

Comparison of Multiplex PCR and Acid Fast and Auramine -Rhodamine Staining for Detection of *Mycobacterium tuberculosis* and Non tuberculosis Mycobacteria in Paraffin- Embedded Pleural and Bronchial Tissues with Granulomatous Inflammation and Caseous Necrosis

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Abstract

Objective

The aim of this study was to compare the sensitivity and specificity of Acid fast and Auramine-Rhodamine staining and Multiplex PCR for the detection of *Mycobacterium tuberculosis* complex and non tuberculosis Mycobacteria on formalin fixed paraffin embedded tissues (FFPE)

Materials and Methods

Forty cases of FFPE pleural and bronchial tissue with chronic granulomatous inflammation and caseous necrosis and 10 cases with bronchogenic carcinoma as controls were investigated. We designed a Multiplex PCR DNA amplification method with two targets: 123bp DNA fragment from IS6110, which is present only in mycobacterium tuberculosis complex and 162bp DNA encoding Ag 85complex which is present in all of mycobacteria. The FFPE also stained by Acid fast and Rhodamine-Auramine staining method.

Results

In 26 samples (65%) 123 bp and 162 bp DNA fragments were detected together (12 in bronchial samples and 14 in pleural samples). The 162 bp fragment wasn't detected alone. The sensitivity of PCR was 65% and the specificity was 100%. Eleven cases were positive for Acid fast staining. There was 27.5% sensitivity and 100% specificity. Thirteen cases were positive for Auramine-Rhodamine staining (A-R-S); there was 32.5% sensitivity and 100% specificity. All of the 10 controls were negative for 123 bp, 162 bp DNA fragments, for Acid fast and Auramine-Rhodamine staining.

Conclusion

Multiplex PCR is a sensitive, specific and rapid method for detection of *M. tuberculosis* in FFPE tissues.

Keywords: Acid fast stain, Auramine-Rhodamine stain, Bronchial tissue, *M. tuberculosis*, Multiplex PCR, Pleural tissue

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Introduction

Despite longstanding effort to conquer tuberculosis, this disease remains an expanding global health crisis with 1.86 billion infected people (1-2). Methods for the diagnosis of tuberculosis have improved in recent years and several molecular techniques for its diagnosis have been introduced for clinical use. Molecular methods provide several advantages, including confirmation of the presence of *Mycobacterium tuberculosis* within 1 to 3 day (2, 3).

The use of DNA amplification for detection of *M. tuberculosis* in formalin fixed paraffin embedded tissue samples would be useful for patients in whom diagnosis depends on tissue examination, rather than detection of *M. tuberculosis* in body secretion (1, 4, 5).

The diagnosis of tuberculosis is largely based on conventional approaches, which rely on clinical features and the result of microscopy and culture. Culture methods are sensitive and specific but they are slow and take 2-6 wks (3, 4). There are frequent occasions when tissue obtained by biopsy is not sent for culture because the diagnosis was not a clinical consideration before the report of findings on microscopic examination of the tissues (6- 8).

Acid fast and Auramine-Rhodamine staining are rapid and inexpensive methods for diagnosis of tuberculosis but their sensitivity is low. The number of bacilli in tissue section stained with Acid-fast and Auramine-Rhodamine staining seems to be much lower than that expected from the sputum smear data or the patient's condition (3, 5, 9).

Infections caused by nontuberculosis mycobacteria (NTM) are increasing in immunocompromised individuals. Effective therapeutic regimens are different for patients infected with *M. tuberculosis* or NTM. Therefore, it is necessary to establish and evaluate PCR assay to differentiate between these two groups of Mycobacteria.

In order to establish sensitive Multiplex PCR, we selected two DNA targets: insertion

sequences 6110 (IS6110) genes which present in multiple copies in the *M. tuberculosis* genome and the gene encoding Ag 85 complex.

We also used Acid fast and Auramine-Rhodamine staining to evaluate the sensitivity of these methods and compare the results with Multiplex PCR.

The aim of this study was to compare the sensitivity and specificity of Acid fast and Auramine-Rhodamine staining and Multiplex PCR for the detection of *M. tuberculosis* complex (MTC) and non tuberculosis Mycobacteria on formalin fixed paraffin embedded tissues.

Materials and Methods

From January 2002 to June 2005, forty formalin-fixed, paraffin-embedded (FFPE specimens) (20 pleural, 20 bronchial samples) with granulomatous inflammation and caseous necrosis were obtained from lung biopsy files at the Department of Pathology of Ghaem Hospital. Two pathologists confirmed that every section included granuloma with caseous necrosis. The patients aged 17 to 78 years, twenty three cases were males and 17 were females. Ten FFPE specimens of patients with bronchogenic carcinoma without any histologic and clinical evidence of tuberculosis considered as negative controls. FFPE sample of a patient with confirmed pulmonary tuberculosis was used as a positive control in each procedure.

Sample processing for Acid Fast and Auramine-Rhodamine staining

Five Micron-thick sections from each paraffin block were cut with a microtome. The paraffin was removed by soaking the slides in xylene, and then the slides were transferred to Tris-EDTA, containing decreasing concentration of ethanol. These smear were stained with Acid-fast and Auramine- Rhodamine staining according to standard confirmed procedures (4) (Figure 1, 2).

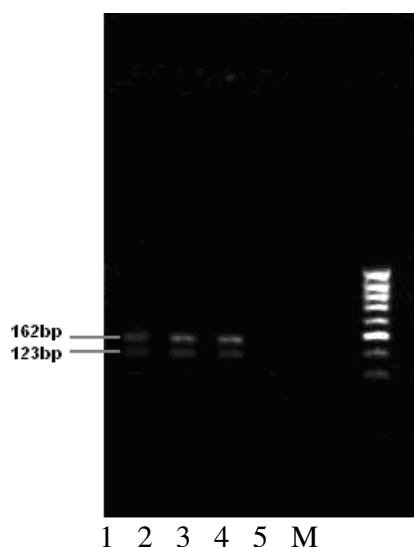


Figure 1. Detection of Multiplex PCR product of *M.tuberculosis* gene on polyacrylamide gel electrophoresis. The lane numbers are as follows: lane M marker of 50-bp ladder; lane 1, 2, 3, positive; lane 4, negative; and lane 5, negative control

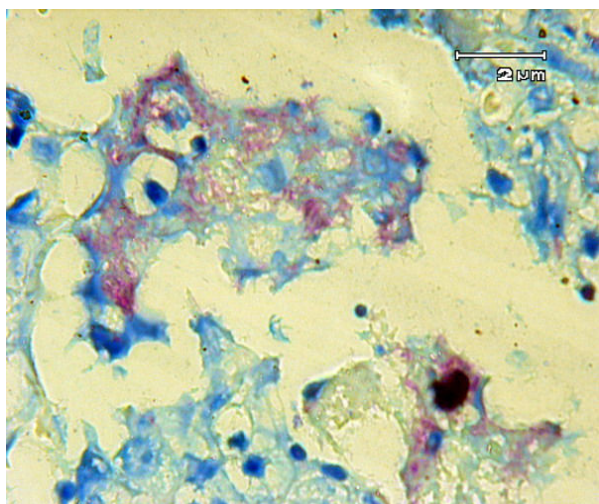


Figure 2. Acid fast staining (1000x) on bronchial FFPE.

We used fluorescence microscopy for A-R stain reporting. The results of Acid- fast and A-R staining were reported after viewing 100 fields according to table 1.

Table 1.Guidelines for reporting smears for acid-fast bacilli.

Report	Number of AFB with A-R stain (450x)	Number of AFB with acid fast stain (1000x)
Doubtful	1-2/70 F	1-2/300F
1 ⁺	2-18 /50 F	1-9/100F
2 ⁺	4-36/10F	1-9/10F
3 ⁺	4- 36/ F	1-9 F
4 ⁺	> 36/F	> 9/F

DNA extraction

Five Micron-thick sections were made from 10% FFPE. To prevent contamination, a fresh blade was used for each sample and the microtome was covered all over with a piece of tape, changed for every sample, and after processing each specimen it was subsequently cleaned with xylene and ethanol (100%). Paraffin was removed from the samples by adding 1 ml xylene, vortexing the mixture, and incubating it at 45°C for 15 minute; this was followed by 10 minutes centrifugation at 14000 rpm. The supernatant was removed and discarded, a further 1 ml of xylene was added to the pellet and the procedure was repeated. To facilitate pelleting and hydration of sample 1 ml of ethanol (100%) was added. After vortexing, the samples were pelleted by 10 minutes centrifugation at 14000 rpm and the supernatant was removed. A further 1 ml of ethanol (100%) was added to the pellet and the procedure was repeated. The supernatant was removed then the pellet was air dried. The samples were resuspended in 400 µl of digestion buffer made up of 1.5 ml NaCl 5M, 0.5 ml EDTA, 0.5 ml Tween 20 and sterilized water up to total volume of 100 ml. Then 40 µl of proteinase k was added and the mixture was incubated at 37° C for 24 hours. The proteinase k was inactivated by incubating the samples at 100° C for 8 minutes and then the pellets were centrifuged at 12000 rpm for 5 minutes and the supernatant removed which was ready to use.

DNA amplification by PCR

Two sets of primer were used to amplify target DNA fragments: The primer pairs MD1- MD2 expected to amplify 162 bp DNA fragments of antigen 85 complexes and the primer pairs KD1- KD2 were expected to amplify 123bp fragment of insertion sequence 6110. The designation and sequence of these primers are given in table 2. The primers were synthesized and cartridge purified by Bioscience Ltd, Heslington, York, England.

Table 2. The oligonucleotide sequence of primers used in Multiplex PCR.

Base number	Oligonucleotide sequence	Oligoname
21	5'- ATC AAC AAC CCG GCG TTC CAG-3'	MD1
20	5'- CGC CAG CTC GCT GGT CAG GA-3'	MD2
20	5'-CCT GCC AGC GTA GGC GTC GG-3'	KD1
20	5'-CTC GTC CAG CGC CGC TTC GG-3'	KD2

Each reaction mixture contained primer KD1, primer KD2, primer MD1, primer MD2, dNTP, 10xPCR buffer, MgCl₂, Taq DNA polymerase, water and 5 μ liter of purified DNA in total volume of 50 μ l. Gene amplification was done by a thermal cycler (Thouchgene, Gradient). Cycling consisted of denaturing at 94° C for 45 sec, annealing at 68°C for 60 sec and extension at 72°C for 6 sec. The total process had 35 cycles. To avoid false positive, separate physical facilities were used for sample preparation, amplification and analysis of the amplified products, in addition positive and negative controls were included. Aliquots of amplified samples were loaded on 2% agarose gels in Tris- Acetate- EDTA (TAE) buffer and subjected to electrophoresis in mini gel boxes for 30 minutes at 80 volts. The gel stained with ethidium bromide at 0.5 mg/mL. Observed under ultraviolet light for specific DNA bands and photographed. The DNA bands were identified according to size by comparing with molecular weight marker (50bp DNA ladder) loaded in separate lane (Figure 3).

Results

Fourty samples (20 from bronchial tissues and 20 from pleural tissues) from the patients with chronic granulomatous inflammation and caseous necrosis were studied, the patients aged 17-78 years and 23 were males and 17 were females. Also, ten patients with bronchogenic carcinoma, as controls, were investigated.

Overall 123bp and 162bp DNA fragments were detected together in 26 samples (65%) (12 in bronchial samples and 14 in pleural

samples). The 162 bp DNA fragment was not detected alone (0%). All of the 10 controls were negative for 123bp and 162 DNA fragments (Table 3).

The sensitivity of PCR was 65% and the specificity was 100%. The positive predictive value of the test was 100% and the negative predictive value was 41%, all the negative samples were also negative for Acid fast and A-R staining.

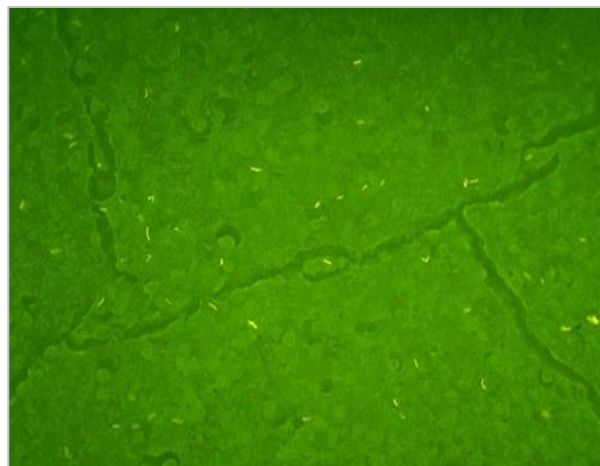


Figure 3. Rhodamine- Auramine staining (450X).

Table 3. Result of Multiplex PCR for 123bp and 162 bp Mycobacterial DNA fragment in pleural and bronchial tissue with caseous necrosis.

	123bp fragment		162bp fragment	
	number	frequency	number	frequency
Negative	14	35	14	35
Positive	26	65	26	65
	40	100	40	100

Eleven cases were positive for Acid fast staining, 2 samples were +++, 4 samples were ++ and 5 samples were +, 15 samples with negative AFS were PCR positive, the sensitivity of AFS was 27.5% the specificity was 100%, the PPV (positive predictive value) was %100 and NPV (negative predictive value) was 27% (Table 4).

Table 4. Result of Acid Fast and Auramine-Rhodamine stain for detection of mycobacteria in pleural and bronchial tissue with caseous necrosis.

Acid Fast stain		Auramine-Rhodamine stain		
frequency	number	frequency	number	
72/5	29	67/5	27	-
%12/5	5	%10	4	+
%10	4	%17/5	7	++
%5	2	%5	2	+++
%100	40	%100	40	Total

Thirteen cases were positive for A- R staining, 2 samples were +++, 7 samples were ++ and 4 samples were +, the A-R negative section included 13 PCR positive cases. The sensitivity of A-R staining, specificity, PPV, and NPV were 32.5%, 100%, 100% and 28.5% respectively (Table 4).

Discussion

Rapid diagnoses of MTC and NTMC have an impact on the management of patients. The detection of MTC in clinical samples leads to initiation of correct treatment, thereby avoiding or diminishing severe complications.

Several amplification methods are available now and are useful for patients in whom diagnosis depends on tissue examination rather than detection of *M. tuberculosis* in body secretion (10, 11, 12). These methods render a feasible, rapid and easy tool to perform retrospective diagnosis of *M. tuberculosis* infection, which would be particularly useful when there is a lack of growth on the culture or when fresh material has not been collected for the culture. Several factors affect the result of PCR in FFPE samples, such as target DNA size, DNA concentration, and target fragment repetitiveness within the *Mycobacterial* genome (13). Many reports have confirmed that PCR amplification in formalin- fixed tissues detects TB DNA when only a few genomes are present (14,15).

We designed a Multiplex PCR with two DNA fragment targets:

1) 123 bp DNA fragment from IS 6110 which is present in organisms of the *M. tuberculosis* complex group (MTB, *Mycobacterium bovis*, *Mycobacterium bovis* bacille calmette-Guerin, *Mycobacterium africanum*, *Mycobacterium microti*) and is absent in other species of *Mycobacterium*. The IS6110 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 MTB complex (16- 18).

2) 162 bp DNA encoding Ag 85 complex which is present in all groups of *Mycobacteria*. The sensitivity of our Duplex PCR was 65%, which is similar to or lower than that of other

reports. Most of the authors reported the sensitivity of 60 to 90 percent and the specificity of 95 to 100 percent for FFPE (3, 4, 5, 6). The specificity of our Duplex PCR was 100%. Our study shows that a properly designed PCR assay can successfully be used to detect *M. tuberculosis* in formalin fixed paraffin embedded tissues. It is evident that further investigation need to be conducted in order to ameliorate and possibly standardize a protocol of DNA amplification from archived material. We believe that TB PCR is a suitable method for the diagnosis of tuberculosis in routinely processed, formalin fixed and paraffin embedded histologic specimens, although application of the PCR method to formalin fixed, paraffin embedded tissue has shown several limitations, including DNA structure change due to prolonged formalin fixation (7). Although PCR reduce the delay in diagnosis, culture remains the gold standard for identification of *Mycobacteria* in tissues. Culture also allows for the testing of antibiotic sensitivity of any isolated species, in this way determining appropriate treatment (3, 8, 9).

In this study, we didn't use *Mycobacterial* culture of tissue specimens as a reference for the TB PCR; instead, we used the pathologic feature of TB granuloma as a comparative reference for TB PCR. The ability of detection of nontuberculosis *Mycobacteria* was the advantage of this Duplex PCR, although NMTC was not detected.

Acid fast and A-R staining are rapid and inexpensive methods for the diagnosis of tuberculosis but in this study these methods sensitivity was not significant (Table 4) which is similar to other reports (3, 6, 10).

Some researchers have suspected that the organic tissue samples might affect the stainability of *Mycobacteria* by Acid fast staining (3, 11, 12).

These hypotheses seem reasonable because the molecular target of the Acid fast staining dyes is the mycolate in the bacterial cell wall. Mycolate is soluble in organic agents (3). However, this result should not discourage pathologists from using Acid fast and A-R staining to evaluate mycobacterial lesions.

Conclusion

Multiplex PCR is sensitive, specific and rapid method for detection of *M. tuberculosis* in paraffin embedded samples. Acid fast and R-A staining are rapid and inexpensive methods. These methods are specific but their sensitivity was not significant in this study.

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