

CELLULAR IMMUNE RESPONSE IN ATYPICAL TUBERCULOSIS DIAGNOSED BY PCR IN PARAFFIN EMBEDDED MATERIAL

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Summary. The aim was to evaluate the cellular immune response in atypical tuberculosis and granulomatous inflammation consistent with tuberculosis (TBC), negative histochemically for acid-fast bacilli and analysed by PCR for *Mycobacterium tuberculosis* (MT) detection in paraffin-embedded tissue. Thirty six samples of differently localized atypical tuberculous lesions and granulomatous tuberculoid lesions negative for acid fast bacilli and 4 positive cases on Ziehl-Nielsen stain were analysed by PCR for MT detection and were tested immunohistochemically (IHC) for the cellular immune response in the granulomas and perigranulomatous tissue. The samples selected were: 7 pulmonary and 33 extrapulmonary specimens, especially lymph nodes. Histologically, the atypical tuberculous lesions contained suppurative necrosis, defective granulomas and cellular polymorphism. The epithelioid cells showed frequent mitoses. The immunoprofile of cells was polymorphous. L26 positive small lymphocytes were found in nodular lymphoid aggregates surrounding granulomas. A significantly increased number of positive UCHL1 cells were found in 33 out of the 40 analysed cases, with a larger percentage of CD 4 positive T cells (81.8% of cases). CD44 was positive in multinucleated giant cells (17.5% of cases), epithelioid cells (60% of cases) and lymphocytes (30% of cases). CD68 was localized in multinucleated giant cells and epithelioid cells, in a 4%, respectively 62.5% of cases. The PCR was performed in all 40 cases; the tissue samples were heterogeneous (lung, lymph nodes, liver, nasopharynx, etc.) and needed a good quality extraction of DNA. Performing a control PCR for Beta Globin tested the extraction; a good result was obtained in 31 cases (77.5%); from these, 19 cases had amplification for IS 6110. The cellular immune response in the atypical tuberculous lesions was similar in cases with and without acid-fast bacilli, but positive for PCR. In the most cases with negative PCR reaction, it was due to a deficient fixation of the material. The T lymphocytes were numerous in all types of tuberculous granulomas, with the prominence of CD4 positive subtype. The immunoprofile of the epithelioid cells, positive for CD44 and CD68, presenting frequently mitoses suggests an activate state in a possible relationship to the T-cell-mediated immune response in tuberculosis.

Key words: tuberculosis, imunohistochemistry, PCR detection of *Mycobacterium tuberculosis*.

INTRODUCTION

Tuberculosis (TBC) still remains a major health problem, with two million deaths and eight million new cases annually. At the same time, one third of the total world population (two billion people) is infected with the etiological agent,

Mycobacterium tuberculosis (MT). Of these, fewer than 10% ever develop disease, although the pathogen is not eradicated but rather contained in discrete lesions [1].

Due to the increased incidence of TBC in the last 5 years and the length of time required by classical diagnostic tests, especially among immunosuppressed patients (HIV-infected), rapid and sensitive tools for the diagnosis of tuberculosis are needed.

As a result, using the polymerase chain reaction (PCR) for the amplification of mycobacterial nucleic acids, the time required for the identification of MT can be considerably shortened (24–48 h) [2, 3], in comparison to the time required by microbiological tests (3–4 weeks on Lowenstein-Jensen culture) [4].

The PCR is especially requested in the retrospective diagnosis of the lymph nodes TBC, on paraffin embedded material, or in absence of MT on culture [5,6], or in a paucibacillar TBC; the PCR is useful also in cases with unexpected histopathological suspicion of a TBC and no material for culturing [7].

MT is a Gram-positive, aerobic, immobile and unsporulated bacillus; it has a waxy wall, with a complex structure which can retain the red dye from Ziehl-Nielsen stain, even after exposure at strong acids, which commonly destroy other bacteria. It does not growth on usual cultures, grows slowly on special enriched cultures (Lowenstein-Jensen), giving rise to only 1 colony per month, being, so far, difficult to study [8].

Its genome is estimated at 3.2 millions base pairs, with a high content of cytosine (C) and guanine (G) in its DNA structure. This high level of CG may be a survival strategy employed by the bacteria: heat-stability of DNA increases with the number of CG bonds.

Unlike other bacteria, MT genome is not supplemented by plasmids, but contains transposons (mobile genetic elements) [9, 10].

Chemotherapy resistant subtypes selection is produced after one single mutation, due to the presence of one gene for rRNA encoding, the same gene leading to slow growth of MT [11].

The MT *efpA* gene encodes a proteic complex (efflux protein A – EfpA), similar in structure with members of QacA transporter family (QacA TF) which mediate antibiotic and chemical resistance in bacteria [12]

The direct repeat (DR) region is characteristic for MT complex and is composed of multiple tandem repeats (each of 36-bp) plus nonrepetitive spacer sequences. DR and the adjacent variable sequence forms a direct variant repeat (DVR) as in the Figure 1.

Polymorphism of DR region (absence or presence of single DVR) [9] permits to distinguish different strains using a spacer oligonucleotide typing (spoligotyping) and may be used in the epidemiology of tuberculosis [13].

The DR locus represents one of the IS6110 insertion preferential loci in the MT genome. The mobile insertion sequence, IS 6110, is an important genetic marker in tracking MT strains [14].

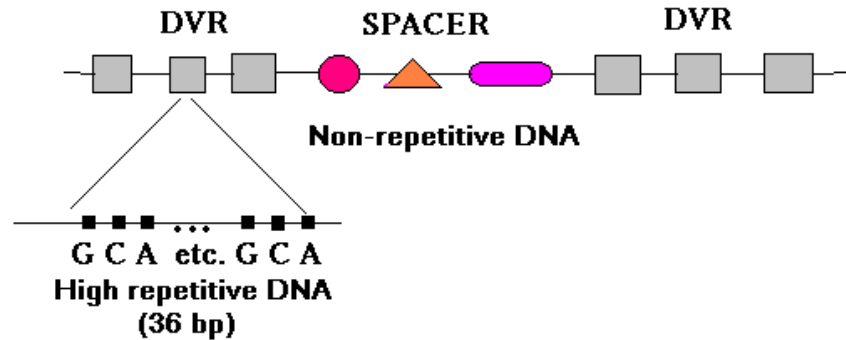


Figure 1 – The DR and DVR of MT complex

Practically, a good result may be obtained by PCR assay, amplifying a 123-base-pair segment within the insertion sequence IS 6110. This is a 1,361-base-pair putative insertion sequence that is repeated multiple times within the chromosome of MT; it is specific for all strains of MT complex; the detection limit is ranged between 1 to 20 bacilli, reflecting a good sensitivity, due to the great number of copies of IS6110 and to the small PCR product (123 bp) [6].

The inflammatory response to MT infection is a hypersensitivity-type granuloma [15], typical for tuberculosis, sarcoidosis or leprosy; the incidence of tuberculosis, the most important of the mycobacterioses is increasing and for HIV-infected persons it becomes a life-threatening disease [16].

In the last years the specific morphology of tuberculosis is changing and becomes similar to other inflammatory granulomas, especially in the lymph nodes (cat-scratch disease, lymphogranuloma venereum, yersinia lymphadenitis, tularaemia, etc.) [5].

The atypical tuberculosis is a polymorphous lesion, containing defective granulomas, suppurative necrosis, or extensive caseous necrosis without granulomas, an increased number of plasma cells, eosinophils, polymorphonuclear granulocytes, etc.

The cellularity of tuberculous lesions is changed and the typing of involved cells is obtained by immunohistochemistry (IHC). MT is an intracellular pathogen, which replicates within alveolar cells; it is engulfed by and subsequently infects alveolar macrophages in the lungs. Tuberculous granulomas consist of T lymphocytes and mononuclear phagocytes, while other infectious granulomas usually have different cellularity (B type lymphocytes, monocytoïd B cells, dendritic cells) [15].

Macrophage activation is achieved by T lymphocytes which are the principle mediator in the immune response against BK. CD4⁺ T cells secrete interleukines involved in the activation of macrophages; the NK-cells are also involved in the

activation of macrophages. In the delayed-type hypersensitivity (DTH) response to MT, CD4+ cells predominate over CD8+ T-cells, which are cytolytic cells that destroy specific target cells.

Helper T-cells are required to recruit and activate new macrophages to the granuloma. CD4+ T cells are divided into Th1 and Th2 subsets depending on the type of cytokines produced. Th1 cells produce IFN-gamma and IL-2 that are important for activation of antimycobacterial activities in DTH response. However it seems that in patients with advanced clinical forms of active TBC, antigens from MT may induce cell activation of T lymphocytes what leads to apoptosis not to proliferation [17]. IFN-gamma specifically activates macrophages and stimulates them to engulf and kill mycobacteria more effectively.

MATERIAL AND METHODS

One hundred and sixty nine formalin fixed, paraffin embedded samples from patients suspected of tuberculosis were re-examined histopathologically at the “Victor Babeş” Institute, Bucharest. The samples had been processed routinely at various hospitals, at the “Marius Nasta” Institute and “Victor Babeş” Institute of Bucharest.

The routine stain used was Haematoxylin and Eosin (H&E); the Ziehl-Neelsen stain was used for microscopic identification of acid-fast bacilli. Forty samples were retained as atypical tuberculosis, after reexamination of H&E; all but 4 of them were negative for Ziehl-Nielsen stain. The samples contained 7 pulmonary and 33 extrapulmonary specimens: 21 lymph nodes, 3 spleen, 2 bone marrow, 1 nasopharynx, 1 chest wall, 1 liver, 1 skin, 1 ovary, 1 prostate, 1 kidney.

The morphologic criteria used for categorizing atypical tuberculosis were: defective granulomas, suppurative necrosis, and extensive caseous necrosis devoid of granulomas, increased number of plasma cells, eosinophils, dendritic cells, and macrophages.

The selected specimens were tested by immunohistochemistry (IHC) for immunocompetent and immunoassociated cells, by means of following antibodies: L26, for B lymphocytes (DAKO, Glostrup, Denmark, dilution 1:100, with termic pretreatment; UCHL1 for T cells (DAKO, dil. 1:100, without pretreatment), CD4 for T helper cells (Neomarkers Fremont, Ca, USA, dil. 1:50), CD8 for T cytotoxic cells (DAKO, dil 1: 50), CD68 for macrophages (DAKO, dil 1:100) and the adhesion molecule, CD 44 (Novocastra, Newcastle upon Tyne, UK, dil. 1: 50), TB-01 for NK-like cells (kindly supplied by Prof. F. Malavasi, Istituto di Genetica Turin, Italy, dil. 1:100).

The immunohistochemistry (IHC) was performed on 5 µm thick sections from 10% formalin fixed paraffin embedded specimens, according to the Avidin-Biotin-Complex method of HSU [18], modified by Bussolati and Gugliotta [19].

Briefly, the procedure was: deparaffinization in xylene and alcohol series, rehydration, washing in phosphate saline buffer (PBS), incubation with normal serum, for 20 min., incubation with primary antibody overnight, LSAB kit (DAKO), washing in carbonate buffer and development in 3-3'-DAB hydrochloride / H₂O₂; nuclear counterstain with Mayer's Haematoxylin.

All 40 samples were prepared for performing PCR.

PREPARING SAMPLES FOR PCR

10 sections 5 µm thick from formalin fixed, paraffin embedded tissue were cut for every case, using a microtome. For preventing contamination among samples, the microtome blade and tools were cleaned with xylene and absolute ethylic alcohol (Wright and Manos), and samples mandatory are manipulated using gloves. For DNA extraction QIAamp DNA Mini Kit was employed [20].

The paraffin was extracted from tissue using 3 successive baths of xylene (vortexed then centrifuged) followed by three baths of absolute ethanol. The pellet resulted was dried in oven, and then mixed with ATL Buffer. The digestion was performed overnight using Proteinase K at 56°C.

The manufacturer's protocol was followed for the procedure; the final dilution volume was decreased to increase the concentration of genetic material. Samples were stored at -20°C. The quality of nucleic acids was evaluated on agarose gels and absorbance was analysed at spectrophotometer for 260 nm and 280nm wavelength (DNA and proteins). A ratio over 1.7 was considered suitable.

The polymerase chain reaction was performed first for Beta Globin gene to assess the presence of extracted DNA, then for IS 6110 sequence.

Primers used were kindly supplied by Prof. Dr. Gianni Bussolati and Dr. Patrizia Gugliotta from Università degli Studi, Turin, Italy. Primers used for beta globin gene, common to all human cells, amplify a specific segment of 251 base pairs. Primers used for mycobacterial detection amplify a 123 bp fragment of the repetitive insertion element IS 6110.

Amplification by PCR was performed in a Perkin Elmer thermocycler, with a reaction mixture of 20 µl final volume.

The initial denaturation consisted in 8 minutes at 94°C, then the mixtures were amplified 35 cycles: denaturation at 94°C for 30 sec, followed by annealing at 53°C for 1 min (Beta globin control) and 62°C (mycobacterial amplification) respectively, extension 7 min at 72°C for 2 min with a final extension of 7 minutes followed by cooling at 4°C.

Detection of PCR products was done by electrophoresis in 2% agarose (BioRad-certified PCR agarose) gel Ethidium-Bromide stained, using a mixture of product and loading dye (bromophenol blue – LKB, Bromma, Sweden) in TRIS-borate-ethylene-diaminetetraacetic acid buffer.

Each PCR run included positive and negative controls. The positive controls consisted of a sample of a microbiologically certified tuberculous lymphadenitis; the negative control contained the reaction mixture without DNA, which was replaced by the equivalent volume of distilled water.

RESULTS

All the 40 samples derived from the files of various hospitals, “Marius Nasta” Institute and “Victor Babeş” Institute were reexamined histologically and were controlled for MT by Ziehl-Nielsen stain. All but 4 cases were negative for Ziehl-Nielsen stain.

The localization of samples was: 7 pulmonary and 33 extrapulmonary (21 lymph node, 3 spleen, 2 bone marrow, 1 nasopharynx, 1 chest wall, 1 liver; 1 skin, 1 ovary, 1 prostate, 1 kidney).

From the patient's data resulted that 3 patients had malignant tumors (mammary carcinoma – 2 and Hodgkin disease – 1) and were polychemotreated; 1 patient was diagnosed with a HIV infection and chemotherapy resistant tuberculosis; 1 patient with hepatic multiple tuberculoid granulomas was previously treated by BCG instillation for urothelial carcinoma.

Microscopically, 15 samples had extensive caseous necrosis without proliferative lesions, 6 cases – suppurative necrosis (Figure 2), 3 cases – only proliferative isolated granulomas without necrosis (Figure 3), 1 case – perinecrotic palisading macrophages, 1 case – tuberculoid granulomas with foamy macrophages, the remainder, 14 cases – tuberculoid granulomas with or without caseous necrosis surrounded by polymorphous cellular infiltrate (plasma cells, eosinophils, foamy macrophages, etc.). The Ziehl-Nielsen stain was positive in 4 cases (3 with granulomatous lesions without or with minimal necrosis, and 1 with suppurative necrosis in a patient infected with HIV).

The cellular immune response inside or around granulomatous tuberculoid lesions presented various aspects. L 26 was positive in frequent small lymphocytes in the follicular lymphoid parenchyma surrounding granulomas. UCHL1 was positive in all cases, well expressed in numerous lymphocytes localized inside or around granulomas. A significantly increased number of positive cells were found in 33 out of the 40 analysed cases. The percentage of CD 4 positive T cells (Figure 4) was larger (81.8%), than that of CD8 positive T cells (Figure 5).

TB-01 was positive in 9 cases (22.5%) in rare lymphocytes around granulomas, but in numerous cells in the remnants of lymphoid follicles in lymph nodes. CD68 was localized in multinucleated giant cells and epithelioid cells in a 4%, respectively 62.5% of cases. In a case of the patient with hepatic granulomas treated by instillation for urothelial carcinoma of the bladder, the positivity for CD68 was observed in epithelioid and in Kupffer cells (Figure 6).

CD 44 was positive in multinucleated giant cells (17.5% of cases), epithelioid cells (60% of cases) and lymphocytes (30% of cases). The IHC reaction was localized at prominently at the membrane of cells (Figure 7).

The PCR was performed in all 40 cases; the tissue samples were heterogeneous (lung, lymph nodes, liver, nasopharynx, etc.) and needed a good quality extraction of DNA.

The extraction was tested by performing a control PCR for Beta Globin (Figure 10); good results were obtained in 31 cases (77.5%); from these, 19 cases had amplification for IS 6110. Every run of PCR contained a positive control, a negative control and a molecular weight marker (Figure 11).

DISCUSSION

The results of the histopathologic study permit to identify an increased number of atypical tuberculous lesions in different localizations. It is to retain the possibility of confounding them with other tuberculoid granulomas like sarcoidosis, or other inflammatory granulomas like cat-scratch disease, lymphogranuloma venereum, yersinia lymphadenitis, tularaemia, etc. [5]. In a case of cutaneous granulomas it was observed an increased number of foamy macrophages among and around of epithelioid cells, a finding requesting a differential diagnosis with the leprosy.

From 15 cases with extensive caseating necrosis, devoid of proliferative granulomas around them, no one had a positive reaction for Ziehl-Nielsen stain. The same negative Ziehl-Nielsen stain was observed in 5 out of the 6 cases with suppurative necrosis, but presenting around, rare or defective granulomas. Ziehl-Nielsen positivity was observed in 3 cases with prominent proliferative granulomas, having no necrosis or incipient necrosis (Figure 8); sometimes the acid-fast bacilli were nested in the multinucleated giant cells (Figure 9).

The paraffin embedding might to destroy the bacilli and this fact may be an explanation for the reduced number of positive cases but the large number of bacteria in the positive cases.

The cases represented by tuberculoid granulomas with polymorphous cellular infiltrate inside or around had different extension of necrosis, but no acid-fast bacilli.

Among the epithelioid and multinucleated giant cells a various number of plasma cells, eosinophils, foamy macrophages or histiocytes were observed. In some cases the necrosis was vaguely suppurative, containing a few polymorpho-nuclear granulocytes.

The multinucleated cells showed distorted forms and a reduced number of nuclei. In the cases with rich proliferative granulomas it was observed an increased number of mitoses in the epithelioid cells and in the peripheral lymphocytes.

The PCR amplification applied in all 40 cases showed unexpected aspects; the tissue samples had a heterogeneous localization (lung, lymph nodes, liver, nasopharynx, skin, etc.) and needed a good quality extraction of DNA.

The extraction was performed once in 22 cases and it was repeated twice or more times, in 18 cases using the QIAGEN Kit. In 9 cases the control PCR for Beta Globin failed to obtain a valid result.

The problem raised by this finding was the processing of the specimens, variable for different laboratories. The formalin, acid or neutral and the duration of fixation can affect the PCR amplification, like the molecular weight of the expected PCR product [2].

All cases followed from the beginning in our laboratory to have a buffered formalin fixation, not more than 24 hours permitted a good extraction even with a negative PCR amplification for IS6110 (in 2 cases of sarcoid granulomas). The positive amplification in 19 cases included all type of lesions, from an extensive caseating necrosis to rich proliferative granulomas; all 4 cases positive for Ziehl-Nielsen stain presented a good PCR amplification.

The immunoprofile of cell populations in the analysed cases revealed a reduced participation of B-lymphocytes; they appeared only outside of granulomas, like nodules or lymphoid follicles, mainly in lymph nodes. In spite of atypical microscopic morphology, like suppurative necrosis, or unusual cell populations as specified in some works [15], the PCR positive cases presented numerous UCHL1 positive T cells, inside and around granulomas.

This fact reflects a T-cell-mediated immune response, supported in the majority of cases by the prominence of CD4 positive lymphocytes. The positivity for PCR in cases negative for Ziehl-Nielsen stain can reflect a good defence mechanism; one of cases in this series presenting extremely numerous acid-fast bacilli belongs to a HIV infected patient.

The activating role of NK and NK-like cells was not observed in this series of cases. The positive cells were rare and in a few cases of granulomatous lymphadenitis with numerous TB-01 positive cells they were situated only in germinal centers of surrounding lymphoid parenchyma.

The positivity for CD68 was well exprimed in the largest part of cases reflecting the macrophagic nature of the epithelioid / histiocyte cells; for the multinucleated cells this positivity was much lesser.

The epithelioid/histiocyte cells are very active cells as it is showed by the numerous mitoses found especially in the proliferative granulomas; in these situations the lymphocytes have also mitoses. The active status of these cells is suggested by a strong expression of CD44, an adhesion molecule and an activating factor; the epithelioid cells and the lymphocytes express it in about 2/3 of cases, in their membrane and cytoplasm.

As it results from this study, the PCR amplification for MT is useful and sometimes necessary for diagnosis and for a correct treatment.

CONCLUSIONS

The cellular immune response in the atypical tuberculous lesions was similar in cases with and without acid-fast bacilli, but positive for PCR. In the most cases with negative PCR reaction, it was due to a deficient fixation of the material.

The T lymphocytes were numerous in all types of tuberculous granulomas, with the prominence of CD4 positive subtype. The immunoprofile of the epithelioid cells, positive for CD44 and CD68, presenting frequently mitoses suggests an activate state in a possible relationship to the T-cell-mediated immune response in tuberculosis.

Analysing the immune response in granulomatous tuberculoid inflammation can offer information about the immunopathological mechanisms of the atypical tuberculous process and an aid in differentiating them from other inflammatory granulomas.

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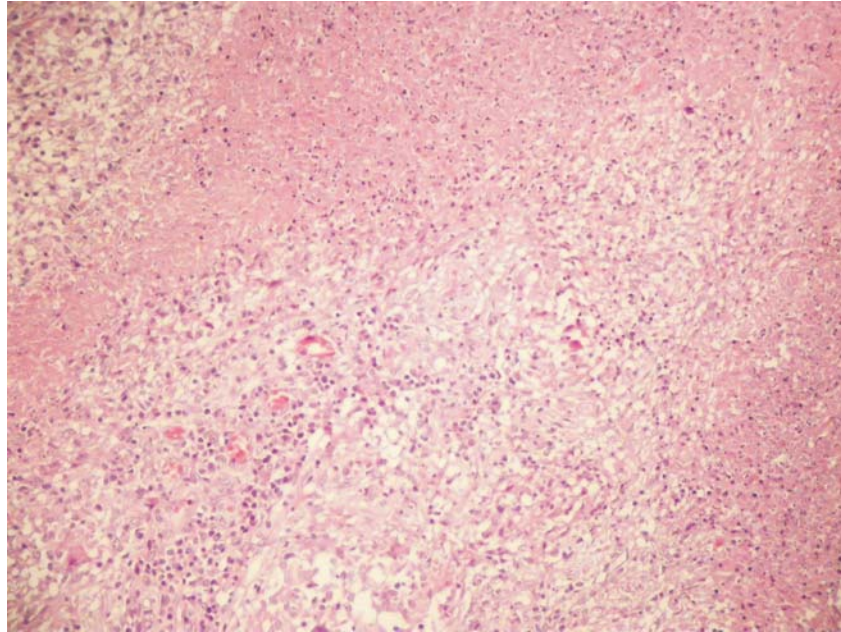


Figure 2 – Suppurative necrosis in an atypical defective granuloma (H&E stain, $\times 100$)

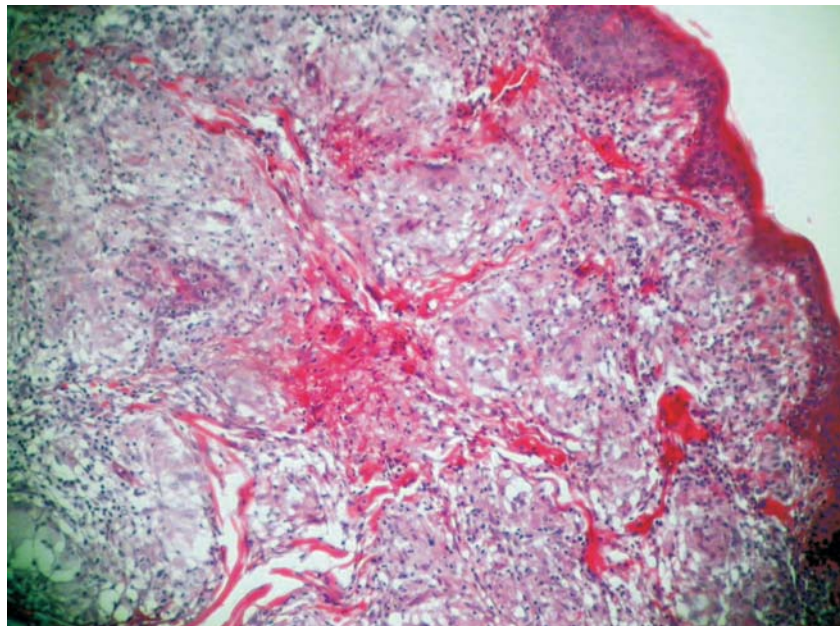


Figure 3 – Tuberculoid granuloma without caseous necrosis (H&E stain, $\times 100$)

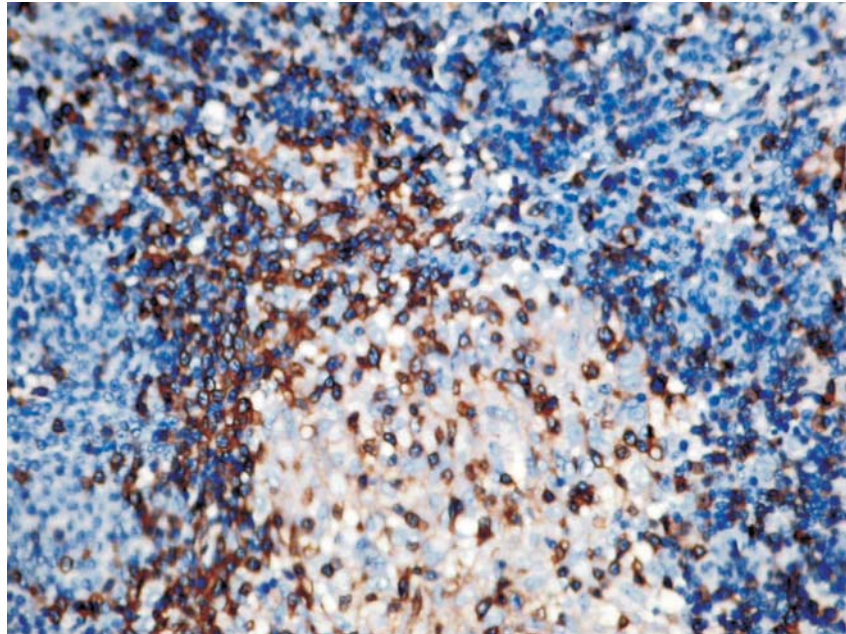


Figure 4 – Cd4 positive lymphocytes inside and around granuloma (ABC-immunoperoxidase technique, $\times 200$)

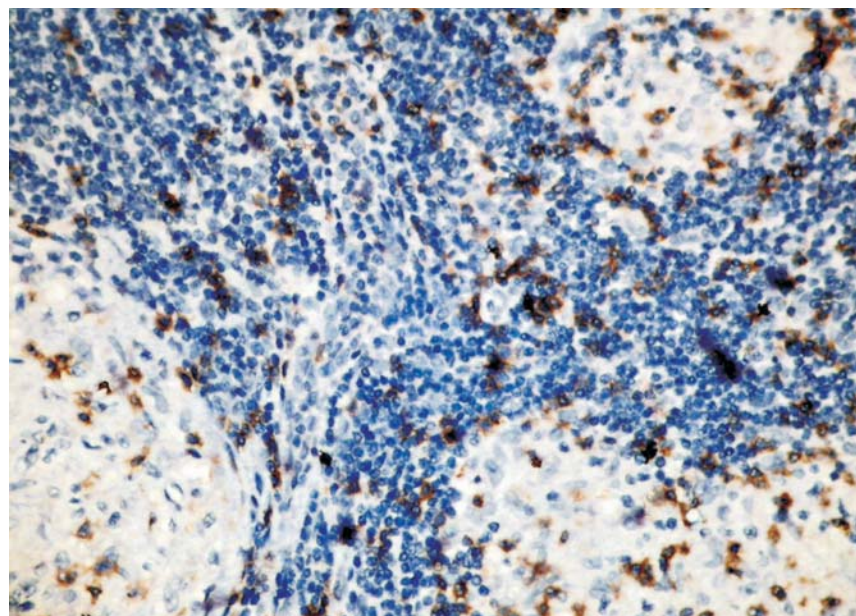


Figure 5 – Rare CD8 positive lymphocytes (ABC-immunoperoxidase technique, $\times 200$)

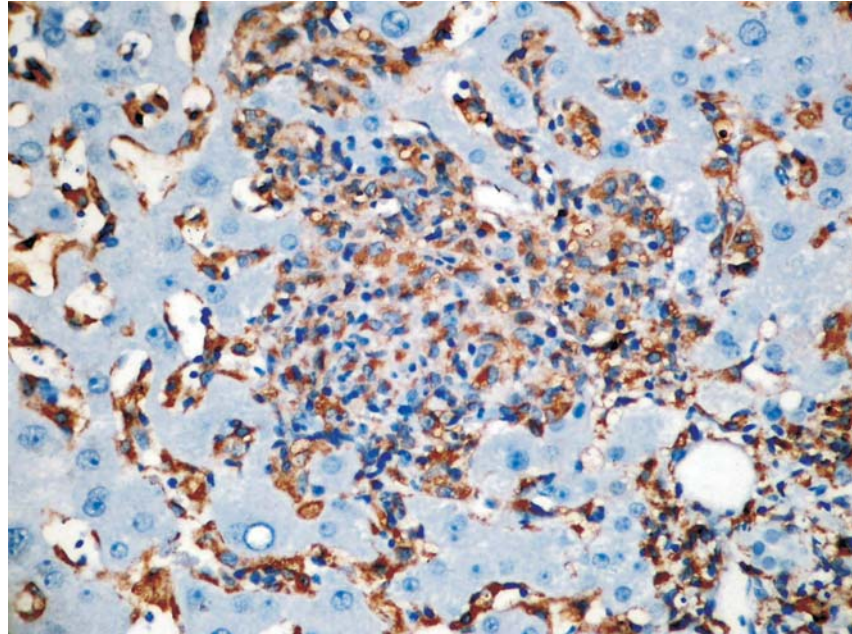


Figure 6 – Hepatic tuberculous granuloma, CD68 positive epithelioid and Kupffer cells (ABC-immunoperoxidase technique, $\times 200$)

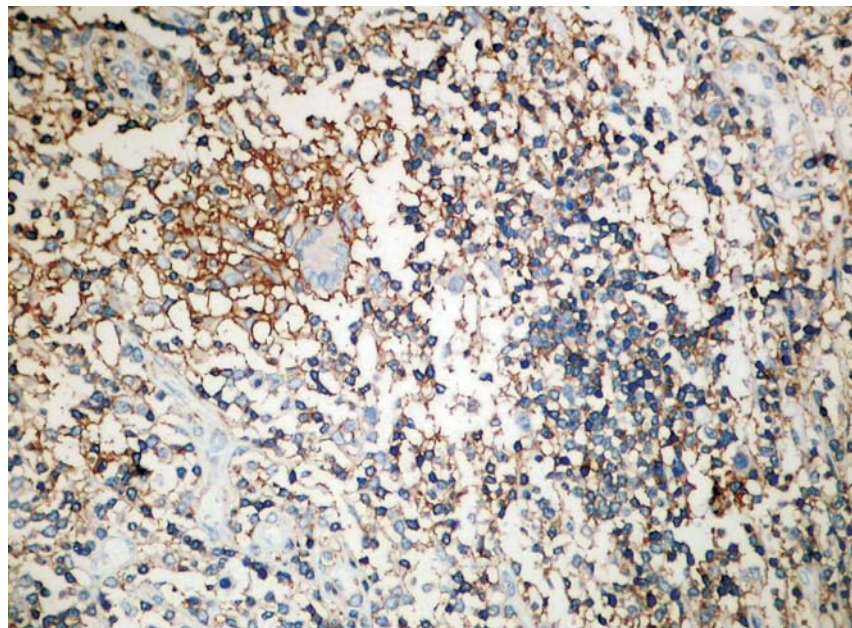


Figure 7 – Tuberculous granuloma, CD44 positive membrane of cells (ABC-immunoperoxidase technique, $\times 200$)

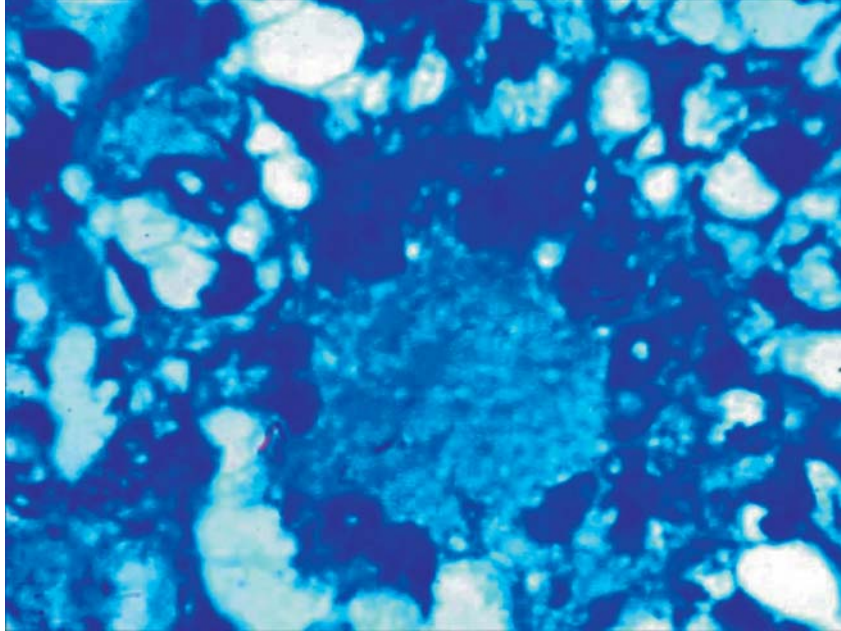


Figure 8 – Numerous MT in a pulmonary tuberculosis (Ziehl-Nielsen stain, $\times 100$)

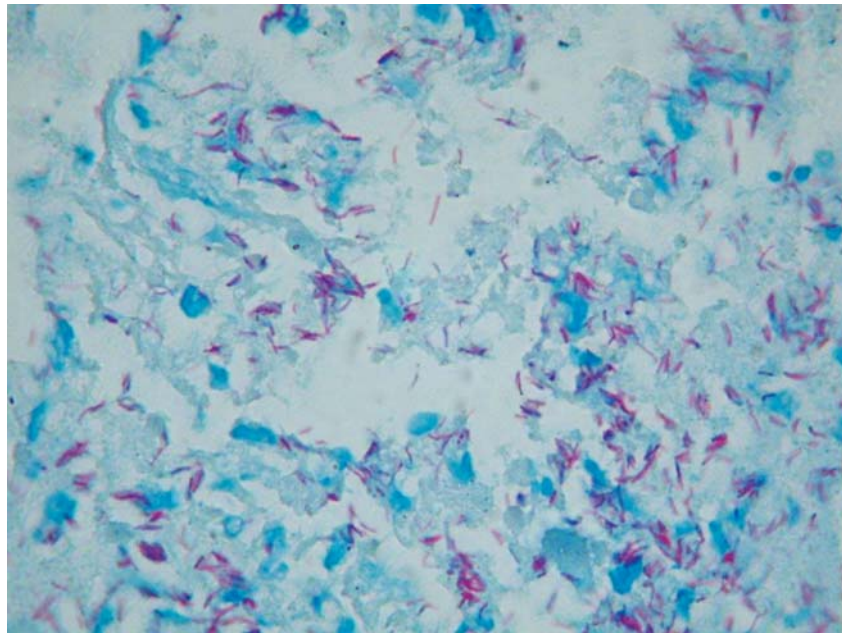


Figure 9 – Acid fast bacilli in a multinucleated giant cell (Ziehl-Nielsen stain, $\times 100$)

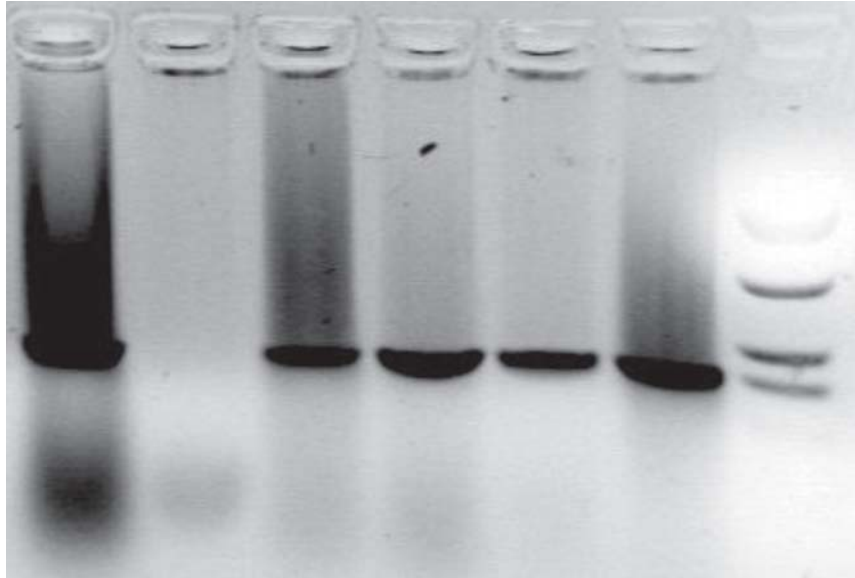


Figure 10 – PCR for Beta Globin, DNA quality extraction control

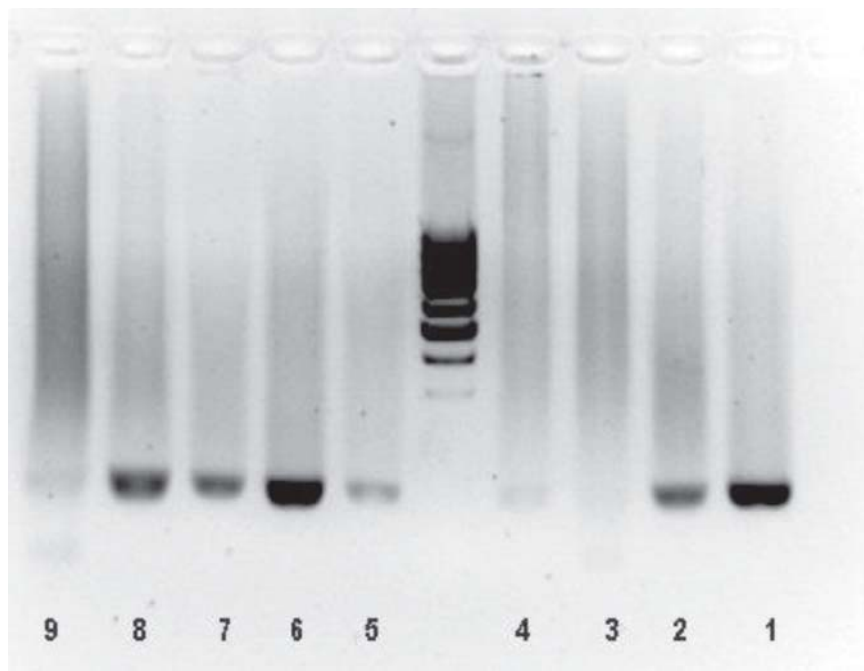


Figure 11 – PCR for IS6110; positive samples for lanes 2, 6, 7, 8; lane 1 positive control; lane 9 negative control; middle lane – molecular weight marker