellulare, M. scrofulaceum*, *M. malmoense*, *M. kansasii* etc) or microorganisms like *Staphylococcus aureus* and *Streptococcus pyogens* (Beiler et al., 1997; Simo *et al.*, 1998). The main infectious agents are *M. tuberculosis* complex and some of the non-tuberculous mycobacteria. It is the most frequent manifestation of extra-pulmonary tuberculosis worldwide (Humphries and Lam, 1998), accounting for about 30% of cases of extra-pulmonary tuberculosis in USA and for more than one half extra-pulmonary TB in immigrants of African and Indian origin in England. In Hong Kong, 62% of extra-pulmonary cases were cervical lymphadenitis (Humphries and Lam,

1998; Sloane, 1996).

Lymphadenitis appears with symptoms ranging from focal lesions to sign of wide spread systemic disease. These include cutaneous and subcutaneous infection, septic arthritis, osteomyelitis, pneumonitis, hepatitis and renal involvement in patients who are immunologically compromised. Cevical lymphadenitis is also a frequent manifestation in immuno-suppressed individuals (Humphries and Lam, 1998; Schwietert and Battegay, 1999).

Lymph node tuberculosis appears as reactivation of primary infections at previously contained foci or by extension from a neighbouring focus (Humphries and Lam, 1998). This implies that the primary infection of the lungs by *M. tuberculosis* complex is disseminated lympho-haematogenously to other organs from where it is reactivated. Reactivation may occur as a result of disease conditions believed to have a direct link to putative cellular immune deficiencies in relation to age, undernourishment, genetic factors, the administration of immunosuppressive drugs and the presence of diseases like diabetes mellitus, lymphoreticular malignancies chronic renal insufficiency, silicosis, and most notably HIV infection which suppress cell mediated immunity (Daniel, *et al.*, 1994).

Immunolgical defects are not a prerequisite, because persons without recorded immunological defects can also be infected. In fact in many countries it is becoming an increasing clinical problem in immunocompetent young children (Haas, *et al.*, 1997).

Lymphadenitis is generally considered as a local manifestation of a systemic infection caused by *M.tuberculosis* .The agent usually enter through the respiratory tract and undergoes lymphohematogenous dissemination to reach the systemic circulation. Generally it presents as

painless neck lesions, which take weeks to months to enlarge. The masses formed frequently involve the jugular, the posterior triangle or supraclavicular lymph nodes (Humphries and Lam, 1998; Sloane, 1996; Wark, *et al.*, 1998).

In contrast, non-tuberculous mycobacterial lymphadenitis is thought to be due to local infection. The pathogens enter through the oropharyngeal mucosa, salivary glands, tonsils or gingival. Non-tuberculous mycobacteria lymphadenitis commonly involves upper cervical lymph nodes. The enlargement of these lymph nodes usually is rapid as compared to lymphadenitis caused by M. tuberculosis and may be associated with fistula formation (Sloane, 1996; Humphries and Lam, 1998; Phillips *et al.*, 1999).

In Butajira, Ethiopia a town 130 Km south of Addis Ababa, a considerable portion of the population is reported to suffer from the disease (Olobo,1998). Unlike lymphadenitis occurring in other countries mostly in children under age 5 (Kawana, *et al.*, 1984; Colvielle, 1993), here it is known mostly to involve young adults. Earlier attempts at AHRI to trace the causative agent described in histological picture, as "granulomas" with necrotic background were able to recover acid-fast bacilli from smears, and rarely culture positive (Kassahun *et al.*, unpublished data). These findings, however, were far from being satisfactory because they would not enable to

rapidly recognize and characterize the causative organism, which is mandatory for appropriate treatment, especially in patients with a complicated clinical course. For example when non-tuberculous mycobacteria lymphadenitis appears, medical therapy with the presently available drugs is not usually successful. The alternative form of treatment at present could be surgical excision of the involved nodes accompanied by chemotherapy.

To take this type of measure requires the correct identification of the causative agent. To this effect several diagnostic methods have been developed. Some of these are: Microscopic diagnosis, PPD skinning test, bacterial culture including biochemical tests, ELISA and PCR. Most of these assays are tedious and time consuming except the last two. Development of rapid, sensitive and specific diagnostic techniques for identification of disease caused by infection with tuberculosis complex and atypical mycobacteria is essential in the control of tuberculosis and other mycobacterioses (Del-Portilollo *et al.,* 1996; Kox *et al.*, 1997). This is to curtail the difficulties present in routine diagnostic methods for identification of the causative agents. A minimum of 5 x 10^3 to 5 x 10^4 bacilli per ml is required for detection by smear under microscope; even if culture detects as few as 10 to 100 viable bacilli, it is time-consuming growth requiring in selective media. On the other hand ELISA requires specific antigens and antibodies for detection with sufficient precision and, this has not been achieved so far as aimed (Del Portillo *et al.,* 1996; Nolte and Metchock, 1995).

Serologic testing for tuberculosis has been extensively studied. However, serologic tests of adequate specificity to give acceptably high predictive values require

availability of selected purified antigens or complex inhibition assays using monoclonal antibodies (Sada *et al.*, 1990).

The most important point that should be born in mind is that the efficiency of ELISA depends upon reagents and preparation (reagents & preparation refer to the selected type of mycobacterial antigen, the concentration of the chemicals, temperature used to kill and the

concentration of the antigen per well).

The MPB70 antigen of the *M. tuberculosis* complex is a well-characterized major soluble secreted protein antigen. Thus, it can be readily prepared in sufficient purity for use in serodiagnosis by simple physicochemical means (Harboe, *et al.*, 1998). MPB70 is a secreted mycobacterial protein with limited species distribution. This protein is highly expressed in *M.bovis* and minimally expressed in *M.tuberculosis*. MPB70 has not been demonstrated in mycobacteria outside the *M. tuberculosis* complex and is an important target antigen of humoral immune responses during infection with bovine and human tubercle bacilli (Wiker *et al.*, 1998)

Immunological diagnosis for detection and identification is helpful. But it requires specific antigens or antibodies for detecting the targeted condition with a high sensitivity and specificity. This is not achieved so far. Thus, the search for less time and high precision giving technique is mandatory to meet what the time demands for treating victims.

The power of the diagnostic test is measured in terms of sensitivity, specificity and predictive value. The sensitivity of an immunoassay refers to as the minimum amount of a particular antigen or antibody that can be detected by a given test. Specificity, particularly with regard to antibodies used in an immunoassay, refers to the ability of the assay to distinguish reactivity with the target antigen from other antigens. The predictive value refers on the other hand to as the probability that the test will correctly indicate the presence or absence of the disease

The clinician practice to commence treatment upon microscopic detection of mycobacteria should be supported with relatively fast diagnostic method (ELISA and PCR) findings on the suspects. Which may avoid unnecessary expense on treatment.

Polymerase chain reaction (PCR)

PCR is a technique, which exploits the amplification of specific sequences of nucleic acids (Baumforth *et al.,* 1999). It is used for rapid differential identification of mycobacteria from clinical samples (Del Portillo *et al.,* 1996).

Before the advent of PCR technology diagnosis of non-respiratory *Mycobacterium tuberculosis* was very challenging, because acid fast staining and mycobacterial culture methods have low sensitivity for detecting non-respiratory tuberculosis, since the specimens collected have a very low quantity of the micro-organisms (Portillo-Gomez *et al.,* 2000). PCR technique operates on amplification of specific sequence of nucleic acids (RNA or DNA), the clinical samples require processing for extraction

of nucleic acids. The introduction of nucleic acid amplification assay into diagnostic mycobacteriology provides with very sensitive, specific and rapid test for the detection of *Mycobacterium tuberculosis* in respiratory and non-respiratory specimens (Gamboa *et al.,* 1998).

The application of PCR in the diagnoses of extra pulmonary tuberculosis, following DNA purification and different *M. tuberculosis* target sequences such as the 32-kDa (85B) antigen (a 506 bp), Insertion sequence element IS6110 (984 -bp). Allele specific amplification method
based on two genetic polymorphisms differentiates *M. tuberculosis* from *M. bovis*.

The 32-kDa (85B) antigens are present in most of the species belonging to the genus *Mycobacterium.* The IS6110 insertion sequence belonging to the *M. tuberculosis* complex has it excessively in *M. tuberculosis* (Del Portillo *et al.*, 1996; Roderiguez *et al.*, 1999).

Rapid differentiation of *M. bovis* from *M. tuberculosis* is based on a characteristic mutation in the bovine pyrazinamidase gene (pncA gene). The *pncA* genes from *M. tuberculosis* and *M. bovis* were cloned and found to have a single point mutation in the pncA gene that appears to be unique to *M. bovis*. This point mutation was the result of a change of C to G at nucleotide position 169 of the *pncA* gene, which causes the substitution of histidine (CAC) with aspartic acid (GAC) at amino acids position 57 in *M. bovis* Pzase (Scorpio *et al.*, 1997; Espinosa *et al.*, 1998).

The available PCR techniques are based either on the amplification of common sequences able to identify mycobacterial DNA or on the amplification of sequences present in specific strains. Using either of the two amplification procedures, some how improved the difficulties on the diagnosis of mycobacterial disease of respiratory and /or extra-pulmonary types in developed countries from either cultured strains or uncultured clinical samples. Extrapulmonary TB such as tuberculous lymphadenitis for which rapid, sensitive and specific diagnosis is needed. This because of the paucibacillary nature of the specimens for which the traditional microbiological methods cannot give positive test result (Singh *et al.,* 2000). Therefore, ELISA and PCR are the choice of procedure for diagnosis to increase sensitivity

and decrease the time necessary to detect the *M. tuberculosis* complex in serological diagno-sis and fine needle aspirate clinical specimens. This condition undoubtedly facilitates rapid initiation of effective anti-tuberculosis treatment for patients.

In view of the need for a rapid, specific and sensitive diagnosis of the causative agents of tuberculous and non -tuberculous cervical lymphadenitis our study was undertaken with the following objectives: -

General objective: - To identify the causative agent(s) of tuberculous lymphadenitis.

Specific objectives:

(1) To assess the relative usefulness ELISA versus FNAC in diagnosis of tuberculous lymphadenitis patients.

(2) To evaluate the usefulness of PCR test on fine needle aspirates with that on fine needle aspirate cytology from the same patients and to compare it

with plus FNAC when they are taken as " gold standard". (3) To compare the sensitivity of ELISA and PCR tests with FNCA

II. Materials and methods

The study was composed of 60 subjects sera and 39 fine needle aspirates from the involved lymph nodes of cervical lymphadenitis patients seen at Butajira Health Center between March 2000 and February 2001. All specimens were collected from Butajira Health Center after getting permission from the Ethiopian science and technology commission (ESTC).

A total of 60 sera were collected: 25 from TBLN patients, 14 from NTBLN patients, 11 from PTB patients, and 10 from healthy individuals.

With the exception of the pulmonary tuberculosis sera obtained at AHRI, the rest were from individuals seen at Butajira Health Center seen individuals. Sera were code-labeled and stored in aliquots at -80^0C until use. Thirty-nine fine needle aspirates from involved lymph nodes were obtained from clinically diagnosed tuberculous lymphadenitis patients. The clinical symptoms suggestive tuberculosis were fever, loss of appetites or weight loss, and lymphadenopathy. Healthy controls were evaluated extensively and found not to have pulmonary tuberculosis or cervical lymphadenitis. The pulmonary tuberculosis patients were confirmed TB patients by sputum smear examination and X-ray.

2.1. Reagents for ELISA

The following reagents and solutions that were used are listed below. All the solutions were prepared according to Molecular cloning A laboratory manual 2^{nd} ed of Sambrok, Fritch and Maniatis(1989).

Purified MPB70: stock solution of 0.5 mg/ml

Antibody: Antihuman immunoglobulin alkaline phosphatase conjugate stock solution 5 mlTween-20

PBS buffer pH 7.4

NaCl	80 g	
Na_2HPO_4	11.6g	
KH_2PO_4	2 g	
KCl	2g	
distilled water	to	10 liters

Blocking buffer (5 mg/ml)

BS (pH 7.4) 500 ml

BSA 2.5mg

Washing buffer

0.1% PBS 10 litters

Tween-20 10 ml

2.2 Reagent for PCR

Tris base	Proteinase K
SDS	Ethidium bromide
EDTA	Boric acid
Lysozyme	Chloroform
CTAB	Isoamyl alcohol
Rnase	NaCl

1x M Tris

Tris base 121.1g

Distilled water 800 ml

10x TE buffer

100 mM Tris

10 mM EDTA

Continued from..

Conc. 42 ml

Dissolve in distilled water

1x TE

Take 1 volume of 10x TE buffer

Dilute it with 9 volumes

Autoclaved, store at room temperature

0.5 M EDTA (pH 8.0)

Na_2 EDTA.$2H_2O$ 186.1g

Distilled water 800 ml

NaOH 20g autoclave

Ethidium bromide (10 mg/ml)

Ethidium bromide 1g

Distilled water 100 ml

Stir several hrs in Aluminum wrapped bottle and store

Lysozyme (10g/ml)

Lysozyme 1g

Distilled water 100 ml

store at -20°C

SDS solution

SDS 10g

Distilled water 100 ml

Dissolve by heating at 65°C for 20 min.

Store at room temperature

CTAB/NaCl solution

NaCl 4.1g

Distilled water 80 ml

While stirring add 10g CTAB

Adjust volume with 100ml distilled water

Proteinase K 100 mg/ml

10 mM Tris HCl (pH 7.5)

20 mM $CaCl_2$

50% glycerol

Store at -20°C

5M NaCL solution

NaCl 29.9g

Distilled water 100 ml

Autoclave

Store at room temperature

TBE buffer

RNase free DNAse

1.Tris base 54g	1. 0.01M sodium acetate (pH 5.2)

Continued from...

2.Boric acid 27.5g	2. Distilled water 100 ml
3.0.5 M EDTA 20 ml	3. Heat to 100° C for 15 min
4.store at room temperature	4.Allow to cool at room temperature adjust the pH by adding 0.1volume of .Tris.Cl (pH 7.4), store at -20°C

The methods used for in the investigation were ELISA (direct antibody detection) and PCR (multiprimers PCR system and allele-specific PCR system).

2.3.Laboratory diagnosis of tuberculous lymphadenitis

2.3.1.ELISA for antibody detection

For direct antibody detection: a) specific antigen is attached to a solid phase surface by adsorption. b) serum to be tested for antibody is added c) enzyme labeled antiglobulin specific for the test serum species is added. d) enzyme substrate is added and the amount of antibody present is determined from the intensity of colour developed. Based on this procedure the sera were tested as follows:

The sera were grouped according to the FNAC finding into TBLN patients' (25), NTBLN patients (14) and controls from pulmonary tuberculosis patients (11) and healthy individuals (10). Purified MPB70 antigen of *M .bovis*, specific for the *M. tuberculosis* complex, was kindly provided by Prof. Morten Harboe Norway.

ELISA was done on flat bottom polyvinyl micro titer 96-well plates. The wells were coated with 100μl of 5mg/ml of MPB70 being incubated at 4^0C for 16 hrs. After four times washing with 0.1% PBS-Tween-20 (pH7.4), blocking with 200 μl of 5% bovine serum albumin (BSA) in PBS (pH 7.4) for 1h at room temperature.

All sera were tested in triplicate at optimal dilution of 1:100 in 0.1% PBS-Tween-20 containing 5% BSA, in parallel with non- coated control wells. Two hours later, the plates were washed as described above. Hundred μl of anti-human immunoglobulins alkaline phosphatase conjugate diluted 1:2000 in 0.1% PBS-Tween 20 was added and kept for 2 hrs at room temperature. The plates were washed four times with 0.1% PBS-Tween-20. A similar volume of p-nitrophenyl phosphate (PNNP) substrate diluted according to the manufacturer's description was then added after four times wash to each well. The substrate reaction was stopped with 25μl of 3M NaOH after 30 min. The optical density was read at 405 nm optical densities on a Titertek Multiskan Plus ELISA reader.

2.3.2 Fine needle aspirates

The laboratory diagnosis of tuberculous lymphadenitis was made either if the aspirate revealed acid fast bacilli (AFB) on Ziehl-Neelson (ZN) staining and/or revealed caseating granulomas with or without multinucleated giant cells /AFB on cytological examination. Those in the non- tuberculous lymphadenitis group were pyogenic and negative for TB on the basis of the fine needle aspirate cytology

(FNAC)/AFB smears. On the basis of mentioned criteria the patients were grouped as tuberculous lymphadenitis (TBLN) and non-tuberculous lymphadenitis (NTBLN).

Aspirates from the involved lymph node were divided into four portions. One portion was smeared on slide directly and subjected to ZN staining at Butajira Health Center. Two other portions were concentrated and made into slide smears air-dried and subjected to ZN and May Grunwald Geimsa staining byDr.Yohannes Negusse at AHRI. If epitheloid cell granulomas,

with or without multinucleated giant cells and caseation necrosis or AFB were seen this was considered positive for TB. The fourth portion was smeared on a slide and sterile distilled water cleaned portion of the samples of the aspirates was poured into eppendrof tubes were rendered for PCR assay. These fourth portions of the aspirates were stored at -70^{0}C until processed for PCR.

2.3.3. PCR

A modified protocol of (Del Portillo *et al.*, 1996 and Espinosa *et al.*, 1998) was adopted: Thirty-nine fine needle aspirates (FNA) from involved cervical lymph nodes of patients were taken.

FNA from cervical lymph nodes of tuberculous lymphadenitis patients (confirmed TB by AFB smears, FNAC or culture) were obtained from Dr. Yohanns Negesse , and known *M. tuberculosis* and *M. bovis* were received from W/zt Haimanot G/Igziabehare of AHRI. .Primers for PCR were obtained from Tib Molbiol (Berlin, Germany) and Ready to go beads for PCR were obtained from Amersham

Pharmacia Biotec (Viena, Austria).

2.3.4.Extraction of mycobacterial DNA

Two hundred micro liters of each specimen was initially heated for 20 min at 80 ^{0}C to kill the mycobacteria. The tubes were centrifuged for 5 min at room temperature at 12000 xg and the pellets were resuspended in 500µl Tris HCl ethylenediaminetetra acetic acid (EDTA) 1x TE (10 mM Tris HCl (pH 8.0) 1 mM EDTA) buffer containing 50µl of lysozyme for cell wall and membrane digestion (10mg/ml) and incubated for 1hr at 37 ^{0}C. Six µl of proteinase K and 70µl of 10% sodium dodecyl sulfate (SDS) were added, and incubated at 65°C for 10 min. To this 100µl of 5M NaCl and 80µl of cetyltrimethylammonium bromine (CTAB)/NaCl solutions were added for complexing with the cell walls,proteins and polysaccarides debris and allowing them to be precipitated by ensuring that the nucleic acidsdo not complex with CTAB. The mixture was vortexed until the liquid content became white ('milky) and then incubated for 10 min at 65^0 C. Here after to the micro tube containing the mix an equal volume (0.7ml) of chloroform /isoamyl alcohol (24:1) was added for denaturation of the proteins, removing lipids components and preventing foaming respectively. It was then vortexed for 10 seconds, centrifuged at room temperature for 5 min at 12000 xg. The supernatant was transferred to a fresh eppendrof tube and 0.6 volume of isopropanol was added to precipitate the nucleic acids. After placing it at least for 1h at -20 ^{0}C, it was spinned for 15 min. at room temperature in a microcentrifuge at 12000xg for recovering DNA. The pellet DNA was washed and resuspended with 1 ml of cold 70% ethanol to remove cetyltrimethylammonium bromine (CTAB) /NaCl for 5 min. at 12000xg. The supernatant was removed carefully and dried for 5 min in the microtubes. Finally the pellet was dissolved in 20µ l of

sterile distilled water and kept at -20^0C until use. (Prior to use in PCR usually the solution is treated with RNase Jaber *et al.,* 1995).

2.3.5.Primer design

The desired target sequence of amplification in PCR relies on both the design and optimal utilization of the primer pair (Baumforth *et al.,* 1999). To this effect multiprimers system, which involves the use of two sets of primers, each annealing to different mycobacterial genomic fragments. The system has allowed us to to amplify the genus *Mycobacterium* and *M. tuberculosis* complex 5µl of DNA extract. And pncA system for discriminating *M.bovis*

from *M. tuberculosis.*

2.3.6.Optimization

From the above procedure one can assume the biochemical reactions in PCR are easy, but involves a basic complex kinetic interaction among essential components. By repetitively rising and lowering the temperature of the mixture components for a considerable length of times attain the desired quantity of amplification. As the result of this the PCR has become a slightly intricate and yet not fully understood dynamic biochemical brew with constantly changing kinetic interactions. There are a number of parameters, which influence better results, despite the complexity of the technology. Adjustment of some of the parameters such as alteration of the concentration of primers and DNA as well as annealing and extension time including the temperature were done to improve specificity and yield of the PCR.

2.3.4.Amplification and Detection of Mycobacterial DNA

The targets for PCR assay were the following genes: One coding the 32-kDa (85B) proteins, the IS6110 insertion sequence and the allele- specific pncA gene were the forward and reverse primers for amplification of these genes. The names and the sequences of the primers are shown below.

Primer name	**Sequence**	**Target to be amplified**
MT1 MT2	F (5'-TTCCTGACCAGCGAGCTGCCG-3') R (5'-CCCAGTACTCCCAGCTGTGC-3')	Gene coding for 32kDa
IS5 IS6	F (5'-GAGACGGTGCGTAAGTGG-3') R (5'-GATGGACCGCCAGGGCTTGC-3')	IS6110 insertion element
pncATB 1.2 pncAMT 2 pncAMB 2	F (5'-ATGCGGGCGTTGATCATCGTC-3') R (5'-CGGTGTGCCGGAGAAGCGG-3') R (5'-CGGTGTGCCGGAGAAGCCG-3')	Allele-specific *pncA* gene

For each allele- specific method DNA samples were subjected to two differential amplifications in two separate tubes. Both reactions were performed with the same forward primer pncATB-1.2 and one of the two-discriminator primers, pncAMT-2 for *M. tuberculosis* or pncAMB-2 for *M. bovis* as shown in Fig.1.

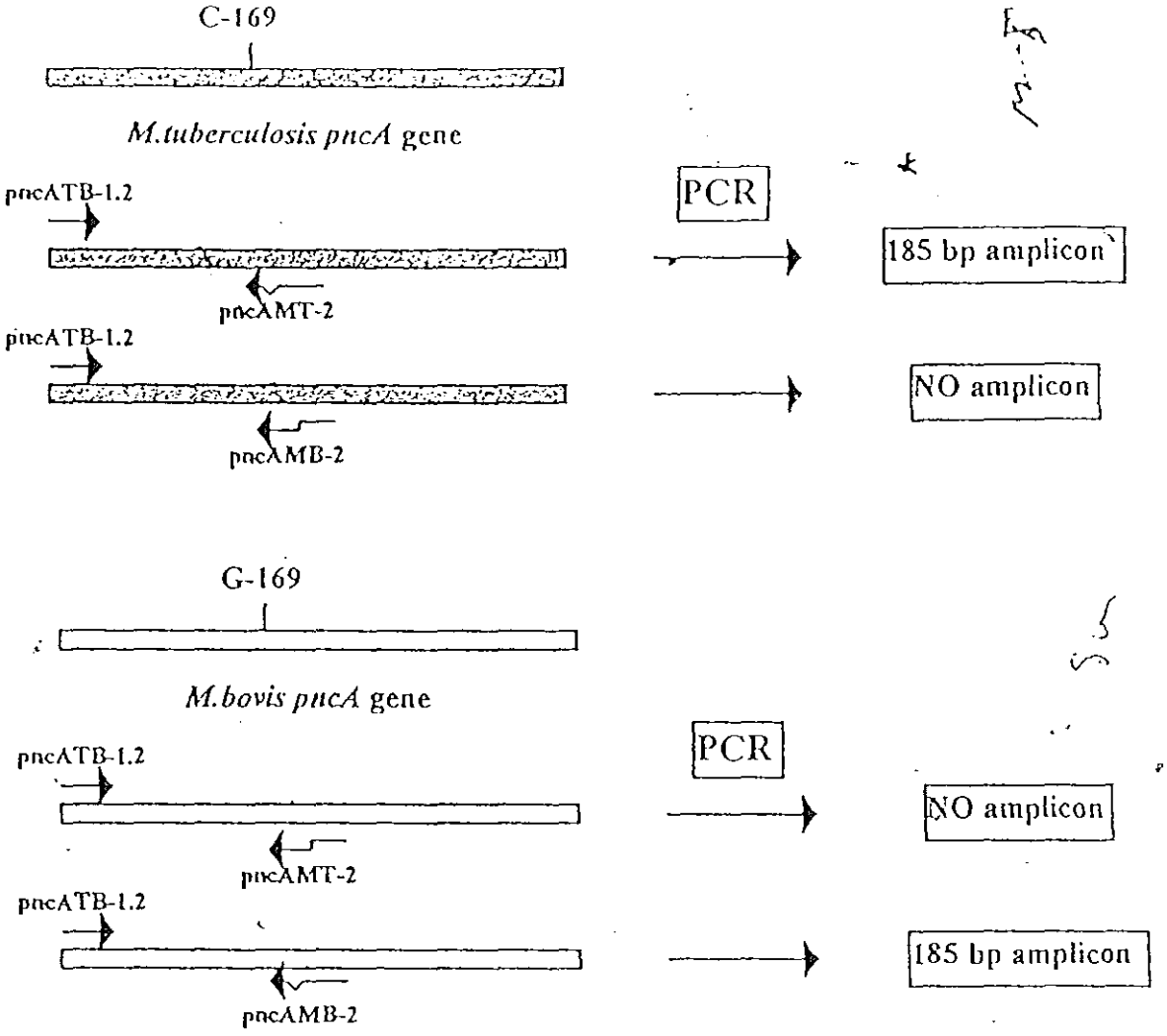

FIG. 1. Diagram showing the principle of the allele-specific PCR method to detect the polymorphism at position 169 in the *pncA* gene. pncATB-1.2 is a forward primer able to hybridize both *M. tuberculosis* and *M. bovis pncA* genes; pncAMT-2 is a reverse primer presenting a single mismatch with the *M. tuberculosis pncA* gene and two mismatches with the *M. bovis* gene; pncAMB-2 is a reverse primer presenting two mismatches with the *M. tuberculosis pncA* gene and a single mismatch with the *M. bovis* gene. Only single mismatches allow PCR amplification. The identical principle was applied to detect *oxyR* polymorphism. (Espinosa et al.,1998)

Amplification was carried out in a final volume of 25µl. The final 25µl volume for amplification of the same template DNA with different primers was constituted by adding into the ready to go bead (Tris HCl, pH8.3, $MgCl_2$, KCl, BSA, dNTP and Taq polymerase with standard recommended concentration) contained 0.5 ml micro-tube.

a) PCR mix for multiprimer system 6µl distilled and sterile water; 3µl (10 pmol) of each forward MT1 and MT2 reverse primers and; 4µ1(15pmol) of each forward IS5 and reverse IS6 primers 5µl of template DNA and 1 drop of mineral oil was added to the ready to go contained in 0.5ml micro-tube to the final volume of 25µl.

b) For pncA PCR system14µl of dH2O; 3µl (25 pmol) of each forward pncATB-1.2 and reverse pncAMT-2 primers or reverse primer pncAMB2 in different tubes; 5µl of template DNA; and 1 drop of mineral oil was added to the ready to go containing 0.5ml micro-tube to the final volume of 25µl

The amplification process was initiated by heating the mix in the thermal cycler for 7 min at 94^0C to separate the template DNA strands and followed by annealing and extension. The extended cycles resume denaturing at 94^0C for 1 min. This was followed by annealing of primers at 71^0 C for 1.5min and primer extension at 72^0 C for 2 min with final incubation at 72^0C for 10 min. A total of 35 amplification cycles were performed for each sample.

The detection of the gene product was carried out by 1.5% and 2% agarose ethidium bromide incorporated gel electrophoreses and subsequent visualization

under ultraviolet light. Positivity in Fig. 2 was determined in reference to the clearly defined bands of molecular size markers and control samples that were run in parallel with the test samples.

III. Results

3.1.ELISA results

The levels of antibodies to MPB70 were assayed in sera from tuberculous cervical lymphadenitis patients, non-tuberculous cervical lymphadenitis patients, and pulmonary tuberculosis patients, and healthy controls (Table 1). There was a wide variation in antibody content in individual sera.

Antibody responses to MPB70 in patients with pulmonary tuberculosis and non-tuberculous patients were higher than the tuberculous cervical lymphadenitis patients (82%, 79% Vs 64% respectively).

Table 1. History of the patients

			H. treatment Antibiotics		Contact with chronic cougher
Patient no.	Sex	Age	other	Anti-TB	
310	M	18	Yes	No	No
315	F	14	Yes	No	No
318	M	20	Yes	No	No
324	M	30	Yes	No	No
347	F	30	No	No	No
348	F	35	No	No	No
356	F	28	Yes	No	Yes
365	F	25	Yes	No	No
368	F	30	Yes	No	No
369	F	17	Yes	No	No
371	M	27	Yes	No	No
374	F	35	Yes	No	No
376	M	40	Yes	No	No
383	M	40	No	No	No
385	F	15	Yes	No	No
388	F	20	Yes	No	Yes
390	F	23	Yes	No	No
389	F	60	Yes	No	No
392	F	13	Yes	No	NO
393	M	18	Yes	No	No
394	F	35	Yes	No	No
395	M	28	No	No	No
398	F	35	Yes	No	No
404	F	18	No	No	No
406	M	22	No	No	Yes
321	M	30	No	No	No
322	M	18	No	No	No
323	M	37	No	No	No
326	M	10	Yes	No	No
329	F	20	Yes	No	No
332	M	15	No	No	No
340	M	18	Yes	No	Yes
344	M	30	Yes	No	No
351	M	25	Yes	No	No
353	M	28	Yes	No	No
311	M	22	Yes	No	No
355	F	30	No	No	No
358	M	20	Yes	No	No
367	M	18	no	No	No

Table 1 (Continued).
H treatment antibiotics = History of treatment with anti-TB drug;Otherher = Anti-biotic other than TB drug

Table 2. Comparison of antibody responses to MPB70 in patients with tuberculous cervical lymphadenitis, non-tuberculosis lymphadenitis, pulmonary tuberculosis and healthy controls.

Group (no. subjects)	ELISA results(MPB70) Mean OD 405 (SD)	No. of positive serum samples
Tuberculous lymphadenitis patients (TBLN) 25	0.277 (0.265)	16 (64%)
Non-tuberculous lymphadenitis (NTBLN) 14	0.403 (0.340)	11 (79%)
Pulmonary tuberculosis (PTB) 11	0.314 (0.202)	9 (82%)
Healthy controls 10	0.063 (0.045)	0

Cut off value for each was calculated as the mean optical density 405 nm (OD405) obtained with sera from 10 healthy controls, plus two standard deviations.

Table 3. ANOVA for ELISA data (n1=25, n2=14 n3=11,n4=10) from OD 405nm

TBLN ΔOD	NTBLN ΔOD	PTB ΔOD	HC ΔOD	$(x_1-x_1)^2$	$(x_2-x_2)^2$	$(x_3-x_3)^2$	$(x_4-x_4)^2$
1.305	0.664	0.552	0.107	1.057	0.068	0.059	0.002
0.255	0.252	0.088	0.037	0.000	0.023	0.049	0.001
0.124	0.076	0.067	0.008	0.023	0.107	0.059	0.003
0.163	1.231	0.747	0.013	0.013	0.686	0.190	0.003
0.545	0.238	0.282	0.069	0.072	0.027	0.001	0.000
0.381	0.152	0.225	0.064	0.011	0.063	0.007	0.000
0.646	0.193	0.449	0.61	0.136	0.044	0.019	0.000
0.056	0.314	0.189	0.026	0.049	0.008	0.015	0.001
0.041	0.646	0.273	0.099	0.056	0,059	0.001	0.001
0.243	0.192	0.236	0.145	0.001	0.045	0.006	0.007
0.113	0.919	0.349		0.027	0.266	0.002	
0.219	0.095			0.003	0.095		
0.267	0.304			0.000	0.010		
0.015	0.366			0.069	0.001		
0.020				0.066			
0.103				0.030			

0.330				0.003			
0.186				0.008			
0.136				0.020			
0.373				0.009			
0.301				0.000			
0.240				0.001			
0.386				0.012			
0.339				0.004			
0.142				0.018			
6.929	5.642	3.457	0.629	1.688	1.502	0.408	0.018

Δ *OD= Optical density.*
OD= The optical density reading of serum plus MPB70 minus the optical density reading of serum without MPB70.
HC= Healthy control.
Cut off value for each was calculated as the mean optical density 405 nm (OD405) obtained with sera from 10 healthy controls, plus two standard deviations.

Table 4. ANOVA for ELISA data

Source variation	sum of squares	degree of freedom	variance	F	Sw
Between groups	0.695	3	0.232	3.569	
Within groups	3.616	56	0.065		0.278
Total	4.311	59			

Critical F0.01 (3,56) =4.15. The computed F, 3.569, being lower than that critical value, is not significant (p>0.01), so, the difference among the four group means is not significant.

Anova was used to test the significance of differences between group means when the groups have been exposed to MPB70 antigen. It investigates whether or not the variance estimate between the groups contains an added treatment variable and distinct from the effects of the individual differences caused by the circulating antibody.

3.2. PCR results

Here a multiprimer PCR system and an allele-specific PCR system were used. Hence, proper design of the primers used is essential for successful DNA amplification and identification of polymorphism at position 169 in the pncA gene. The following aspects were taken into consideration: (i) that the size of the amplified fragments should be different enough to be discriminated in agarose gels, (ii) that the primers should not have potential matching sequences for non specific target sites, and (iii) that the optimal DNA-primer annealing temperature should be nearly equal for all template-primer combinations. An ethidium bromide stained gel of *M.tuberculosis* DNA and *M.tuberculosis* pncA gene amplified by the designed multiplier system is shown in Fig.2.

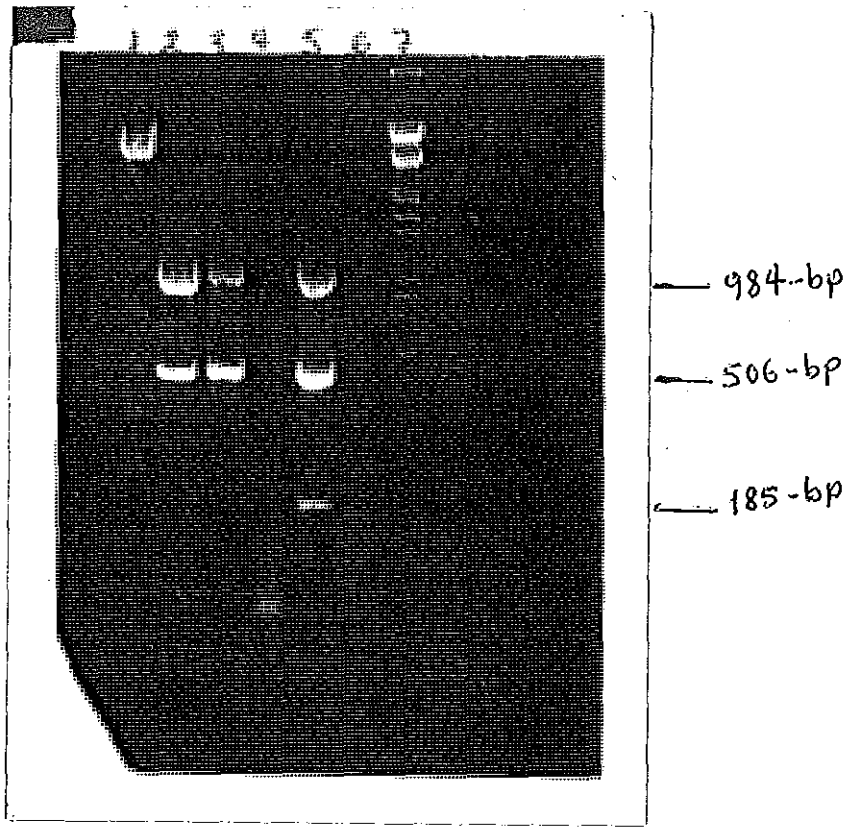

Fig 2.A 1.5 agarose gel electrophresis of amplified DNAs of Mycobacterium by using primers MT1/MT2, IS5/IS6 and pncATB/pncAMT Lane 5 shows as expected, three bands, corresponding to 185-bp resulting from the allele-specific PCR, to a 506-bp amplification fragment from the 32 kDa alpha antigen gene, and to a 984-bp amplification fragment from the IS6110 insertion sequence present in all bacteria from the complex. Lane 1 is the 123 DNA ladder. Lane 2 and lane 3 show the amplification of *M.tuberculosis* and *M.bovis* (32Kda and IS6110). Lane 4 sample 302 (32 kDa and IS 6110). Lane 6 is the negative control; lane 7 is λ DNA molecular weight marker.

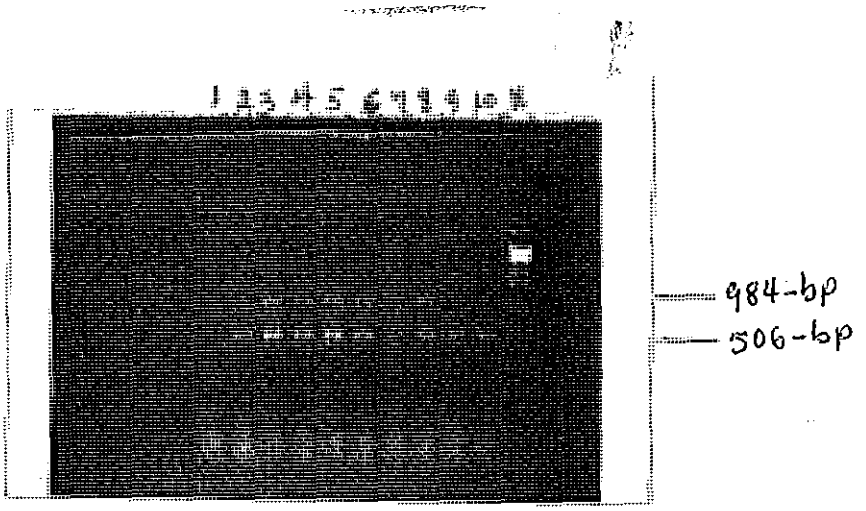

Fig. 3. A 1.5% agarose gel electrophoresis of amplified DNAs of *Mycobacterium* and clinical samples by using primers MT1/MT2 & IS5/IS6 . Multiprimer PCR with Clinical samples. After standardization of the multiprimer PCR assay with M.tuberculosis and M. bovis, we wanted to analyze 39 fine needle aspirates with which a diagnosis had been made by fine needle aspirate cytology (FNAC) and enzyme linked immunosorbent assay (ELISA) techniques. The results are shown in Fig.3 seven of the samples (2,3,4,6, 8,9, and 10) showed the pattern of amplified bands expected for *M.tuberculosis* and *M. bovis*. Samples 3,4,5,6, and 8 had been identified as positive in ELISA and FNAC. No visible bands were observed in samples, which were also negative in ELISA and FNAC tests.

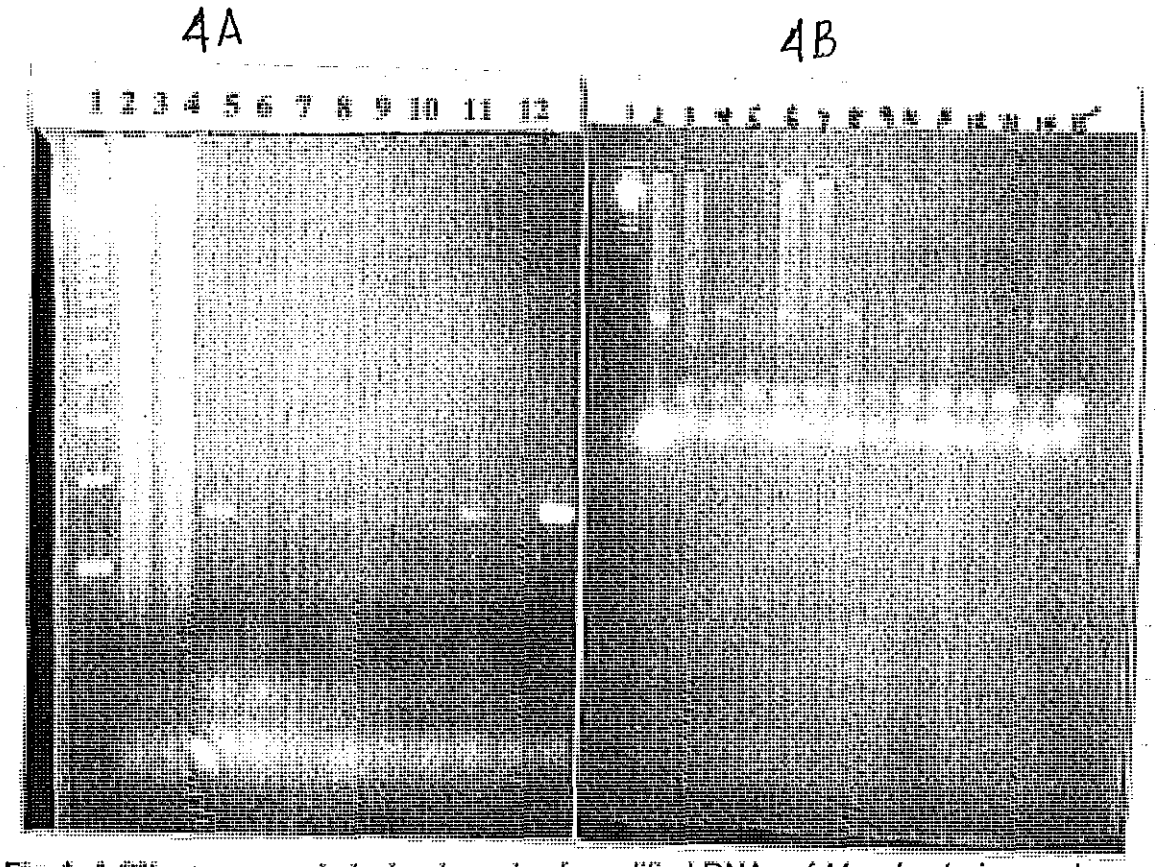

Fig.4. A 2% agarose gel electrophoresis of amplified DNAs of *Mycobacterium* and clinical samples using primers pncATB 1.2/pncAMT 2 & pncATB 1.2/pncAMB 2. Fig. 4A. Lane 1 shows the 123 DNA ladder. Lane 2 shows smears with band positive control for *M. tuberculosis* and lane 3 shows negative control for *M. tuberculosis*. Lane 4, 5,6,7,8,9,10,11 &12 are clinical samples. Lanes 8&9 co-amplify for *M.tuberculosis* and *M. bovis.* Fig 4 B Lane 1 shows 123 DNA ladder, lanes 2 and3 positive and negative controls for *M. bovis*, lanes 4 ,5,6,7,8,9,10,11,12,13,14,and15 are clinical samples. Lanes 4,6 8 10,and 14 show positive for *M. tuberculosis*. Lanes 5,7,9,11, and13 show negative for *M bovis*. Lane 12 shows negative *for M.tuberculosis*

Table 5. The results of the diagnostic tests performed on patients with tuberculous cervical lymphadenitis are summarized in the following table.

Pat,no.	Age	Sex	FNAC	ELISA	Genus level	Mt. Cplx	Mt	Mb	BCG scar	AFB
310	18	M	+	+	+	+	+	+	no	+
315	14	F	+	+	-	-	-	-	no	-
318	20	M	+	-	-	-	-	-	no	+
324	30	M	+	+	-	-	-	-	no	+
347	30	F	+	+	+	+	+	+	NA	+
348	35	F	+	+	+	+	+	-	no	+
356	28	F	+	+	+	+	+	-	yes	+
365	25	F	+	-	-	-	-	-	no	-
368	30	F	+	-	+	+	+	+	no	+
369	17	F	+	+	+	+	+	+	yes	+
371	27	M	+	-	+	+	-	+	no	-
374	35	F	+	+	+	+	+	-	no	-
376	40	M	+	+	+	+	-	+	no	-
383	40	M	+	-	-	-	-	-	no	NA
385	15	F	+	-	+	+	+	-	no	-
388	20	F	+	-	+	+	+	-	no	-
389	23	F	+	+	-	-	-	-	no	-
390	60	F	+	+	+	+	+	-	no	-
392	13	F	+	-	+	+	+	-	no	-
393	18	M	+	+	+	+	+	-	no	+
394	35	F	+	+	+	+	+	-	NA	+
395	28	M	+	+	+	+	+	-	yes	+
398	35	F	+	+	-	-	-	-	yes	-
404	18	F	+	+	+	+	+	+	no	+
406	22	M	+	-	+	+	-	+	no	+
321	30	M	-	+	+	+	+	-	no	NA
322	18	M	-	+	-	-	-	-	no	+
323	37	M	-	+	+	+	+	-	no	+
326	10	M	-	+	+	+	+	-	no	NA
329	20	F	-	+	+	+	+	-	no	-
332	15	M	-	+	+	+	+	-	no	-
340	18	M	-	+	+	-	+	-	no	-
344	30	M	-	+	-	-	-	-	no	-
351	25	M	-	+	+	+	+	-	no	NA
353	28	M	-	+	+	+	+	-	no	NA
311	22	M	-	-	+	+	+	+	no	+
355	30	F	-	+	-	-	-	-	yes	-
358	20	M	-	-	+	+	+	+	no	NA
367	28	M	-	-	+	+	+	+	no	+

NA = not available; Mtcplx = M. tuberculosis complex.;Mt = M. tuberculosis Mb = M. bovis; BCG = Bacillus almette-Guerin ;AFB = acid-fast bacillus

Table 6. Characteristics of fine needle aspirate diagnostic tests in 39 patients with a clinical diagnosis of tuberculous lymphadenitis.

Patient no.	FNAC	ELISA	PCR
310	+	+	+
315	+	+	–
318	+	–	–
324	+	+	–
347	+	+	+
348	+	+	+
356	+	+	+
365	+	–	–
368	+	–	+
369	+	+	+
371	+	–	+
374	+	+	+
376	+	+	+
383	+	–	–
385	+	–	+
388	+	–	+
389	+	+	–
390	+	+	+
392	+	–	+
393	+	+	+
394	+	+	+
395	+	+	+
398	+	+	–
404	+	+	+
406	+	–	+
321	–	+	+
322	–	+	–
323	–	+	+
326	–	+	+
329	–	+	+
332	–	+	+
340	–	+	+
344	–	+	–
351	–	+	+
353	–	+	+

311	–	–	+
355	–	+	–
358	–	–	+
367	–	–	+

Definition of abbreviations: FNAC= fine needle aspirate cytology;

ELISA = enzyme immunosorbent assay; PCR = polymerase chain reaction.

Mean age was 25.8 yrs (range 10 to 60 yrs) (Table in the appendix). There were 21 males and 18 females. A BCG vaccination scar was observed in five of the patients clinically diagnosed as tuberculous and non-tuberculous lymphadenitis. Of these 39 clinically diagnosed tuberculous lymphadenitis FNAC was positive in 25 (64%) and ELISA in 27 (69%). Mycobacterial genome was detected by PCR in 29 of the 39 patients with cervical lymphadenitis (74%). Of the 29 mycobacterial DNAs 29 0f them found to have *M.tuberculosis* complex. Further detection for distinguishing *M.tuberculosis* from *M. bovis*. PCR was done with primers pncATB 1.2 and pncAMT-2 for M. tuberculosis and pncATB1.2 –pncAMB-2 for M. bovis. Thus, *M. tuberculosis* genome was detected in 18 of the 29 (62%), *M. bovis* 3 genome was detected of the 29 (10%) and *M. tuberculosis* complex and 8 genomes were found to co-amplify (28%).

Table 6 summarizes the diagnostic tests results, which was performed on 39 clinically diagnosed tuberculous cervical lymphadenitis patients. The mean age was 25.8 years (10 to 60 yrs). There were 18 females and 21 males. The FNAC test was positive in 25 (64%) of the 39 patients. Of the 39 patients clinically diagnosed with tuberculosis lymphadenitis, ELISA with MPB70 antigen was positive in 27 (69%). PCR positive in 29 (74%) patients.

Table 7. Comparison of final results of FNAC, ELISA, and PCR with Clinical diagnosis

Clinical diagnosis	FNAC	ELISA	PCR
Tuberculous lymphadenitis 39	25 positive	16 positive 9 negative	18 positive 7 negative
	14 negative	11 positive 3 negative	11 positive 3 negative

Table 8. PCR test taking FNAC as assumed gold standard

	+Ve	-Ve
+Ve	18	11
-Ve	7	3

Sensitivity, 72%; specificity, 21%; test efficiency, 54%; positive predictive value, 62%; and negative predictive value, 30%

Table 9: ELISA test taking FNAC as assumed gold standard

	+Ve	-Ve
+Ve	16	11
-Ve	9	3

Sensitivity 64%; specificity, 21% test, efficiency, 49%; positive predictive value, 59% and negative predictive value 25%.

Table 10. Occurrence of *M. tuberculosis* and *M. bovis* in 29 patients with cervical lymphadenopathy and a positive PCR

Mycobacteria	No. of positive
M .tuberculosis	18 (62%)
M. bovis	3 (10%)
Co-amplification	8 (28%)

The results of PCR amplification for *M. tuberculosis* complex in fine needle aspirates from these 39 patients are compared with clinical diagnosis in Table 7. Thirty-nine of the patients had a clinical diagnosis of tuberculosis lymphadenitis; the fine needle aspirate cytology was positive in 25 of these 39 cases and ELISA for *M.tuberculosis* complex was positive in 27. PCR of the fine needle aspirates for *M.tuberculosis* complex DNA was positive in 29 patients of the 39 patients who had a clinical diagnosis of tuberculosis lymphadenitis.

The results of ELISA for *M.tuberculosis* complex are compared with the fine needle aspirate cytology taking them as "a gold standard". Three were negative for both ELISA and PCR diagnosis for *M.tuberculosis* complex in Table 8 and 9. ELISA sensitivity for *M.tuberculosis* complex was 64% and 21% specific, having a positive predictive value of 59% and a negative predictive value of 25% (Table 7).

The PCR results were compared with the fine needle aspirate cytology are shown in Table. Taking them as "a gold standard ". PCR for *M.tuberculosis* complex DNA in 39 fine needle aspirate specimens described here was 72% sensitive and21% specific, having a positive predictive value of 62% and negative predictive value 30%.

From table 10 one can say 62% of the identified causative agents are *M.tuberculosis* and 10% are *M. bovis*. Beside this, 28% are found to be co-amplification of *M. tuberculosis* and *M. bovis.*

V. Discussion

The MPB70 antigen of *M. bovis* used in this study is well characterized (Wiker, et al., 1998). It is found only in *M. tuberculosis* complex, and it has a considerable potential for improved diagnostic test for tuberculosis (TB). It is an important target antigen of humoral and cellular immune responses during infection with bovine and human tubercle bacilli (Wiker et al., 1996) and thus has been used in serodiagnosis of *M. tuberculosis* complex. The diagnostic utility of ELISA with this antigen is not impaired by cross-reactivity with other mycobacteria.

The results of our study are encouraging. No positive reading was observed among the 10 healthy control subjects, when an optical density reading cut off value > 0.153 was used.

The sensitivity of 0.820 in patients with pulmonary tuberculosis is promising. It was higher than that seen with other parts of mycobacterial extracts (Khomenko et al., 1996). The sensitivity of 0.667 in cervical lymphadenitis patients is higher than seen with other (Kp-90 antigen) antigen with sera of extra-pulmonary TB (Julian et al., 2000).

All of the patients in this study were treated as cervical lymphadenitis patients (39) and pulmonary tuberculosis (11). Of the 39 clinically diagnosed cervical patients, ELISA has detected 27 (69%) as positive for tuberculous lymphadenitis. But FNAC has detected 25 (64%) as positive. From FNAC detected as positive, ELISA has shown 16 (64%) as positive. Of the 14 FNAC showed as negative ELISA has

detected 11 (79%)as positive.

Fine needle aspirates (FNA) of lymph nodes is preferable diagnostic procedure to biopsy, when obtainable, in documenting tuberculous lymphadenitis. This is because incisional biopsy is often associated with sinus tract and fistula formation. In addition to this, biopsy requires a more involved procedure including the application of anesthesia. Biopsy is best reserved for the patients in whom FNA fails to be diagnostic or in selected patients for treatment (Sloane, 1996). Thus FNA is a safe and applies limited procedure. In addition, its sensitivity and specificity are comparable with biopsy (Sloane, 1996). But the aspirates require thorough diagnosis of AFB staining and culture besides cytological findings for confirmation.. Of the 14 FNAC negative the ELISA and PCR assays have detected in common 8 (57%) as positive. Therefore, both the assays have detected in common as positives from the 39clinically tuberculous cervical lymphadenitis 20 (51%) as tuberculous lymphadenitis.

Of the 39 clinically said tuberculous cervical lymphadenitis, FNAC, ELISA, and PCR diagnostic tests detected in common 12 (31%) as tuberculous lymphadenitis.

When we come to the part of the study, which is identification of the causative agents using primers specific to the genus mycobacteria, *M. tuberculosis* complex and species specific (*M. tuberculosis* and *M. bovis*).

The grouping of non-tuberculous lymphadenitis based on observation of purulent (pyogenic) appearance from the lymph node aspirate and cytological findings alone cannot hundred percent help to conclude as non-tuberculous lymphadenitis. This is

because tuberculous lymphadenitis in immunosuppressed patients may resemble an acute pyogenic bacterial infection. In addition to this , the majority of the patients in the study seems to have HIV co- infection because of the following reasons: as the most frequent site of extra-pulmonary tuberculosis in patients infected with HIV is lymph node, 94% of the cases had tuberculous lymphadenitis; the majority of the patients were young adults where prevalence of HIV is at its peak like our country Ethiopia, more than one third of the said NTBLN were noted to have abscess in the FNAC, which is a common picture seen in HIV/AIDS patients (Wondwosen and Alemayehu,2000). This statement was supported by the tests we conducted on the said non-tuberculous lymphadenitis i.e., of the "14 NTBLN" 8 of them were positive for TBLN in both ELISA and PCR tests. This finding indicates that is why the specificity and the predictive value were very low. When the FNAC was assumed as a gold standard.

It is believed f that in the developing countries cervical lymphadenitis is mainly caused by *M. bovis* (Sloane, 1996). This general assumption cannot go with our findings, i.e., the major causative agent of cervical lymphadenitis in Butajira, Ethiopia is *M. tuberculosis*.

The other belief is that in populations with the high rate of tuberculosis, lymphadenitis has a great incidence in early child hood (Humphries and Lam, 1998). But this assumption cannot go with our observation in Butajira, Ethiopia i.e., here most of the victims are young adults. Among 29 PCR detected tuberculous lymphadenitis, 18 (62%) were found to be *M. tuberculosis*, 3 (10%) *M. bovis* and 8 (28%) co-

amplification of the two.

The argument of the co-amplification could be either due to a single point mutation in pncA gene of *M. tuberculosis* at a nucleotide position 169 (a change of C to G) i.e., resistant strains for pyraziamide , which is unique to *M. bovis*.

V. Conclusion and recommendation

Mycobacterial lymphadenitis is caused by *M.tuberculosis* complex and non-tuberculosis mycobacteria (NTM). The latter are commonly opportunistic AND cause lymphadenopathy in immuno-compromised individuals. Cervical lymph nodes are the common site of involvement

In Butajira, Ethiopia cervical lymphadenitis patients are in a considerable number the reason for this is not clear yet. Here the young adults are found to be highly affected than children and older people.

To know the causative agents the current definitive method for distinguishing *M.tuberculosis* from *M. bovis* still depends on testing of several biochemical parameters, which is tedious and time consuming. In our study we used ELISA and rapid allele-specific pncA gene tested based on detection of characteristic stable point mutation in the M. bovis pncA gene to differentiate *M.tuberculosis* from *M. bovis*. This is because *M. bovis* strains are known to have defective pyrazinamidase indicative of the pncA mutation.

Distinguishing *M.tuberculosis* from *M. bovis* based on this mutation is particularly useful, because the *M.bovis* disease should not be treated with pyrazinamide to which the bovine bacilli are naturally resistant.

VI. References

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