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An improved microculture method for diagnosis of cutaneous leishmaniasis

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Abstract This study evaluated the performance of three diagnostic methods for cutaneous leishmaniasis (CL). Patients who came to the Health Center Laboratory of Gonbad-e-Qabus in Golestan Province, Iran, were enrolled in the study. Skin scraping smear, improved microculture (IMC) and polymerase chain reaction (PCR) were performed. A total of 303 subjects were recruited, among whom 273 subjects fulfilled the criteria for CL. Sensitivity and specificity were 88.8 % (95 % CI = 84.2–92.2 %) and 100.0 % for smears, 98.4 % (95 % CI = 96.1–99.1 %) and 100.0 % for IMC, both of them 100.0 % for PCR. Although, PCR was relatively more sensitive than the IMC, the high correlation (agreement = 96 %, Kappa = 0.82) between IMC and PCR along with the advantages of simplicity, rapidity, adequate sensitivity and as a needle free method, offers the IMC as a valuable alternative diagnostic method for PCR in diagnosis of CL.

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Introduction

Leishmania is the vector-borne protozoan parasite that infects animals and humans and causes different forms of diseases such as: visceral, cutaneous and muco-cutaneous leishmaniasis. Cutaneous leishmaniasis (CL) is a major public health problem although its mildest clinical form can cause considerable morbidity and after healing it often leaves disfiguring scares. CL is a widespread infectious disease that is commonly caused by *Leishmania tropica*, *Leishmania major* and *Leishmania aethiopica* in the Old-World (Reithinger et al. 2007). The disease typically presents as ulcerated or crusted nodules and plaques. However, it may present atypical clinical presentations. (Karamian et al. 2008) Golestan Province, Northern Iran, is a hyperendemic region of CL. In this area, common causative agents of CL had been recognized as *L. major* (Rassi et al. 2008; Mirzaei et al. 2011; Pagheh et al. 2012).

CL various and complex manifestations depends on host immunity, in addition, CL resemblance to other dermal disorders such as leprosy, cutaneous mycoses and skin cancers, makes distinguishing and diagnosis of CL a troublesome issue (Reithinger et al. 2007). However, definitive diagnosis of CL remains challenging, particularly in areas where the disease is highly endemic. A timely and definitive diagnosis of CL is important for initiating appropriate clinical management and treatment of this disease.

Rapid, simple and easy to use microscopic examination, commonly applied to CL diagnosis which is based on the demonstration of *Leishmania* amastigote in tissue biopsies or smears. But several studies demonstrated that microscopic

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examination usually has low sensitivity and cannot identify even some confirmed CL cases. In contrast, in visceral leishmaniasis (VL), serological tests are infrequently used for CL diagnosis (Bensoussan et al. 2006; Shahbazi et al. 2008). Molecular techniques are particularly valuable in low parasite density, asymptomatic or misdiagnosed lesions have more than 95 % sensitivity and specificity. Although, different molecular assays have consecutively been evaluated for diagnosis and also identification of *Leishmania* species, these methods are limited since they are costly, involve a great deal of time and require advanced equipments and expert personnel (Motazedian et al. 2008; Bensoussan et al. 2006; Shahbazi et al. 2008).

Parasites cultivation has many advantages, for instance it allows genotyping, species identification and testing for drug resistance (Sharquie et al. 2002). In Adana, Turkey, Allahverdiyev et al. (2004) has developed a microcapillary culture method (MCM) for the diagnosis of CL. The MCM is rapid (4–7 days), simple and cost-effective compared to traditional culture (TC), and is more sensitive than smears and TC for the diagnosis of CL. However, the recent to be introduced MCM has some disadvantages such as invasiveness and inadequate satisfactory regarding sampling method. For the reason that the sample is obtained using a 26-gauge needle and syringe containing 0.1–0.2 ml of sterile saline which its inserted intradermally into the outer border of the lesion.

Subsequently, the aspirated materials were inoculated into microhematocrit capillary tubes (Allahverdiyev et al. 2004). To date, in all performed investigations for MCM evaluation, the samples have been obtained using the aspiration puncture by the syringe. In addition to, these studies had showed varied performance by sensitivity and saving incubation time (Boggild et al. 2007, 2008, 2010). Therefore, further evaluation and also modification for MCM usefulness in the diagnosis of CL in other endemic countries is needed. Hence, for the first time we have developed and evaluated an improved microculture (IMC) method without using needle and syringe, as a non-invasive method, and compared it with PCR and smear for diagnosis of CL in an endemic focus.

Materials and methods

Patients

A total of 303 patients clinically suspected of CL, living in the endemic regions of Golestan Province, northeast of Iran enrolled in our study. They were referred to Health Center Laboratory of Gonbad-e-Qabus in Golestan Province during October 2010 to March 2011. Samples were obtained through skin scrapping, under sterile conditions, and used for smear scraping (Giemsa staining), PCR and microculture (IMC) methods. The study was approved by the Ethics Committee of Mazandaran University of Medical Sciences.

Microculture preparation

First skin lesions were cleaned and disinfected with alcohol 70 %. After scraping the lesion with a sterile lancet, the fluid materials (serosity of the lesions) were inoculated into capillary tubes for incubation under the following conditions. Under sterile conditions, microculture sampling was performed directly by bringing the tip of sterile non-heparinized 1 × 75 mm glass capillary tube close to outer border of the lesion and then the serosity was inserted into the tube. Afterward, fluid materials were loaded with about 50–70 µl of RPMI 1,640 medium with L-glutamine (Sigma, St. Louis, MO), buffered with 2 mM NaHCO3, supplemented with 10 % fetal bovine serum (FBS) (heat inactivated at 56 °C for 30 min), and gentamicin (80 µg/ml). Subsequently, the ends of the capillary tubes were sealed with melted candle wax. For each patient two capillary tubes were prepared. The inoculated tubes were incubated at 24 °C under standard atmospheric condition and were regularly examined every 1–7 days thoroughly under an inverted microscope to monitor the *Leishmania* promastigotes growth and presence of contaminations. Capillary tubes can also be examined under a light microscope if no inverted microscope is available. In this method four capillary tubes are placed on a microscope slide. Another slide is added on top and the gap between the slides is filled with distilled water. This allows the promastigotes, if present, to be visible in the tube. The magnification is usually 100× (×10 objective and ×10 eyepiece). All cultures were kept and examined for 30 days before considered negative.

Smear preparation

At once, following microculture preparation for each patient, Giemsa stained smears were prepared by using material scraped from the ulcer base and border with a sterile lancet, air-dried, fixed with 100 % methanol and then examined under a compound light microscope for the amastigote forms. Smear amastigote density was quantitated as described using the method of Chulay and Bryceson (1983). Two smears were provided separately for each lesion.

PCR assay

In order to extract the DNA of 303 clinical samples the immersion oil which was used to slide microscopic examination was whipped off by tissue paper. Then in a 1.5 ml tube, smear scrapings were added to 200 µl lysis

buffer (50 mm Tris–Hcl pH = 8.0, 1 mm EDTA pH = 8.0, 1 % Tween 20) containing 8.5 µl proteinase K solution (19 mg/ml). The tubes were incubated for 2 h at 56 °C then 60 µl of 6 M NaCl was added. After being shacked slowly, the tube was centrifuged at 14,000×*g* for 15 min. Finally DNA that floated in the supernatant solution was precipitated with 400 µl cold ethanol. After trapping the DNA and drying its ethanol, we added 50 µl double distilled water to each tube and stored them at 20 °C until tested for *Leishmania* kDNA (Aljanabi and Martinez 1997).

Two PCR assays that amplify conserve and variable regions of *Leishmania* kDNA were used. Therefore, each specimen was analyzed by using two different pairs of PCR primers and infection with *Leishmania* spp. detected by PCR protocol that was previously described by Lachaud et al. (2001).

At this stage, primers RV_1 (5-CTTTTCTGGTCCCGCG GGTAGG-3) and RV_2 (5-CCACCTGGCCTATTTTACA CCA-3) were used to amplify a fragment of 145 bp present on the highly multicopy of kinetoplast deoxyribonucleic acid (kDNA) minicircles. The set of primers LIN-4 (forward: 5′-GGGGTTGGTGTAAAATAGGG-3′) and LIN-17 (reverse: 5′-TTTGAACGGGATTTCTG-3′) were used for species specific PCR to characterization of *Leishmania* species, the protocol that was described by Aransay et al. (2000). It is 650 and 720 bp for *L. major* and *L. tropica*, respectively.

Statistical analysis

The degree of agreement was determined by calculating Kappa (k) values with 95 % confidence intervals using SPSS version13.5 (SPSS Inc., Chicago, IL). Chi square test was applied to find the differences between the groups. *P* value <0.05 was considered statistically significant. Kappa values express the agreement beyond change and a k value of 0.21–0.60 represents a fair to moderate agreement, a k value of 0.60–0.80 represents a substantial agreement, and a k value of ≥ 0.81 represents almost perfect agreement (Altman 2001). Moreover, in each individual study, the composite reference standard against which each diagnostic test was compared was two of three or four tests with positive results, where tests refer to smears, culture by either method, or PCR. For evaluation of performance characteristics of each test, the lesion was the unit of analysis, as previously described by Boggild et al. (2007, 2008).

Results

Using the composite reference standard applied in each study (at least two of three positive test results), we recruited a total of 303 patients clinically suspected of CL, among whom 273 fulfilled criteria for the diagnosis of CL. The 273 subjects included 139 (50.9 %) males and 134 (49.1 %) females. They were aged 6 months to 75 years-old with the mean of 24 years old. The CL was observed more frequently in children <5 years old (30.3 %) while it was less prevalent among 30–35 year olds patients. Among 273 patients, 25 (9.1 %) were found with atypical presentation of CL such as diffused type, erythematous and papular ulcers. Moreover, the majority of skin lesions (88.3 %) were acute (duration $\leq$3 months) and 11.7 % were chronic (duration >12 months).

Overall, 234 (85.7 %) of the CL patients were found to be positive in all three diagnostic tests. A total of 238 patients were positive by smears, giving a sensitivity and specificity of 88.8 % (95 % CI = 84.2–92.2 %) and 100.0 % for smears (see Table 1). However, 35 of the subjects were smear-negative. The lesions were located principally on the hand (36.9 %), foot (30.6 %), face (20.2 %) and other organs (12.3 %).The mean duration of lesions was 3 months. Among patients 115 (42.1 %) had multiple lesions. The median amastigote density was grade 3 in smear-positive lesions (1–10 amastigotes/10 high-power microscopic fields).

Two hundred and sixty-two patients were positive by IMC, indicating a sensitivity of 98.4 % (95 % CI = 96.1–99.1 %) and specificity 100 %. However, only 11 of the 273 patients were negative by IMC. In addition, 24 samples obtained from lesions that were negative by smear were also IMC positive. In lesions that fulfilled reference standard criteria, median smear density was higher in those that were also IMC positive (density grade = 3, 1–10 amastigotes/10 high-power microscopic fields) compared with those that were IMC negative (density grade = 1, 1–10 amastigotes/1,000 high-power microscopic fields) ($P < 0.003$). The median time to culture positivity was 2 days for IMC; no bacterial and fungal contamination was observed in the specimens. The emergence of promastigotes in the IMC showed a correlation with the amastigote density; median time to culture positivity was lower in smears with large number of amastigotes. Also, we observed promastgotes which remain frequently viable for up to 8 weeks.

A total of 273 patients were positive by genus-specific PCR, giving a sensitivity and specificity of 100 % in both. However, all 273 patients fulfilling criteria for CL were found to be positive by PCR. Thus, the PCR was positive for all grades and independent of amastigote density in the lesions. Moreover, all atypical forms of the skin lesions were positive by both PCR and IMC methods.

All PCR positive cases produced specific bands with species-specific primers corresponding to *L. major*. Data analyses of three diagnostic tests that used in the

Table 1 Analysis of three diagnostic tests used for evaluation of 273 patients that fulfilled criteria for cutaneous leishmaniasis

Test	No. of positive (%)	No. of negative (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Smear	238 (87.2)	35 (12.8)	88.8	100	100	68.4
Microculture	262 (96)	11 (4.03)	98.4	100	100	88.2
kDNA PCR	273 (100)	0 (0)	100	100	100	100

PPV positive predictive value, *NPV* negative predictive value

examination of 303 patients suspected of CL are shown in Table 1.

Our study showed the highest sensitivity and specificity observed in PCR and the lowest sensitivity (88.8 %) in the smear. The highest degree of agreement (Kappa = 0.82) was found between PCR and IMC. The degree of agreement between PCR and IMC was fairly close to IMC and smear (Kappa = 0.69). The results showed a fair agreement (Kappa = 0.57) between smear and PCR, as only 238 of CL patients were positive in both tests.

Discussion

In the present study, we have demonstrated that the IMC method is highly more sensitive than the scraping smear offering a simpler, economical and sensitive alternative to PCR in the diagnosis of CL. The sensitivity of the smear varies in different reports, depending on the lesions duration and appearance, parasite strain and localities, even within the same region (Weigle et al. 2002). Positive diagnosis of CL by the smear also varies greatly with the number of amastigotes in aspirate materials and sometimes requires an incubation period of up to 15–30 days (Navin et al. 1990; Weigle et al. 1987). In contrast, the IMC is much less susceptible to these parameters and provides a fast reading. Allahverdiyev et al. (2004) showed that the positive rate and the period for promastigote emergence with MCM were 83–97 % positive in 4–7 days. In our study, positive rate and the time for promastigote emergence were 96 % in 2–3 days. The aforementioned study (2004) also expressed that the high sensitivity of the MCM might be explained by the use of capillary tubes, which concentrate the sample material and provide microaerophilic conditions favorable for transformation of the amastigotes to promastigotes. The sensitivity and specificity of the IMC was found to be 98.4 and 100 %, respectively which is comparable with microculture results achieved by previous studies (Allahverdiyev et al. 2004; Ihalamulla et al. 2005, 2006; Boggild et al. 2007, 2008, 2010).

Although molecular techniques such as PCR are sensitive and becoming increasingly popular (especially in research settings), definitive diagnosis still rests on demonstration of the parasite by microscopy or culture, both of which are widely used in clinical laboratories. (Marques et al. 2006; Bensoussan et al. 2006) Thus, culture-based testing such as IMC is more practical, particularly in under resourced settings. In addition to being more economical and accessible, the IMC has the advantage of isolating organisms for drug susceptibility testing and genotyping, which are important in countries where CL is endemic.

The MCM developed by Allahverdiyev et al. (2004) is invasive. While the present study used a new improved, noninvasive, safer (needle free), simpler, faster and more sensitive microculture method compared with studies carried out so far (Ihalamulla et al. 2005, 2006; Boggild et al. 2007, 2008, 2010) as sampling was taken from the ulcer borders directly and without aspiration by needle.

In our study, we found 11 false negative cases using IMC. The false negative results of the IMC may be explained as a result of failed sampling and/or also low amastigote density in non-ulcerative and chronic lesions. However, negative results of IMC should be confirmed by tests with higher sensitivity such as PCR. Marques et al. (2006), Bensoussan et al. (2006) suggested using PCR for chronic cases and when other methods had failed to detect the disease. Hence, the IMC results may not be reliable in favor of chronic skin lesions.

Surprisingly, we found that capillary tubes could be examined under a light microscope if no inverted microscope is available. Therefore, the IMC method can be universally used in current diagnostic laboratories. Also observation of the developmental cycle of *Leishmania* promastigote into capillary tubes, which is naturally found in sand fly host, can be used for educational purposes and also making images from different forms by photography.

In conclusion, the high correlation between IMC and PCR along with the advantages of simplicity, rapidity, adequate sensitivity as a needle free method, offers the IMC as a valuable alternative diagnostic method for PCR in diagnosis of CL, especially atypical clinical presentations. Thus, the IMC method is recommended as a confirmatory test for accurate and reliable diagnosis of CL especially for patients with negative smear in endemic areas. Finally, we hope IMC method could increase patient satisfaction. Moreover, further evaluations should be carried out to prove its usefulness in the diagnosis of CL in other endemic regions of the world.

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