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Original Article

Comparative Analysis of Microscopy, Leishmanin Skin Test (LST), and Direct Agglutination Test (DAT) for the Diagnosis of Human Cutaneous Leishmaniasis

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Abstract

Background: Cutaneous Leishmaniasis (CL) is a highly infectious parasitic disease in Iran. Although various diagnostic methods are available, identifying a sensitive and specific approach remains essential for effective treatment and disease management. We aimed to compare microscopy, the *Leishmanin* Skin Test (LST), and the Direct Agglutination Test (DAT) for diagnosis of human CL.

Methods: Fifty samples were obtained from clinically suspected CL cases. The results of conventional methods, including microscopy, the LST, and the DAT, were then compared among these samples. For microscopical examination considered a gold standard, smears were stained with Giemsa 10% and then examined for the observation of amastigote forms for the LST, 0.1 ml of standard *Leishmanin* solution was intradermally injected into positive patients, and then indurations were measured after 48-72 hours; an induration of ≥5 mm was considered positive. Additionally, anti-*Leishmania* antibodies were detected using DAT on positive collected serum samples.

Results: Out of 50 samples collected from individuals suspected of CL, 66% (33/50) of them showed positive results using microscopic examination. Among these 33 patients diagnosed with CL, 9% (3/33) tested positive in the LST, and only 1 (3%) patient showed specific antibodies against *Leishmania* using DAT.

Conclusion: The microscopy method appears to be more suitable for diagnosing CL. However, there is a clear need for additional diagnostic methods with more validity for CL.

Introduction

Leishmaniasis is a vector-borne parasitic disease in tropical regions, caused by various species of the genus *Leishmania* (1). Leishmaniasis presents in three clinical forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL) (2). CL is transmitted through the bite of the female mosquito of *Phlebotomus* species (3). Although this disease is typically non-fatal, it is necessary to use chemical drugs or natural products to treat this disease due to the chronic and disfiguring lesions it causes (4-6). Currently, CL is present in over 98 countries globally, with an estimated prevalence affecting 12 million people (7, 8). Diagnosing CL is challenging due to the varied symptoms, the stage of the lesions, and the involvement of different species (9, 10). Various methods are employed in diagnosing CL, including direct smear examination (microscopic), culture, molecular, and immunologic techniques (11-13).

The microscopic method is considered the gold standard for diagnosis (12, 14). The sampling location, the lesion stage (chronic or active), and the individual collecting the sample are all crucial factors to consider (15).

The *Leishmanin* Skin Test (LST) is a method utilized in diagnosing chronic CL in some regions. In this test, typically 0.1 ml of *Leishmania major* antigen containing one million *L. major* promastigotes, which are rendered inactive by 1% Mertiolate, is intra-dermally injected into the forearm area (16-18). After 48-72 hours, the resulting induration is measured, often characterized by a raised line upon stretching. This test is primarily employed in diagnosing cutaneous, mucosal, and mucocutaneous leishmaniasis, as well as in epidemiological studies related to CL (16, 18, 19).

The Direct Agglutination Test (DAT) serves as a routine serological method for diagnosing VL. Notably, one of its advantages lies in its simplicity, as it requires no complex equipment and offers easy interpretation of results (20-22). The DAT test demonstrates high sensitivity and specificity in diagnosing VL (9, 23) but it has not assessed for the diagnosis of CL. Iran stands out as one of the most significant epicenters of CL worldwide (24-27). Therefore, we aimed to investigate and compare the effectiveness of the microscopic method, DAT test, and LST for diagnosing human CL in Iran.

Materials and Methods

Sample collection and patients

Samples were collected from 50-suspected patients of CL in Iran. These patients were referred to the center in Golestan Province, the most important endemic focus of CL in Iran, between August 2023 and March 2024. Alongside demographic data, information regarding the age, sex, and lesion number was gathered and documented in the questionnaires.

Microscopic diagnosis

The patients received a diagnosis based on clinical characteristics and parasitology methods, including microscopic examination. For microscopic diagnosis, suspected CL lesions were scraped using a sterile scalpel, and the exudate materials were fixed with 100% methanol, allowed to dry, and subsequently stained with Giemsa 10% stain for microscopic examination. The diagnostic criteria for CL were established upon the observation of amastigotes within the smear under a light microscope with a magnification of 1000X.

LST

We administered 0.1 ml of *Leishmanin* liquid intradermally into the alcohol-cleansed volar surface of the patient's forearms. The *Leishmanin* solution was derived from *L. major* (MRHO/IR/75/ER) Pasteur Institute of Iran (28). After 48-72 hours, the induration was measured along two diameters using the ball-pointed pen technique. An induration with a diameter of 5mm or more was deemed indicative of a positive test result (17, 29).

Blood sampling

Two ml blood samples were obtained from CL patients. The blood was then centrifuged at 800 g for 5-10 minutes, and the sera were separated and subsequently stored at -20°C. DAT was performed on all the serum samples.

DAT

The DAT antigen was procured through a multi-step process involving the mass cultivation of promastigotes of the Iranian strain *L. infantum* (MCAN/IR/07/Moheb-gh (GenBank Accession No. FJ555210)) in RPMI-1640 medium (Bioidea, Iran) supplemented with 15% FBS (Gibco, Germany). Following parasite trypsinization, the promastigotes were stained with Coomassie Brilliant Blue R-250 (Sigma, USA) and subsequently fixed with 1.2% formaldehyde. For DAT testing, serum was initially screened at a dilution of 1/800. In the event of a positive reaction, further titration was performed up to a dilution of 1:102,400. The experimental plates utilized for this purpose featured 96 (8 × 12) V-shaped wells.

During the screening procedure, 8-well rows were allocated for each sample. Conversely, in the case of titration, 12-well rows were designated for one specimen. To prepare serum dilutions, 90 µl of human sera diluting agent and 10 µl of sera were combined in the first well to achieve a 1:10 dilution. Following this, 10 µl of this mixture was transferred to the second well and mixed with 90 µl of human sera diluting agent to attain a 1:100 dilution. In the remaining wells, 50 µl of human sera diluting agent was mixed with 50 µl of human sera to achieve dilutions of 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600, 1:51200, and 1:102400, respectively. Subsequently, 50 µl of DAT antigen was added to the specific well containing the 1/800 dilution during the screening process. The plate was then incubated at ambient temperature for 13–18 hours. It is worth noting that positive and negative control sera were included in each set of experiments. When comparing the results with these controls, compact blue dots were interpreted as negative, whereas large diffuse blue mats were indicative of a positive reaction. The test results underwent independent examination by two individuals. According to previous studies, human sera showing specific anti-*Leishmania* antibodies at a titer of ≥1:3200 were considered positive cases (20).

Statistical analysis

Data analysis for this study was performed using the SPSS software version 24 (IBM Corp., Armonk, NY, USA). Nonparametric statistical test (chi-square) was applied to analyze statistically significant relationship among microscopic examination, LST, and DAT in a bivariate table. The probability (*P*) value was considered as statistically significant if $P < 0.05$.

Results

Out of 50 patients with suspicious CL, 33/50 patients (66%) tested positive in the direct microscopic examination. A significant relationship was observed between the diagnosis of the CL using the microscopic method ($P< 0.05$).

Demographics

Out of the 33 patients, 18 were male. The age range of patients was 11 to 56 years, with a mean age of 35.3 years (Table 1). No significant correlation was found between age, sex, and the number of lesions.

Table 1: Demographic Information of Patients with CL in Golestan Province

Variable	Number
Age (yr)	
10-25	10
26-40	13
>40	10
Sex	
Male	18
Female	15
Number of lesions	
1-3	12
4-6	13
>6	8

LST

Out of 33 positive CL patients, 3 tested positive for the LST after 48-72 hours (9%) (Table 2). No statistical correlation was found between LST results and age, sex, and number of lesions.

Table 2: Information of LST-positive results in CL patients

Patient's No.	duration of Lesion (month)	LST
1	1-3	+ (5 mm)
2	3-6	+ (9 mm)
3	3-6	+ (7 mm)

DAT

Out of 33 positive CL patients, only one patient in the acute form of CL showed a seropositive rate (SPR) and had anti-*Leishmania* antibodies at titers of 1:3200 using DAT.

There was no significant relationship between the microscopic method, LST, and DAT test in the positive CL patients.

Discussion

Cutaneous leishmaniasis (CL) is highly prevalent in Iran, with several provinces acting as focal points for this disease (24, 30, 31). There are many reliable laboratory diagnostic methods for CL, including direct smear examination, culture, and molecular techniques (15, 18, 10). The primary diagnostic method for CL is the direct smear examination, also known as the microscopic test, which uses smears of dermal scrapings obtained from a skin biopsy (32, 33). This test is inexpensive, accessible, and highly reproducible. Alternatively, due to the wide clinical spectrum of leishmaniasis and its various clinical forms, it is essential to find a highly sensitive diagnostic method (11). Even today, microscopic identification remains a primary diagnostic tool in many regions where leishmaniasis is endemic. The detection of amastigotes in smears under microscopy has long been considered the gold standard and is highly specific for diagnosing CL (27, 34). However, its sensitivity varies between 42% and 70%, depending on the expertise and skill of the technicians performing the test. The diagnostic sensitivity of the microscopic examination observed in our study is 66% in suspected CL patients. Similarly, Rasti et al. reported a positivity rate of 66.9% for CL using the microscopic method (10). Weigle et al. reported in contrast to our results with low sensitivity (32.7%) (35). Navin et al reported a significant increase in the sensitivity of this method, from 40% to 80%, by increasing the number of samples collected from each lesion from one to four (36). On the other hand, Ramirez et al. reported the sensitivity was 90.4% in only a single sample (33). The site of sample collection significantly impacts the sensitivity of the microscopic method (37-39).

In this study, a combination of the microscopic method and the *Leishmanin* Skin Test (LST) has been utilized for the diagnosis of CL patients. The LST, also known as Montenegro's test, is an important tool for the diagnosis and epidemiological surveys of CL. The first study of this test was conducted by Montenegro in 1926 in Brazil (40). Due to its high sensitivity in CL, the LST test has been widely used for diagnosis (12, 41). LST can remain positive from a few days after infection to several months (18). Therefore, positive LST results were not associated with either acute or chronic forms or the number of lesions (16). In Colombia, LST was employed to diagnose leishmaniasis in chronic lesions. This combined approach's reported sensitivity, speci-

ficity, and overall efficiency were 92%, 70%, and 87%, respectively. This study emphasizes the importance of integrating LST into diagnostic protocols to improve specificity, complementing other methods (35). Although the LST cannot differentiate between current and past infections, it remains valuable for diagnosing leishmaniasis in endemic regions. In addition to its diagnostic role, the LST has been a valuable tool in epidemiological studies for monitoring exposure and immunity to *Leishmania*, as well as in vaccine research, where it serves as a surrogate marker of immunity (42, 16). The efficacy and safety of Iranian *Leishmanin* have been previously documented (29). The sensitivity and specificity of LST may vary depending on specific geographic areas, as the response can be influenced by various factors related to both the parasite and the host (17). Dostrovsky and Sagher suggested that the LST becomes positive very soon after *Leishmania* infection. However, they conceded that this rapid conversion might not occur in natural infections (43). In a study, LST showed a 90.4% positivity rate. Most patients with early lesions had positive LST results and, sex, age, and duration of the lesions had no significant impact on the test's positivity (44). The use of LST can be very helpful in confirming the CL diagnosis in early-suspected lesions. Some studies have found a relationship between the positivity of LST and the duration of the lesion. For example, a study in Pakistan evaluated 100 patients with CL using LST. The LSTs were positive in 78% of patients 2 weeks after diagnosis, increasing to 98% after 6 weeks. This sensitivity increases to 94% and 98% at 4 and 6 weeks of disease duration, respectively. Therefore, the test can be confidently employed even in cases presenting with early lesions (17). In our study, we evaluated the LST in CL patients. The results indicated that 3 out of 33 patients were positive. The results of our study showed no significant relationship between the number of CL lesions, age, and sex in the positivity of the LST. Recently, the use of the LST has declined due to the lack of a standardized and reliable *Leishmanin* product. Despite efforts over the past few decades, *Leishmanin* antigen is no longer produced under good manufacturing practice (GMP) conditions anywhere in the world. As a result, the LST has been increasingly replaced by serological and molecular tests in epidemiological studies (16). In the early 1990s, effort to standardize the LST was initiated when the Special Programme for Research and Training in Tropical Diseases (TDR) at the WHO requested *Leishmanin* submissions from institutions worldwide (45). TDR began the wide distribution of Iranian leishmanin, addressing the need for a standardized and reliable antigen. However, for unknown reasons, the distribution of Iranian *Leishmanin* was eventually discontinued. Some institutions briefly continued producing *Leishmanin* on a smaller scale. Although Iranian *Leishmanin* is still used in some studies (18, 45).

The DAT is a routine diagnostic test for VL due to its simplicity, affordability, and high specificity 95-96% and sensitivity 95% in endemic areas (21, 46). In this study, the measurement of DAT titers in CL patients was conducted. The DAT titer showed positivity in 3% (1/33). A previous study indicated that DAT may be a useful test for serologic diagnosis of Ethiopian CL patients, demonstrating that 60% of CL cases could be confirmed using this test (23). Hailu et al. reported a 90% positivity rate of DAT titers in CL patients (9). In another study, DAT was a valuable diagnostic tool for CL in Sudan. DAT has shown high positivity in the CL form (47). In this study, we did not expect cases that are more positive in CL patients. In Iran, *L. infantum* is primarily used to prepare the DAT antigen for diagnosing VL (20). Therefore, in this study, we utilized *L. infantum* antigen for CL patients, as *L. major* antigen was not available for the DAT test. We aimed to examine the sensitivity of *L. infantum* antigen for diagnosing CL. The

limited number of positive cases observed may therefore be related to the type of antigen used. According to this claim, Mengistu et al. reported strong sensitivity and specificity of DAT using *L. major* antigen (23).

Using a larger sample size could have yielded more accurate and reliable results. Additionally, the antigen used in the DAT test was specific to *L. infantum*. DAT is recommended to be used with *L. major* and *L. tropica* for the evaluation of diagnosis CL.

Conclusion

The overall positivity rate of the microscopic test was 66% in the suspected CL patients. In this study, the positive rate of both the DAT and LST was very low for CL patients. Therefore, we recommend the development of a suitable test specifically for CL detection. Overall, it can be concluded that the microscopic method is more suitable for diagnosing CL in Iran.

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Conflict of interest

The authors declare that they have no conflict of interest.

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