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Original Article

Comparison of Polymerase Chain Reaction, Ziehl-Neelsen Staining and Histopathologic Findings in Formalin-fixed, Paraffin-Embedded Tissue Specimens for Diagnosis of Tuberculosis

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ABSTRACT

Background and Objective: **Tuberculosis is still a major health problem, involving about 1/3 of the world´s population. Diagnosis is difficult when we only use Ziehl-Neelson staining. Many cases may be missed. A more rapid and sensitive diagnostic method is necessary. PCR may be helpful. The aim of this study is to compare PCR, Zieh-Neelsen staining and histopathologic findings in diagnosis of tuberculosis on formalin-fixed paraffin-embedded tissues.**

Methods: **Paraffin blocks of the submitted specimens of the patients clinically suspicious for tuberculosis or containing granuloma were selected. Ziehl-Neelsen Staining & TB-PCR (IS6110 element) were carried out. The results of the tests were compared by using the McNemar test. Statistical significance was accepted when the *P* value was less than 0.05.**

Results: **Forty five specimens were included in the study, 35 had granulomas (19 with caseous necrosis). Acid-fast bacilli were identified in 17 specimens (37.8%). TB-PCR was positive in 16specimens (84%) with caseating granulomas, 11 specimens (68.8%) with non-caseating granulomas & 6 specimens (60%) without granulomas. (*P* value = 0.59).**

Conclusions: **TB-PCR on paraffin–embedded tissue is a potentially useful approach for early, rapid and sensitive diagnosis of tuberculosis. It is especially useful when granuloma is seen in tissue section, while acid-fast stain is negative. If there was no facilities for PCR, histopathological diagnosis with clinical correlation is more reliable in comparison to AFB results.**

Keywords: **PCR, Staining, Pathology, Tuberculosis**

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Introduction

Tuberculosis (TB) is an important health problem worldwide (1). In 2008, the World Health Organization (WHO) estimated that yearly about 1.7 million people die because of TB (2).The incidence of all forms of TB in Eastern Mediterranean sub region countries such as Iran is 104 per 100000 and the incidence of cases which were smear positive is 24 per 100000 populations (3). In Iran, most of the new TB cases occur among immigrants especially in Afghan immigrants (4).

The reasons for this may be attributed in part to the increasing number of immunocompromised individuals with human immunodeficiency virus (HIV) infection and emergence of many multi-drug resistant strains of mycobacteria (5). Early diagnosis and complete treatment of TB is important for effective control and prevention of the spread. Despite the fact that TB is an important infectious disease with varying degrees of prevalence around the world, the clinical diagnosis of this disease remains a true challenge due to its varying localizations and presentations (6).Therefore the disease may remain undiagnosed and untreated (7).

Early diagnosis of infection is important before administration of antituberculosis chemotherapy. In terms of histopathologic diagnosis, TB can be diagnosed only as a chronic granulomatous inflammation, suggestive for tuberculosis "on a routine pathologic slide (8, 9). Histopathologic features of chronic granulomatous inflammation can be found in various conditions and diseases other than TB, such as foreign body reaction, fungal infection, sarcoidosis, cat scratch disease, leprosy and brucellosis(8). In addition, Ziehl-Neelsen (ZN) staining for the diagnosis of tuberculosis (TB) as employed in most low-income countries is easy to use and cost effective, but its low sensitivity is a major drawback (4,10). Diagnostic methods of tuberculosis have improved in recent years, and several molecular techniques such as PCR have been introduced for its diagnosis. Many studies suggest PCR as a sensitive and rapid test for diagnosis of tuberculosis especially in well-formed granuomatous lesions (11, 12, 13).

Due to clinico-sosial significance of rapid and reliable diagnosis of TB this study was performed to compare three diagnostic methods including: histopathology (H&E staining), Ziehl-Neelsen staining and PCR on formalin-fixed, paraffin-embedded (FFPE) tissue suspicious of TB and choose the best of them.

Materials and Methods

In this cross sectional study materials were obtained from the archives of Pathology Department "Taleghani" Hospital, Kermanshah University of Medical Sciences between 2007 and 2009. Inclusion criteria were the clinical and/or pathological findings compatible with tuberculosis. We retrieved formalin-fixed, paraffin-embedded (FFPE) tissue blocks from 45 different specimens (17 pleura, 13 lymph nodes, 5 lungs, 5 bones, 3 synovia, 1 peritoneum and 1 intervertebral disc tissues). We performed the following tests on the samples:

1. Histopathology examination

Tissue samples were processed by standard wax techniques. The FFPE tissue blocks were cut in 4 μm serial sections. The sections were stained with H&E method (14). Then slides were evaluated microscopically at increasing magnifications (×100, ×400).

Histopathologic classifications (15, 16):

• **Caseating granulomas**: Tuberculosis granuloma displaying central necrosis with or without mineralization surrounded by macrophages, lymphocytes, plasma cell, neutrophils, epithelioid cells, and langhan's giant cells and enclosed partly or completely by a thin capsule (Fig. 1A).

• **Non- caseating granulomas:** Lesion characterized by irregular unencapsulated clusters of

epithelioid macrophages but not langhan`s –type multinucleated giant cells and necrosis, consistent with an initial stage (Fig. 1B).

• **Without granulomas:** Features not consistent with tuberculosis granuloma, including significant eosinophilic infiltrates, lymphoid hyperplasia and presence of bacterial colonies within necrotic area or tumors.

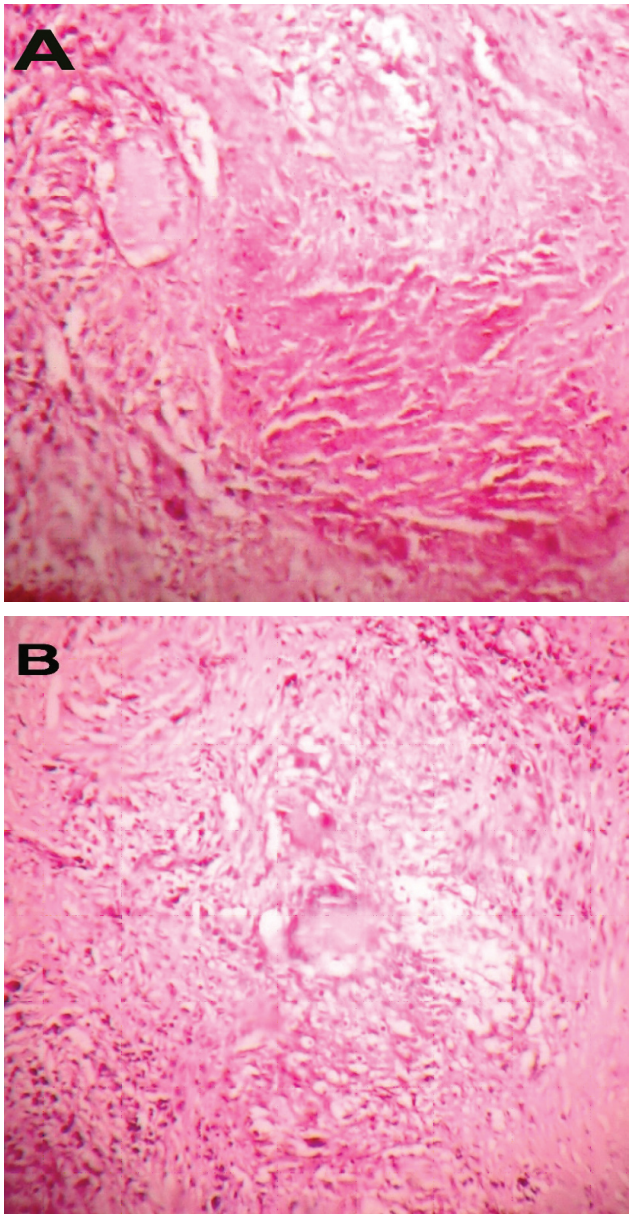

Fig.1: A) Caseating granulomatous necrosis; **B)** non-caseating granulomatous necrosis (Hematoxylin and Eosin Staining ×100)

2. Ziehl-Neelsen staining

Two or three sections were stained with ZN method (14). Ziehl- Neelsen carbol- fuchsin was used after treatment with periodic acid solution 1%. The entire areas of each section of all slides were examined at ×100 magnification carefully. They were considered positive when one or more acid- fast bacteria were detected in at least one section of the sample (Fig. 2).

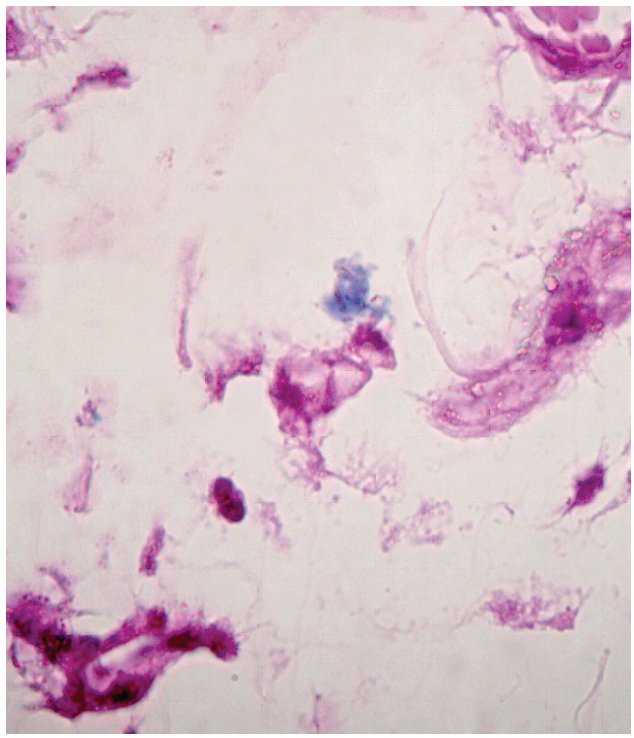

Fig.2: Zeihl-Neelsen staining ×1000

3. PCR

a- Tissue processing for DNA extract

Five serial sections (5µm thick) were cut from each paraffin-embedded tissue block by microtome blade (Leica 2135). For prevention of other contamination the microtome blade was cleaned with xylene and absolute ethanol after each sample sectioning. Tissue section was deparaffinized by xylene and heat (70- 80 °C by oven) 3 to 4 times. Total DNA was extracted by standard proteinase K digestion followed by salting- out method (17). As a negative control, a sample was used with a histolopathological diagnosis other than mycobacterial infection and without granuloma. The amount of extracted DNA was determined by spectrophotometer.

b-ThenTB-PCR (IS6110 element) was performed

on extracted DNA by Detection kit of Mycobacterium tuberculosis (Rob Gene).

c- PCR analysis

Specimens were considered PCR- positive for M. tuberculosis when the length of DNA fragment present on gel (2% agarose stained with ethidium bromide) were 182bp and were declared PCR – negative when this fragment was absent. Each set of PCR reactions contained a positive control. (Fig.3)

Statistical analysis

We compared the TB-PCR results with histopathological findings and the results of AFB by using McNemar Test. (Stata software) Statistical significance was accepted when the *P* value was less than 0.05.

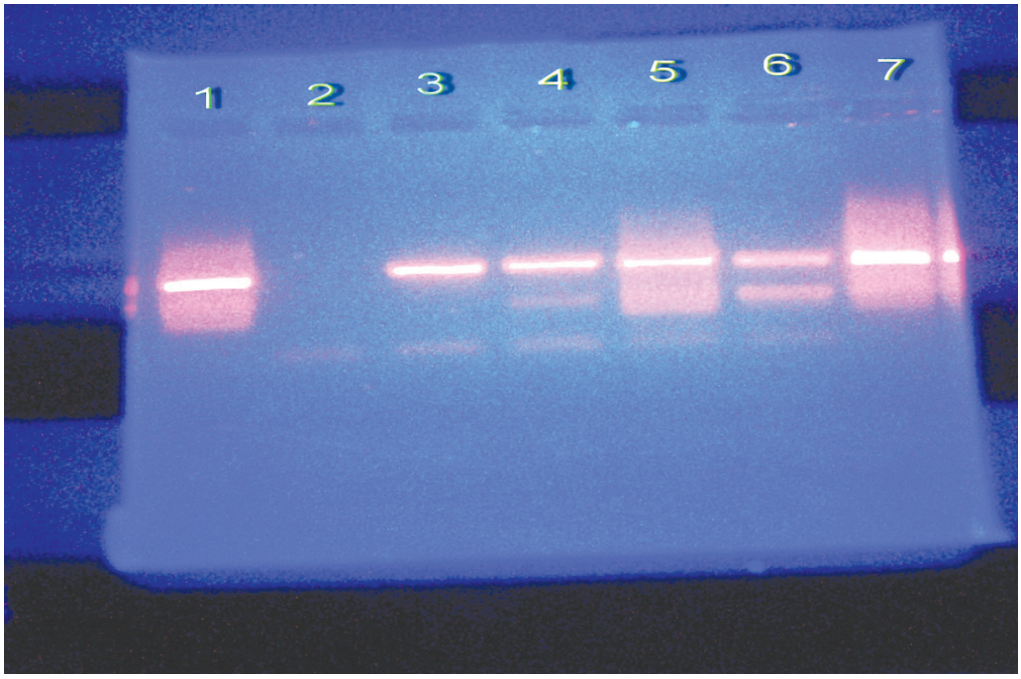

Fig.3: Agarose gel electrophoresis of TB-PCR products

Lanes1&7: Positive control Lane2: Negative control Lane3: sample 34 (Positive)
Lane4: sample 43 (Positive) Lane5: sample 45 (Positive) Lane6: sample 39 (Positive)

Results

Our Results showed that most of the specimens were from pleura (37.8%). The mean age of the subjects was 45/87±22 years (ranges 1-95 years) and 55.6% were men. We reviewed all forty-five samples histopathologically and 35 cases (77.8%) showed microscopic lesions compatible with granulomas including 19 (42.2%) with caseous necrosis.

Sixteen cases (35.6%) were positive with Ziehl-Neelsen method. The number of AFB was extremely low in all samples (Fig.2). Comparisons of histopathologic finding and ZN staining are shown in Table1. There was a statistically significant difference between histopathologic findings and the ZN staining results by McNemar Test (P value=0.0001).

Table 1- Comparison of histopathologic finding and Ziehl-Neelsen staining

		Histopathology Result	
		Granulomas n (%)	**Without granulomas n (%)**
Ziehl-Neelsen staining	Positive	14 (40)	2 (20)
	Negative	21 (60)	8 (80)
	Sum	35 (100)	10 (100)

The FFPE samples from various locations (45cases) were analyzed by PCR for detection of TB- DNA. Thirty-three cases (73.3%) were positive for TB-PCR and twelve cases were negative (Figure3). presents the results of PCR for M. tuberculosis. Twenty-seven cases (77.1%) with granulomas and six cases (60%) without granulomas had TB-PCR positive (Table 2).

Table 2- Comparison of histopathologic finding and PCR results

		Histopathology Result	
		Granulomas n (%)	**Without granulomas n (%)**
PCR	Positive	27 (77.1)	6 (60)
	Negative	8 (22.9)	4 (40)
	Sum	35 (100)	10 (100)

Sixteen cases (84.2%) of caseating granulomatous necrosis and 11 cases (68.8%) of non-caseating granulomatous necrosis were positive for TB-PCR. There was not a statistically significant difference between histopathologic findings and PCR results. In other words, both were in agreement, by McNemar Test (*P* value=0.56).

Comparison of PCR results with histopathologic findings showed that all AFB positive cases were TB- PCR positive and all PCR- negative were AFB negative (Table 3). Comparison between the histopathologic findings and their TB-PCR results and the Ziehl-Neelsen staining results are summarized in Table 4.

Table 3- Comparison of PCR results and Ziehl-Neelsen staining

		Ziehl-Neelsen staining Result	
		Negative n (%)	**Positive n (%)**
PCR	Positive	16 (100)	17 (58.6)
	Negative	0 (0)	12 (41.4)
	Sum	16 (100)	29 (100)

Table 4- Comparison of three tests (PCR, Histopathology & Ziehl- Neelsen stainin.g)

Histopathology	**Ziehl-Neelsen staining**	**PCR**		**Sum n (%)**
		Negative n (%)	**Positive n (%)**	
Caseating granulomas	Negative	4 (40)	4 (40)	8 (80)
	Positive	0 (0)	2 (20)	2 (20)
	Sum	4 (40)	6 (60)	10 (100)
Caseating granulomas	Negative	3(15.8)	9 (47.4)	12 (63.2)
	Positive	0 (0)	7 (36.8)	7 (36.8)
	Sum	3 (15.8)	16 (84.2)	19 (100)
Non- Caseating granulomas	Negative	5 (31.1)	4 (25)	9 (56.3)
	Positive	0 (0)	7 (43.8)	7 (43.8)
	Sum	3 (31.3)	11 (68.8)	16 (100)

Discussion

In this study, 27 out of 35 specimens with granulomatous inflammation were positive for *Mycobacterium tuberculosis* demonstrated by PCR study. Furthermore 16 acid-fast positive and 17 acid-fast negative specimens showed TB-PCR positive results.

The results indicate a high concordance rate between the presence of granuloma and positive PCR for mycobacterium tuberculosis, while there is a low rate between acid-fast staining and PCR. Many studies suggest PCR as a sensitive and

rapid test for diagnosis of tuberculosis especially in well-formed granuomatous lesions (11-13).

Other studies have shown high specificity but low sensitivity (respectively 100% and 33.9%) for acid-fast staining which can be attributed to low survival rate of mycobacteria in the center of caseation or loss of bacterial structure due to immune responses in granulomatous inflammation in mycobacteriosis. They suggested that histopathologic examination is a reliable tool for rapid diagnosis in countries where active tuberculosis eradication programs allow the prompt identification and elimination of reactor cattle (16).

In a study of 6 cases with proven tuberculosis, acid fast stain provided a 50% sensitivity (3/6 cases) while PCR showed 66% sensitivity for detection of *M. tuberculosis* on paraffin-embedded tissue and no false positives were detected, giving a specificity of 100% for PCR in diagnosis of tuberculosis. Other studies that have reported a few false positive cases, suggest a possible contamination during sample collection (18).

In another study on 95 formalin-fixed, paraffin-embedded tissue blocks from 81 patients who were clinically suspected of having tuberculosis, there was one false negative case which even on repeated PCR with two fold larger aliquot remained negative. False negative results can be due to sampling errors, paucibacillary nature of the specimen, inefficient extraction of DNA, presence of PCR inhibitors and degraded DNA(due to high storage temperatures of paraffin blocks) (8).

Positive reaction for acid-fast bacilli in histological specimens is dependent on load of infection with a wide range of AFB positively from 0% to as high as 75% (8).

Conclusion

Because PCR is a highly time consuming method in comparison with acid-fast staining, we recommend PCR usage only in highly suspicious acid –fast –negative specimens.

If there was no facilities for PCR, histopathological diagnosis with clinical correlation is more reliable in comparison to AFB results.

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