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Original Article

PCR-Based Diagnosis of *Leishmania* Species in Chronic Granulomatous Dermatitis in Mashhad, Iran

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Abstract

Background: Cutaneous leishmaniasis (CL) is a chronic granulomatous dermatitis (CGD). Approximately, 90% of CL patients are from seven countries including Iran. We explain *polymerase chain reaction (*PCR) diagnostic technique for chronic granulomatosis dermatoses including CL in Mashhad, Iran.

Methods: This study enrolled 64 patients within 2009-2013 with chronic granulomatosis dermatitis referred to dermatology and pathology departments of Imam Reza Hospital, affiliated to Mashhad University of Medical Sciences (MUMS), Mashhad, Iran. We gathered demographic data from archived folders. Histological light microscopic evaluation and parasitological tests were done on selected specimens. We used PCR diagnostic test on specimens. Statistical analysis was done by SPSS version 15.

Results: Generally, 7 out of 64 specimens had *Leishmania* DNA and other samples had no *Leishmania* DNA. The mean age of patients was 46 ± 18.77 years; disease duration was 7 ± 6.73 months ranging from 1-24 months. Most of the lesions were located on face and upper limb. Totally, 5 out of 7 samples were *Leishmania major* and 2 out of 7 samples were *L. tropica*. Tuberculoid granuloma was present in *L. tropica* samples and 3 of *L. major* samples. Other light microscopic changes were as follow: 42 suppurative granuloma, and epidermal changes including atrophy, pseudoepitheliomatous hyperplasia, and parakeratosis with dermal changes including, plasma cell, involvement of papillary and reticular dermis, and distribution of granuloma to hypodermis.

Conclusion: Our results addressed PCR-based diagnosis of chronic cutaneous leishmaniasis, which is resulted from *L. major* and *L. tropica*.

Introduction

Granulomatous dermatoses are a broad spectrum of skin disorders with different etiologies and clinical patterns but common histological morphology defined as predominance of histiocytic accumulation surrounded by various other inflammatory cells including macrophages (1, 2). Previously, some papers noted the classification as infectious and non-infectious granulomatous dermatoses (1). A study revealed the classification based on presence of necrotizing (suppurative and caseous) and broad range of non-necrotizing differential diagnoses including sarcoidosis, tuberculosis and leishmaniasis (2).

Leishmaniasis appears in tropical and subtropical areas worldwide, which is transmitted by female phlebotomine sand fly bite (3, 4). The incidence of cutaneous leishmaniasis (CL) is about 1.8 million cases with 350 million people at risk of infection, annually (5). Every year, about 600,000 to one million new CL patients are detected worldwide (6). Based on recent literature review, about 95% of CL patients live in the following countries: Americas, the Mediterranean basin, the Middle East and Central Asia. Within 2018, more than 85% of new CL patients located in 10 countries including Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran (Islamic Republic of), Iraq, Pakistan, the Syrian Arab Republic and Tunisia (7).

Reports showed CL in some cities of Iran including Bam, Mobarakeh, Natanz, Kashan, Ardestan, Mehran and Dehloran. Also, both types of CL have been reported in Iran as the following: *anthroponotic cutaneous leishmaniasis*, caused by *L. tropica* and transmitted by *Phlebotomus sergenti*, and zoonotic cutaneous leishmaniasis, caused by *L. major* which is transmitted by *P. papatasi (8)*.

Leishmania has a wide range of clinical patterns from subclinical infection to fatal visceral infection in humans (9). *Leishmania* diagnosis needs clinicopathological correlation with other paraclinical tests (1). The *Leishmanin* skin test is an appropriate diagnostic test with high sensitivity and low specificity when used in endemic area because it cannot distinguish between acute and chronic infection (9). The most common diagnostic technique in Iran is still low sensitivity microscopic examination in order to demonstrate the parasite in skin biopsies and smear specimens or by culture in-vitro. However, culture isolation needs more viable amastigotes and longer incubation period (3). Furthermore, as the duration of infection increase, parasite load decreases. Therefore, some other diagnostic tests including molecular genetic testing and immunohistochemistry can be appropriate (1). The noticed problems motivated us to evaluate other diagnostic tests such as polymerase chain reaction (PCR) in either conventional or real time formats to increase sensitivity and specificity (3, 4). PCR is able to amplify minute nucleic acid quantities (10).

The current paper summarized the ability of PCR technique to evaluate presence of *Leishmania* deoxyribonucleic acid (DNA) in paraffin-embedded skin biopsies with confirmed chronic granulomatous dermatitis diagnosis.

Materials and Methods

Sampling and parasitological tests / Light microscopic examination

The current study included 64 patient archived folders diagnosed as chronic granulomatosis dermatitis in Dermatology and Pathology departments of Imam Reza Hospital, affiliated to Mashhad University of Medical Sciences (MUMS), Mashhad, Iran; from 2009-2013. We used 64 paraffin-embedded blocks of patients with chronic granulomatosis dermatitis with unknown etiology. We used DNA extraction and PCR on samples to demon-

strate *Leishmania* in specimens. We enrolled 34 males (53%) and 30 females (46.9%) in the study. Also, we collected some associated data in our research project including: patients name, folder number, age, gender, location of lesion, duration of skin lesion formation, season the patient referred to clinic, and type of granuloma and also slides with paraffin blocks were gathered. Hematoxylin and eosin (H&E), Periodic-Acid-Schiff (PAS), Giemsa, and Ziehl–Neelsen staining were used in order to re-evaluate granuloma type, fungal elements and acid-fast bacilli. In addition, we used polarized light to assess foreign bodies. Regarding to inclusion criteria, all specimens with negative results for acid-fast bacilli, fungal elements, foreign bodies, and Leishman body, and no history of cutaneous lupoid leishmaniasis in clinical manifestations were enrolled the study and named as granulomatous dermatitis with unknown etiology. Then, for 64 blocks, we prepared five µm sections from paraffin-embedded blocks, put them into sterile micro-tubes, and referred them to microbiology laboratory for PCR assessment. Furthermore, we assumed a cutaneous *Leishmania* paraffin block for positive control and some paraffin blocks as negative controls as follow: foreign body granuloma, necrobiotic granuloma, pyogenic granuloma, annulare granuloma, and granulation tissue. PCR was done on samples according to the following procedure:

Deparaffinization

First, we added 1200 microliters xylene to all micro tubes and mixed them. Then, we used micro centrifuge for 5 minutes in room temperature (15 to 25 centigrade degree) with maximum speed. We used biological hood because of toxic effects of xylene and omitted upper liquid in micro tubes. Then, 1200 microliters ethanol 96% added to specimens in order to delete the xylene remnants and mixed them again. We centrifuged them again for 5 minutes in room temperature and for maximum speed. We used pipette to remove remnant of ethanol from surface of specimens. Again, we repeated the mentioned steps in order not to any xylene remained. We incubated micro tubes in room temperature for 10 minutes with open door.

DNA extraction

DNA extraction was performed by DNA extraction kit (QIAGEN, Kit, Germany) according to manufacturer's instructions. Some material, which we used in DNA extraction are in Table 1.

Table 1: The materials needed for DNA extraction*.

Material	*Implication*
Buffers ATL and AL	Lysis solutions that break open tissue, cell, and nuclear membranes
Proteinase K	This enzyme catalyzes cellular protein breakdown to amino acids and peptides
Ethanol 96%-100%	For DNA precipitation
Buffer AW1 and AW2	Wash solutions for DNA attached in the column membrane of contaminants
Buffer AE	A solution that allows stable storage of DNA for many years in the freezer or refrigerator

* All steps done under biological hood of laboratory.

Polymerase chain reaction amplification

The quantity and quality control of the DNA extraction done by using beta globin to verify efficacy of PCR diagnosis. Polymerase chain reaction was performed with beta globin pattern as **GH20**: 5'-GAA GAG CCA AGG ACA GGT AC-3' and **PC04** 5'CAA CTT CAT CCA CGT TCA CC-3' (Sina Clon Company, Tehran, Iran). PCR was performed as initial denaturation for 5 min at 94 ºC, fol-

lowed by 35 cycles of 94ºC for 30 seconds, 55 ºC for 45 seconds and 72 ºC for 45 seconds, and last extension for 5 minutes at 72 ºC. A *Leishmania* genus specific primer performed amplification of DNA. The primer was obtained from Sina Clon Company, Tehran, Iran. Furthermore, a 600-bp fragment for *L. major* and 800-bp fragment for *L. tropica* were amplified in PCR for molecular target (Fig 1). In each round of PCR, definite *Leishmania* specimen was used as a positive control and distilled water was characterized as the negative control. Data was analyzed in SPSS-15 and chi-square test.

Gel electrophoresis

Gel electrophoresis of PCR product on the 1.5% agarose gel was performed. In addition, all steps were re-evaluated histopathologically.

Data analysis

Statistical analysis was done by SPSS version 15. Chi-square test with the level of significance defined as ($P < 0.05$).

Results

Among all samples, seven samples had *Leishmania* DNA and the others had no *Leishmania* DNA. Totally, 5 out of 7 samples were *L. major* and 2 out of 7 samples were *L. tropica*. Tuberculoid granuloma was present in *L. tropica* samples and 3 of *L. Major* samples. Among 64 samples, 13 specimens had plasma cells and 8 samples with pseudoepitheliomatous hyperplasia. Among PCR positive samples, 5 specimens had tuberculoid granuloma and other 2 samples showed suppurative granuloma. In contrast, the negative PCR samples showed different types of granuloma as follow: 39 samples (60.9%) with tuberculoid granuloma, 10 specimens with sarcoidal granuloma and 8 samples with suppurative granuloma.

Comparing PCR results with light microscopic evaluation for pseudoepitheliomatous hyperplasia is as follow: 57 patients out of 64 patients had negative PCR (7 patients had pseudoepitheliomatous hyperplasia and 50 patients (78.1%) did not have pseudoepitheliomatous hyperplasia); other 7 patients had positive PCR results (3 patients with pseudoepitheliomatous hyperplasia and 4 patients did not have pseudoepitheliomatous hyperplasia).

Comparing PCR results with light microscopic evaluation for granuloma formation is defined here: 57 patients out of 64 patients had negative PCR (39 patients (60.9%) had tuberculoid granuloma, 10 patients with sarcoidal granuloma and 8 patients with suppurative granuloma; other 7 patients had positive PCR results (5 samples had tuberculoid granuloma and 2 specimens with suppurative granuloma).

Regarding to comparing PCR results with light microscopic evaluation for plasma cells, (57 patients (89.5%) out of 64 patients had negative PCR (46 patients (71.9%) had no plasma cell and 11 samples with plasma cell); other 7 patients had positive PCR results (4 specimens without plasma cells and 3 samples had plasma cells).

The electrophoresis for PCR results is shown in Fig. 2 and Fig. 3. The clinical and pathological features of PCR positive specimens are shown in Table 2.

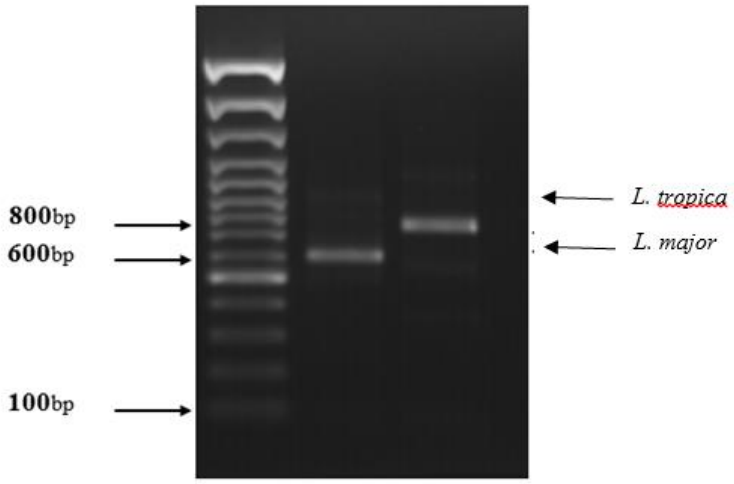

Fig. 1: Electrophoresis of DNA samples obtained from positive control *L. major* and *L. tropica* compared to marker band

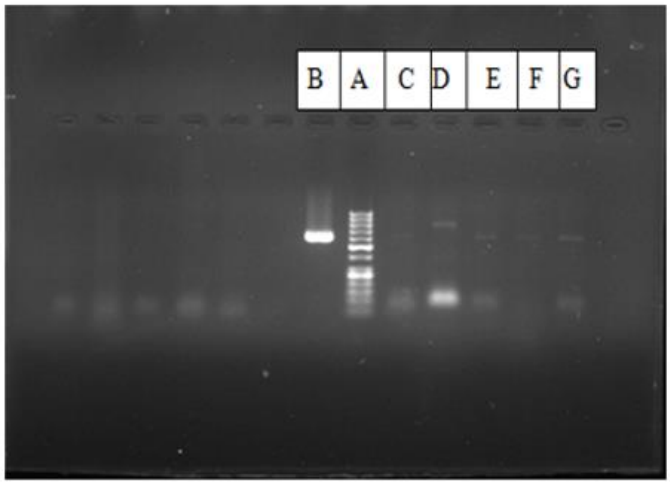

Fig. 2: PCR results. B: stands for positive control *L. major* (600bp); D stands for positive control *L. tropica* (800bp); A stands for marker; others including C, E, F, G are positive *L. major* specimens in study.

Fig. 3: PCR results. H band shows positive specimen for *L. major* and I band shows positive specimen for *L. tropica*. A band is our marker.

Table 2: Clinical and pathological characteristics of PCR[1] positive samples.

Pathologic diagnosis	*Clinical diagnosis*	*Duration of disease*	*Location of lesion*	*Gender*	*Age (years)*	*Location*
Tuberculoid granuloma, with plasma cell and pseudoepitheliomatous hyperplasia	Leishmaniasis	One year	Face	Male	41	Mashhad
Tuberculoid granuloma, without plasma cell and with pseudoepitheliomatous hyperplasia	Leishmaniasis	One year	Arm	Female	51	Mashhad suburbs
Suppurative granuloma with abundant plasma cell	Leishmaniasis	Three months	Face	Female	65	Mashhad suburbs
Tuberculoid granuloma	Leishmaniasis	One month	Face	Female	53	Mashhad suburbs
Suppurative granuloma with abundant plasma cell and pseudoepitheliomatous hyperplasia	Leishmaniasis/foreign body granuloma/ bowen	Two years	Finger	Female	58	Mashhad
Tuberculoid granuloma with abundant plasma cell	Leishmaniasis	One year	Face	Female	65	Mashhad suburbs
Tuberculoid granuloma	Leishmaniasis, DLE[2]	6 months	Cheek	Male	11	Mashhad suburbs

1: PCR, Polymerase chain reaction; 2: DLE,* Discoid lupus erythematosus.

Some other pathologic light microscopic features were epidermal changes including atrophy, pseudoepitheliomatous hyperplasia, and parakeratosis. The dermal changes had several items: tuberculoid granuloma, suppurative granuloma, plasma cells, involvement of papillary and reticular dermis, and distribution of granuloma to hypodermis. Just in one case, the granuloma composed of epithelioid cells with foamy clear cytoplasm extended to arrector pili muscle. Season distribution in lesions is as follow: 22 patients (34%) in spring, 19 patients in winter, 15 patients in autumn and less percentage in summer with 8 patients. The most involved age range distribution was 20-39 years and less in 80-99 years. Sixty-two out of sixty-four patients had one lesion, and two had more than one lesion. In addition, 59 patients living in Mashhad, others in Mashhad suburbs. Facial involvement with 27 patient and upper limb involvements with 26 patients were most common locations. Lower limb with 9 patients and trunk with 2 patients were as next areas. The mean age of patients was 46 ± 18.77 years, ranging from 11-87 years. Disease duration was 7 ± 6.73 months ranging from 1-24 months. In histopathologic evaluation, 57 samples (89.5%) were without necrosis and 7 specimens showed necrosis. In our study, there was no statistically significant relationship between the PCR results and gender, lesion location, age, granuloma type, necrosis, plasma cell, and pseudoepitheliomatous hyperplasia. (*P* value>0.05). Furthermore, we found no statistically significant relationship between gender and lesion location (*P* value>0.05).

Discussion

Our paper summarized *Leishmania* detection in chronic granulomatous dermatitis with no definite infectious etiology, using PCR diagnostic modality. Based on previous reports,

we used PCR diagnostic method for *Leishmania* species on extracted DNA that were obtained from skin biopsies.

Regarding to literature review, there are some differential diagnoses for chronic amastigote-negative CL that presenting as tuberculoid granulomatous dermatitis, including mycobacterial infection, borreliosis, rosacea granuloma, syphilis and pseudolymphomatous pattern of CL (11). It is necessary to distinguish these diagnoses from each other by some highly sensitive tests for appropriate management. For example, in pseudolymphomatous CL as a rare disorder, some papers have found no amastigote in 2 out of 4 skin biopsies, but PCR have confirmed the infection for diagnosis as our study. In addition, they demonstrated PCR diagnostic test for paraffin-embedded formalin fixed tissue (PEFF) with chronic CL diagnosis that have no parasite in histopathologic examination (11).

On physical examination, CL has a red-crusted nodule appeared on face or extremities as a typical clinical manifestation. Unlike typical granuloma formation in light microscopic changes, some studies have depicted that CL sometimes has different clinical and histopathological presentations. They concluded that in such cases PCR could make a correct diagnosis between differential diagnoses. Some papers showed that CL may be similar to granuloma annulare, sarcoidosis, lupoid rosacea, and atypical mycobacteriosis, and they used PCR method for *Leishmania* DNA as a reliable diagnostic tool (2, 11-12). In recent years PCR have been used for infectious disorders including viruses, bacteria and protozoa in clinical specimens (10). Diagnosis of *Leishmania* species can be helpful for their treatment regimens and prevention manners.

L. major was the main cause of CL species in Varzaneh, Mehran, Gonbad-e-Qabus, Esfahan, and Qom (13-18), in agreement with our paper. In addition, another research suggested *L. major* is responsible for CL in Tehran, Shiraz, Khuzestan, and Kashan (19-22).

Some researchers have used PCR diagnostic test for paraffin-imbedded biopsies and reported kDNA in 53% of 20 culture-positive samples and 48% of clinically suspected samples for lupoid leishmaniasis (9, 23). Although some authors have used conventional methods with low sensitivity (24, 25), others have found PCR as a highly sensitive method (9). In contrast, Aviles *et al.* (26) found PCR as 100% specific for CL diagnosis. Although, Pirmez *et al.* reported PCR has the greatest impact in mucosal lesion diagnosis (27). Laskay *et al.* (10) used the same diagnostic PCR method as our work.

Our report showed that most patients were male. These findings are similar to those reported by other studies (28). This can be due to dry and warm weather in south and west of Khorasan province. The men who work in deserts and wear less are susceptible to contact with sand flies during the evening.

The age range 20-39 years was the most common spectrum in our patients afflicted with chronic granulomatosis dermatitis. There are a number of similarities with our work and other papers, in which some reported 21–30 years (29) and 10–19 years as the most affected age ranges (13). In contrast, some other papers reported patients under 20 years as the most common age range afflicted with cutaneous leishmaniosis (30, 31). Generally, it may be because of more exposure to sand flies in people more than 20 years.

Our report showed that most cases were appeared in spring season and a few were related to summer season, in contrast to other studies with high incidence in last summer and first autumn annually (32, 33). Occupational jobs as farmers may be the reason of the mentioned results. In addition, we concluded that solitary lesions are more common than multiple lesions the same as Namazi *et al.* study (13), in which this matter may belong to biting habits of sand flies. Regarding to CL control and

diagnosis, there is few data on health care system for its epidemiological condition. Besides, epidemiological surveys are helpful for control programming (34).

Our study limitations are as follow: 1) insufficient specimen in paraffin blocks 2) Probability of DNA destruction and breakage in older blocks. Further data collection is required to determine exactly molecular methods to get the practitioners more insights about diagnostic methods. Some long-lasting lesions also with few numbers of microorganisms are difficult to diagnosis specially when there are no organisms, so that it is necessary for practitioners to utilize additional diagnostic methods including PCR (11).

Therefore, we designed a clinincohistopatholgical study for specimens with the diagnosis of chronic granulomatous dermatitis with no definite etiology in light microscopic examination and special staining for finding *Leishmania* infection, via PCR method.

Conclusion

The current paper concluded PCR as a method for the diagnosis of chronic granulomatous dermatitis with no definite etiology in light microscopic examination and special staining for finding *Leishmania* infection.

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Conflict of interest

The authors declare no conflict of interest.

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