

# MOLECULAR EPIDEMIOLOGY OF CUTANEOUS LEISHMANIASIS IN SOUTHERN BELT OF KHYBER PAKHTUNKHWA, PAKISTAN

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## ABSTRACT

Cutaneous leishmaniasis is a vector-borne disease caused by the flagellate parasite *Leishmania tropica*. Diagnosis by PCR is becoming a 'gold standard' technique. This study was aimed to investigate the prevalence of Cutaneous Leishmaniasis and to determine the usefulness of kDNA-PCR for the diagnosis of *Leishmania tropica*. Exudates smears/ skin biopsy of 133 suspected individuals of Cutaneous Leishmaniasis were included in this study. All the samples were analysed with microscope and kDNA-PCR for detection of *Leishmania tropica*. The study population in the endemic regions suggested the highest sensitivity of 78.8% of CL in men than women 72.2% by kDNA-PCR. While the microscopy findings were 60.5% in males and 40.4% in females, followed by 94.1% confirmation by kDNA-PCR and 70.5% detection by microscopy in male's age group 21-30 years while 75.0% kDNA-PCR confirmation and 41.6% microscopy positivity in same age group of females. Face was found to be more infected 88.5% with lesion duration 4-6 months having sensitivity of 85.2% by kDNA-PCR as compared to 65.4% detection but sensitivity of 61.8% by microscopy. The highest epidemics 86.4% and 61.4% of *Leishmania tropica* were found in North Waziristan Agency by kDNA-PCR and microscopy respectively. For sampling methods, kDNA-PCR showed greater sensitivity 81.4% for dermal scraping than 69.9% Whatman sterile paper.

**Keywords:** kDNA-PCR, Cutaneous Leishmaniasis, *Leishmania Tropica*, Khyber Pakhtunkhwa

## INTRODUCTION

LEISHMANIASIS is endemic with 1.5–2 million new cases annually in 88 countries and threatens more than 350 million people at risk in the world [1]. At least 20 species and subspecies of *Leishmania* have been recorded as being infective to humans; many of them cause extensive morbidity and are responsible for a wide spectrum of clinical signs and symptoms in the world [2]. Correct diagnosis of the disease is very important for the selection of appropriate treatment and reduces its complications [3]. As Cutaneous Leishmaniasis (CL) is clinically similar to other skin diseases, various techniques have been introduced to diagnose its agent in affected lesions [4].

Diagnosis of CL due to its varied symptoms with different species is difficult. The classic laboratory diagnosis methods, such as examination of skin lesions by using smears, cultures and histopathological examinations, are highly specific but its sensitivity is low and variable [5] and in addition, they are time consuming, usually requiring experienced technicians and cannot identify the species of *Leishmania* parasite [6]. Microscopic examination of skin scrapings, though rapid and low-cost has limited sensitivity, particularly in chronic lesions [7]. Leishmanin Skin Test, (LST) is another diagnostic technique but is of less value for anthroponotic CL than the zoonotic form [8]. Molecular techniques, such as the Polymerase Chain Reaction (PCR), offer an alternative approach to the demonstration of parasites [9] and for detection of low amount of *Leishmania* DNA in clinically obtained samples [10], [11], [12], such as skin biopsy, blood, bone marrow puncture, or samples prepared from Giemsa-stained slides even in paraffin block [13]. The PCR is a sensitive and rapid technique, which can be adapted for use under conditions of limited resources by applying a low cost approach [14].

The need for more sensitive methods has prompted the development of DNA-based diagnostic techniques. The kinetoplast, an organelle unique to the kinetoplastids, contains approximately 10,000 small circular DNAs, known as kinetoplast DNA (kDNA) minicircles, which are between 600 and 800 bp in members of the genus *Leishmania* [15]. The abundance and other characteristics of these molecules have made them the target for a number of PCR-based techniques [15]. The minicircle of kinetoplast DNA (kDNA) and ribosomal DNA are ideal targets for amplification because they are present in multiple copies and have both conserved and variable regions [16], [17]. In this study, we compared the sensitivity and specificity of kDNA PCR assay used for parasite identification with microscopic detection in order to validate PCR techniques for molecular diagnosis of CL.

## 1. METHODS

**1.1. Samples Collection:** Samples were randomly collected by using aseptic precautions from 133 suspected CL patients of both sexes having variable age groups and examined by microscopy and PCR in Molecular Parasitology and Virology Research Laboratory, Department of Zoology, Kohat University of Science and Technology, Kohat. Different endemic districts like Kohat, Karrak, Bannu and North Waziristan Agency of Khyber Pakhtunkhwa were visited.

**2.2. Smears Preparation:** The patients' lesions and adjacent normal looking skin around infected areas were cleaned with disinfectant. Samples were then taken for smear preparation according to the method of Bensoussan, *et al.*, 2006 [5]. The stained smears were examined under the Binocular microscope (Olympus CX31, Japan) with a 40x lens and with a 100x oil immersion lens. If at least one intra- or extra-cellular amastigotes with a distinctive kinetoplast was found, the smear was declared as positive. When no amastigotes were seen after 15 minutes the smear was declared as a negative.

**DNA Extraction and PCR amplification:** DNA was extracted both from skin scrapings and Whatman filter paper with the help of GF1-Kit (Vivantis Germany) according to the manufacturer procedure. PCR amplification was performed with 4 $\mu$ L of cDNA by outer sense Primer 13A (5/-GTGGGGAGGGGCGTTCT-3/) and 13B (5/-ATTTTACACCAACCCCCAGTT-3/) were used to amplify the 120-bp fragment of minicircle kinetoplast DNA that is specific to all *Leishmania* species [19]. While forward primer JW11 (5/-CCTATTTTACACCAACCCCCAGT-3/) and reverse primer JW12 (5/-GGGTAGGGGCGTTCTGCGAA-3/) were used to amplify 116bp of the conserved region of kDNA minicircle of specific *L. tropica* by PCR [20]. The reaction mixture for a single reaction consisted of 10X PCR Buffer 2.0 $\mu$ L, MgCl<sub>2</sub> (25mM) of 2.4 $\mu$ L, dNTPs (100 $\mu$ M) 1.0 $\mu$ L, outer Sense Primer 1.0 $\mu$ L, outer Antisense Primer 1.0 $\mu$ L, dH<sub>2</sub>O up to 20.0 $\mu$ L, *Taq.* DNA polymerase (5U/ $\mu$ L) of 0.4 $\mu$ L and extracted DNA 4.0 $\mu$ L. Reaction buffer without Leishmanial DNA was also included as a negative control. The reaction was carried out in Thermal Cycler (Nyxtech USA). The PCR products were then electrophoresed in 2% agarose gel under Ultra Violet (UV) light.

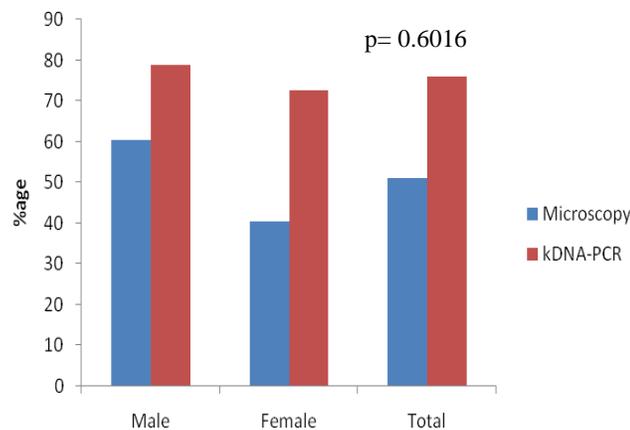
**1.2. Statistical Analysis:** The obtained data after experimental work was explored by using Statistics 9 Soft ware. The Chi Square t test showed no significant difference ( $P > 0.05$ ) between the two diagnostic tools.

## 2. RESULTS

A total of 133 patients, 53.4% (n= 71) men and 46.6% (n= 62) women of age 1-50 years expected to CL infection were randomly selected and studied by two diagnostic methods. Results of each assay were compared for sensitivity and specificity according to the patients clinical features based on gender age/Sex, duration of lesion, site and number of lesion and geographical distribution. The positive *Leishmania* samples were determined by identifying a cDNA bands 116 bp comparing with the 100-bp DNA ladder (Gibco BRL) used as DNA size marker (Figure 1).

Out of 133 specimens 75.9% (n= 101) and 51.2% (n=68) were found CL positive while 24.1% (n=32) and 48.87 % (n=65) were found CL false negative by kDNA-PCR and microscopy respectively. However 32 more samples were confirmed positive by kDNA-PCR than microscopy showing greater reliability and sensitivity of 24.81%. Males were found to be more infected 78.8% (n= 56) than 72.5% (n= 45) females by kDNA-PCR followed by low microscopic detection 60.5% (n= 43) in males than females 40.4% (n=25) (Figure 2).

**Figure 2**  
**Overall Gender Wise Prevalence of *Leishmania tropica* by Microscopy and kDNA-PCR**



Both genders age groups were variably infected by *Leishmania tropica* being highest 94.1% CL infection detected by kDNA-PCR and 70.5% detection by microscopy in male's age group 21-30 years as compared to 75.0% detection by kDNA-PCR and 41.6% microscopy positivity in same females age group. The duration of lesion (average, 7-9 months; mean, 8 months; gap period, 1-3 months history of onset of lesion at the time of diagnosis) being minimum duration was below one month and maximum above 12 months showed that kDNA-PCR has high detection rate 85.2% (n =29/34) for 4-6 months old lesion than 61.8% (n= 21/34) microscopy (Table 1).

**Table 1**  
**Duration of Lesion Wise comparison of Microscopy vs PCR for Detection of *Leishmania tropica***

Lesion Duration (Months)	Samples	Positive Results Analysis		Chi Square test
		Microscopy n (%)	kDNA-PCR n (%)	
<1	12	07 (58.4)	09 (75.0)	P = 0.9999
1-3	25	15 (60.0)	21 (84.0)	
4-6	34	21 (61.8)	29 (85.2)	
7-9	27	13 (48.2)	20 (74.1)	
10-12	21	11(52.4)	14 (66.7)	
>12	14	05 (35.8)	08 (57.1)	
<b>Overall</b>	<b>133</b>	<b>68 (51.2)</b>	<b>101 (75.9)</b>	

In this study a brief survey for CL was also carried out in different Southern districts like Kohat, Karrak, Bannu and North Waziristan Agency of Khyber Pakhtunkhwa. North Waziristan Agency was found to be more epidemics 86.4% (n=38/44) for CL by kDNA-PCR and 61.4% (n= 27/44) by microscopy (Table 2). The difference of undetermined 33 cases (24.9%) between PCR and microscopy revealed the highest diagnostic sensitivity of kDNA-PCR as compared microscopy, while those patients false negative detected by microscopy and improper medication also contributed in the epidemics of CL. It was also observed that kDNA-PCR sensitivity for dermal scraping was 81.4% (n= 57/70) and 69.9% (n= 44/63) for Whatman filter paper for whom the one-tailed p value equivalent to 0.2039 showed the association between rows and columns outcomes and was considered to be statistically non significant.

**Table 2**  
**District Wise Comparison of Microscopy and PCR for Epidemiology of *Leishmania tropia***

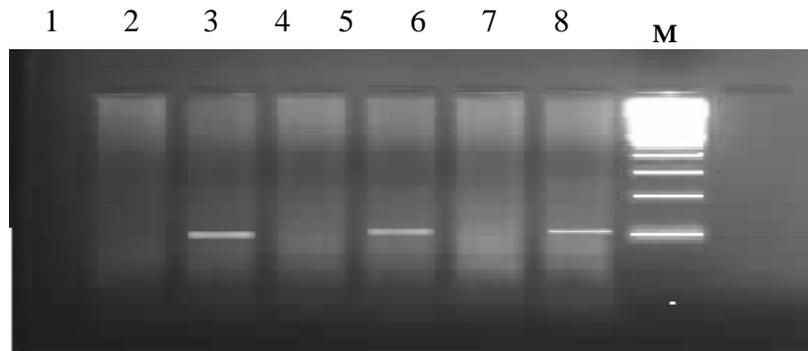
Epidemics Districts	Samples	Positive Results Analysis		Chi Square test
		Microscopy n (%)	kDNA-PCR n (%)	
Kohat	18	7 (38.9)	13 (72.3)	
Karrak	21	9 (42.9)	14 (66.7)	
Hangu	23	10 (43.5)	17 (73.9)	
Bannu	27	15 (55.6)	21 (77.8)	
North Waziristan Agency	44	27 (61.4)	38(86.4)	
<b>Overall</b>	<b>133</b>	<b>68 (51.2)</b>	<b>101 (75.9)</b>	

There were 184 (average; 1-6; mean, 10) CL lesions observed on different body sites among those face was found to be more infected and the detection rates were 88.5% (n= 23/26) and 65.4% (n= 17/23) confirmed by kDNA-PCR and microscopy respectively (Table 3).

**Table 3**  
**Comparison of Microscopy and PCR for Detection of *Lishmania tropica* on the Basis of Lesion Sites**

Sites of Lesion (On various Body Parts)	Samples	Positive Results Analysis		Chi Square test
		Microscopy n (%)	kDNA-PCR n (%)	
Forehead (23)	14	08 (57.2)	10 (71.5)	
Cheeks (Face) (35)	26	17 (65.4)	23 (88.5)	
Nose tip (11)	13	04 (30.8)	10 (76.9)	
Ear (18)	17	05 (29.5)	13 (76.5)	
Lips (12 )	9	04 (44.4)	07 (77.8)	
Hands (37)	19	07 (36.8)	15 (79.0)	
Forearms (21)	11	04 (36.3)	08 (72.7)	
Legs (17)	15	06 (40.0)	12 (80.0)	
Abdomen (10)	9	03 (33.3)	05 (55.5)	
<b>Overall (184)</b>	<b>133</b>	<b>68(51.2)</b>	<b>101(75.9)</b>	

**Figure 1**  
**Amplified kDNA bands (116 bp) of *L. tropica* by PCR**



Lane 1, 2, 4 and 5: Negative samples (No DNA amplified)  
Lane 3:6 Positive samples (116bp DNA band)  
Lane 8: Positive Control (Cocktail of microscopic positive samples)  
Lane M: 100bp DNA Ladder Marker  
Lane 7: Negative Control

### 3. DISCUSSION

*Leishmaniasis* is one of the highest incident infectious parasitic diseases in the world [20]. The diagnostic methods available at present for *Leishmaniasis* are based on clinical and epidemiologic features, parasitologic detection (stained smears, culture and histopathology) and immunological methods [15]. These traditional laboratory methods have several limitations; prominent among them is low sensitivity, specificity and variability regarding the species involved in the disease pathogenesis [21], [22], [23]. The use of PCRs has slowly become the preferred way for diagnosing leishmaniasis since conventional parasitological methods are not sufficiently sensitive [4].

PCR can detect the *Leishmania* DNA parasites in a variety of clinical samples (e.g., skin biopsy, ulcer material, blood, bone marrow and lymph node aspirates). Some studies showed that PCR methods had positive results in more than 90% of CL cases [17], [23] [24], while several other authors have reported 100% specificity with increasing overall sensitivity of 92% and 98% [4], [8], [25]. Other groups have reported sensitivities of 85-92% of diagnosing of CL using PCR based on kDNA [26] [27], [28]. While the sensitivity of microscopic techniques, i.e., histopathology and tissue smears, touch preparations and exudates has been reported to range from 17 to 83% for CL [26], [29], [30], [31]. It was reported by others that positive detection rates of CL were 84.6% - 96.5% by kDNA- PCR and 38.5% - 58.6% by direct visualization of smear [22], [32]. Our study show quite similarity in respect of limitation and sensitivity of microscopy 51.2% and above 80% sensitivity, authenticity and specificity by kDNA-PCR.

Keeping above in view a survey was conducted in Southern districts of Khyber Pakhtunkhwa to identify CL by microscopy and kDNA-PCR. The highest infection of CL 61.4% was found in North Waziristan Agency and lowest 38.9% was found in Kohat by microscopic while by kDNA-PCR the highest 86.4% and lowest 66.7% infections were found respectively North Waziristan Agency and Karrak. Another study carried out by WHO in Kurram Agency NWFP, Pakistan, 738 cases were found CL positive mostly in local population and in addition to, 1500 cases in Afghan refugee camps [33]. Similarly 15/ 20 samples were found CL positive in the Timaragara Afghan refugee camp in Dir NWFP, Pakistan during a prevalence survey by Nested PCR based method [34]. In Israel, the West Bank, other Middle Eastern Region and South America smear microscopy CL findings were 63% (58/92) showing 74.4% sensitivity while kDNA-PCR confirmed CL positive cases were 90.2% (83/92) showing 98.7% sensitivity [5]. In Rajasthan state India the sensitivity of kDNA-PCR was 96.6% while direct smear positivity was 65.5% for CL [21]. Our findings show quite similarit to these studies, but CL was mostly prevalent in North Wazirstan Agency (NWA). It might be due to like Kurram Agency and District Timaraga, NWA is near to Afghanistan boarder and more Afghan refugees influxed frequently to this area after America attack on Afghanistan.

It was also found out in this study the microscopy detected CL infection was 60.5% (n= 43/71) in male and 40.4% (n= 25/62) in females collectively being 51.2% (n= 68/133), while by kDNA-PCR CL in male was 77.5% (n= 55/71) and in females 72.5% (n= 45/62) collectively was 75.2% (n= 101/133). Other studies showed that CL affects both sexes at all ages to varying degrees [35], [36] and the disease is evenly distributed amongst both genders [33]. In other study incidence ratio of CL in male to female was found to be 72:28 on the basis of microscopy [37] and the infection is more prevalent in men 56.7% - 62.24% than in women 37.75% - 43.3% [23], [38]. Our findings are quite in comparison to the mentioned studies. In males we found out more infection of CL by Microscopy and kDNA-PCR than in females. It was because that females are mostly present at homes and traditionally they are well covered when visit outside the doors followed by microscopy and kDNA-PCR highest infection rates of CL 58.62 % (n= 17/29 cases) and 82.8 % (n= 24/29 cases) respectively by microscopy in the male age group of 21-30 years than females.

The duration of lesion length was between 1-12 months (average 7, mean 6) with modification in to each 3 months period of time. The highest 29.6% (n= 10/34) CL infection was found in 7-9 months, while lowest 21.4% (n= 3/14) CL positivity found when lesion duration was above 12 months by microscopic examination. Similarly highest 81.4% (n= 22/27) and lowest 66.7% (n= 8/14) infection rates were found in the periods of 7-9 and 10-12 months respectively by kDNA-PCR. In other studies CL was confirmed 61.5% positive in 1-6 month interval of lesion between onset of the lesion and the time when CL was diagnosed and only few cases were diagnosed in less than one month lesion duration since the time of onset [36]. Our results are quite similar to these studies on the basis of smear positivity by microscopy but more authentic and reliable for each group by kDNA-PCR.

On different body sites of 133 suspected individuals of CL a total of 184 (average range, 1-6) lesions were observed. Most of the patients had a single lesion; some have 2 or 3 lesions, while multiple lesions were found on cheeks and hands. Highest infection of CL 65.4% (n= 17/26) was found in samples collected from cheek with total of 39 lesions and lowest 33.3% (n= 3/9) was found in samples collected from abdomen with total of 10 lesions by microscopy. Same samples when analyzed by kDNA-PCR the infection rates were 88.8% (23/26) and 55.5% (5/9) for cheek and abdomen respectively. In other studies CL lesions on skin ranging from single to multiple ulcers and ranged between 1-8 per person [39]; [36]. In a study majority 65% (105) patients had a single lesion and 35% (56) patients had 2 lesions being most 78% lesions on face and 26% on upper limb [36]. Some others reported that face was found to be more involved 25% - 41% in CL as compared with limbs 30% -37%, Legs 11.5% - 22% and other sites [37], [38], while CL lesions also showed a marked variation of 0%- 21.9% [39]. We have also found out similar findings related to these studies but variability in the number of lesions was due to the mode and severity of infection caused by *L. tropica*.

In this study we also analyzed the results of DNA extraction for kDNA-PCR from dermal scraping and exudate on sterile Whatman filter paper. The sensitivity of kDNA-PCR for dermal scraping was 81.4% while for Whatman paper was 69.9%. In other studies the detection rate of leishmanial DNA by PCR in dermal scrapings proved to be higher than in blood smears [40], [41]. It is known that skin biopsies have a higher detection rate than dermal scrapings if examined microscopically [26], but with PCR the sensitivity and specificity was high enough using dermal scrapings [7]. In other study PCR revealed 100% sensitivity by using Whatman sterile paper than microscopy using direct smear [8]. Our findings are similar to the above mentioned studies but we have shown that kDNA-PCR of dermal scraping is more sensitive than direct microscopic examination. It was also observed statistically by Chi Square test that there is no significant difference ( $P > 0.05$ ) between the two samples procedure using either dermal scraping or exudate on sterile Whatman paper for DNA extraction and analysis by kDNA-PCR. But for the long term preservation, filter paper proved to be especially suitable for field studies due to very good conservation of the DNA, without cooling, with little storage space and low expenses.

**Conclusion:** PCR has increased the speed and sensitivity of *leishmania* diagnosis compared to the microscopy. However, PCR-based protocols urgently need standardization and optimization and efforts should be made to make PCR platforms more user-friendly and cost-effective in the remote areas of southern belt of Khyber Pakhtunkhwa.

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## REFERENCES

- [1] Desjeux P: Leishmaniasis: current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* 2004, 27: 305.
- [2] Pearson RD, Sousa AQ: Clinical spectrum of leishmaniasis. *Clin. Infect. Dis.* 1996, 22: 1–13.
- [3] Alvar J, Croft S, Olliaro P: Chemotherapy in the treatment and control of leishmaniasis. *Adv Parasitol* 2006, 61:223–274).
- [4] Vega-Lonez F: Diagnosis of cutaneous leishmaniasis. *Curr Opin Infect Dis.* 2003; 16(2):97-101.
- [5] Bensoussan E, Nasereddin A, Jonas F, Schnur FL, Jaffe LC: Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. *J Clin Microbiol* 2006, 44:1435–1439.
- [6] Profeta MZ, Rotondo da-Silva A, Oliveira FS, Caligiorne BR, Oliveira E, Rabello A: Lesion aspirate culture for the diagnosis and isolation of *Leishmania* spp. from patients with cutaneous leishmaniasis. *Mem Inst Oswaldo Cruz Rio de Janeiro* 2009, 104:62–66.
- [7] Belli A, Rodriguez B, Aviles H, Harris A: Simplified Polymerase Chain Reaction detection of New World *Leishmania* in clinical specimens of cutaneous leishmaniasis. *Am. J. Trop. Med. Hyg* 1998, 58(1): 102–109.
- [8] Fata A, Dalimi-asl A, Jafari MR, Moha-jery M, Khamesipour A, Valizadeh M: Clinical appearance, Leishmanin Skin Test and ELISA using Monoclonal Antibody in diagnosis of different forms of cutaneous leishmaniasis. *Med J Mashhad Univ Med Sc.* 2004, 47(1):19-27.
- [9] White T, Madej R, Persing D: The Polymerase Chain Reaction: Clinical applications. *Adv Clin Chem* 1992, 29: 161–196.
- [10] Schallig HD, Oskam L: Molecular biological applications in the diagnosis and control of leishmaniasis and parasite identification. *Trop Med Int Health* 2002, 7:641– 651.
- [11] Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I: Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. *Journal of Clinical Microbiology* 2003, 41: 3147–3153.
- [12] Brustoloni YM, Lima RB, da Cunha RV, Dorval ME, Oshiro ET, de Oliveira AL, Pirmez C: Sensitivity and specificity of Polymerase Chain Reaction in Giemsa-stained slides for diagnosis of Visceral Leishmaniasis in children. *Mem Inst Oswaldo Cruz* 2007, 102: 497-500.
- [13] Blum J, Desjeux P, Schwartz E, Beck B, Hatz C: Treatment of Cutaneous Leishmaniasis among travelers. *J. Antimicrob. Chemother* 2004, 53: 158–166.
- [14] Harris E, Belli A, Agabian N: Appropriate transfer of molecular technology to Latin America for public health and biomedical sciences. *Biochem. Educ* 1996, 24:3–12.
- [15] Rodrigues EHG, De-Brito MEF, Mendonc MG, Roberto P, Werkhauser, Coutinho EM, Souza WV, Albuquerque, MFPM, Jardim ML, Frederico GCA: Evaluation of PCR for Diagnosis of American Cutaneous Leishmaniasis in an Area of Endemicity in North-Eastern Brazil. *J Clin Microb* 2002, 40(10): 3572–3576.
- [16] De-Bruijn MHL, Labrada LA, Smyth AJ, Santrich C, Barker DC: A comparative study of diagnosis by the Polymerase Chain Reaction and by current clinical methods using biopsies from Colombian patients with suspected leishmaniasis. *Trop. Med. Parasitol* 1993, 44:201–207.

- [17] Richard R, Jean-Claude D: Molecular diagnosis of leishmaniasis: Current status and future applications. *J Clin Microbiol* 2006, 45:21–25.
- [18] Rogers WO, Wirth DF: Kinetoplast DNA minicircles: regions of extensive sequence divergence. *Proc. Natl. Acad. Sci* 1987, 84: 565–569.
- [19] Nicolas, L.; Milon, G.; Prina, E.; Rapid differentiation of Old World *Leishmania* species by Light Cycler Polymerase Chain Reaction and melting curve analysis. *J. Microbiol. Methods* 2002, 51: 295–299.
- [20] Venazzi EAS, Roberto ACBS, Barbosa-Tessmann IP, Zanzarini PD, Lonardonni MVC, Silveira TGV: PCR with lesion scrapping for the diagnosis of human American Tegumentary Leishmaniasis. *Mem Inst Oswaldo Cruz, Rio de Janeiro* 2006, 101(4): 427-430.
- [21] Wilson SM: DNA-based methods in the detection of *Leishmania* parasites: field applications and practicalities. *Ann. Trop. Med. Parasitol* 1995, 89 (1): 95-100.
- [22] Aviles H, Belli A, Armijos R, Monroy FP, Harris E: PCR detection and identification of *Leishmania* parasites in clinical specimens in Ecuador: a comparison with classical diagnostic methods. *J Parasitol* 1999, 85:181-7.
- [23] Kumar R, Bumb R, Ansari AN, Mehta R, Salotra P: Cutaneous leishmaniasis caused by *Leishmania tropica* in Bikaner, India: Parasite identification and characterization using molecular and immunological tools. *Am J Trop Med. Hyg* 2007, 76: 896–901.
- [24] Mahmoodi MR, Mohajery M, Tavakkol AJ, Shakeri MT, Yazdan-panah MJ, Berenji F, Fata A: Molecular identification of *Leishmania* species causing cutaneous leishmaniasis in Mashhad, Iran. *Jundishapur J Microbiol.* 2010, 3(4): 195-200.
- [25] Reithinger R, Dujardin JC: Molecular Diagnosis of Leishmaniasis: Current Status and Future Applications. *J Clin Microbiol* 2007, 45(1): 21–25.
- [26] Andresen K, Gaafar A, El-Hassan AM, Ismail A, Dafalla M, Theander TG, Kharazmi A: Evaluation of the polymerase chain reaction in the diagnosis of cutaneous leishmaniasis due to *Leishmania major*: A comparison with direct microscopy of smears and sections from lesions. *Trans R Soc Trop Med Hyg* 1996, 90: 133–135.
- [27] Matsumoto T, Hashiguchi Y, Gomez EA, Calvopina MH, Nonka S, Saya H, Mimori T: Comparison of PCR results using scrape/exudates, syringe-sucked fluid and biopsy samples for diagnosis of cutaneous leishmaniasis in Ecuador. *Trans R Soc Trop Med. Hyg* 1999, 93: 606–607.
- [28] Safaei A, Motazedian MH, Vasei M: Polymerase Chain Reaction for diagnosis of Cutaneous Leishmaniasis in histologically positive, suspicious and negative skin biopsies. *Dermatology* 2002, 205:18–24.
- [29] Medeiros, A. C.; Rodrigues, S. S.; Roselino, A. M. Comparison of the specificity of PCR and the histopathological detection of *Leishmania* for the diagnosis of American cutaneous leishmaniasis. *Braz. J. Med. Biol. Res* 2002, 35: 421–424.
- [30] Faber WRL, Oskam T, Van-Gool NC, Kroon KJ, Knecht-Junk H, Hofwegen AC, Van der Wal, Kager PA: Value of diagnostic techniques for cutaneous leishmaniasis. *J. Am. Acad. Dermatol.* 2003, 49:70–74.
- [31] Disch J, Pedras MJ, Orsini M, Pirmez C, De Oliveira MC, Castro M, Rabello A: *Leishmania (Viannia)* subgenus kDNA amplification for the diagnosis of mucosal leishmaniasis. *Diagn. Microbiol. Infect. Dis.* 2005, 51:185.
- [32] Atik E, Kuk S, Inandi TJ: Diagnostic approach and significance of inducible nitric oxide positivity in human cutaneous leishmaniasis caused by *leishmania tropica*. *International Journal of Dermatology* 2007, 46:273–277.

- [33] World Health Organization. Afghanistan Crisis-Special Report. Leishmaniasis in Pakistan. January, 2001.
- [34] Noyes HA, Reyburn H, Bailey W, Smith DA: Nested-PCR-based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan. *J.Clin. Microbiol* 1998, 36(10): 2877-2881.
- [35] Uzun, S, Uslular, C, Yücel, A, Acar MA, Özpoyraz M, Memis-og̃lu HR: Cutaneous Leishmaniasis: Evaluation of 3,074 cases in the Çukurova region of Turkey. *Br. J. Dermatol* 1999, 140: 347–350.
- [36] Sharma NL, Mahajan VK, Kanga A, Sood A, Katoch VM, Mauricio I, Singh C, Parwan U, Sharma V, Sharma R: Localized Cutaneous Leishmaniasis due to *Leishmania donovani* and *Leishmania tropica*: preliminary findings of the study of 161 new cases from a new endemic focus in Himachal Pradesh, India. *Am. J. Trop. Med. Hyg* 2005, 72(6): 819–824.
- [37] Khan J: Present situation of Cutaneous Leishmaniasis in Balochistan, Pakistan. *Pak. J. Biol. Sci* 2004, 7(5): 698-702.
- [38] Hajjaran H, Vasigheh F, Mamishi S, Mohebbali M, Charedar S, Rezaei S: Direct Diagnosis of Leishmania Species on Serosity Materials Punctured From Cutaneous Leishmaniasis Patients Using PCR-RFLP. *Journal of Clinical Laboratory Analysis* 2011, 25:20–24.
- [39] Brooker S, Nasir M, Adil K, Agha S, Reithinger R, Kolaczinski J: 2004. Leishmaniasis in refugees and local Pakistani populations. *Emerging Infect Dis.*, 10: 1681–1684
- [40] Belli A, García D, Palacios X, Rodriguez B, Valle S, Videa E, Tinoco E, Marin F, Harris E. Widespread atypical Cutaneous Leishmaniasis caused by *Leishmania chagasi* in Nicaragua. *Am. J. Trop. Med. Hyg* 1999, 61(3): 380-385.
- [41] Rodriguez N, Guzman B, Rodas A, Takiff H, Bloom BR, Convit J: Diagnosis of Cutaneous Leishmaniasis and species discrimination of parasites by PCR and hybridization. *J. Clin. Microbiol* 1994, 32(9): 2246-2252.