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Diagnosis of Cutaneous Leishmaniasis: Why Punch When You Can Scrape?

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Abstract. Cutaneous leishmaniasis (CL) has been introduced to the *Leishmania* under-endemic Lebanese population in an uncontrolled manner as a result of recent large-scale displacement of refugees from endemic Syria. Accordingly, a quick and reliable method to diagnose CL is essential. Matched punch biopsies and air-dried scrapings on 72 patients were obtained. Scrapings were collected in two forms: thick drop ($N = 33$) or thin smear ($N = 39$). Clinical information was recorded. Sections of punch biopsies and scrapings were stained and examined microscopically. Polymerase chain reaction (PCR) was performed on both scraping forms and biopsies. The diagnostic sensitivity of the tests performed revealed that microscopy in conjunction with PCR on punch biopsies was the most sensitive test (93%) overall. However, taken individually, microscopy and PCR yielded the highest sensitivities when performed on drop scrapings (63% and 85%, respectively), and not smear scrapings (38% and 56%, respectively) as compared with the punch biopsies (44% and 83%, respectively). Microscopic concordance for punch biopsies and drop scrapings was present in 25 of 33 cases. Concordance was predicted only by the high/low parasitic index (PI: 3.1 ± 1.7 and 0.4 ± 0.5, respectively; $P < 0.05$). Herein, we optimized a novel rapid method for reliable diagnosis of CL based on drop scrapings with good agreement with the gold standard punch biopsy technique.

INTRODUCTION

Leishmaniasis, a neglected tropical disease, is a poorly studied and monitored infectious entity. At the time, disease burden and epidemiology are poorly surveyed and treatment of the disease needs further study and development.[1,2] Leishmaniasis can occur in three forms: visceral, mucocutaneous, and cutaneous leishmaniasis (CL). Globally, > 90% of CL cases are reported in Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, Iran, Brazil, and Peru.[2–4] The CL in the Middle East is predominantly caused by the *Leishmania tropica* species.[5] Although not a cause of high mortality, CL can be chronic and disfiguring, producing multiple lesions that induce serious secondary infections and can involve vital sensory organs.[4]

With the influx of over 1,500,000 refugees from *Leishmania*-endemic areas in Syria seeking shelter from the armed conflict in their country[6,7]; Lebanon, a *Leishmania* under-endemic country, is threatened by an epidemic outbreak of CL. To prevent such a public health hazard from occurring, large-scale diagnosis and treatment of CL in the Syrian refugees has been a necessity. Because lack of experience with CL makes diagnosis depending on clinical presentation unreliable, and that treatment can be costly and toxic,[2] diagnosis has to be confirmed by collecting punch biopsies. Punch biopsy is the most commonly performed reliable diagnostic procedure, as the alternatives—culture or polymerase chain reaction (PCR)—require more tedious and technically demanding procedures.[2] However, collecting punch biopsies in the setting of a social, economic, and health care crisis has shown numerous issues: unwillingness to tolerate the procedure because of its invasive nature and inability to take samples from vulnerable anatomic locations, e.g., eyelid, aside from the high cost and instrumentation (sterilization requirements, sutures and local anesthesia, fixative and transportation media, processing, embedding, sectioning, and staining).[8]

Therefore, collecting a scraping from a lesion instead of a punch biopsy is considered as an alternative sampling method. Scrapings circumvent many of the problems listed previously because they are brisk, non-invasive, collected on a slide as an air-dried smear or drop, stable at room temperature, safe, and easy to transport. However, data comparing the diagnostic sensitivity of parasitological diagnosis, whether by light microscopy or PCR, on samples in the form of punch biopsy versus scraping has not been well examined.[8] In this study, we compared matched scrapings and punch biopsies for microscopic and PCR testing in an epidemic of CL.

MATERIAL AND METHODS

Patient demographics and sample collection. This study was approved by the American University of Beirut Institutional Review Board and patient data used in this study was anonymized. One-hundred and thirty-three individuals suspected to have CL were guided to an outpatient department medical center near their camps in North Lebanon and Bekaa area. Epidemiological data and clinical information collected include: sex, age, duration of disease in months, skin location of lesion, number of lesions, and lesion type (wet or dry).

On the outpatient department premises, a team of trained physicians collected punch biopsies and concurrent multiple scrapings of the lesions (Figure 1B and C). Scrapings were made with the help of a scalpel, pushing in one direction until blood oozes from the inflamed border of the lesion. Multiple drops of the oozing blood were distributed on multiple glass slides. In thick-drop scraping, the blood drop was kept without smearing until it dried at room temperature. In smeared scraping, the blood drop was spread using a spreader slide and left to dry at room temperature. Biopsies were stored in tightly sealed formalin-filled tubes, whereas skin scrapings were stored at room temperature (Figure 1C).

Sample preparation. A formalin-fixed paraffin-embedded (FFPE) tissue block was prepared from each punch biopsy, and two hematoxylin and eosin (H&E) stained slides were obtained for each block. On the other hand, four different types of stains: Papanicolau (PAP) stain, Diff Quick, H&E,

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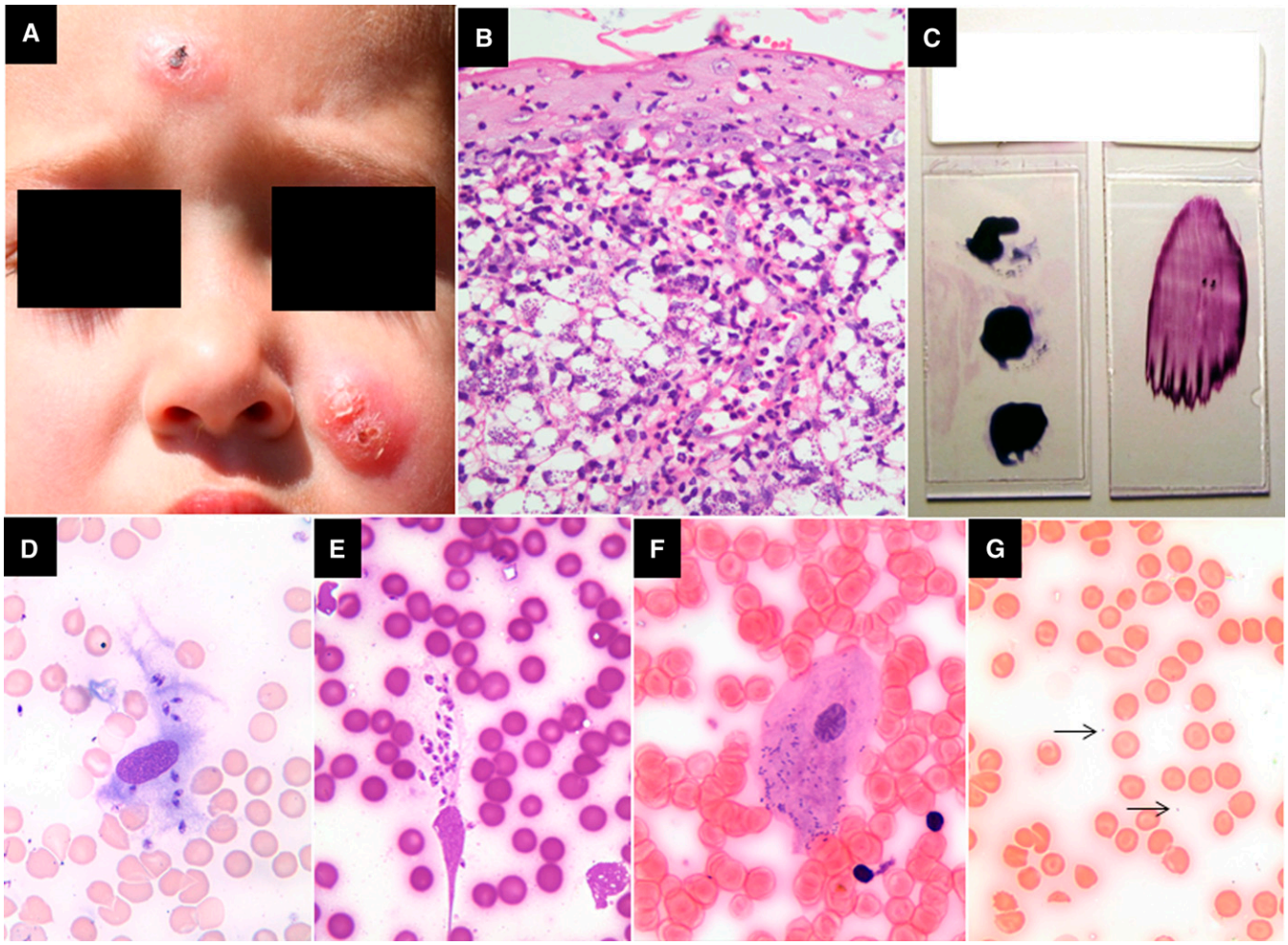

FIGURE 1. Nodular erythematous facial lesion in a 5-year-old child with cutaneous leishmaniasis (**A**). A punch biopsy (**B**), showing numerous amastigotes is collected in addition to thick drop and thin smear scrapings (**C**). Smears stained with Wright Giemsa (**D**), Diff quick (**E**), H&E (**F**), and Papanicolau (PAP) stain (**G**) are portrayed (×200).

and Wright Giemsa, were performed on scrapings from each patient (Figure 1D–G).

Microscopic examination. The stained biopsy sections and scrapings were examined by two pathologists (IK and MS). Biopsy slides were examined for the presence or absence of amastigotes, and parasitic index (PI) determined based on modified Ridley's PI, which quantifies the parasitic load of amastigotes in cutaneous lesions and has a numerical score from 1 to 6.[9] Scrapings (drop or smear) were examined for presence or absence of amastigotes.

Inclusion/exclusion criteria. One hundred thirty-three patients with CL had punch biopsies. Cases with sufficient clinical data and material (a matched punch biopsy, and a scraping "drop or smear") for diagnosis confirmation by PCR were included in the study ($N = 72$). Positive diagnosis of CL was confirmed by the presence of one or more of four parameters: positive PCR on punch or scraping, or microscopic detection of amastigotes in punch biopsies or in scrapings. Samples that did not have matched punch biopsies and scrapings were excluded from the study ($N = 61$).

Molecular confirmation and speciation. *DNA extraction from FFPE punch biopsy blocks.* The DNA extraction from paraffin-embedded punch biopsies was performed according to the protocol used by Yehia and others 2012.[10]

DNA extraction from scrapings (smears and drops). The DNA extraction was done using the Pel-Freez (Invitrogen, Carlsbad, CA) kit according to the manufacturer's instructions. The DNA samples were incubated at 65°C for 30 minutes and then stored at 4°C until analyzed by PCR. The resultant DNA was then quantified using the Biomate spectrophotometer (Thermo, Glasgow, UK).

PCR amplification and agarose gel electrophoresis. All cases were sent for confirmation by PCR. In brief, PCR was performed using primers specific for the *Leishmania* ribosomal internal transcribed spacer 1 (ITS1-PCR). The PCR amplicons were then analyzed by agarose gel electrophoresis (1.5% TBE gels stained with ethidium bromide) and compared with a standard 100 base pair (bp) DNA ladder. Samples (25 μL were electrophoresed at 90 V in 1× TBE buffer (0.04 M Tris-acetate and 1 mM EDTA, pH 8.0) for 30 minutes. The bands were visualized under UV light. Images were then captured using the DigiDoc-IT TM Imaging System (UVP, Upland, CA). A DNA band of 300–350 bp was considered as a positive indicator for the presence of *Leishmania*.

Molecular subspeciation. The digestion of the ITS1-PCR amplicons with restriction enzyme *HaeIII* was performed for restriction fragment length polymorphism (RFLP) analysis and consequent subspeciation. The ITS1 RFLP technique used allowed the identification of all clinically significant strains, including *Leishmania tropica*, *Leishmania major*, *Leishmania braziliensis*, *Leishmania donovani*, *Leishmania aethiopica*, and *Leishmania infantum*.

Statistical analysis. After determining the study subjects, 72 cases were confirmed to have CL. All of the 72 cases had punch biopsies (72 cases). Matched scrapings were present in two forms: thick drop (N = 33) and thin smears (N = 39). Continuous variables were analyzed by t test or Mann-Whitney rank sum test as appropriate. Categorical variables were analyzed using the χ^2 test. A two-tailed $P < 0.05$ was required for statistical significance. Analyses were performed using SPSS version 19 (IBM Inc., Somers, NY).

RESULTS

Patient demographics and clinical presentation. Patients' age ranged from 1 to 61 years (mean: 18 years) and included 33 males and 39 females. The average duration of the observed lesions was 6 months. Lesions varied in location, and were found on the head and neck (31%), upper extremities (29%), lower extremities (11%), or a combination of these locations (29%). The number of lesions ranged from 1 to 15 (mean = 6). Thirty-five patients had lesions with plaque/nodule appearance, whereas 37 patients had ulcer/verrucous lesions.

Stain evaluation and selection. To determine the efficiency of different stains for amastigote detection on scrapings, four CL positive cases were selected randomly. The average time for detection was recorded for every stain. Recorded times were as follows: 176 seconds for PAP stain, 85 seconds for H&E stain, 66 seconds for Diff Quick stain, and 42 seconds for Wright Giemsa stain (Figure 1D–G).

Amastigote kinetoplasts showed intense staining with Diff Quick and Wright Giemsa only (Figure 1F and G). However, staining with Wright Giemsa produced a lighter background, and thus better contrast with peripheral blood elements, making the amastigotes readily apparent.

Microscopic confirmation. Out of the 72 CL cases with a punch biopsy, 33 had a corresponding scraping in the form of drops, and 39 in the form of smears. The detection rates of amastigotes by light microscopy were 44.4%, 63.6%, and 38.6% for the punch biopsies, drop scrapings, and smear scrapings, respectively. Drop scraping was superior when it comes to light microscopy and this was statistically significant ($P < 0.005$, Figure 2).

Molecular confirmation and speciation. Molecular confirmation by PCR was obtained in 83.3%, 85%, and 56.4% for the punch biopsies, drop scrapings, and smear scrapings, respectively. *Leishmania tropica* subtype was identified in 69 patients, *L. major* in 1 patient, and 2 patients had degraded DNA; precluding molecular confirmation and speciation. Drop scrapings and punch biopsies showed relatively similar sensitivity by PCR testing, which was not statistically significant ($P > 0.05$, Figure 2).

Diagnosis confirmation by PCR and microscopy combination. The combined detection rate (i.e., detection of amastigotes by microscopy and/or PCR) was 93%, 85%, and 59% for the punch biopsies, drop positive scrapings, and smear scrapings, respectively. The difference in the combined detection rate between drop scrapings and punch biopsies was not statistically significant ($P > 0.05$, Figure 2).

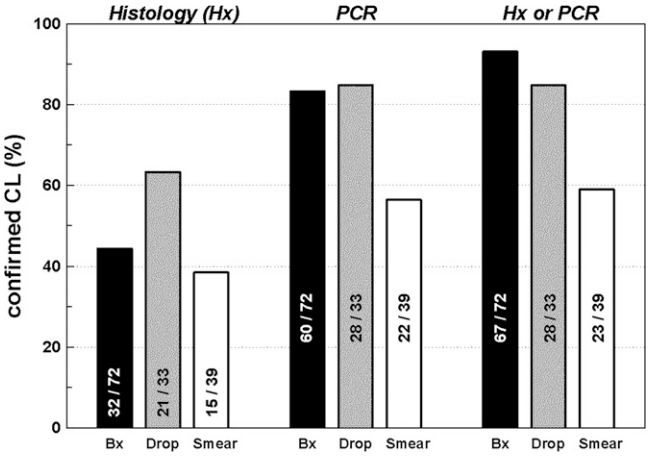

FIGURE 2. The detection rate by light microscopy showed superiority of thick drop scraping over other diagnostic techniques ($P < 0.05$). However, the combined detection rate by polymerase chain reaction (PCR) and light microscopy or PCR alone was relatively similar between drop scrapings and punch biopsies. Such findings suggest using thick drop scraping as a first approach towards diagnosis.

Predictors of concordance. The PI was the only predictor of agreement between detection of amastigotes on drop scrapings and punch biopsies. In 25 samples, where amastigotes were detected in punch biopsies and drop smears (concordant), the average PI was 3.1 ± 1.7, whereas for the remaining 8 discordant samples a PI of 0.4 ± 0.5 (P value < 0.05) was recorded.

DISCUSSION

Recently, the field of molecular biology has been extensively related to the diagnosis and control of infectious diseases, especially in clinically broad parasitic disease such as CL. The CL can clinically mimic a wide range of inflammatory and neoplastic skin diseases.[11,12] The two basic conventional techniques used for confirmatory diagnosis of CL are microscopic identification of amastigotes and culture.[13] The sensitivity of these techniques is low and can be highly variable, depending on the PI in biopsy samples, technical expertise, and culture media.[10] On the other hand, CL diagnosis by PCR, is very promising; potentially becoming the golden standard as a result of its high sensitivity.[4,8,10]

Experience with triaging patients having CL by running different confirmatory diagnostic tools is not well investigated. Although multiple studies have been performed, no standardized protocol has been developed for the management of *Leishmania* epidemics. The PCR appears to be the most promising technique in such a situation; nevertheless, its relatively high cost, sample type, and preparation remain major variables.

The high sensitivity of PCR in diagnosing CL has been reported in several studies in patients with *L. tropica*, and compared with microscopy and culture. Abdel El-Salam and others,[4] performed PCR using DNA extracted from media-cultured dermal scrapings. The group reported sensitivity up to 87.6% when culture is combined with PCR, 53.9% for microcopy, and 46.9% for culture alone.

The PCR has also been performed directly on skin biopsies, fixed and frozen. Yehia and others[10] performed ITS1-PCR on DNA extracted from 122 FFPE punch biopsies. Microscopically, 44.3% of cases were positive for CL. All cases were subjected to ITS1-PCR and 100% turned out to be positive. In another study, PCR performed on DNA from frozen skin biopsies, showed 81% sensitivity.[14]

Another type of sampling for PCR is using scrapings. Schönian and others[15] report 64% sensitivity of PCR performed on scrapings (blood smears) for detection of amastigotes. Using Giemsa-stained smears from Palestinian patients, Al-Jawabreh and others[16] extracted DNA from the slides and performed PCR showing 87% sensitivity and 100% specificity. The authors suggest that using ITS-PCR on slides is an accurate technique for use in *Leishmania*-endemic areas.[16,17]

Management of CL epidemic outbreaks has been reported multiple times; however, the high variability in testing techniques makes it very difficult to determine the best way to manage a CL epidemic.

During an epidemic outbreak of canine CL in Colombia caused by *L. braziliensis* and *L. panamensis*, culture, microscopy, and the indirect immunofluorescent antibody test (IFAT) were used for diagnosis confirmation. Using a combination of the techniques, 81% sensitivity was reported.[18]

In 1997, CL outbreak occurred in 9,200 Afghan refugee settlements in north-west Pakistan. To confirm the diagnosis, smears of the examined lesions were evaluated. Amastigotes were detectable by microscopy in only 36% of lesions. Forty-eight percent of slide-negative cases produced positive cultures. A number of cases negative to both microscopy and culture were positive by PCR. The overall detection rate was 80%.[19]

During an epidemic outbreak of CL in Colombia, the Montenegro Skin test (MST) and IFAT were used for confirmatory diagnosis. The MST and IFAT were positive in 77% and 54% of patients, respectively.[20]

In our study, aside from describing confirmatory tests to diagnose CL; we also compared matched skin punch biopsies and scrapings for each patient. The value of each diagnostic modality was not limited to simple light microscopy, we further extended our experience beyond what we previously standardized (PCR on FFPE punch biopsies)[10] to include a detailed PCR protocol on room temperature stored scrapings. Different scraping preparations, i.e., drops and smears, were also compared, and different types of stains were evaluated. Our findings suggest a novel and rapid method to handle CL in epidemics.

Herein, four diagnostic modalities were performed for each patient (microscopy/PCR for biopsy and scraping). A positive result by at least one of the methods was considered confirmatory of CL infection. Despite the high sensitivity and specificity of punch biopsy, it remains an invasive procedure having multiple requirements including instrumentation, storage, transport conditions, and processing before reaching the final product, i.e., H&E slides. On the other hand, scraping is a simple procedure that does not share the previously mentioned inconveniences of the punch biopsy. Performing a scraping in the form of a thick drop or smear requires limited instrumentation and minimal skills and can be applied in all anatomic sites. Moreover, scrapings do not need fixation (air dried) and can be stored and transported at room temperature.

Our results show that the highest sensitivity was achieved when a combination of microscopic and molecular tests (PCR) were performed on punch biopsies (93%). However, as a single diagnostic modality, PCR on drop scrapings showed the highest sensitivity (85%). The PCR performed on punch biopsies showed 83% detection rate followed by microscopic examination of drop scrapings (63%). Smear scrapings fared the worst across all three methods of detection (light microscopy, PCR, or combined tests, Figure 2). High PI was the only predictor of concordance between punch biopsies and scrapings. Such findings suggest using thick drop scraping as a first approach toward diagnosis. If amastigotes are not found by light microscopy, PCR can be performed on prepared drop scrapings. Such an approach may eliminate using invasive costly procedures such as punch biopsy in most of the cases.

Our rationale for applying the scrapings as thick drops or thin smears was that the latter should clearly show amastigotes under the light microscope. On the other hand, the advantage of the thick drop is that it provides a sample that is easy to peel off the slide for PCR analysis. Contrary to our prediction, drop scrapings showed better resolution and detection of amastigotes under light microscopy. This is probably a result of higher concentrations of amastigotes in drop scrapings.

We went further to standardize our protocol by performing four different stains on the scrapings: PAP, H&E, Diff Quick, and Wright Giemsa. Staining with Wright Giemsa was rapid, produced a light background, and thus better contrast with peripheral blood elements. This can best be explained because the Wright Giemsa lyses the red blood cells, which emphasizes the amastigote-laden macrophages on the slide. Additionally, the intensely stained kinetoplasts allowed us to clear any confusion between platelets and amastigotes that may be encountered because of similarity in shape and size.

In this study, we optimized a novel rapid method for reliable diagnosis of CL based on microscopic and molecular testing performed on drop scrapings in good agreement with the gold standard punch biopsy technique. A practical algorithm to triage the diagnosis of CL should be based initially on microscopic examination of a drop scrapping followed by PCR on the DNA extracted from the drop scrape and later punch biopsy if necessary.

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