Female Genital Tuberculosis in Ethiopia: Occurrence and Immunodiagnosis

By
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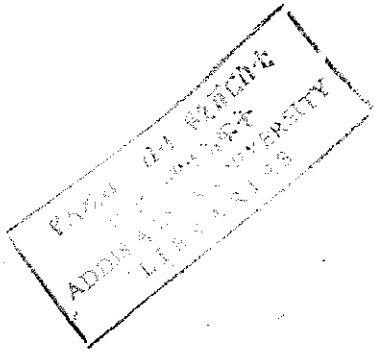
A Thesis presented to the School of Graduate Studies of the Addis Ababa University in partial fulfillment of the requirements of the degree of Master of Science in Biology



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FEMALE GENITAL TUBERCULOSIS IN ETHIOPIA: OCCURRENCE AND IMMUNODIAGNOSIS

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List of tables and figures

Abbreviations

AFB	Acid Fast Bacilli
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immuno Deficiency Syndrome
ALP	Alkaline Phosphatase
B&D	Becton Dickinson
BCG	Bacille Calmette Guerin
CD	Cluster Differentiation
CMI	Cell Mediated Immunity
CPM	Count Per Minute
ELISA	Enzyme Linked Immunosorbent Assay
EPTB	Extra Pulmonary Tuberculosis
FACScan	Fluorescence Activated Cell Sorter
FGTB	Female Genital Tuberculosis
HIV	Human Immuno-deficiency Virus
HSG	Hysterosalpingography
IL	Interleukin
LJ	Lowenstein-Jensen
LST	Lymphocyte Stimulation Test
MAB	Monoclonal antibody
MB	*Mycobacterium bovis*
MTB	*Mycobacterium tuberculosis*
NHS	Normal Human Serum
OD	Optical Density
ON	Over Night
OPD	o-Phenylenediamine Dihydrochloride
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PHA	Phyto Hemo Agglutinin
PLTB	Pulmonary Tuberculosis
p-NPP	p-nitrophenyl phosphate
PPD	Purified Protein Derivative
R & D	Research and Development
RNI	Reactive Nitrogen Intermediates
ROI	Reactive Oxygen Intermediates
RT	Room Temperature
SI	Stimulation Index
TB	Tuberculosis
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor

Abstract

Female genital tuberculosis (FGTB) causes severe and irreversible damage to the reproductive organs, most commonly the fallopian tubes and the uterus, ultimately resulting in infertility. Diagnosis of FGTB is often difficult because of the inaccessibility of the affected organs, requiring invasive procedures including surgery. There is a strong need for a simplified and reliable diagnostic technique. This is even more urgent today than in the past because of the spread of HIV/AIDS and the unknown magnitude of FGTB under the current epidemic situation. We studied twenty-five gynecological patients diagnosed clinically as FGTB at the Black Lion Hospital for laboratory evidence of etiology and for possible associated immunodiagnostic indicators. Biopsy and curettage samples were taken from each patient and investigated with histopathology, smear microscopy, culture and polymerase chain reaction (PCR) for Mycobacteria. Culture positive samples were examined for the type of species. Peripheral blood mononuclear cells were stimulated *in vitro* with mycobacterial antigens for recall responses with lymphocyte stimulation Test (LST). Cytokines: IL-10, TNF-α and INF-γ were measured from the supernatant of cultured PBMC. CD4:CD8 ratio in blood was evaluated by flow cytometry. Serum IgG, IgA and IgM levels to Mycobacterial antigen (MPT59) were also measured by ELISA. The study subjects were all in child bearing age (18-39). Of the 17 patients whose infertility status was known, 6 (35.3) had primary while 11 (64.7) had secondary infertility. Among the 25 gynecological patients investigated, only 1 was AFB smear positive, 3 were culture positive, 7 were histology positive and 12 were positive by PCR (a total of 16 positives). CD4:CD8 ratio was not helpful indicator for FGTB. The serum antibody level did not distinguish between laboratory 'positive' and 'negative' cases. However, all 'patients' had detectable level of one or more of serum IgG, IgA and IgM to MPT59. 'Patients' and 'controls' showed remarkable difference in their proliferative response to PPD suggesting its diagnostic value. The result clearly showed that FGTB is a rather common clinical problem among Ethiopian gynecological patients and its causative agent is mainly MTB. As far as this work goes, the only diagnostic method to support the clinical suspicion is the LST to PPD.

1. Introduction

1.1. General

Tuberculosis is an infectious disease characterized by chronic granulomatous disease with long incubation period and dormant infection (latency) (Kaufmann, 1993; Enarson *et al.*, 1996). It is usually caused by *Mycobacterium tuberculosis* (MTB) and rarely by *Mycobacterium bovis* (MB) (Crofton *et al.*, 1992). The route of infection is mainly through uptake of aerosolized droplet nuclei in MTB and intake of milk in the case of infection by MB (McDonough *et al.*, 1993). The site of primary infection is usually the lung, but when infection is from MB, the tonsils, intestine and at times the skin and any of the regional lymph nodes could as well be involved (Crofton *et al.*, 1992).

Tuberculosis is one of the leading causes of death in the world (McDonough *et al.*, 1993). Globally, more than three million people die from this disease and about 10 million new cases arise each year (Kaufmann, 1993). Over 95% of the cases and 98% of the deaths due to TB are found in the developing countries. The age distribution of the disease in developing and developed countries is quite contrasting. Nearly 75 % of patients in the developing countries belong to the economically productive age group (15-50 years) while in the developed countries, 80% are above 50 years (Chan *et al.*, 1992; WHO, 1997).

Despite the endeavor to control the disease, it is still on the rise. The projection of the disease based on the 1995 data estimates that the global incidence could reach 10.2 million in the year 2000 and 11.9 million in 2005 (Raviglione *et al.*, 1995; Dolin *et al.*, 1994).

The pathology is an undesired result from the host attempt to kill the bacilli. A large proportion of infiltrating phagocytes as well as parenchymal cells die producing a characteristic solid caseous necrosis. The solid caseous necrotic tissue could further liquefy as a result of hydrolase enzymes released from inflammatory cells and facilitate proliferation and further dissemination of the bacilli (Crofton *et al.*, 1992).

Protective immunity to tuberculosis generally depends on cell-mediated immunity (CMI) that involves recruitment and activation of monocytes that directly kill and wall off the intracellular bacilli from the rest of the body through granuloma formation (Johnson *et al.*, 1998; Cooper *et al.*, 1997). In the process, $CD4^+$, $CD8^+$, γδ T cells and natural killer cells contribute their share in clearing infected macrophages that harbor the bacilli (Bloom and Murray, 1992; Ottenhoff *et al.*, 1998). This is effected by cytokines like IL-12, IFN-γ and TNF- α. IL-12 is a cytokine that has the ability to initiate the differentiation of uncommitted Th0 cells into the Th1 phenotype. It also potentiates the Th1 and natural killer cells to produce IFN-γ, a cytokine that has a great role in granuloma formation (Cooper *et al.*, 1997; Ellner, 1997). IFN-γ and TNF- α play role in limiting growth and dissemination of bacilli by recruiting and activating macrophages to produce reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (Ellner, 1997). Patients who lack these cytokines fail to produce ROI and RNI to kill the bacilli and show a severe and disseminated form of TB. This is mainly due to poorly differentiated granuloma and often result in a rapid and fatal course of TB (Flynn *et al.*, 1993; Cooper *et al.*, 1995).

The above Th1 cytokines are important in protective immunity against MTB. However, the host regulates their production by producing other cytokines that counter their effect. Among

these cytokines, TGF-β and IL-10 are the major ones. They suppress blastogenesis and IFN-γ production and play a role in down regulating protective immunity to TB (Ellner, 1997). Diagnosis of TB involves various techniques. The simplest and rapid method is the detection of Acid fast bacilli (AFB) by microscopy (Wilkins and Ivanyi, 1990). The other conventional method, culture, is also an option. These two methods are considered the most reliable ones. Skin testing (delayed type hypersensitivity reaction to PPD) and Radiology (X-ray) provide supportive evidence (Bloom and Murray, 1992; Enarson *et al.*, 1996).

Another possible method of diagnosis is the use of a molecular method, Polymerase Chain Reaction (PCR) to detect mycobacterial DNA using specific primers. It is relatively specific and sensitive (Bloom and Murray, 1992) but requires high tech and well trained personnel. Immunological methods, using humoral or cellular responses to infer the presence of infection/disease, is another potential area under investigation more recently (Pottumarthy *et al.*, 2000).

1.2. Extra-pulmonary Tuberculosis (EPTB)

Extra-pulmonary tuberculosis is a type of tuberculosis affecting organs other than the lungs, most commonly the pleura and lymph nodes but also the spine, genito-urinary tract, nervous system and intestine (Enarson *et al.*, 1996). It is a result of hematogenous or lymphatic spread of the bacilli from the site of primary infection, usually the lung or due to the reactivation from other organs (Crofton *et al.*, 1992).

Because of its ability to destroy the immune system, HIV has emerged as the most important risk factor in the progression of new or dormant TB infection to clinical disease (Daley *et al.*, 1992). The annual risk of progression to active TB among individuals co-infected with HIV is

5 to 15% (Raviglione *et al.,* 1995; WHO, 1997). Prior to the epidemic of HIV infection, TB involving extra-pulmonary sites occurred in only 15% of the cases (Farer *et al.*, 1979). With the onset of HIV epidemic, the occurrence has increased both in absolute and relative numbers. For example a study by Small *et al.* (1991) showed that TB patients with advanced HIV disease had 62% extra pulmonary involvement. This is further complicated by the negative influence of the virus on the disease (TB). The virus catalyzes the spread of multi drug resistant strains, alters the clinical expression of TB to less cavitary and more disseminated form causing problems in diagnosis, treatment and control of the disease (Ellner, 1997). Even BCG all alone is causing disseminating infection in these subjects (North and Izzo, 1993; Ottenhoff *et al.*, 1998).

The diagnosis of EPTB is more difficult than that of pulmonary tuberculosis (PLTB). This is primarily because of the inaccessibility of the sites and the small number of bacilli involved in the process. Besides these, it is mostly asymptomatic so is difficult to diagnose clinically (Lal and Wright, 1999).

1.3. Female Genital Tuberculosis

Female genital tuberculosis (FGTB) is one of the presentations of EPTB. It affects the female genital organs most commonly the fallopian tubes, endometrium, ovary, and rarely the cervix and the vagina (Varma, 1991). The fallopian tubes are the primary foci (90%) of infection. From it the bacilli often disseminate up and down to involve the ovaries (10-30%), endometrium (50%), cervix (<5%) and the vagina and vulva (<1%) (Varma, 1991; Arora *et al.*, 1994). In 90% of the cases, infection is caused by MTB and only occasionally by the *M. bovis* (Winfred *et al.,* 1977). The disease in 80-90% of the cases affects young women between 20 and 40 years of age and is an important cause of infertility (Crofton *et al.*, 1992;

Varma, 1991). It is often a secondary complication that results from reactivation of a " silent bacillemia " i.e., an extension of a lesion, primarily pulmonary, but in some instances also renal, intestinal, etc. (Simon *et al.,* 1977). Primary infection of the female genital organs, though very rare, could also occur through semen of an infected male partner (Richards and Angus, 1998). The bacteria upon reaching the genital organs through the various means (hematogenous and lymphatic spreading or via direct contiguity with an intra-abdominal or peritoneal foci), can cause active disease (Arora *et al.*, 1994; Simon *et al.*, 1977).

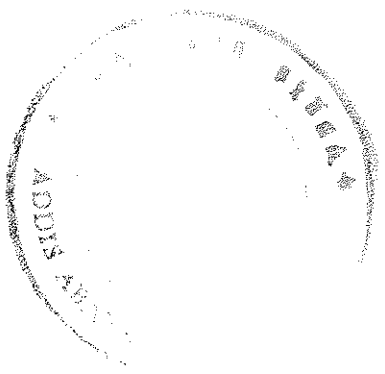
The incidence of FGTB from infertility clinics shows marked variations in different countries ranging between 1-19% (Varma, 1991). The establishment of the true incidence and prevalence of FGTB is difficult because asymptomatic latent cases predominate over symptomatic ones (Schaefer, 1976). However, HIV/AIDS is expected to increase the incidence and prevalence of FGTB for they are frequently symptomatic (Giannacopoulos *et al.*, 1998; Rom and Garay, 1996).

The most frequent symptoms are: infertility, lower abdominal distention and pain, disturbance of rhythm of menstruation, abscess formation of the fallopian tube, ectopic pregnancy, loss of weight and appetite, a mass felt in the pelvis and signs of TB elsewhere in the body (Crofton *et al.,* 1992).

Diagnosis of FGTB is usually based on clinical examination (Simon *et al.*, 1977). But when facilities are available, microbiological (AFB staining and culture), histopathological (Winfred, 1977), radiological methods can be used. Radiology in this case specially refers to Hysterosalpingography (HSG). HSG exhibits features like multiple constrictions and obstruction of the fallopian tubes, endometrial adhesion, obliteration or distortion of the

uterine cavity (Schaefer, 1976; Rom and Garay, 1996). PCR and immunological methods are also expected to provide valuable information for the diagnosis of FGTB.

FGTB is an important gynecological disease needing early consideration and management. However, this often fails because its Epidemiology as well as the method of diagnosis have not been given a due consideration. The available TB diagnostic methods are often insufficient for FGTB. That is because the accessible materials for diagnosis are the endometrium and menstrual blood, which are not diagnostic enough on their own (Klein *et al.*, 1976). One can not take biopsy for diagnostic sake for it creates a complication. Other approaches that could be tried are all non confirmatory. Physical examination is of little help in diagnosis (Simon *et al.*, 1977) and histological examination is less reliable (Winfred, 1977). Bacteriology (culture) needs a long time (about 40 days) to see 75% of growth and AFB staining requires a minimum of 10^5 bacilli per ml of a specimen for positivity and this is difficult to obtain (Varma, 1991). Culdoscopy, Laparoscopy, etc., carry a significant risk of bowel injury. Chest X–ray for genital tuberculosis is less effective since the majority of pulmonary lesions are arrested by the time genital tract involvement becomes obvious. The tuberculin skin test fails to discriminate between past and present active infection, and may be affected by various systemic illnesses and immuno-suppression (Varma, 1991). Molecular methods were highly specific but are expensive. Surgery is disadvantageous as it carries a risk of activating silent infection (Gurgan *et al.*, 1996). Even when the abdomen is opened, diagnosis is not always easy because the tubes may appear normal to the naked eyes unless a tubal biopsy is made, which in turn may aggravate the disease (Winfred, 1977). In general, genital tuberculosis will remain as a problem as long as pulmonary and intestinal tuberculosis are prevalent and will continue to be the cause for a significant proportion of infertility (Sheikh, 1996).



In an attempt to develop a rapid, reliable and non-operative diagnostic method this project was set with the following general and specific objectives.

General Objectives

- To develop an immunological method for the diagnosis of female genital tuberculosis
- To identify the causative agent

Specific Objectives

This Thesis will address the following specific objectives:

- To identify the genital tuberculosis cases among gynecological patients undergoing surgical procedures or dilatation and curettage.
- To examine immunological responses at various levels

  A/ to measure the serum antibody level and

  B/ to measure the proliferative response of PBMC to mycobacterial antigens
- To identify the possible causative agent

  A/ to detect the Mycobacterium and determine if it is in the MTB complex or not using specific primers in PCR.

  B/ to identify the causative agent at the species level using biochemical tests
- To explore from the immunological findings the one that could be of diagnostic value

2. Materials & Methods

2.1. Study subjects

Twenty-five gynecological patients from the Black Lion hospital were included in the study subject. These patients came to the hospital for reasons mainly of infertility and were clinically suspected to have FGTB. Tissue sample for diagnosis was taken from the fallopian tubes (biopsy) or from the endometrium (curettage). The study subjects were divided into two as patients when they were confirmed positively by any one of the four laboratory diagnostic methods and controls when negatively confirmed by all the tests.

2,2. Antigens

Purified MPT59, which is a major protein from *M tuberculosis* culture filtrates containing reactive B cell epitopes (Andersen, 1997) and BCG sonicate were a kind gift from Prof. Morten Harboe. The antigens were aliquoted and frozen at -20^0C until use. Tuberculin purified protein derivative (PPD) was purchased from Staten's Serum Institute (Denmark) and stored at 4^0C.

2.3. Specimen collection

Blood and biopsy specimens were taken from each patient and directly transported to cell laboratory at AHRI. About 10 ml of blood was drawn in a heparinized vacutainer while the biopsy materials were brought to the lab in two vials; one in 10 % formalin, for histological examination, and the other in PBS to use for cell extraction, AFB staining, culture and PCR.

2.4. Diagnostic methods

The tissue kept in PBS was homogenized manually using a mortar and pistil. Then, 1 ml of the homogenate was added into two tubes, one for AFB staining and the other for culture.

2.4.1. AFB staining (Ziehl-Neelsen)

One ml of hypochlorite and 8 ml of sterile distilled water were added to the 1 ml homogenate and left for 15 minutes on a shaker. It was then centrifuged at 3000 rpm for 15 minutes. The supernatant was removed gently and a drop from the pellet was smeared on duplicate slides. After air-drying it for 2 hrs, AFB staining was performed according to Vestal (1975). Briefly, a smear from the pellet was prepared on a slide and heat fixed. Then it was covered with carbol fuchsin and heated to steam and allowed to stand for 10 minutes. After washing with tap water, it was decolorized with acid (H_2SO_4, 25 %) and alcohol (70 %). Following this, it was flooded with methylene blue and washed again. Finally, it was air-dried and was examined for red stained bacilli under microscope.

2.4.2. Culture

Three ml of 4% NaOH was added to the 1 ml homogenate and left for 15 minutes on a shaker. Then it was centrifuged at 3000 rpm for 15 minutes. Subsequently, the supernatant was removed and the pellet was resuspended by adding 1% Bromocresol until the color changed to yellow and then added into two Lowenstein-Jensen (LJ) culture medium in duplicate. This was put in slant for one week and then upright for up to 8 weeks at 37^0C. The growth of the bacilli was checked every week. After the end of the 8^{th} week, AFB staining was performed and the results were recorded.

2.4.3. C. Histology

Paraffin embedded tissue sections were prepared and stained with haematoxylin-eosin at AHRI histopathological unit and examined under microscope for granulomatous reaction by a pathologist at AHRI.

2.4.4. Polymerase Chain Reaction (PCR)

2.4.4.1. DNA extraction

The mycobacterial genomic DNA was isolated according to Van soologen *et al.* (1995). Briefly, 200 µl of homogenized tissue was incubated at 80^0C for 20 minutes and diluted with 500 µl TE buffer and lysed with 50 µl of 10 mg/ml of lysonzyme and vortexed and incubated for 1 hr at 37^0C. The lysonzyme treated samples were incubated at 65^0C for 10 minutes with the presence of 10 mg/ml of proteinase K and 10% SDS. 5M NaCl and CTAB were added to the sample and chloroform/isomylalchol (24:1) extraction was performed. Then, the DNA was precipitated by adding Isopropanol (0.6 times the volume of to the aqueous phase). After 1 hr storage at -20^0C, the DNA was collected by centrifugation at 12000 rpm for 15 minutes and washed with 70% ethanol, and resuspended at 30 µl of distilled water, and the sample was treated by 3 µl of Rnase (2 mg/ml) (volume. 1:10 ratio of Rnase to sample) for 1 hr at 37^0C and stored at -20^0C.

2.4.4.2. Polymerase Chain Reaction (PCR)

The sequence for ESAT-6 protein encoding gene was selected to be amplified in our PCR system. Because others have showed that ESAT-6 is present in MTB complex except in BCG. ESAT-6 is a low mass secreted protein (6 *kDa*) identified due to its high reactivity with T cells (Andersen, 1997). Therefore, it could help us to exclude BCG induced tuberculosis cases. Primers for ESAT-6 protein encoding gene sequence were purchased from Gibco BRL. The forward primer was (5′-CCCCGGATCCCATGACAGAGCAGCAGTGG-3′) and the reverse was (5′-CTCGGAATTCCCCTATGCGAACATCCC-3′). Ten µM of each primer, 100 ng mycobacterial DNA and dH_2O were added to the beads (Pharmacia P-L Biochemicals Inc., Uppsala, Sweden) to a total volume of 25 µl. The mixtures were then covered by mineral oil and subjected for amplification in three steps as follows: First step denaturing at 95^0C for

2′ one cycle; second step denaturing at 95^0C, 15″; primer annealing was done at 60^0C, 30″; and primer extension at 72^0C, 1′for 25 cycles and third step, prolonged primer extension at 72^0C, 7′ for one cycle. The PCR reaction was run using a Hybid Omini-gene PCR machine. The test system was sensitive to detect as low as ten bacilli (Dawit, K. Personal communication).

2.4.4.3. DNA agarose gel Electrophoresis

Two percent gel was prepared as follows: Three gm of Agarose (sigma)(w/v) was dissolved into 150 ml of 0.5x TBE buffer (5.9 gm Tris base, 2.75 gm boric acid, and 2 ml 0.5M EDTA pH 8.0 for 1 liter) and melted in microwave oven. When the temperature of the gel mixture was around 50^0C, 8 µl of ethidium bromide (10 mg/ml) was added and the gel mixture was poured into a gel tray. Sample buffer (10% glycerol w/v, 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1% bromophenol blue) was mixed in 1:1 ratio with the PCR product and loaded into the wells of the gel. Electrophoresis was carried out using an electrophoresis power supply machine (Pharmacia, Biotech). DNA was visualized under UV light and the gel was photographed using Polaroid film cassettes.

2.5. Species Identification

In order to identify the Mycobacterial species, cord formation as well as biochemical (Niacin and Pyrazinamidase) tests were performed on positive cultures.

2.5.1. Cord Forming Test

In order to investigate cording structure, a loop full of growth was emulsified in physiological saline on microscope slides according to Heifts (1982) and cording was examined at 100x magnification (oil immersion).

2.5.2. Niacin Test

This test was performed according to the protocol set by the manufacturer (Difco laboratories, Detroit, Michigan, USA) as follows: A loop full from the primary culture, was sub-cultured into LJ media and incubated for three weeks at 37^0C. On the 4^{th} week the culture was flooded with 1.5 ml of sterile distilled water and stabbed with pipette to release the Niacin in to the liquid. The tubes were then incubated in a slant position at 37^0C for 30 minutes. Then, about 0.6 ml of the wash was carefully transferred into sterile tubes and left to stand for 20 minutes. Using a flamed forceps, Niacin stuffs (DIFCO) were put into the tubes that contain the washes and shook three times for a total of 15 minutes after which time the result was read against a white background by comparing the color change. Those that produce yellow color were identified as Niacin positive.

2.5.3. Pyrazinamidase Test

Pyrazinamidase test was performed to distinguish among species of mycobacteria specifically between MTB and MB based on the ability to hydrolyze Pyrazinamide to Pyrazinoic acid. Pyrazinoic acid reacts with Ferrous ammonium sulfate to form a pink ferrous-Pyrazinoic acid complex. The presence of this pink band in the agar was taken as a marker of positive reaction to MTB and the absence of it as MB. Based on this criterion the test was performed according to the protocol set by Barabara and Howard (1987). Briefly, Pyrazinamidase agar (6.5 gm Dubos broth base + 0.1 gm Pyrazinamide + 2 gm Sodium Pyruvate + 15 gm Agar in one liter (pH 7.0) was inoculated from the positive cultures and incubated at 37^0C for 7 days. Then 1 ml of 1% ferrous ammonium sulfate solution (fresh) was added and kept at 4^0C for 4 hrs. Finally, the result was read against white background.

2.6. Immunological status

To characterize the immunological status of patients the following tests were done:

2.6.1. FACScan (Fluorescence activated cell sorter)

For FACScan analysis monoclonal antibodies (MAB) for CD45/CD14 (Leukogate), CD4/CD8 and mouse IgG1/IgG2a (controls) were purchased from Becton Dickinson (B&D) and the experiment was performed according to the manufacturer prescription. Briefly, 20 µl of each MAB and 100 µl of well-mixed whole blood were added to pre chilled tubes and incubated for 30 minutes on ice. Two ml of FACS lysing solution (B & D) was added to each tubes, and incubated for 10 minutes at RT. Following this, it was centrifuged at 1500 rpm for 5 minutes and the supernatant was removed leaving 50 µl of the pellet to which 1 ml of PBS azide was added and centrifuged as above. The supernatant was then aspirated leaving 50 µl pellet. Finally, 0.5 ml of 0.5% formaldehyde was added to each tube and kept at 4^0C until FACScan was run. Cell acquisition and phenotype analysis were done using 2 color analysis and CELLQuest software computer programs (B&D)

2.6.2. HIV antibody testing by ELISA

The study subjects were screened for HIV antibody using microelisa system kit (Vironostika, HIV Uni-Form 11 Plus, Organon Teknika GmbH, and Eppedlheim) in accordance with the manufacturer instruction. Briefly, 100 µl of specimen diluent was added into wells, including control wells. Then, 50 µl of sample or control was added into assigned wells and agitated for 15 seconds using a micro shaker and incubated for 1 hr at 37^OC. Then, it was washed 6 times with phosphate buffer and 100 µl of the substrate was added into each well. This was

incubated for 30 minutes and the reaction was stopped by adding 100 µl of H_2SO_4 and the result was read at 450 nm.

2.7. Blood processing

2.7.1. Serum

The blood in the vacutainer was centrifuged at 1500 rpm for 5 minutes and the serum was collected in nunc tubes and stored at -20^0C until use.

2.7.2. Lymphocyte Isolation

The blood was diluted with RPMI-1640 in 1:1 ratio and layered over Ficoll-Hypaque (Pharmacia, Sweden) in the ratio of 3:1 blood to Ficoll. Then, it was centrifuged for 30 minutes at 1800 rpm at RT, break released. Peripheral Blood Mononuclear Cells (PBMC) at the interphase were collected into 15 ml polystyrene tubes and washed 3 times with RPMI-1640, each time centrifuged at 1500 rpm for 5 minutes at 4^0C. PBMC in the pellet were resuspended in 2 ml of 5% Normal Human Serum (NHS) and counted before use.

2.8. ELISA (Enzyme Linked Immuno Sorbent Assay)

Flat bottomed 96 well plates (Immulon -2 Dynatech, Virginia, USA) were coated with 0.5 µg/ well of purified antigen, MPT59 in PBS (pH 7.2) for ON at 4^0C. Then the plates were washed 4 times with 0.1% PBST and blocked with 100 µl of blocking buffer (5mg BSA/ml in PBS) and incubated for 2 hrs at RT. Next, standards, IgG, IgA and IgM antibodies each having 1mg/ml concentration were serially diluted starting from 500 µg/ml concentration and samples diluted 1:50 for IgG and IgM and 1:10 for IgA were prepared and 100 µl/well was added and incubated for 1 hr at RT. After washing 4x with 0.1% PBS-T, 100 µl of peroxidase conjugated secondary antibody (anti-IgG/IgA/IgM) (1 mg/ml) diluted 1: 10,000 was added

and further incubated for 1 hr at RT. Finally, 100 µl/well of the substrate o-Phenylenediamine Dihydrochloride (OPD) was added and incubated for 30 minutes. The reaction was stopped by adding 50 µl/well of stop solution (1M H_2SO_4) and the result was read at 492 nm in an ELISA reader (Titertek multiskan plus, Finland).

2.9. Lymphocyte Stimulation Test (LST)

PBMC were cultured in U-bottomed 96 well tissue culture microtiter plates (Flow laboratory, USA) at a concentration of 10^5 cells/well in the presence of 1 µg PHA/well or 10 µg /well of PPD or BCG in 5% NHS supplemented with 5% glutamine and 5% Penicillin-Streptomycin in RPMI as culture medium. The plates were then incubated at 37^0C in 5% CO_2. 100 µl of the supernatant was collected at 48 hrs from plates containing PHA and 72 hrs from plates containing BCG and PPD and replaced with an equal volume of the enriched medium. Then, the cells were pulsed with Tritiated thymidine (1µCi/well) at day 3 (PHA) and day 6 (BCG and PPD). After 18 hrs the cells were harvested by an automatic cell harvester (Skatron, Norway) on to a filter mat (cat. No. 11731). Finally, proliferation was measured on a β-liquid scintillation counter (LKB Wallac 1216 Rackbeta II) and the stimulation index (SI) was calculated by dividing the CPM (count per minute) of antigen stimulated cells with that of non stimulated ones.

2.10. Cytokine assay

Cytokines (TNF-α, IFN-γ and IL-10) in supernatants collected 48 hrs (from PHA) and 72 hrs (from BCG and PPD) post incubation of PBMC were assayed using Sandwich ELISA as described below. The monoclonal antibodies (MAB) were purchased from R&D and aliquots were made and stored frozen after reconstitution according to the manufacturer prescription.

2.10.1. IFN-γ

IFN-γ assay was done using a kit (Mabtech, Nacka, Sweden). Briefly, 96 well plates (Immunol 2 Dynatech, Virginia, USA) were coated with 100 ul (2 μg/ml) of primary monoclonal antibody to human IFN-γ (MAB 1-DIK) and incubated overnight (ON) at 4^0C. After washing 3 times with PBS-Tween (0.05% Tween-20), plates were blocked with 200 ul of the blocking buffer and incubated at RT for 1 hr. At the end of incubation, 100 μl/well of the standard human IFN-γ (Gibco laboratories) 3 ng/ml serially diluted (positive control) and culture supernatants diluted 1:50 were added in parallel and incubated at RT for 2 hrs. After further washes with saline-Tween, 100 μl (100 ng/ml) biotinylated secondary monoclonal antibody (7-B6-1.biotin, Mabtech) was added and incubated for an hour. Then Streptavidin Alkaline Phosphatase (ALP) diluted 1:1000 in PBS-T was added to each well after another washing step and incubated for 1 hr at RT. After further washes, 100 μl/well of the substrate, p-nitrophenyl phosphate (p-NPP) (Sigma, UK) was added and incubated for 30 minutes at RT covered with aluminum foil. The reaction was stopped by adding 50 μl of NaOH. The absorbance was read at 405 nm by ELISA reader (Titertek multiskan plus, Finland). The results were given as the mean OD of duplicate wells.

2.10.2. IL-10 and TNF-α

MAB specific for IL-10 and TNF-α (R & D) were added in 100 μl volume (3 μg/ml) per well of a 96 well plate (Immulon 2 Dynatech, Virginia, USA) and incubated ON at RT. After washing three times with 0.05 % PBS-Tween, plates were incubated with blocking buffer (PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3) for 1 hr. Then, 100 μl standards (4 ng/ml serially diluted) and culture supernatants were added into the respective wells and incubated at RT for 2 hrs. After washing 3 times with PBS-Tween, 100 ng/ml of peroxidase

enzyme conjugated MAB specific for IL-10 and TNF-α (R & D) were added (200 µl/well) and incubated at RT for 1 hr. After 3 washes with PBS-T, 100 µl of the substrate (OPD) (Sigma, UK) was added to each well. Then the plate was incubated for 30 minutes at RT covered with aluminum foil. Finally, 50 µl of stop solution (2N H_2SO_4) was added and the optical density (OD) was read at 492 nm in an ELISA reader (Titertek multiskan plus, Finland).

2.11. Statistical analysis

Statistical analysis was performed using excel and sigma stat statistical packages mainly descriptive statistics. Student's t – test was used to compare differences among groups and considered to be significant when the *P*-value is less than or equal to 0.05.

3. Results

3.1. Description of the study subjects

Twenty-five female patients who came to the Black Lion teaching hospital mainly for infertility but also other gynecological complications were included in the study. All patients were clinically suspected to have mycobacterial infection. The clinical symptom of our patients was mainly infertility. Of the 17 patients whose infertility status was known, 6(35%) had primary and 11 (65%) had secondary infertility. Other symptoms like lower abdominal pain, irregular menstrual bleeding, pelvic mass, adhesion, etc. were observed in some of the patients. Constitutional symptoms like sweating and weight loss were not observed, yet it caused local organ dysfunction of the endometrium and the fallopian tubes as shown by bilateral tubal blockage by HSG examination. Their age ranged between 18-39 years and the median age was 28. Sixty two percent of them were less than or equal to 28. The majority of them (80%) were between the ages 20-34, which are the optimal child bearing age. The overall distribution of study subjects by age group is shown in table 1.

Table. 1. The distribution of study subjects by age

Age group	**<19**	**20-24**	**25-29**	**30-34**	**35-39**	**≥40**
N	1	5	7	4	3	0

Biopsy materials from surgically operated patients were taken from 14 patients and endometrial curettage from 11 patients. These specimens were examined for mycobacterial infection by four different methods namely, AFB staining, culture, histopathology and PCR.

Among the twenty five patients investigated, only one (4%) was AFB smear positive, three (12%) were culture positive, seven (28%) were histology positive and 12 (48) were positive

by PCR, which gave a total of 16 (64%) positive. The number of positive cases recovered from the biopsy specimen was much higher than that from the curettage specimen. This is shown in the table below.

Table 2. The number of positive cases by the four laboratory techniques in the two different samples (Biopsy and Curettage)

	N	AFB	Culture	Histology	PCR
Curettage	11	0	0	2	4
Biopsy	14	1	3	5	8
Total	25	1	3	7	12

3.2. HIV/AIDS status

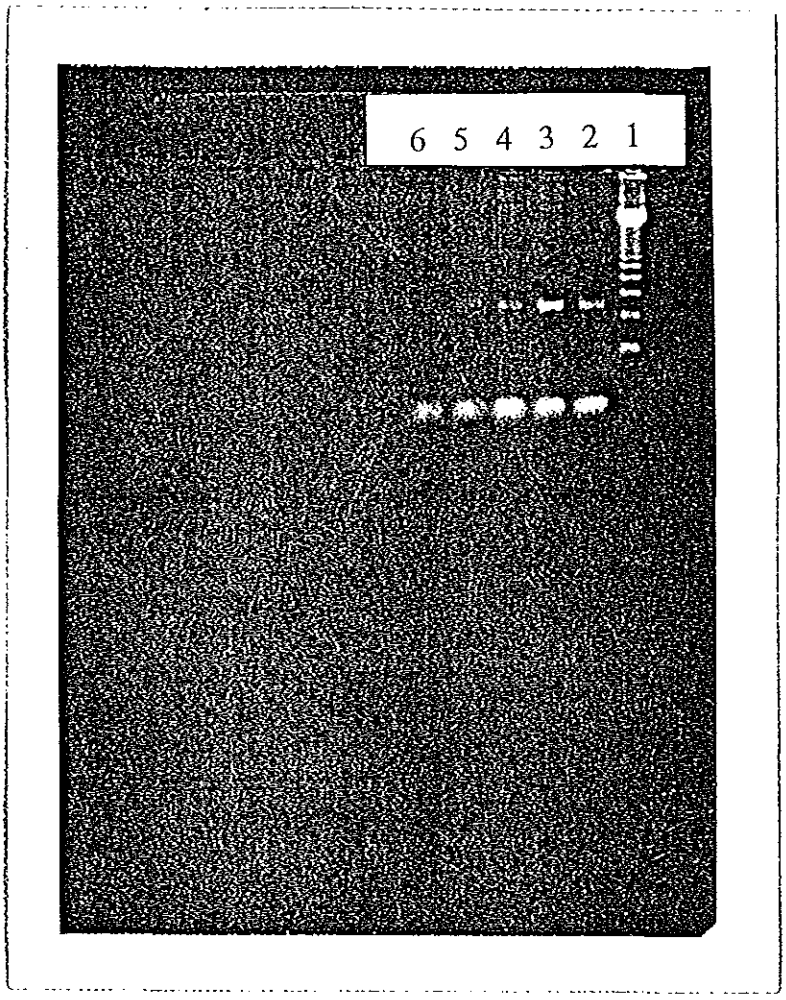
To determine the HIV/AIDS status, ELISA-HIV for 22 samples was done. Of these, FACScan analysis was performed only for 14 patients. The result showed ten of the twenty-two (45.4 %) were HIV positive while the percentage of $CD4^+$ and $CD8^+$ T cells ranged between 19.0% - 50.7% and 13.7% - 53.0% respectively. The percentage of CD4 as well as CD8 T cells in most of the study subjects were in the normal range set by the manufacturer of the MAB i.e., 28 -58 % for CD4 T cells and 19 - 48 % for CD8. The table (Appendix iv) shows the mean percentage of these cells in different groups of subjects.

3.3. Polymerase Chain Reaction (PCR)

Of the twenty-five clinically suspects, 16 were confirmed by one and/or the other methods. Of which, twelve were positive by PCR for ESAT-6 protein encoding gene. Eight patients could be confirmed by PCR but not by other methods. The following picture (Fig. 1) shows the PCR

positive clinical samples. The positive band is shown by the 300 bp amplified gene (esat-6) located between the 246 and 369 bp in the 123 DNA ladder.

Fig. 1. PCR amplified ESAT-6 protein encoding gene



Lane 1, 123 DNA ladder; Lane 2-4, clinical samples (DNA extracted from tissue);

Lane 5, positive control (known MTB DNA); and Lane 6, negative control (distilled water)

3.4. Species identification

Three methods (Cord formation, Niacin and Pyrazinamidase tests) were performed for the two culture positive samples in order to identify the type of species. Mycobacteria that belonging to the MTB complex group form tight cording (bacilli arranged in parallel). The result

showed that the two culture positive samples were cord forming confirming that they belong to the *M. tuberculosis* complex (Table 3). To identify further at the species level, the Niacin and Pyrazinamidase tests were performed. Both tests confirmed that the two culture positive samples were MTB and not *M bovis*. Table 3 shows the summary of the results found by the three methods.

Table 3. Identification of Mycobacterial species

Mycobacterium	Cord formation	Niacin	Pyrazinamidase
FGTB-022	Tight cording	+	+
FGTB-023	Tight cording	+	+
Control H37Rv	Tight cording	+	+
Control *M. avium*	No cording		
Control *M. bovis*		–	–

FGTB-022 and 023 cultures tested; *M avium* was negative control for cording;

M. bovis negative control for Niacin and pyrazinamidase tests and H37Rv was used as positive control.

3.5. Immunological assays

3.5.1. Criteria

Immunoglobulins were analyzed using three different ways following different approaches as criteria to define patients (laboratory confirmed) and controls (laboratory negative). These were:

Criterion A. Patient– when a sample was positively confirmed by any one of the four laboratory

diagnosis and control –when negative by all methods

Criterion B. Samples were divided into two groups as biopsy and curettage and both groups were analyzed separately as in criteria A

Criterion C. When patients were grouped into two based on HIV status irrespective of laboratory positivity

The four laboratory diagnosis results are given in the tables below (Table 4 - 6) according to the above criteria.

Table 4. Results of laboratory diagnosis according to Criterion A

Patient	AFB	Culture	Histology	PCR	N = 16
M-003			+		
M-006			+		
M-012	+	+	+	+	
M-017				+	
M-020		+		+	
M-024		+	+	+	
M-028				+	
M-032				+	
M-037				+	
M-049				+	
M-053			+	+	
M-057				+	
M-066				+	
M-069			+		
M-086			+		
M-089				+	
Control					N = 9
M-007					
M-041					
M-045					
M-060					
M-063					
M-072					
M-077					
M-081					
M-093					
Total	1	3	7	12	

Table 5. Results of laboratory diagnosis according to Criterion B

Biopsy	**Patient**	AFB	Culture	Histology	PCR
N=11	M-003			+	
	M-006			+	
	M-012	+	+	+	+
	M-017				+
	M-020		+		+
	M-024		+	+	+
	M-028				+
	M-032				+
	M-057				+
	M-066				+
	M-086			+	
Control					
N = 3	M-007				
	M-060				
	M-063				
Curettage	**Patients**				
N = 5	M-037				+
	M-049				+
	M-053			+	+
	M-069			+	
	M-089				+
Control					
N = 6	M-041				
	M-045				
	M-072				
	M-077				
	M-081				
	M-093				

Table 6. Results of laboratory diagnosis according to Criterion C

Patient	AFB	Culture	Histology	PCR	**HIV-, N=12**
M-003			+		
M-006			+		
M-007					
M-012	+	+	+	+	ND
M-020		+		+	ND
M-028				+	
M-045					
M-049				+	
M-053			+	+	
M-057				+	
M-063					
M-066				+	
M-077					ND
M-086			+		
M-089				+	
					HIV+, N=10
M-017				+	
M-024		+	+	+	
M-032				+	
M-037				+	
M-041					
M-060					
M-069			+		
M-072					
M-081					
M-093					

3.5.2. Antibody Assay

Antibody assay was performed using MPT59 as antigen on the solid phase in ELISA. All the clinically suspected cases were found to be seropositive (IgG, IgA, IgM) to this antigen except that there was difference in OD among them.

The OD reading for the three antibodies according to the three criteria are summarized below (Table 7) and the details are in Appendix II and III.

Table 7. Summary of the mean OD of the three antibodies according to the different criteria

CRITERIA	Mean IgG			Mean IgA			Mean IgM		
A	Patient	Contl	P-value	Patient	Contl	P-value	Patient	Contl	P-value
	0.396	0.287	0.676	0.371	0.363	0.895	0.264	0.304	0.383
B									
Biopsy	0.451	0.275	0.597	0.337	0.332	0.673	0.26	0.272	0.597
Curettage	0.286	0.293	0.968	0.438	0.378	0.540	0.295	0.304	0.646
C									
HIV^+/TB^+	0.320		0.195	0.359		0.854	0.345		0.222
HIV^+/TB^-	0.179			0.380			0.296		
HIV^-/TB^+	0.279		0.712	0.340		0.609	0.237		0.022
HIV^-/TB^-	0.330			0.306			0.416		
HIV^+	0.250		0.410	0.370		0.974	0.260		0.653
HIV^-	0.438			0.331			0.282		

According to criteria A and B, none of the three antibodies showed statistically significant difference between 'patients' and 'controls' ($P > 0.3$). The mean OD of anti MPT59 IgM in the 'control' groups was greater than from that of the 'patients'. When the data was analyzed based on the HIV status (criterion C), there was a statistically significant difference only in the level of IgM between HIV negative 'patients' and 'controls' ($P = 0.02$). The figures given below show the serum level of the three antibodies based on the different criteria.

Fig. 2. The level of IgG, IgA and IgM antibodies in the serum of 'patients' and 'controls'

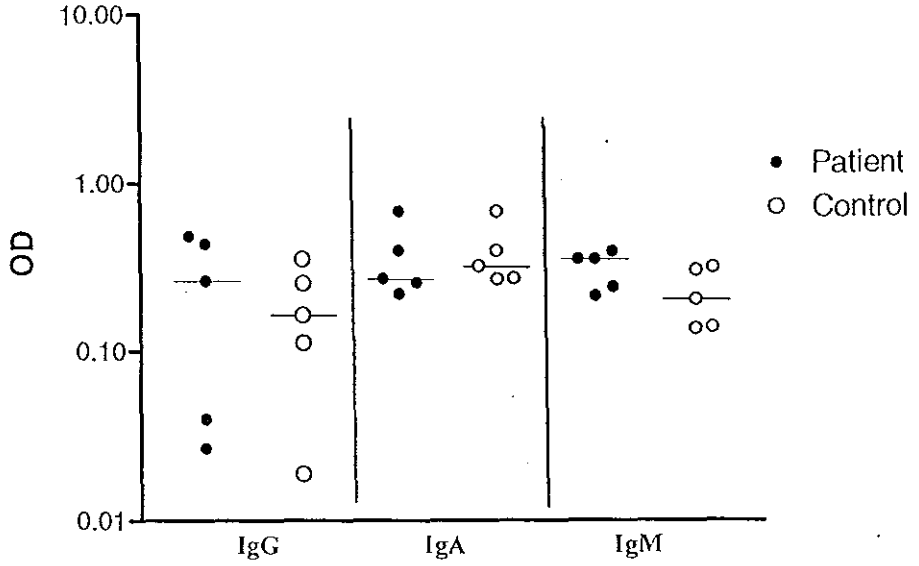


Fig. 3. The level of IgG, IgA and IgM antibodies in the serum of HIV^+ and HIV^- subjects

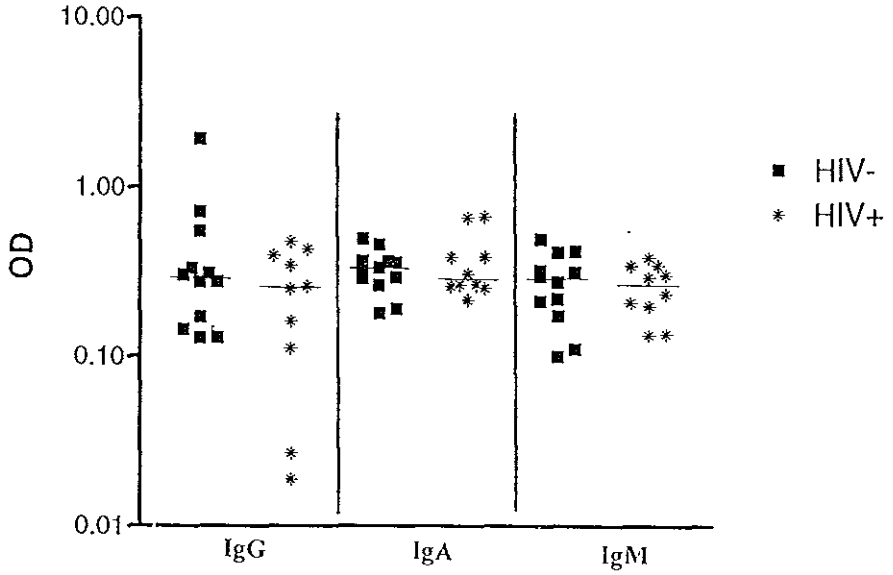


Fig.4. The level of IgG, IgA and IgM antibodies in the serum of HIV^+ 'patients' and 'controls'

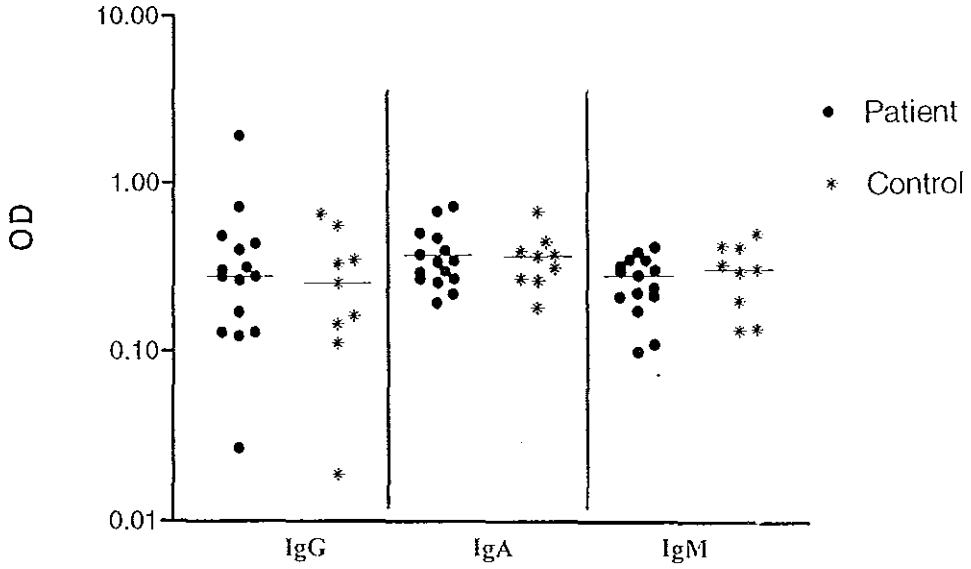
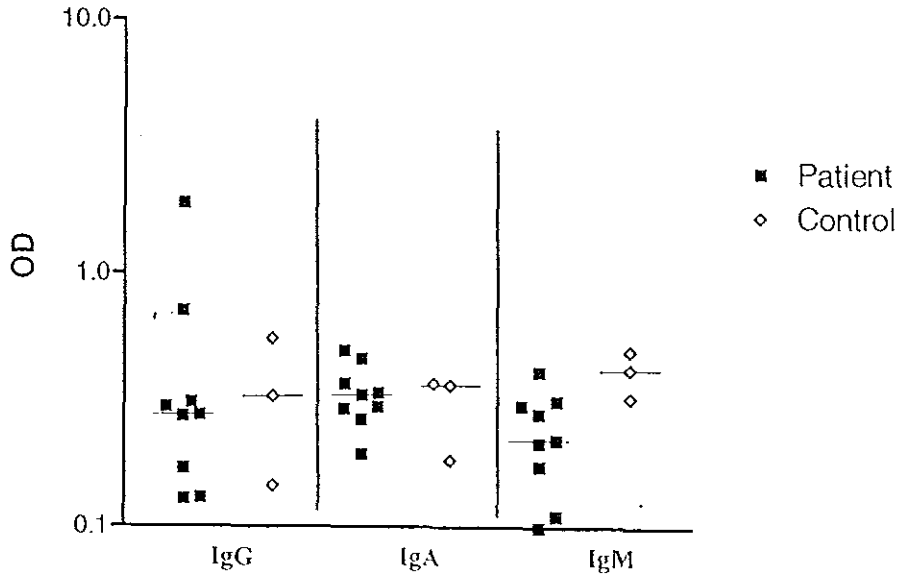


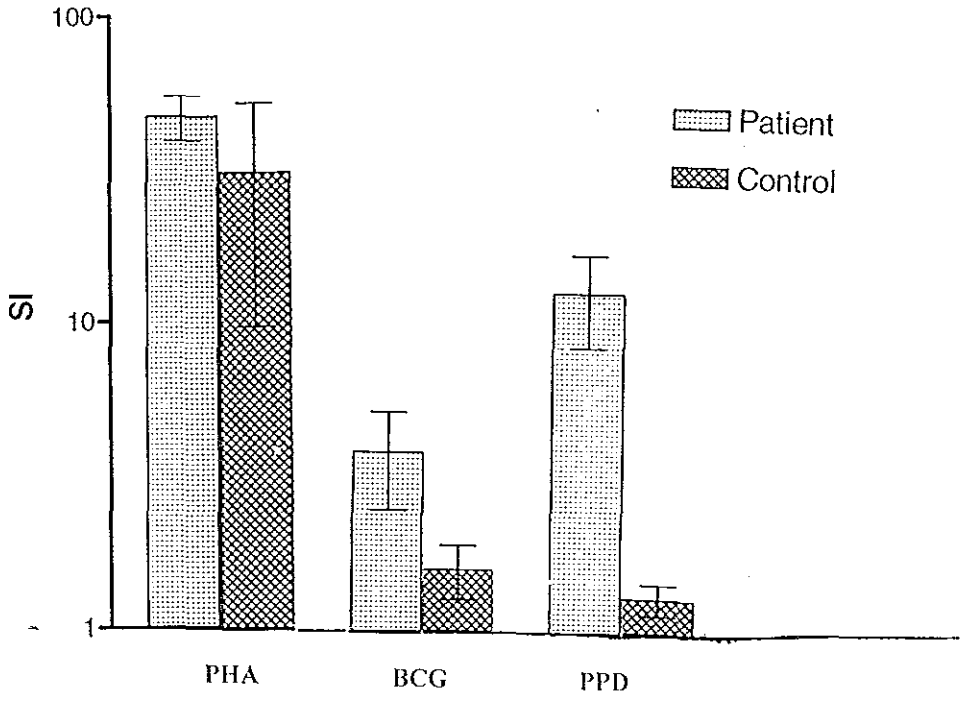
Fig.5. The level of IgG, IgA and IgM antibodies in the serum of HIV^- 'patients' and 'controls'



3.5.3 Lymphocyte stimulation Test

PBMC were subjected to LST using mytogen (PHA) and mycobacterial antigens, BCG and PPD. The raw data is given in (Appendix I). The graph given below (Fig. 6) shows the mean proliferative response of 'patients' and 'controls' according to criterion A with out considering the HIV status.

Fig. 6. The mean proliferative response of PBMC in 'patients' and 'controls'

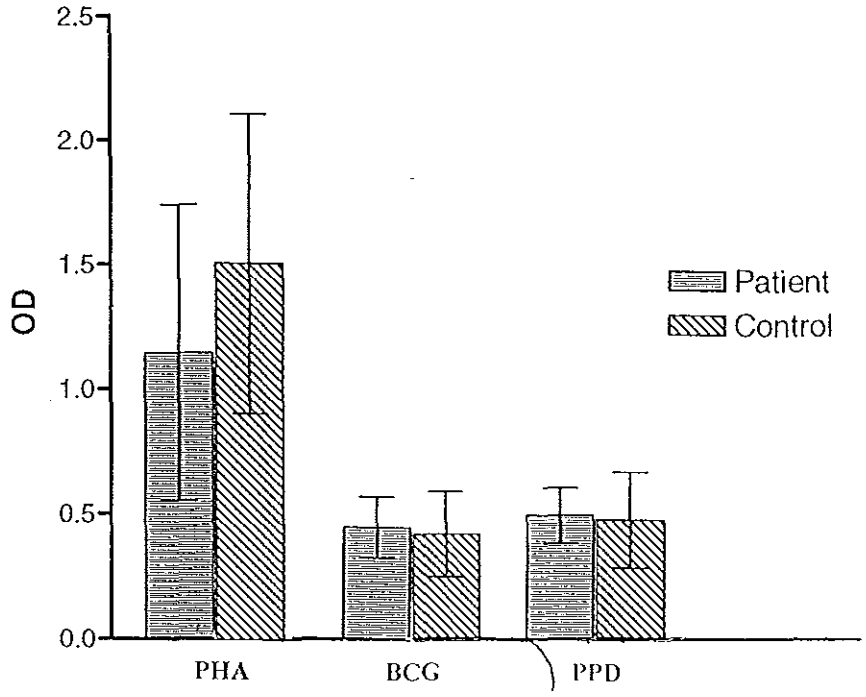


Although there was variation in the degree of stimulation, all subjects responded to PHA. There was a contrasting difference between the 'control' and the 'patient' response to BCG and PPD. This difference was statistically significant. The P value was 0.05 and 0.01 for BCG and PPD respectively. Although it is not statistically significant, the mean SI for PPD was greater than that for BCG (P = 0.43).

3.5.4. Cytokine Assay

In vitro production of IFN-γ, TNF-α and IL-10 following stimulation of PBMC with PHA, BCG and PPD was performed but detectable level was obtained only for IFN-γ. The level of IFN-γ to PHA was higher than to BCG and PPD and no clear difference was observed in the level of IFN-γ production between BCG and PPD stimulated PBMC. This is shown by the graph below (Fig. 7)

Fig. 7. The level of IFN-γ in the supernatant following stimulation of PBMC with PHA, BCG and PPD



4. Discussion

FGTB is an important gynecological problem in most countries where PLTB is prevalent. Nevertheless, efforts as to the diagnosis of this disease were not equally worked out. This may be because for the last two to three decades PLTB itself showed a reduction due to effective chemotherapy, and the same was observed regarding FGTB (Rom and Garay, 1996). But, now all forms of TB are showing up once again. A recent report by the National TB and Leprosy Control program of Ethiopia showed that the prevalence of PLTB and EPTB are now becoming equal (Dr. Birhanie, Personal communication). Literatures are now giving attention to this disease, which they had forgotten for decades. However, in these days too, except in case reports there are no immunological breakthroughs regarding the diagnosis of FGTB. Therefore, we intended to address this issue in addition to seeing into the occurrence and strain identification of this disease in Ethiopia.

According to our findings, the disease represents an important gynecological problem in Ethiopia. Although all samples were from clinically suspected patients, the laboratory diagnosis revealed that sixteen out of the twenty-five to be positive, which is a very big number.

Of the four laboratory diagnostic techniques that we used, AFB staining was the least sensitive (1/25; 4% were positive) and it was expected to be so because the samples were from extra-pulmonary sites.

Culture also was not diagnostic enough (3/25; 12%) though it was better in sensitivity than AFB staining. However, it requires longer time to see the growth (Varma, 1991). In our work the minimum number of days to see colonies was not less than 30.

The histopathological results also were not satisfactory as applied in several diagnostic laboratories. In our study we could detect only 7/25 (28%) patients to be positive by histology. Surprisingly, 4 of the 7 positives were not supported by PCR (more sensitive method). Therefore, one could think of false positive results from either PCR or histopathology. One patient was found to be negative by histology at the first day of curettage sample and became positive at the second sampling. This could show that histology suffers from false negative results. This may be because of the representativeness of the tissue, way of taking biopsy, period of taking curettage, preparation of tissue slices for microscopy, HIV co-infection and the like.

HIV co-infection could also be responsible for the absence of a clear (classical) granuloma formation (Bloom and Murray, 1992; Enarson, 1996). In our study, we could find a classical granuloma only in one patient out of the seven histology positives.

In order to increase the sensitivity of the diagnosis we included the PCR. When we used PCR as an additional tool, we got a total of 16 positives. Of these, eight were positive by PCR alone and the other eight were positive by any one of the other three techniques.

Although it is not logical to compare biopsy and curettage samples because they were not taken from the same patient, biopsy specimens taken from the fallopian tube gave higher positivity (78%) than the endometrial curettage (45%). This was also shown by others (Nogales *et al.*, 1979; Varma, 1991). But, according to Abebe and Wondwessen (1999), the highest proportion of positive cases was from endometrium (56%) and fallopian tube was second (27%), which is in contrast to our observation. The endomtrium provides less

diagnostic usefulness because, it is sloughed monthly and the time for granuloma formation at this site will be inadequate. Therefore, diagnosis based on histology from endometrial curettage alone and a report based on a single biopsy could result in false negativity.

FGTB occurs in relatively young females in the reproductive age group. This was also the case in our patients, the highest was between the group 24-29. This could be because, after puberty the blood supply to the pelvic organs is so much increased (Crofton *et al.*, 1992) as a result more bacilli could reach at the site and infect the reproductive organs.

Although the cultures tested were very few, the species identified as the causative agent was MTB. Therefore, it is logical to think that infection to the genital organs was secondary to PLTB. This was in line with the information found in the literatures that in 90% of the cases FGTB is secondary to PLTB (Winfred, 1977).

The serology studies were interesting in that all the 25 clinically suspected patients were serologically positive to mycobacterial antigen, MPT59. This was also shown by (Parikh *et al.*, 1997) where by they could confirm all the 20 clinically positive patients by using the three antibodies (IgG, IgA and IgM) to a different antigen called A60. The 100% positivity by serology could be achieved when the three antibodies were tested for a single patient. Because, a patient negative for IgG could be positive for IgM or IgA and vice versa.

In fact serology could not specifically tell that the infected organ is genital or pulmonary or kidney etc. (not site specific). What is important then is, if a gynecologist diagnoses a patient to be clinically positive for FGTB, seropositivity to any one of the three antibodies to a

specific mycobacterial antigen could suggest that the patient was highly likely to be a patient of that kind.

The other advantage could be that any patient who is suspected of this disease could easily be tested for the procedures are not invasive.

In using serology as a diagnostic tool, measuring a single antibody could result in false negative/positive results. For example, measuring IgG alone could not tell recent infection and a patient could be misdiagnosed as false positive. This in fact depends up on the sensitivity of the test.

In this experiment, we could not determine the cut-off values to the antibodies because all of the samples were at least clinically positive.

The HIV positivity of these patients was high i.e., 10/22 (45.4%). However, these patients were not at the AIDS phase as shown by their percentage of CD4 T cells (Appendix iv), which are in the normal range set by the manufacturer of the MAB (B&D). According to B&D, the normal range for the percentage of $CD4^+$ T cells is between 28 - 58% and for $CD8^+$ Tells, 19 - 48%. From this we can generalize that FGTB could have been established early and cause local organ dysfunction prior to progression of HIV infection to the AIDS phase. The high percentage of $CD8^+$ T cells in most of our patients could be due to coinfection with other microorganisms or parasites that could cause immune activation (Bentwich *et al.*, 1996). However we did not investigate the presence of coinfection.

A striking result was obtained from the proliferative response of patients to PPD. That is, the SI for FGTB positive patients (3.4 - 40) was at least two fold higher than that for the control

groups (1-1.5). There for, as far as this work is concerned, LST is the most likely candidate test system for the diagnosis of FGTB. Our impression is that "the test system" should further be evaluated on larger scale and more systematic approach to answer the remaining questions.

5. Conclusion and Recommendations

5.1. Conclusion

The study clearly showed that this disease, FGTB, does occur in Ethiopian gynecological patients and the prevalence and incidence could be high as Ethiopia is one of the high TB endemic country. Early diagnosis of the disease is crucial because once the bacilli damaged the tubes, reverting the tubal patency is difficult if not impossible. Early diagnosis usually fails mainly because the methods that we are using until now are either low in sensitivity or procedurally invasive. On the other hand, patients are not aware to seek medical help before such damages occur. Physicians too should be aware of the magnitude of the disease and suspect when they encounter the clinical symptoms. As diagnosis based on clinical manifestations alone is not reliable, searching for a better means of diagnosis is badly needed. On our side we propose LST as a potential diagnostic tool. Saying this, we recommend the following points for future investigation.

5.2. Recommendations

1. Epidemiological studies to see the magnitude of the problem using autopsy and/or biopsy
2. Further study on serology by taking well defined clinical samples and controls
3. Study on other accessible materials (menstrual blood)
4. Further investigation on LST
    - using different antigens to find the most immunogenic and TB specific
    - to study the immunological picture in PLTB, PLTB-FGTB, FGTB and other EPTB patients

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Appendix

Appendix I. The proliferative response of PBMC from patients and controls following stimulation with PHA, BCG sonicate and PPD

Patient	PHA	Control	SI	BCG	Control	SI	PPD	Control	SI
M-017	18719	421	44	296	243	1.2	2997	243	12.3
M-020	17640	235	75	848	180	4.7	1084	180	6
M-024	5843	340	17	353	268	1	909	268	3.39
M-026	13580	795	17	2156	233	9.2	9460	233	40.6
M-037	13625	316	43	294	278	1	1639	278	5.89
M-o49	7008	182	38.5	186	143	1.3	1146	143	8
M-086	5566	152	36	431	148	3	1479	148	9.9
M-041	22823	374	61	3498	290	12	4617	290	15.9
M-045	14990	360	41.6	660	495	1.3	ND	ND	ND
Controls									
M-077	712	177	4	235	132	1.7	190	132	1.4
M-081	2308	145	16	131	120	1	133	120	1
M-093	19196	263	72.9	268	125	2.1	190	125	1.5

Appendix II. The OD of anti MPT59 antibodies in the serum of patients and controls according to criterion A

Patient	HIV	IgG	IgA	IgM	N = 15
M-003		0.312	0.298	0.221	
M-006		1.908	0.369	0.174	
M-012	ND	ND	ND	ND	
M-017	+	0.398	0.266	0.386	
M-020	ND	0.122	0.711	0.297	
M-024	+	0.263	0.256	0.349	
M-028		0.275	0.267	0.215	
M-032	+	0.480	0.219	0.237	
M-037	+	0.431	0.664	0.345	
M-049		0.130	0.462	0.111	
M-053		0.715	0.340	0.413	
M-057		0.171	0.294	0.301	
M-066		0.300	0.194	0.316	
M-069	+	0.027	0.391	0.210	
M-086		0.279	0.500	0.100	
M-089		0.129	0.334	0.281	
Controls					N = 9
M-007		0.330	0.364	0.423	
M-041	+	0.112	0.670	0.296	
M-045		0.557	0.183	0.498	
M-060	+	0.349	0.261	0.137	
M-063		0.145	0.370	0.326	
M-072	+	0.163	0.270	0.135	
M-077	ND	0.654	0.445	0.416	
M-081	+	0.253	0.390	0.306	
M-093	+	0.019	0.311	0.200	

Appendix IV. The mean percentage of $CD4^+$ and $CD8^+$T cells in the peripheral blood of the study subjects

	N	$CD4^+$ T cells	**$CD8^+$T cells**
HIV +	6	32.7	33.5
HIV –	7	33.3	35.0
FGTB +	8	33.6	33.9
FGTB –	6	30.0	32.3
HIV+/TB+	3	30.6	31.6
HIV-/TB+	5	35.4	35.3
HIV+/TB-	3	34.8	35.3
HIV-/TB-	2	28.3	34.5