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Direct and Rapid Diagnosis of Extra-Pulmonary Tuberculosis in Archival Tissue by Nested and Touchdown PCR

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Authors' contributions

Author LFN designed the study, wrote the protocol, supervised the laboratory experiments, interpreted the results and wrote the first and final manuscript. Author ZAL carried out the laboratory experiments and analyzed the data. Author IM examined the biopsy blocks and interpretation of results all authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To identify and differentiate mycobacterium spp. in archival formalin-fixed, paraffin-embedded tissue by PCR to supply additional differential diagnostic method for *Mycobacterium* infections, where tuberculosis had been tested for by histopathological methods but without culturing.
Place and Duration of Study: Department of Medical Laboratory Sciences, Department of Pathology and Microbiology (Faculty of Medicine) and King Abdulla University Hospital, between January 2004 and July 2010.
Methodology: Fifty-six extra-pulmonary specimens of formalin-fixed, paraffin-embedded tissue obtained from patients showing granulomatus inflammation and/or other histopathologic features. Specimens were analyzed by a hemi-nested PCR assay targeting the gene encoding for 16S ribosomal RNA, which is common to all mycobacteria spp. The PCR positive specimens were amplified by a touch-down PCR targeting a fragment in the insertion sequence IS6110 specific to *Mycobacterium tuberculosis* complex. Results were compared to acid fast bacilli stain and with histopathology of each specimen.
Results: *M. tuberculosis* complex DNA was detected in 27 (48.2%) specimens, and non-

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tuberculous mycobacteria in four specimens, compared to 10 (18%) specimens that were positive by acid fast staining. The positive cases were observed more in the lymph nodes, and pleural specimens.

Conclusions: This study is among few studies to use the touch-down PCR assay as a promising auxiliary tool for the diagnosis of extra-pulmonary tuberculosis in archival tissue specimens. It could be used in conjunction with routine laboratory tests (e.g., cultures, acid fast staining) and clinical criteria of the patient to increase the accurate diagnosis of such cases. It is also recommended that culture be routinely done for all tuberculosis suspected cases.

Keywords: Formalin-fixed; paraffin-embedded tissue; granuloma; mycobacterium tuberculosis complex; PCR.

1. INTRODUCTION

Despite the fact that tuberculosis (TB) is a highly prevalent infectious disease, the clinical diagnosis posses a true challenge due to its varying symptoms, localization and appearance. Although the predominant site of TB is the lung, other sites are known to have this infection. Depending on the immune status of the patient, extra-pulmonary TB (EPTB) has been reported in almost every site of the body [1,2].

EPTB is often diagnosed late because of the mostly atypical clinical presentation [3]. The CT scan, MRI, laparoscopy and endoscopy have tremendously helped in anatomical localization of the EPTB [4]. A presumptive diagnosis of TB can be made if a tissue has characteristic histopathologic changes such as granuloma and caseous necrosis, and acid-fast organisms. However, definitive diagnosis requires culture and identification of the causative *Mycobacterium* species, which takes several weeks to complete [5]. A definitive diagnosis in EPTB cases requires biopsy material with granulomas and caseation in addition to acid fast staining and culture [6].

Determining the causative agent might be difficult in histopathologic sections especially when biopsy material is scanty and fails to confirm or rule out TB [7].

The Incidence of tuberculosis (per 100;000 people) in Jordan was last reported at 5.4 in 2010, according to a World Bank report published in 2012. Incidence of tuberculosis is the estimated number of new pulmonary, smear positive, and extra-pulmonary tuberculosis cases [8]. In a study that was conducted in Jordan, 19% of the tested isolates were drug resistant with different mutation types, and 6% were classified as multidrug resistant to at least rifampicin and isoniazid, which are considered the backbone of short-course TB chemotherapy [9]. The Ministry of health (MOH) is facing new challenges with the discovery that five out of 50 TB cases among Syrian refugees are multi-drug resistant. These TB imported cases will affect Jordan's efforts to completely eliminate the disease.

A rapid, reliable diagnosis of TB is essential to initiate the correct treatment, avoid severe complications, and prevent transmission since conventional microbiological methods may not be the best methods for histological examination if specimens are formalin-fixed and paraffin-embedded (FFPE) [10,12].

The current study used nested and touchdown PCR assays for the diagnosis of TB in FFPE archival tissue specimens with granulomas and other pathological features to supply an

additional differential diagnostic method for infections with the *Mycobacterium* species where TB had been tested for using histopathological methods but without culturing.

2. MATERIALS AND METHODS

2.1 Formalin- fixed, Paraffin- embedded Tissue Specimens (FFPE)

A retrospective study was conducted on a total of 56 FFPE tissue specimens having granuloma, and histpathological features such as caseous necrosis and Langhans giant cells that were selected from the archives of the Department of Pathology at King Abdulla University Hospital in Irbid, Jordan. The specimens were collected between 2004 and 2010, one from each patient. These specimens were not sent for culture, because EPTB was not suspected in these patients prior to the microscopic examination of the tissue specimen. However, the presence of TB had been suggested after microscopy and was tested for by histopathological methods. The presence of granuloma with or without caseous necrosis, or Langhans giant cells in the tissue sections was confirmed by an experienced pathologist.

The 56 tissue specimens included lymph nodes (13), lung/pleural biopsies (9) axillary mass (9), abdominal mass (9), neck mass (5), spinal mass (4), genitourinary (4), breast mass (1), knee mass (1), skin mass (1). These specimens were initially stained by Ziehl Neelsen (ZN) acid-fast stain and were subjected to molecular DNA analysis in this study.

2.2 Sample Preparation

Three 20-µm-thick sections from each block were cut with a microtome. A carryover contaminating DNA was prevented by using a fresh blade for each specimen, the microtome overlay was covered with a piece of adhesive tape changed for every specimen, and subsequently cleaned with xylene and 100% ethanol after processing. DNA was extracted from the FFPE tissue using QIAmp DNA kit, (Qiagen, Germany) according to the manufacturer's protocol. Prior to nucleic acid purification, the paraffin in the samples was dissolved in xylene to allow exposure of the sample to proteinase K. Residual xylene was then removed by washing three times with absolute ethanol. The extracted DNA was eluted into 50 µl elution buffer, and stored in Eppendorf tubes at –20ºC until used.

2.3 Detection of *Mycobacterium* in Tissue Samples

Three PCR assays were performed; the primer sequences used in the three PCR methods are listed in Table 1. A brief description of the PCR assays is as follows:

2.3.1 PCR 1

A hemi-nested PCR targeting the gene encoding for the 16S rRNA that is common to all mycobacteria spp. was performed on all 56 tissue specimens. The outer primers in the first round were primer KY 18 and KY 75 that amplifies a fragment of 584 bp in the 16S rRNA gene. The inner primers in the second round were primers KY 18 and nested KY 75 that amplified a fragment of 439 bp. The first step PCR reaction was carried in a total volume of 50 µl. The reaction mix contained 25 µl master mix (Promega, USA), 2 µl of each forward and reverse primer (5 pmol), 16 µl nuclease free water and 5 µl of genomic DNA. The second round PCR reaction was carried in a total volume of 25 µl. The primer sequences and amplification conditions were performed as previously published [11]. The PCR products

were electrophoresed on a 3% agarose gel containing 1 µg/ml of ethidium bromide per ml. DNA bands were visualized under UV light.

2.3.2 PCR 2

This touch-down PCR assay was performed only on the specimens that were positive in PCR1. The primers IS1 and IS2 were used to amplify a 123 bp fragment in the IS*6110* sequence, specific to the members of the *M. tuberculosis* complex (MTC) genome, and differentiate between the MTC from other non-tuberculous mycobacteria. The primer sequences and amplification conditions were performed as previously published [12]. The PCR products were electrophoresed on a 3% agarose gel containing 1 µg/ml of ethidium bromide per ml. DNA bands were visualized under UV light.

2.3.3 PCR controls

As a positive internal control for monitoring successful DNA extraction of each specimen, a 250 bp fragment of the β-globin gene was amplified using the primers GH21, and PCO3 [13]. A successful amplification was confirmed by electrophoresis of the PCR products on 3% agarose gel. Several *M. tuberculosis* DNA that were previously isolated from confirmed pulmonary TB by culture and PCR were used as positive controls in all PCR assays. A PCR mix with no DNA template was included as a negative control in all PCR amplifications. All PCR amplifications were carried out in GenAmp 9700 (Perkin Elmer, USA).

Several steps were taken to avoid false-positive results and carryover contamination, these included preparing the reaction mixtures and setting up of the amplification procedures in separate sterile areas. Mixes were prepared in a laminar flow hood with continuous decontamination after each step. In addition, sterile filter tips, DNase and Rnase free were used.

Since these specimens were not cultured, which is the gold standard for the diagnosis of tuberculosis; the positive PCR results for *M. tuberculosis* complex were verified using the clinical and histological diagnosis of the patients including the results of the ZN acid fast stain.

Table 1. Sequence of primers used in the PCR assays

PCR/target gene	Primers	Oligonucleotide sequence	Product size (bp)	Ref. #
PCR1/16S rRNA First round	KY 18	5'-CACATGCAAGTCGAACGGAAAGG-3'	584	11
	KY 75	5'-GCCCGTATCGCCCGCACGCTCACA-3'		
PCR1 Second round	KY 75 (nested)	5'-TGCTTCTTCTCCACCTACCGTCAA-3'	439	
PCR2/ IS*6110* sequence	T4	5'-CCTGCGAGCGTAGGCGTCGG-3'	123	12
	T5	5'-CTCGTCCAGCGCCGCTTCGG-3'		
PCR control β-globin gene	GH2	5'-GGAAAATAGACCAATAGGCAG-3′	250	13
	PCO3	5'-ACACAACTGTGTTCACTAGC-3'		

3. RESULTS

3.1 The Hemi Nested-PCR for the 16S rRNA Gene (PCR1)

Of the 56 specimens, 31 (55.4%) tested positive for a mycobacterium species showing the 439 bp fragment amplified from the 16S rRNA gene Fig. 1.

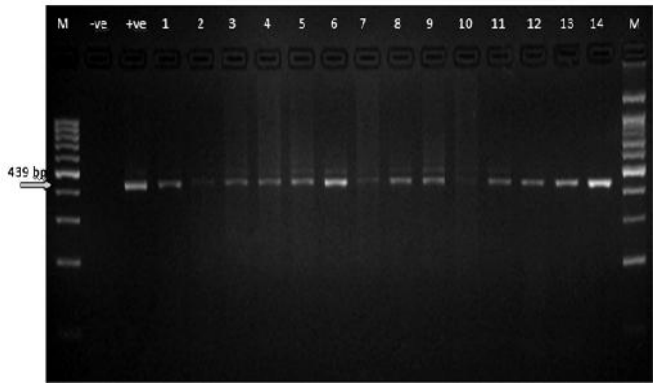

Fig. 1. PCR products obtained in the nested-PCR for the 16S rRNA gene (3% agarose gel). M: 100 bp DNA molecular weight marker, -ve: negative control, +ve: positive control. Lanes: 1-14 are mycobacteria spp. Positive cases

3.2 PCR for the IS6110 Insertion for M. Tuberculosis Complex (PCR2)

Out of the 31 positive specimens in the above PCR, 27 specimens were positive for the *M. tuberculosis* complex showing the 123 bp band amplified from the IS*6110* insertion sequence Fig. 2. Therefore, diagnosis of TB due to a member of the *M. tuberculosis* complex was confirmed in 27/56 (48.2%) patients. The other 4/31 (12.9%) specimens showing only the 439 bp band were considered as non-tuberculous mycobacteria (NTM).

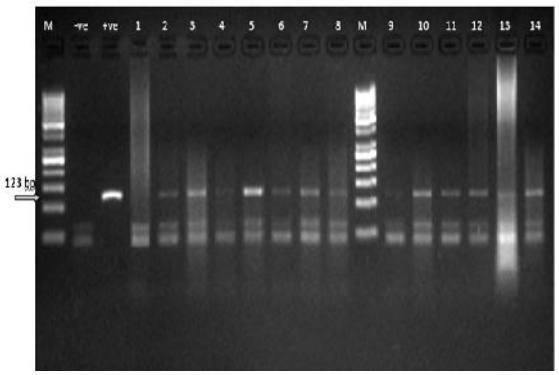

Fig. 2. PCR products obtained in the IS6110 insertion sequence (3% agarose gel). M: 50-bp DNA molecular weight marker, -ve: negative control, +ve: positive control. Lanes: 2-8, and 10-14 are examples of positive cases for *M. tuberculosis* complex. Lanes: 1 and 9 are negative cases for *M. tuberculosis* complex

3.3 Comparing PCR Results with Histological Diagnosis

There was a correlation between the PCR results of the 27 positive cases for *M. tuberculosis* complex and the assessed histopathologic features. They were as follows: 17(63%) had granuloma, caseous necrosis, and Langhans giant cells, 6 (22.2%) had both granuloma and caseous necrosis, 2 (7.4%) had both granuloma and Langhans giant cells, while 2 (7.4%) had only granuloma.

Only 10 out of the 56 tested specimens (18%) were acid-fast bacilli (AFB)-positive cases, seven of these specimens were also positive by both PCR assays, accounting for 22.6% (7/31) of the total PCR positive specimens.

Comparing the positive PCR results for *Mycobacterium* species in all 56 specimens with the ZN acid fast staining, (31:10), PCR detected 3.1 times more cases than acid-fast stain.

3.4 PCR Assays of Various Specimen Types

The highest positive cases for the *M. tuberculosis* complex were detected in the lymph nodes 10/14 (71.3%), and the lung/pleural specimens 6/8 (75%). The other EPTB positive specimens are shown in Table 2.

Table 2. Correlation of various specimen types with PCR assays results

Types of specimen n = 56	TB positive *M. tuberculosis* complex (%)	Atypical Mycobacteria n = 4 (%)
lymph nodes = 14 cases	10 (71.3)	0 (0)
Lung/pleural biopsies = 8 cases	6 (75)	0 (0)
Axillary masses = 9 cases	2 (22.2)	3/9 (33.3)
Abdominal masses = 10 cases	3 (40)	0 (0)
Neck masses = 5 cases	2 (40)	0 (0)
Genitourinary = 3 cases	1 (33.3)	1/3 (33.3)
Spinal masses = 4 cases	2 (50)	0 (0)
Breast mass = 1 case	1 (100)	0 (0)

4. DISCUSSION

Despite the fact that tuberculosis is a highly prevalent infectious disease, the clinical diagnosis posses a true challenge due to its varying symptoms, localization and appearance [14-17].

Histopathological diagnosis of TB usually depends on the presence of granulomatous inflammation or necrosis and other histopathological features. However, this is not specific for TB as it could be present in a variety of other conditions such as syphilis, leprosy, sarcoidosis, Crohn's disease, systemic lupus erythematosus, and rheumatoid arthritis [18]. Cases of EPTB are more often culture-negative than cases of pulmonary TB and when culture is positive, growth on solid media may require as long as 8 weeks [1]. Unfortunately, there are frequent occasions when tissue obtained by biopsy is not sent for culture because the diagnosis of TB was not a clinical consideration prior to the microscopic examination of the tissue. Hence, the result of microscopic examination of AFB cannot be confirmed by positive bacterial isolation. These cases can only be reported as granulomatous

inflammation, consistent with mycobacterial infection [19]. Therefore, to confirm the diagnosis of TB in a tissue biopsy, acid-fast staining and culture of tissues for *M. tuberculosis* must be performed. However, not all tissues that are sent for histopathology are sent for culture because it is a very laborious procedure and because of its limitation in terms of time (4-6 weeks). In addition, these tests have poor sensitivity because of paucibacillary tissue specimens [20]. Ziehl-Neelsen acid-fast staining is a key technique for the diagnosis of mycobacterial infections, but it lacks sensitivity in pulmonary and extra-pulmonary specimens and requires 10^4-10^6 bacilli/ml of tissue or fluid specimens to yield a positive result [21,22], while as little as 5 to 10 fg of purified mycobacterial DNA could be detected by the nested PCR assay [23].

Although there has been improvements and increasing use of PCR for the diagnosis of mycobacterial infections in fresh clinical specimens, there is still a need to validate the use of a more sensitive PCR assays for the identification of mycobacteria species in archival FFPE tissue specimens. Detection of organisms by PCR is not restricted to the presence of viable organisms in the specimens; this makes the diagnosis of TB in archival material possible in the absence of culture results and whose storage in formalin severely hampers processing by usual bacteriological methods [24].

The current study PCR results revealed the presence of *M. tuberculosis* complex DNA, which includes several human-associated lineages in a significant percentage (48.2%) of the specimens with chronic granulomatous inflammation compared to (18%) that were positive by acid fast staining. These findings demonstrated that a high percentage of the TB positive cases that were AFB negative could be missed in the 3–5 µm sections, and that an AFB stain negative case does not rule out the presence of TB [25]. Our results are in agreement with a previous study of pulmonary and extra-pulmonary TB, which reported 55% positivity of IS*6110* PCR in smear negative specimens [26]. In addition, PCR helped make the final diagnosis of TB in 59% of patients, where granulomatous inflammation was present in tissue specimens, but without demonstrable acid-fast bacilli in ZN staining [27].

The highest percentage of the EPTB tested specimens confirmed by both PCR assays was observed in the lymph nodes, followed by the pleural specimens. These results supported the fact that lymphadenitis is the most commonly occurring form of EPTB [2,6,28] followed by pleural effusion and virtually every site of the body could be affected [4].

The three AFB positive specimens that were negative by both PCR assays might have resulted from a low efficiency of the mycobacterial cell wall lyses or uneven distribution of bacilli in the specimens [10,29,12,30]. The PCR negative specimens indicate that even when all the histopathologic features of the submitted tissue are consistent with TB, the same features may also be present in conditions other than TB.

5. CONCLUSION

The nested and touch-down PCRs methods proved to be sensitive, specific and rapid for the detection of mycobacteria in routinely processed FFPE histological samples. They can be used to increase diagnostic accuracy in cases where EPTB was not initially suspected, or had confusing results associated with a granulomatous tissue response that would delay the patients' treatment. Thus enabling definite diagnosis of TB in significant numbers of ZN stain negative biopsies, differentiate between members of the MTC from other non-tuberculous mycobacteria and differentiate TB from other types of granulomas better than acid fast stain alone. The results also suggest that such methods have potential use in detecting

mycobacteria in archival material and could be useful in retrospective epidemiological studies. Although, PCR cannot completely substitute the conventional methods, but it offers additional useful tool for TB diagnosis in patients when clinical diagnosis is uncertain. It is also recommended that culture be routinely done for all tuberculosis suspected cases.

CONSENT

Not applicable, this is a retrospective study on archival tissue.

ETHICAL APPROVAL

The study protocol was approved by the Internal Review Board (IRB) at Jordan University of Science and Technology, Irbid, Jordan.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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