



Full Length Research Paper

PCR Versus Microscopic Examinations for Detection of *Mycobacterial tuberculosis* in formalin fixed Histologic Specimens

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Diagnosis of tuberculosis (TB) in formalin-fixed, paraffin-embedded tissue specimens depends on microscopic examination (Zeihl-Neelsen stain (ZN-stain), and histopathology) but ZN-stain sensitivity is still of primary limitation and histopathological features of chronic granulomatous inflammation can be found in various diseases other than TB. Accurate and early diagnosis of tuberculosis in formalin-fixed, paraffin-embedded tissue samples is important for effective management. The present study aimed to compare between TB-polymerase chain reaction (PCR), ZN-stain, and histopathological examination for diagnosis of TB in formalin fixed histologic specimens. Out of fifty different tissue specimens clinically diagnosed as TB, ZN-stain was positive in (34%), histological examination was positive in (52%) and TB-PCR was positive in (64%). TB-PCR was the most sensitive (100.0%) with specificity of 90.0% while sensitivity of ZN-stain was the lowest (50%). It was concluded that the PCR assay was more rapid, specific and reliable for TB diagnosis. In addition it should be implemented as routine test in histopathology as well as microbiology laboratories for the diagnosis of TB.

Keywords: Mycobacteria tuberculosis, DNA, Formalin fixed, ZN-stain, PCR.

INTRODUCTION

The World Health Organization (WHO) Global Tuberculosis Programme reported that about that one-third of the world population is infected with the TB (Onyango, 2011). It was estimated globally that there were 9.27 million incident cases of TB, 13.7 million prevalent cases, 1.32 million deaths from TB in HIV-negative and 0.45 million deaths in HIV-positive persons (Onyango 2011; WHO 2008). Microscopy is the oldest, easiest, most rapid, and inexpensive procedure that can

be performed in the laboratory to diagnose TB. Examination of stained clinical material can be helpful because it provides a presumptive diagnosis of mycobacterial disease and can be used to follow the success of chemotherapy of tuberculosis patients. It can also confirm that cultures growing on media are indeed acid-fast. But the ZN-stain (AFB stain), sensitivity is poor in extra-pulmonary TB specially in diseases caused by mycobacterium other than tuberculosis and in HIV-infected TB patients (WHO 1998; Dezemon et al., 2014). In terms of histopathology, TB can be diagnosed only as "chronic granulomatous inflammation, suggestive of tuberculosis" (Mert et al., 2001). The inflammation produced with TB infection is granulomatous, with

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epithelioid macrophages and Langhans giant cells along with lymphocytes, plasma cells, fibroblasts with collagen, and characteristic caseous necrosis in the center (Ulrichs and Kaufmann 2006; Dheda et al., 2005). However 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 (Collins 1999; Kitinya et al., 1994; Vassalli 1992). PCR has been reported to be reproducible, sensitive as well as specific. A variety of PCR methods have been developed for MT and other mycobacteria (Soini and Musser 2001). These PCR assays target either DNA or rRNA. They are now extensively being used all over the world. In recent years PCR amplification is introduced for detection of TB in formalin-fixed, paraffin-embedded archival or fresh tissues and has been widely demonstrated in various tissue specimens (Do et al., 2002). Application of TB-PCR in formalin-fixed, paraffin-embedded tissue samples is useful for TB diagnosis in histopathology laboratories. Sometimes diagnosis of tuberculosis is not a clinical consideration before the report of findings on microscopic examination of the tissue. PCR amplification of MT DNA can provide much needed help in these circumstances because this methodology can detect MT in tissue samples even though the tissues have been preserved in formalin or other substances that preclude the possibility of culture (Foulds and Brien, 1998) but the type and duration of fixative, and the molecular weight of the expected PCR product have important effects on the PCR amplification (Greer et al, 1991; Fiallo et al., 1992). The present study aimed to compare between TB-PCR, ZN-stain, and histopathological examination for diagnosis of TB in formaline fixed histologic specimens.

MATERIALS AND METHODS

The study was carried on a total of fifty tissue samples collected from patients who had been diagnosed as having tuberculosis. Only thirty of these different tissues biopsies were previously known to be positive for TB-culture, which were considered as gold standard. The tissues studied included lung (n = 8); brain (n = 2); skin and subcutaneous tissue (n = 6); lymph node (n = 25); liver, and gastrointestinal tissues (n = 4 each); spleen, bone, and bone marrow (n = 3 each); and urinary bladder (n = 2 each).

Tissue processing for histopathology

Each of the 50 formaline-fixed specimens was labeled in a cassette, then collected together and processed using TissueTek VIP (2000) machine. The processing steps include fixation in 10% neutral formalin for 3 hours, dehydration in ethanol (starting from 50% through 70%,

80%, 95% (2 changes), till 100% ethanol (2 changes) each for two hours, then clearing in 3 changes of xylene each for two hours, then impregnation in 3 changes paraffin wax at 60 C° each for two hours. After that, each specimen was embedded and blocked in paraffin wax. From each block, thin sections about 4 µm in thickness were cut using a disposable sterile blade for each block. All sections were dewaxed by heating and then transferred into xylene solution for about 15 minutes (3 changes), then transferred into 100% ethanol for 10 min (2 changes). The sections hydrated by transferring into 95%, 80%, 70%, 50% ethanol, then distilled water, each was for 5 minutes. All sections were stained with Harris haematoxylin for 5 minutes and differentiated with 3% acid alcohol, washed and transferred into running tap water for 10 minutes. The sections were stained with 1% aqueous eosin for 2 minutes then were dehydrated into ascending alcohol starting from 50%, through 70%, 80%, 95% till 100% (2 changes) each step was for 2 minutes. These sections were cleared into xylene solution (3 changes) for 15 minutes all of these steps were done by SAKURA DRS-601 (automatic slide stainer) and after that they were mounted with D.P.X and examined by pathologist.

ZN-stain

All sections were dewaxed by heating and then transferred into xylene solution for about 15 minutes (3 changes). Then transferred into 100% ethanol for 10 min (2 changes). The sections hydrated by transferring into 95%, 80%, 70%, 50% ethanol, then distilled water, each was for 5 minutes. Then they were stained in the pre-heated solution of carbol fuchsin for 15 minutes then transferred and washed into running cold tap water for 3 minutes. All sections were differentiated in 3% acid alcohol until no more colour runs from the slide and then were washed briefly in water to remove acid alcohol and were counterstained with 0.25% methylene blue in 1% acetic acid for 15 to 30 seconds. Then the sections were washed in water, dehydrated in ascending alcohol (as above), cleared with xylene and mounted in DPX. A known positive and negative control sections were used and stained in the same time and the same procedure. All the sections were examined under microscope for AFB (red colour).

DNA Extraction and TB-PCR

From each paraffin-embedded tissue block, 5-µm-thick sections were cut with a disposable microtome blade. The paraffin from each tissue section was dewaxed using xylene and 100% ethanol. After removing the solvent in a decreasing series of alcohol (99-50%), the sections were rehydrated with distilled water and removed from glass slides with 100 µl digestion buffer (200 µg/ml proteinase K, 50 Mm Tris-HCl, PH 8.5, 1 Mm ethylenediamine

Table 1. Results of ZN-stain histopathology findings and TB-PCR among the 50 studied cases.

Description	ZN-stain	Histopathology	TB-PCR
	No (%)	No (%)	No (%)
Positive	17 (34%)	26 (52%)	32 (64%)
Negative	33 (66%)	24 (48%)	18 (36%)
Total	50 (100%)	50 (100%)	50 (100%)

Table 2. Correlation of ZN-stain, histopathology, and TB-PCR in comparison to TB-culture results.

Description	ZN-stain	Histopathology	TB-PCR
Sensitivity	50.0%	80.0%	100%
Specificity	90.0%	90.0%	90%
PPV	88.23%	92.3%	93.75%
NPV	54.54%	75%	100%

tetraacetic acid (EDTA) and 0.5% Tween 20). The mixture was incubated at 56° C overnight. After centrifugation, the supernatant was removed and lysis reagent (containing 1% solubilizer, 0.2% sodium hydroxide and 0.05% sodium azide as preservative) added to the pellet. After vortexing, the suspension was incubated at 60 °C for 45 minutes to allow for complete lysis of the mycobacterial cells. The lysed material was then neutralized by the addition of neutralizing reagent (Tris-HCl solution containing 0.05% sodium azide as preservative). All samples were screened for the IS 6110 region by PCR, with specific primers (sense: 5-CCT AGC GCG TAG GCG TCG G-3 and antisense: 5- CTC GTC CAG CGC CGC TTC GG-3). The primers amplified at 123-bp fragment (Cortez et al., 2011). A 50µl PCR mixture containing 8 µl of DNA template, 1µl (100 pmol) of each primer and a 25µl of Taq PCR Master Mix polymerase containing 100mM Tris-HCl, 500mM KCl at pH 8.3 at 20°C, 1.5 mM MgCl₂, 200M of each of deoxyribonucleoside triphosphate and 0.025U Taq polymerase (Qiagen, USA) was prepared. Amplification was performed using Mastercycler PCR machine (Eppendorf, Germany). The cycling program was performed with an initial denaturation for 5 min at 95°C, then 35 cycles of denaturation for 30 sec at 95°C, annealing for 1 min at 60°C and extension for 30 sec at 72°C and final extension for 5 min at 72°C. About 25µl of the PCR products were mixed 10µl of loading dye and analyzed by electrophoresis in 1% agarose gels (for 35 minutes at 90 V using 5 X TBE running buffer. Also, 100 bp DNA ladder was included in each run and DNA bands were viewed under UVP BioDoct It Imaging System after staining with ethidium bromide (2 g/ml).

Quality control

The positive control for PCR was formalin-fixed paraffin-embedded tissue from lymph nodes that was both ZN-stain and TB culture positive and that produced a 123–base pair PCR product. The negative control sample was formalin-fixed, paraffin-embedded tissue that showed no features of granulomatous inflammation and was AFB (acid-fast bacilli) negative and TB culture negative. Additional negative controls included substitution of distilled water for the test template and a previous test sample that had yielded negative results.

RESULTS

AFB stain was positive in 17 (34%), histological examination was positive in 26 (52%) and TB-PCR was positive in 32 (64%) as in Table.1, figures 1,2,3 and 4. The AFB stain was positive in 15 of the 30 specimens that were positive for TB-culture, figures 2, and positive in 2 of the 20 specimens that were negative for TB-culture and negative in 33 other specimens. The histological examination was positive (consistent with TB), in 24 of the 30 specimens that positive for TB-culture and positive in 2 of the 20 specimens that were negative for TB-culture and negative in other 24 specimens. TB-PCR was positive in all of the 30 specimens that were positive for TB-culture and positive in 2 of the 20 specimens that were negative for TB-culture. The result of ZN-stain, histopathology, TB-PCR in comparison to TB-culture as a gold standard shows that the TB-PCR was the most sensitive 100.0% and of

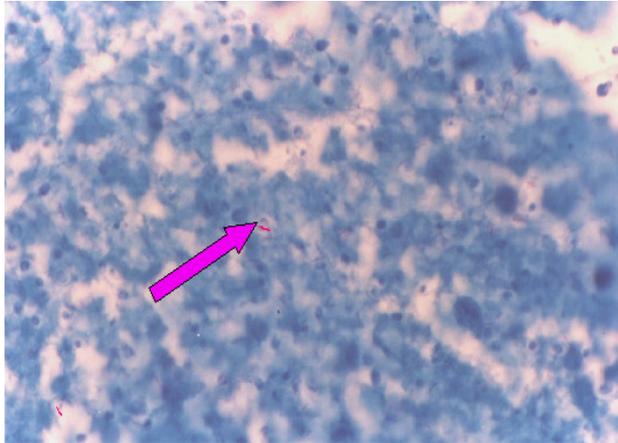


Figure 1. Positive ZN- stain (acid-fast)

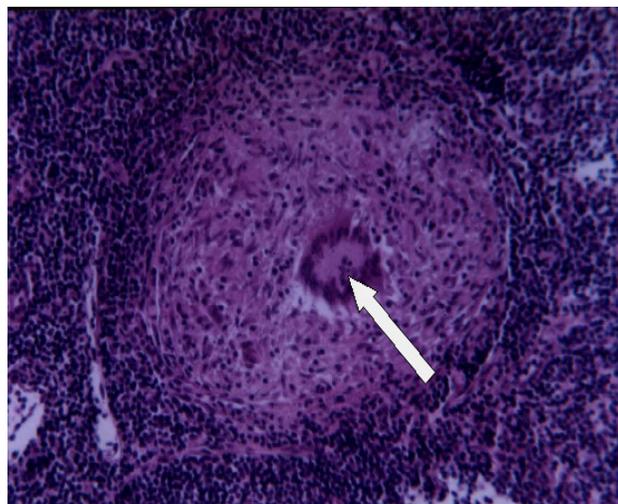


Figure 2. TB Positive histologic picture (Non-caseating granuloma showing langhan's cell . (Haematoxylin and Eosin)

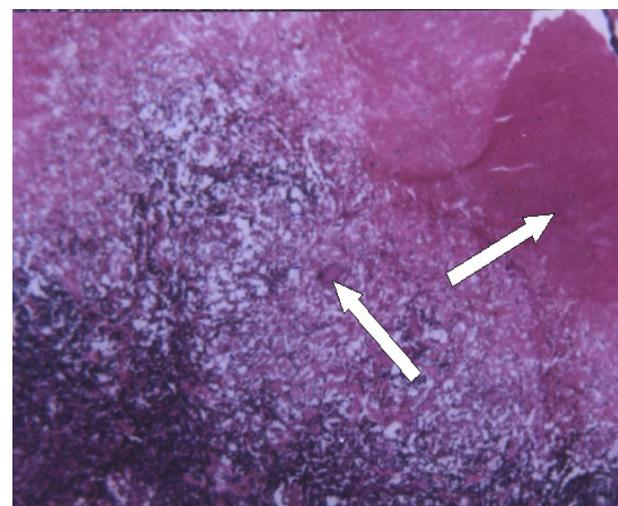


Figure 3. TB Positive histologic picture (well formed granuloma showing caseation and langhan's cell (Haematoxylin and Eosin).

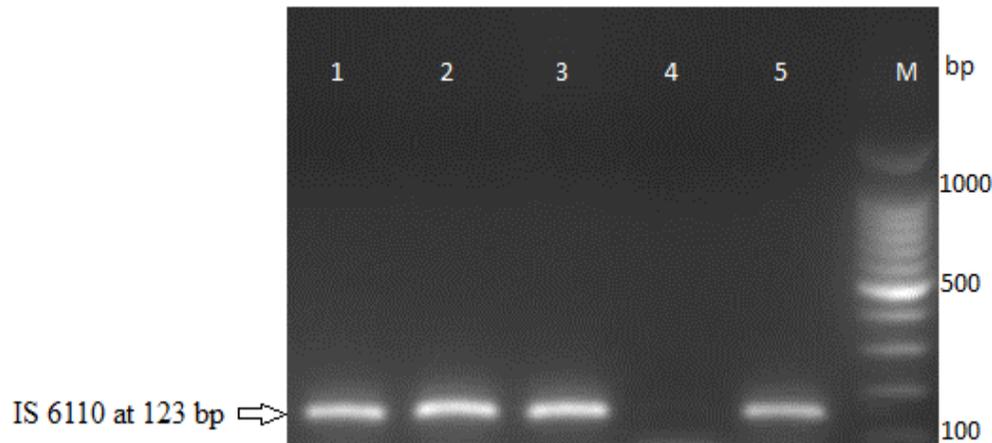


Figure 4. The IS 6110 region after PCR on 1% agarose gel electrophoresis. Lane 1: Control positive. Lanes 2, 3, and 5: positive IS 6110 region for MTB strains (123 bp). Lane 4: control negative. Lane M: 100-bp DNA ladder.

specificity 90.0%. The sensitivity of ZN-stain was the lowest (50%) with specificity of 90.0% as in Table 2.

DISCUSSION

Infection caused by MTB complex remains one of the most important global public health issues. Accurate and rapid diagnosis is the key to control the disease. The results of the traditional tests are either inaccurate or time consuming as in TB-culture. The present study used the PCR and these regular methods for detection of MT in 50 histological specimens suspected for mycobacterium TB infection, thirty of them were previously known as positive for TB-culture. Results showed that out of these 50 specimens, ZN-stain was positive in 17 (34%), histological examination was positive in 26 (52%) and TB-PCR was positive in 32(64%). Many reports have confirmed that PCR amplification in formalin-fixed paraffin-embedded tissue detects TB DNA even few (Diaz et al., 1996; Berk et al., 1996). In the present study the TB PCR used for MTB detection was MT IS6110 sequence. This sequence is a repetitive mobile genetic element and is a good target for diagnosis because of its specificity and its presence in high copy numbers in most strains of the MT {14}. The findings of ZN-stain showed that out of the 30 TB-culture positive specimens, 15 were positive, and of the 20 TB-culture negative, two were ZN-stain positive. These two were TB-PCR negative while the 15 ZN-stain positive, were also TB-PCR positive. This can be explained by infection of Mycobacterium species other than MT. When compared to the TB-PCR, sensitivity of AFB stain were significantly low (50%) while specificity was high (90.0%) suggesting that the ZN-stain is less sensitive but when positive, it is too specific. That is very close to many studies (Murray et al., 1980; Rickman and Moyer, 1980) who detected specificity of

ZN-stain near to 99% or more and the sensitivity ranges from about 25% to about 75%. Although several limitation have been reported (Greer et al., 1991; Karlsen et al., 1994), TB-PCR still is considered suitable method for the diagnosis of TB in routinely processed, formalin-fixed and paraffin-embedded histologic specimens (Diaz et al., 1996; Popper et al., 1994; Ghossein et al., 1992; Salian et al., 1998). Diaz et al., 1996 found that PCR amplification was 100% sensitive and 100% specific which is similar to other reports (Popper et al., 1994; Ghossein et al., 1992). In this study, the corresponding specificity of TB-PCR was 90% which is in agreement to Salian et al., 1998 who got sensitivity and specificity of 100 % and 93% respectively, while others reported that TB-PCR can be 100 % sensitive and specific when both AFB stain and TB-culture are positive (Salian et al., 1998). In view of histological diagnosis in comparing with TB-culture, out of the 30 positive cases, it was found that 24 (80%) were shown to be with chronic granulomatous inflammation with caseous necrosis that consistent with TB. The sensitivity was of 80% and specificity was of 90%. These high significant results of histopathology findings suggest that, the classic histopathology features of chronic granulomatous inflammation are very near to TB- PCR results (Do et al., 2002). In contrast previous studies showed that, more than one third of cases with chronic inflammation but without definite granulomatous lesion were positive by TB-PCR (Do et al., 2002). This can be explained by immunosuppression or sample size. For example in patients infected with HIV, the tissue reaction to TB include a spectrum of changes not involve the formation of granuloma (Kitinya et al., 1994). Also the size of submitted histologic specimens is usually small and the histopathologic features of TB are incompletely seen. The major advantage of histopathology is the production of the results within few days. Furthermore, the technique has a high specificity because it can

histomorphologically characterize lesions unrelated to mycobacterial agents (e.g., parasites, neoplasia). It could be concluded that the use of PCR in paraffin embedded formalin-fixed histologic samples is more rapid, specific and reliable for TB diagnosis and will increase the rates of TB detection and therefore should be implemented as routine test in histopathology as well as microbiology laboratories for the diagnosis of TB.

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