Original Article

A Comparison Study between Parasitological and Molecular Methods for Diagnosis of Acute and Chronic Anthroponotic Cutaneous Leishmaniasis

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Introduction: Anthroponotic cutaneous leishmaniasis (ACL) is still a major public health problem in the northeast and central parts of Iran. This study was designed to compare microscopy and cultivation methods with PCR amplification of kinetoplast DNA and ITS1 followed by RFLP analysis for diagnosis of acute and chronic ACL. **Methods:** In this study, 66 patients with ACL including 24 acute and 42 chronic forms were analyzed. Chronic forms (n=42) were divided into lupoid (n=18) and non-lupoid forms (n=24). The exudates from patient's lesions were examined by parasitological and molecular methods. **Results**: Out of 24 acute ACL cases, 24 (100%), 20 (83.3%), 24 (100%) and 23 (95.8%) were positive with direct examination, cultivation, kDNA-PCR, and ITS1-PCR-RFLP, respectively; while among 42 chronic forms, 29 (69%), 12 (28.5%), 27 (64.2%) and 16 (38%) were positive with the above mentioned methods. The most positivity rate was obtained with the direct examination for all clinical forms of ACL. In comparison with the direct examination as a gold standard, the kDNA-PCR showed the highest sensitivity of 100% and 64.2% in the diagnosis of acute and chronic forms, followed by the ITS1-PCR with lower sensitivity (95.8% and 38%) and then cultivation (83.3% and 28.5%). Also, all of the *Leishmania* isolates were identified as *Leishmania tropica* based on clinical symptoms and molecular methods. **Conclusion:** Our results recommend application of direct examination for the diagnosis of both acute and chronic forms of ACL. Moreover, the molecular method using kDNA-PCR was proposed for the diagnosis of ACL; while ITS1-PCR-RFLP can be utilized as a useful technique for the *Leishmania* species identification of CL. *J Med Microbiol Infec Dis, 2015, 3 (3-4): 52-56.*

Keywords: *Leishmania tropica,* Acute, Chronic, Iran.

INTRODUCTION

Cutaneous leishmaniasis (CL) is still a major public health problem in many parts of the world, especially the Eastern Mediterranean region. CL is more widely distributed, with about one-third of cases occurring in each of three epidemiological regions, the Americas, the Mediterranean basin, and western Asia from the Middle East to Central Asia [1]. The ten countries with the highest estimated case counts, *i.e.* Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru, together account for 70% to 75% of global estimated CL incidence [1, 2]. In Iran CL is mostly caused by *Leishmania tropica* and *Leishmania major* which are responsible for Anthroponotic cutaneous leishmaniasis (ACL) and zoonotic cutaneous leishmaniasis (ZCL), respectively [3-5]. Both of the mentioned forms of CLs can result in acute and chronic infections. The lupoid form is one of the Long-standing chronic forms of ACL, which was described in 1923 [6]. One to two years after the healing of acute form of ACL, new papules and nodules appear around the old scar [7]. The frequency rate of lupoid form has been reported 0.5 to 6.2 in CL endemic areas of Iran [8].This form is frequent in ACL endemic areas such as the northeast (Khorasan-Razavi province) and southeast (Kerman province) of Iran [6, 9]. Another chronic form including both lupoid and non-lupoid forms were unresponsive to treatment with at least two full courses of systemic meglumine antimoniate (Glucantime®) [10-12].

Correct detection and identification of parasites are important for prognosis and appropriate treatment strategies. The routine diagnostic methods are mainly based on clinical symptoms and observation of the *Leishman* bodies in stained smears or promastigotes in specific culture media.

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However, due to the low density of parasite in chronic lesions, the sensitivity of conventional laboratory methods is varied in detecting the parasite [13]. Molecular techniques are sensitive and specific for diagnosis and epidemiological studies [14, 15]. The north east of Iran is one of the main endemic areas for ACL [16] with a high frequency of chronic cases. Therefore, early diagnosis and treatment of leishmaniasis are important in these regions. In this study, we compared PCR methods for amplification of kinetoplast DNA (kDNA) and internal transcribed spacer 1 (ITS1) regions, with parasitological examinations for detection of *L. tropica* in chronic and acute ACL.

MATERIAL AND METHODS

Sampling method. Samples were taken from 66 patients referred to the Dermatology and Leishmaniasis Research Center, Imam Reza Hospital in Mashhad city (Khorasan-Razavi province), north-east of Iran during 2011-2012. In the present study, 66 patients including 24 acute and 42 chronic forms were analyzed who had been diagnosed previously based on clinical manifestation and microscopic exam. All the patients with less than six months duration of lesion considered as an acute form while cases with a duration of lesion > six months and unresponsive to treatment with at least two full courses of intramuscular administration of meglumine antimoniate (Glucantime®) were classified as chronic forms [17]. Chronic forms (n=42) of ACL were divided to lupoid forms (n=18) and non-lupoide forms (n=24). In lupoid forms, patients had exhibited new papules and nodules around the old scar one to two years after the healing of acute form of ACL. Also, non-lupoid forms were cases with treatment failure against at least two full course**s** of meglumine antimoniate (Glucantime®). After obtaining informed consent and completed questionnaires and physical examinations, exudate materials of the lesion were collected using an insulin syringe containing 0.5 ml sterile saline.

Microscopic examination. Microscopical slides were prepared from each patient and were stained with Giemsa, [18]. After staining, slides were examined under a light microscope with magnification at 1000X. Samples were transferred to the Leishmaniasis Laboratory, Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences.

Parasite culture. Approximately 0.05 ml of the exudates were inoculated into RPMI-1640 medium (Gibco, Life Technologies GmbH, Frankfurt, Germany) supplemented with 10% fetal bovine serum (Gibco, Germany), 100 U/ml penicillin and 100 ug/ml streptomycin (Gibco, Germany). All the cultivated samples were transferred to the Leishmaniasis Laboratory, Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences and incubated at 24-25°C. Since the number of parasites in the first culture was often scarce, the samples were subcultured to obtain adequate parasite for DNA extraction. Five days after the last sub-culture, parasites were harvested, washed in sterile PBS, pH: 7.2-7.4 and kept at -20°C [9].

DNA Extractions. DNA was extracted from 4-5 × 10^6 parasites/ml. DNA extractions were conducted using the High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) as recommended by the manufacturer.

PCR Amplification. ITS1-PCR-RFLP and kDNA-PCR were used for detection of parasites DNA in the lesion exudates obtained from patients and determination of the species of *Leishmania* in the cultivated parasites.

To detect and identify the species of parasites, we used PCR for amplification of both ITS1 and kDNA targets. ITS1-specific primers were as the followings: LITSR (5′-CTGGATCATTTTCCGATG) and L5.8S (5′-TGATACCACTTATCGCACTT) [19]. For RFLP analysis Amplification reactions were performed in 25 µl containing 12.5 µl of PCR buffer (Ampliqon, Denmark), 15 pMol of each primer, and 10 ng of genomic DNA. The PCR conditions were as follows: initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 49°C for 30 s, and extension at 72°C for 45 s with a final extension at 72°C for 10 min, in a thermocycler (Peqlab, Biotechnology, Gmbh, Germany). The PCR products were digested with *HaeIII* (Fermentas, Life Sciences, Germany) and the digested products were separated on 3% agarose gels in TAE buffer and visualized after staining with safe red stain [9].

The specific primers for kDNA were designed based on *L. tropica* kinetoplast DNA, non-protein coding region, partial sequence, isolate Iran JW trop, (Accession Number: AB678350) by AlleleID software (Ver.6). The primers sequences were as follows: (DRDF): 5-GCTGACTGTGTTAATATGC-3 as the forward primer and (DRDR): 5-GTTACCTATGATATACCTATGC-3 as reverse primer.

All of the PCR reaction mixtures consisted of 12.5 µl of PCR buffer (Ampliqon, Denmark), 15 pMol of each primer, and 10 ng of genomic DNA. After the initial denaturation (5 min at 94°C), 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 1 min at 72°C were carried out, and the PCR was terminated by final extension at 72°C for 10 min. The PCR products were analyzed on 2% agarose gel by electrophoresis. In each set of tests, a standard strain of *L. tropica* (MHOM/IR/02/Mash10) and negative control were included.

Statistical analysis. Data were analyzed by chi-square test using the SPSS-19 software. The sensitivity of the diagnostic methods was calculated considering direct microscopic examination as the standard method (True positive). The sensitivity of the cultivation, kDNA-PCR, and ITS1-PCR-RFLP methods were calculated with the formula: Sensitivity = TP/(TP+ FN)×100% where TP represents true positive, and FN represents false negative. The level of statistical significance was $P<0.05$.

RESULTS

Based on the results shown in table 1, from 66 ACL cases, 53 (80.3%) and 32 (48.5%) cases were positive in

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direct microscopy and culture tests, respectively. From 24 acute forms, 24 (100%) samples with direct microscopy and 20 (83.3%) with culture were detected to be positive. Whereas, direct diagnosis became positive in 12 (66.7%) chronic lupoid cases and 17 (70.8%) chronic non-lupoid forms, and culture was positive in 4 (22.2%) chronic lupoid and 8 (33.3%) in chronic non-lupoid cases.

Out of 66 ACL cases, ITS1-PCR was positive in 39 (59.1%) samples, including 23 (95.8%), 3 (16.7%) and 13 (54.2%) of acute, chronic lupoid and chronic non-lupoid, respectively. In PCR-RFLP the positive samples yielded an amplicon of 300-350 bp. Digestion of amplicons with *HAEIII* (BsuRI) produced two profiles, two bands of 220 bp and 140 bp indicative of *L. major*, and three bands of 200 bp, 60 bp and 50 bp indicative of *L. tropica* [9] (Figure 1). In the present study, all the species of *Leishmania* isolates identified as *L. tropica.*

The results of kDNA-PCR in different clinical forms showed that from 66 ACL cases, in 51 (77.2%) cases, *Leishmania* DNA samples were positive, including 24 (100%), 9 (50%) and 18 (75%) in acute, chronic lupoid and non-lupoid forms (Table 1). Also, in kDNA-PCR, the positive samples yielded an amplicon of 276 bp (Figure 2). As the kinetoplast primers were designed based on the database specific for *L. tropica*, no band was observed in other species such as *L. major* or *Leishmania infantum* (Data not shown).

Overall, higher sensitivities of 83.3% (95% CI: 64.1-93.3), 100% (95% CI: 100.0) and 95.8% (95% CI: 87.2-100.0) were determined in cultivation, kDNA-PCR, and ITS1-PCR-RFLP methods in acute cases of ACL compared to the direct diagnosis as a standard gold method. While the sensitivity rates of the mentioned methods compared to direct diagnosis in chronic ACL (lupoid and non-lupoid forms) were obtained 41.3% (95% CI: 25.5-59.2), 93.1% (95% CI: 86.6 -99.6) and 55.2 % (95% CI: 37.0 -73.2), respectively. In detail, the sensitivity rates of the methods for chronic lupoid forms were 33.3% (CI: 13.8-60.9), 75% (CI: 46.7-91.1) and 25% (CI: 8.8-53.2); and for chronic non-lupoid samples were obtained 47% (CI: 26.1-69), 100% (CI: 81.5-100) and 76.4% (CI: 52.7-90.4), respectively.

Table 1. Comparison of the diagnostic assays in acute and chronic anthroponotic cutaneous leishmaniasis

Clinical symptoms	Total No. Cases	Direct diagnosis Positive No. (%)	Culture Positive No. (%)	KDNA-PCR Positive No. (%)	ITS1-PCR Positive No. (%)
Acute	24	24 (100%)	20 (83.3%)	24 (100%)	23 (95.8%)
Chronic (Lupoid)	18	12 (66.7%)	4 (22.2%)	9 (50%)	3 (16.7%)
Chronic Non-Lupoid	24	17 (70.8%)	8 (33.3%)	18 (75%)	13 (54.2%)
Total	66	53 (80.3%)	32 (48.5%)	51 (77.2%)	39 (59.1%)

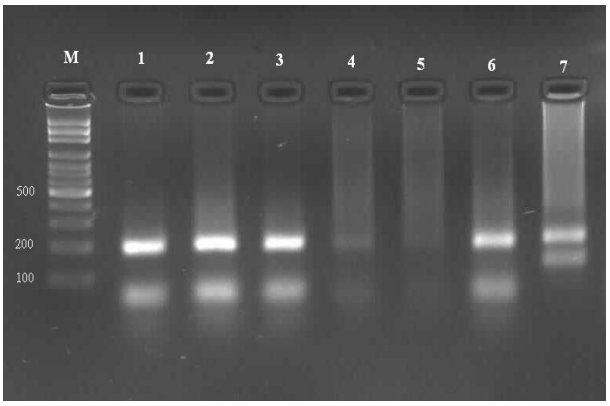

Fig. 1. Lanes 1-5, ITS1-PCR RFLP banding patterns were obtained from samples of patient; lane 6, *L. tropica* stock strain; lane 7, *L. major* stock strain; lane M, molecular size standard marker (100 bp)

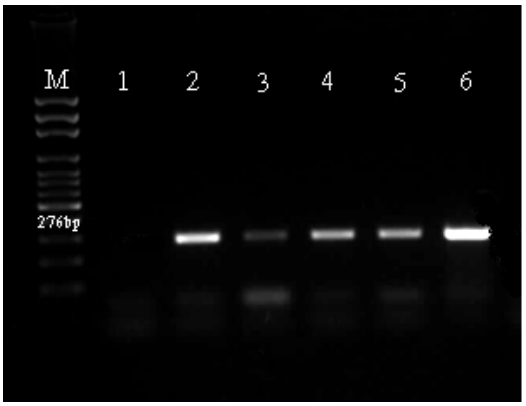

Fig. 2. kDNA-PCR patterns obtained from *Leishmania* stocks and patients samples. Lane M, 100 bp size marker (Fermentas); lane1, negative control; lane 2, *L. tropica* (stock strain); Lanes 3-6, *L. tropica*

DISCUSSION

Diagnosis of ACL using microscopic observation and cultivation are possible; however, the parasitological diagnosis in chronic cases is very difficult and time-consuming due to the low parasite load in lesions [20]. Besides, these methods can not determine the species of parasites because of the morphological similarity.

In recent decades, DNA-based methods are increasingly employed for the detection and identification of *Leishmania* species [20, 21]. Weigle *et al.* (2002) applied the PCR-based method for diagnosis of acute and chronic cutaneous leishmaniasis caused by *Leishmania* (*Viannia*). They concluded that PCR assay in the diagnosis of chronic lesions was more sensitive than conventional methods and should be considered as the preferred diagnostic method for chronic CL [22]. In this context, several studies reported that diagnosis of chronic CL like lupoid forms in the old world, caused by *L. major* and *L. tropica*, is also hampered by the scarcity of amastigotes in the lesion [23, 24].

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In this study, parasitological methods including microscopy and culture as well as PCR-based methods targeting ITS1 and minicircle kDNA were conducted to compare these approaches with microscopy as a standard method for diagnosis of ACL. All the samples were derived from ACL endemic region and identified as *L. tropica* using kDNA-PCR and ITS1-PCR-RFLP assays.

Our results showed that in acute forms, from 24 cases, direct diagnosis and kDNA-PCR we were positive in 100% of cases. Also, culture and ITS1-PCR was positive in 83.3% and 95.8% of cases. On the other hand, from 42 chronic cases (lupoid and non-lupoid) 69%, 28.6%, 64.3% and 38.1% were positive with the direct examination, culture, kDNA-PCR and ITS1-PCR-RFLP methods, respectively. Direct diagnosis and kDNA-PCR methods which have the same results (100%) were more useful than the ITS1-PCR procedure in the detection of acute ACL. In chronic ACL, the positive rates of direct diagnosis and kDNA-PCR were approximately twice more than ITS1-PCR-RFLP and culture regarding scarcity of amastigote forms of *Leishmania* parasites in the culture media.

Overall, sensitivities of 100%, 83.3%, 100% and 95.8% were determined in cultivation, kDNA-PCR and ITS1-PCR-RFLP methods for detection of acute ACL when compared to direct diagnosis; suggesting kDNA-PCR as the best method with the highest sensitivity. Similarly, in chronic ACL direct examination has the highest sensitivity of 69%; however, the sensitivity rates of cultivation and ITS1-PCR methods were 41.3% and 55.2 %, which are lower than that of acute ACL. Also, the sensitivity rates of direct examination, cultivation, kDNA-PCR and ITS1-PCR-RFLP methods for chronic lupoid forms were 66.7%, 22.2%, 50%, 16.7%, and for chronic non-lupoid samples were 70.3%, 33.3%, 75%, 54.2%, respectively; which was higher than those of lupoid forms. These results are in harmony with those of a previous study in which PCR was positive in 12 out of 20 chronic lupoid leishmaniasis cases (60% of cases); showing that a significant percentage of lupoid forms become negative even with a molecular method due to the low density of parasite in lupoid forms [25].

In general, analysis of two molecular methods revealed that PCR using kDNA was more useful than ITS1-PCR-RFLP for diagnosis of lupoid and non-lupoid chronic forms as the sensitivity of kDNA-PCR was 75% and 100% whereas for ITS1-PCR was 25% and 76.4%, respectively. Likewise, Nasereddin *et al.* (2008) reported that kDNA-PCR could identify 100% confirmed positive ACL samples and was more sensitive than ITS1-PCR (85%) [26]. Moreover, Bensoussanet *et al.* (2006) showed that the kinetoplast gene was more useful for diagnosis of ACL while PCR using ITS1 was more suitable when the rapid characterization of species is required [27].

It was demonstrated that the positive rates of microscopic and culture examinations for detection of acute forms were 100% and 83.3%, whereas in chronic forms was 69% and 28.5%, respectively. The low sensitivity of culture could be due to the low number of parasites in the lesion. Furthermore, the sensitivity of kDNA-PCR and ITS-PCR for detection of acute forms of ACL were 100% and 95.8%, while there were 64.2% and 38% for chronic forms, respectively. The reduced sensitivity of molecular methods for detection of chronic forms might result from the low parasite count in samples or technical problems. It seems that modification in samples and optimizing of molecular methods could enhance detection of such cases [28]. Our results showed that the positive rates of the microscopic examination and kDNA-PCR in the diagnosis of acute and chronic cases of ACL was more than cultivation and ITS1-PCR. Consistently, Ovalle Bracho *et al.* (2007) suggested PCR with kDNA as a molecular target for diagnosis and ITS ribosomal DNA for species identification [29]. KDNA in comparison with other genetic markers such as ITS1 appears to be a proper target for diagnosis of ACL especially in chronic forms [22, 30]. Although direct diagnosis method is considered as a “gold standard” method for identifying parasites in endemic areas, the molecular methods can be utilized as useful tools in the diagnosis and epidemiological issues.

Based on the results, we recommend the application of direct examination for the diagnosis of both acute and chronic forms of ACL. Furthermore, kDNA-PCR was suggested as an appropriate method for diagnosis of ACL in endemic areas and ITS1-PCR for rapid characterization of *Leishmania* species.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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