Incidence of Mycobacterium tuberculosis detection in formalin fixed- paraffin embedded granulomatous dermatoses with Multiplex PCR comparing with fluorescent microscopy and acid fast staining in eastern Iran

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Citation

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Abstract

Introduction: Distinguishing cutaneous tuberculosis to from other granulomatous dermatoses is usually difficult. We used polymerase chain reaction (PCR) to evaluate the incidence of cutaneous tuberculosis and comparison of PCR with other conventional methods in formalin-fixed, paraffin-embedded tissues (FFPE) having unspecified granulomatous inflammation.

Materials and Methods: A total of 30 consecutive specimens which had been collected from 30 different patients and fulfilled the criteria for tissues described above were used in this study. Samples obtained from skin biopsy archived blocks of Pathology Department of Ghaem Hospital-Mashhad, Islamic Republic of Iran from January 2000 to June 2003 . Two different primer pairs of MD1-MD2 and KD1-KD2 targeting the gene of of 162 bp (common to all mycobacteria) and of 123 bp DNA (specific for M. tuberculosis complex) were used in the PCR assays.

Results: Of the 30 specimens, 6 were PCR positive for the 123 bp DNA. All of these 6 cases, were PCR positive for 162 bp DNA too. Cutaneous tuberculosis could be diagnosed in these 6 cases (20.0%).). After reviewing their clinical presentation, 5 cases were considered as lupus vulgaris. No cases was positive only for 162 bp gene.. There were 4 and 5 positive specimens in fluorescent and acid fast staining respectively.

Conclusion: These results show that in cutaneous tuberculosis with unclassical clinical and histological presentation, PCR provides rapid and sensitive detection of M. tuberculosis DNA in FFPE specimens. In areas like Iran, where prevalence of extrapulmonary tuberculosis is still high, lupus vulgaris is common form of cutaneous tuberculosis and are seen more frequently than atypical mycobacterial infection. Finally, conventional methods of tissue staining have somewhat lower potency from PCR in distinguishing mycobacteria

INTRODUCTION

Granulomatous dermatoses are a diagnostic challenge in dermatopathology as they encompass a wide range of noninfectious and infectious diseases([1]). In infectious diseases category, cutaneous tuberculosis and atypical mycobacteria infection are especially difficult to diagnose due to the limitations of traditional diagnostic methods. Direct examination of acid-fast bacilli (AFB) by microscopy is rapid but has a low sensitivity, requiring about 10^4 bacteria per milliliter of tissue specimen([2]). Furthermore it lacks specificity as it cannot discriminate different species of mycobacteria. Culture is essential for a definite diagnosis; however, it takes weeks for identification, and its sensitivity is also relatively low in cutaneous tuberculosis because of paucibacillary conditions ([3,4]). Recently, several systems using polymerase chain reaction (PCR) have been reported for the molecular detection of mycobacterial DNA([5,6]). It has become a valuable tool in the rapid identification of slow

growing organisms such as Mycobacteria tuberculosis and fastidious organisms such as atypical mycobacteria. DNA can also be successfully extracted from formalin-fixed, paraffin-embedded tissue([7,8]), thus enabling the use of archival tissue for retrospective study.

The aim of this study was to determine whether M. tuberculosis or atypical mycobacteria could be identified in specimens from patients with a pathologic diagnosis of granulomatous dermatitis and comparing this modality with conventional acid fast staining (AFS) and fluorescent microscopy (FM).

MATERIALS AND METHODS

Thirty consecutive formalin-fixed, paraffin-embedded (FFPE) skin specimens which had been collected from 30 different patients with pathologic diagnosis of granulomatous inflammation and without definitive etiological histology were obtained from skin biopsy archived blocks of Pathology Department of Ghaem Hospital-Mashhad, Islamic Republic of Iran from January 2000 to June 2003 . Two pathologists confirmed that every section included granuloma .The clinical records of the patients were also reviewed and data on age, clinical location, and histopathologic findings were collected. Suspected clinical diagnoses were as fallow: 9 Lupus Vulgaris, 5 cutaneuse leishmaniasis, 2 Necrobiosis Lipoidica, 2 Granuloma Annulare, 1 Sarcoidosis, 1 Tuberculosis verrucosa cutis and 10 without any clinical diagnoses.

5 cases of confirmed cutaneous leishmaniosis served as negative controls throughout the assay. FFPE samples, from a patient with confirmed cutaneous TB, were used as a positive control in each procedure.

SAMPLE PROCESSING FOR ACID FAST AND AURAMINE RHODAMIUNE STAINING

5 Micron- thick sections from each paraffin block were cut with a microtome. The paraffin was removed by soaking the slides in xylene, 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 ([9]).

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.

Figure 1

Table 1: The oligonucleotide sequence of primers in Multiplex PCR which used in this study.

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

DNA EXTRACTION& AMPLIFICATION BY PCR

Two to three sections (8 µm) were cut from each paraffin-embedded tissue block and placed into an Eppendorf tube. To avoid cross-contamination of the specimens, the microtome blade was changed between each block. Tissues were deparaffinized by adding xylene and centrifuged. The supernatant was removed, and was re-suspended in 300 µL of PCR lysis buffer (200 µg/mL proteinase K, 50 mM Tris-hydrochloride, 0.3% SDS, 125 mM NaCl) at 55 °C. 200 µL of chelex-100 was added, followed by boiling for 10 min. The sample was then centrifuged at 13,000 r.p.m. for 10 min. DNA was extracted with phenol-chloroform extraction followed by ethanol precipitation.

Two different oligonucleotide primer pairs were used to amplify target DNA from the multiple copy genes (Table 1). The primers were synthesized and cartridge purified by Bioscience Ltd,Heslington, York, England. Using M. tuberculosis DNA as a template, the primer pairs MD1-MD2 and KD1-KD2 were expected to amplify 162 bp, and 123 bp DNA, respectively.

All reactions were performed with Taq polymerase and on a PCR system thermocycler (Thouchgene, Gradicent).Finally the PCR products were fractionated on 2% agarose gel in the 0.5X TAE buffer, stained with ethedium bromide, and visualized under UV light..

RESULTS

The age range was 8–65 years (mean: 31.2 years).patients were 22 female and 8 male.

Of the 30 specimens (from 30 patients),6 generated a 162-bp amplification product. All of these 6 specimens also showed amplification of a 123-bp sequence of M. tuberculosis complex. The PCR results are summarized in Table 2.

Figure 2

Table 2: Frequency of 162bp and 123bpDNA fragments detection in this study

	162bp DNA fragment	123bp DNA fragment
Negative	24	24
Positive	6	6
total	30	30

The simultaneous presence of two different amplification bands in 6 of the 30 specimens indicated tuberculosis. No samples showing only a 162-bp band as atypical mycobacteria infection. The detection limits of the two primer pairs were 7×10^{-6} mg/mL of mycobacterial DNA. All of our 5 negative controls were negative for 123bp and 162 DNA fragment.

The clinical presentation of the 30 specimens considered to have a cutaneous granulomatous reaction are summarized in Table 3.

Figure 3

Table 3: Clinical localization of the 30 specimens considered to have a cutaneus granulomatosis

	Frequency	Percent	Valid percent	Cumulative percent
Upper extremities	9	25.7	25.7	25.7
Head & neck	13	37.1	37.1	62.9
Lower extremities	8	22.9	22.9	85.7
Trunk	5	14.3	14.3	100.0
Total	35	100	100	

We used fluorescence microscopy for A-R staining .The results of Acid- fast and A-R staining were evaluated as the presence of Acid fast or fluorescent bacilli among the estimated number of total bacilli and the results recorded according to table 4.

Figure 4

Table 4: Guidelines for reporting smears for Acid Fast Bacilli with Acid Fast Staining.

Number of AFB with acid fast stain (1000x)	REPORT
1-2/300field	-
1-9/100 field	1^+
109/10 field	2^+
1-9 field	3^+
> 9 field	4^+

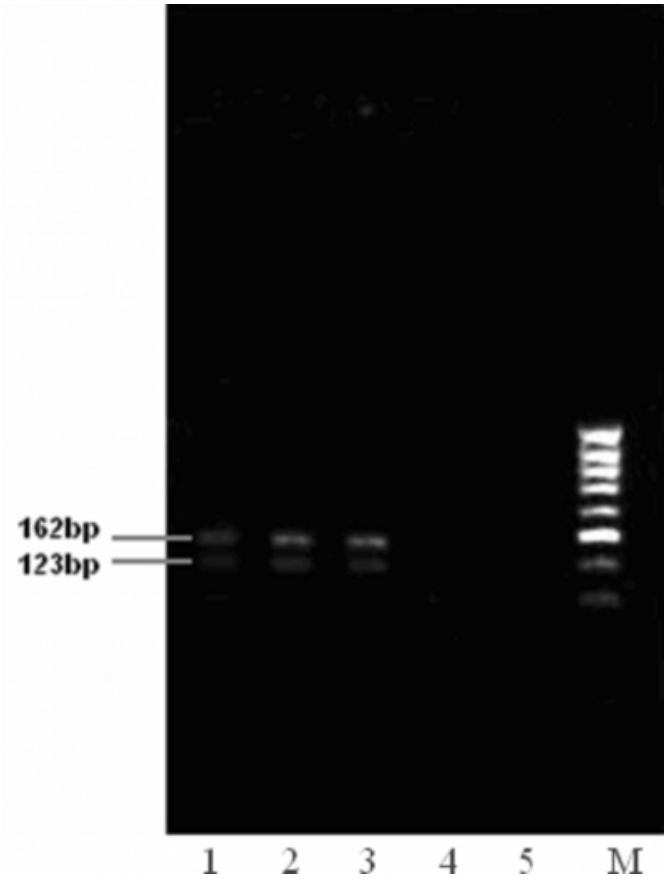
In AFS, 5 specimens were Ziehl-Neelsen positive but there were positivity from + to +++ and there were controversy on positiveness between pathologists from these 5 specimens ,4 specimens were positive in PCR and one specimen was negative.

In A-R fluorescent microscopy 4 sample were positive but there were controversy between examiners from these 4 specimens ,3 specimens were positive in PCR and one specimen was negative.

In our study also we seen granulomatous dermatosis were 13 in head and neck, 9 in upper exterimities,8 in lower extremities and 5 in trunk.

Figure 5

Figure 1: (A) Detection fo Multiplex PCR product of M. tuberculosis gene on polyacrylamide gel electrophoresis. The lane numbers are as follows: lane M marker of 100-bp ladder; lane 1,2,3, positive ; lane 4, negative; and lane 5, negative control.



DISCUSSIONS

Cutaneous tuberculosis and nontuberculous mycobacteria infection are often missed because of their rarity and lack of clinical suspicion. Both of these infections show considerable variability in their clinicopathologic features and should be included in the differential diagnosis of granulomatous dermatoses. Experiences in other countries have been showed that there is a generally low yield from tissue cultures for both M. tuberculosis and atypical mycobacteria. When definite criteria for diagnosis are not fulfilled, their presence can only be confirmed by successful empiric therapy in highly suspicious cases.

PCR has entered as a promising tool in the diagnosis of various forms of cutaneous tuberculosis due to its high sensitivity and speed([10,11]). The sensitivity of PCR has been shown to be better than that of microscopic examination and comparable with that of culture. PCR can also aid in differentiating M. tuberculosis from non tuberculous mycobateria when appropriate primers are chosen ([5,12,13]).

In this study, we chose two pairs of primers to amplify. IS6110 is an insertion sequence present in multiple copies within the genomes of all members of the M. tuberculosis complex, thus accounting for its high sensitivity. Its specificity has also been confirmed in various studies([10,14,15]). Discrimination between M. tuberculosis complex and other mycobateria species can also be achieved with this method.

Reviewing the clinical information of the 6 specimens with a positive result for tuberculosis by PCR, there was a wide range of age distribution was 8–65 years (mean: 31.2 years) among the patients, and the lesions could be found in different locations. Five patients presented as one or several erythematous plaques, in which lupus vulagris was suspected and one patient presented with suspicious Tuberculosis verrucosa cutis. This finding is also compatible with the view that because most people in our country are sensitized hosts, secondary tuberculosis manifesting as lupus vulgaris are seen more frequently. Tuberculosis verrucosa cutis was the most common form of cutaneous tuberculosis in Asia (16 but lupus vulgaris represents a significant proportion in our patients.

Despite an existing high prevalence of pulmonary tuberculosis in Iran, our local experience is that cutaneous tuberculosis is uncommonly seen. However, the high incidence of cutaneous tuberculosis in unclassified granulomatous dermatitis shown in this study alerts us that many of these cases have been missed by the traditional diagnostic methods. With the application of PCR in this group of patients, these cases will not be overlooked and can receive appropriate treatment rapidly.

Reviewing the histopathological findings in these 6 specimens, we found most of them showed nonspecific granulomatous inflammation without the characteristic tubercles. Focal caseous necrosis could be found in only one specimen. Three types of granuloma including sarcoidal and necrotizing granulomas could be identified in the cases, indicating the diversity of histopathologic spectrum of tuberculosis. When we encounter cases showing sarcoidal or necrotizing granulomas, and fail to demonstrate the presence of tubercle bacilli with traditional methods, cutaneous tuberculosis should still be included in the list of differential diagnosis.

PCR sensitivity is decreased when used with paucibacillary samples. This may be explained by the loss of DNA during extraction, failure to sample target DNA during sectioning([18]) or the presence of inhibitory substances. Fixative has also been reported to diminish the PCR signal particularly when the fixation time is prolonged([19]). The overall sensitivity of PCR for paucibacillary specimens has ranged from about 50%([20]) to72%([9])in different studies using IS6110 primers. Although Tan et al.([11]) concluded that PCR was of limited diagnostic value in paucibacillary forms of cutaneous tuberculosis, many cases of lupus vulgaris have been confirmed with PCR in previous studies([10,21,22,23]). However, experiences regarding tuberculosis verrucosa cutis were relatively limited. The detection limits of the current study were 7×10^{-6} mg of mycobacterial DNA, which are consistent with those of previous studies. Appropriate dilutions of samples and incorporation of controls were also implemented in this study to reduce the influence of inhibitory substances. Because in two conventional methods (AFS &FM)neither positive results, nor suspect results were the same and some of the positive results in one method was negative in other and vise versa therefore although we must appreciate partial capabilities of this simple methods but with wide accessibility of PCR in our country and looking straightforward results of it we ,according to our results suggest application of PCR for suspect biopsy samples of granulomatous dermatosis .In our study that we didn't have any correct etiological histopathologic diagnosis unless 5 probable diagnosis ,finally we had 6 documented cutaneous MTB. Also we found that in 4 consecutive years of study incidence of granulomatous dermatosis have some raise.

CONCLUSIONS

In unspecified granulomatous inflammation, PCR provides rapid and sensitive detection of M. tuberculosis and other mycobacteria DNA. In areas like Iran, where prevalence of pulmonary tuberculosis is still high, lupus vulgaris and tuberculosis verrucosa cutis are common forms of cutaneous tuberculosis and are seen more frequently than atypical mycobacterial infection. Our results demonstrate that PCR not also can successfully be used to fast and correct diagnosis of a sample as mycobacterial infected but also can differentiate between M. tuberculosis and NTM in FFPE cutaneous specimens. These are clear cut and correct results that can be used by clinicians.

In the other hand conventional methods such as AFS and A-R FM according our results have acceptable concordance with PCR and in absence of it can be used for etiological diagnosis of granulomatous dermatosis and as a comment (not exactly) come in pathologic report of these type of disorders.

Although multiplex PCR in this study was more sensitive than A-R FM and AFS, but we according to our results recommend that for better detection of Mycobacterium tuberculosis and increasing sensitivity in FFPE samples it is better that all of this methods are done together.

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