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Performance of immunohistochemistry as a useful tool for the diagnosis of cutaneous leishmaniasis in Panama, Central America

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ABSTRACT

Cutaneous leishmaniasis (CL) is one of the most frequent parasitic zoonoses in Panama. Currently, conventional, molecular and histopathological tests are performed to diagnose CL. Immunohistochemistry (IHC) has proven to be a valuable tool to facilitate the diagnosis of leishmaniasis and to study the cellular immune response developed during the infection. Therefore, considering the absence of IHC in the diagnostic routine in Panama, the objective of this study is to demonstrate the usefulness of this test as a complementary diagnostic tool for improving the sensitivity of histopathology (HP) and helping to study the cellular immune response of patients. Samples from patients with suspected CL were analysed by intradermal reaction of Montenegro (IDRM), smears, culture, PCR (*Viannia*, Hsp-70), HP and IHC. According to the diagnostic criteria, 95.8% of patients were positive for *Leishmania* sp., that was characterized as *Leishmania (V.) panamensis* by PCR-HSP70/RFLP. From positive samples, *Leishmania* was detected by the tested diagnostic methods in the following degrees: 100% by IDRM, 60% by smears, 93.3% by culture, 100% by kDNA PCR, 78.3% by PCR Hsp-70, 50% by HP and 73.9% by IHC. Although IHC had a poor correlation (k = 0.191) with the diagnostic criteria, the sensitivities of both HP (76.1%) and smears (89.1%) were improved by combining them with IHC. IHC considerably improved the detection of the *Leishmania* parasites in the histopathological sections, supporting the need to implement this diagnostic tool in Panama. In addition, immunohistochemistry allows evaluation of the patient's immune response and thus provides new guidelines for the treatment and control of CL in Panama.

1. Introduction

Cutaneous leishmaniasis (CL) is one of the most frequent parasitic zoonoses in Panama. Approximately 3000 cases are registered annually, but a high underreporting of cases has been described [1,2]. Because no effective measures are currently available to prevent the disease, CL control in Panama relies mainly on the detection, diagnosis and specific treatment of human cases [3]. Treatment is complicated and is usually associated with adverse effects; therefore, the Panamanian Ministry of Health and the Pan American Health Organization recommend confirming the diagnosis before starting therapy [2,4].

A complete identification of the *Leishmania* sp. parasite in human cases has a fundamental importance in deciding the therapeutic scheme, the management of the patient and the prognosis of the disease [2,5,6]. CL diagnosis is not always easy [7], and it must usually be accompanied by clinical-epidemiological analyses and laboratory tests [8]. In the absence of vaccines and effective preventive measures, diagnostic methods play an important role in the control and adequate treatment of this infection [6–9]. At present, the treatment of most patients in the country with suspected leishmaniasis is based on clinical–epidemiological criteria because a significant number of cases occur in rural and remote areas, where formal health care and resources for laboratory diagnosis are limited; even in the main health centres of the city, clinical-epidemiological diagnosis and histopathology are the only available testing methods, and it is often difficult to clearly observe the parasite using these methods. Therefore, improving the CL diagnostic quality is necessary to implement the treatment guidelines in the different endemic regions of Panama [3].

Laboratory CL diagnosis tests include parasitological, immunological, molecular, histopathological and immunopathological tests [10,11]. In Panama, only parasitological methods, such as lesion smears stained by Giemsa and cultured in biphasic medium and immunological methods, as intradermal reaction of Montenegro (IDRM)

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and histopathology using paraffin section stained by haematoxylin-eosin and Giemsa are performed by the country's health institutions for the diagnosis of CL [3,12]. However, not all health institutions perform these tests; in the areas with difficult access to supplies but that possess a microscope and trained personnel, only lesion smears are performed [4,7]. Molecular tests, such as polymerase chain reaction (PCR), are used only in reference centres, such as the Gorgas Memorial Institute of Health Studies [7,10]. Additionally, in research institutions, Hsp-70 PCR is used, which allows the characterization of species through the use of restriction enzymes (RFLP) [13] and histopathological tests are limited to hospitals with pathology units.

Techniques involving immunohistochemistry (IHC) have proven to be valuable tools to facilitate the diagnosis of leishmaniasis, as well as to study the cellular immune response developed during the infection [14,15]. The diagnostic sensitivity of IHC can reach 80%, in each case improving the diagnostic sensitivity of other methodologies, such as histopathology [10,15,16]. To date, IHC has not been used in Panama for the diagnosis of CL. It is necessary to implement immunohistochemistry as a tool to not only improve the diagnosis of CL but also to improve the studies of patient immune responses; currently, no immunopathological studies have been developed in the country to develop new strategies for the control and treatment of CL. Therefore, the objective of the present study is to demonstrate the usefulness of IHC testing as a complementary tool in Panama for the diagnosis of CL and to compare IHC with the current routine diagnostic tests, strengthening the histopathological diagnosis of leishmaniasis and offering clear, up-to-date knowledge of the diagnostic realities when choosing the methodologies implemented in the different levels of health care centres in Panama.

2. Materials and methods

2.1. Experimental design

Forty-eight skin scraping and skin biopsy samples from patients with lesions suggestive of CL attending the Clinic of Tropical Medicine at the Gorgas Memorial Institute of Health Studies were analysed. All patients were adults and accepted, freely and voluntarily, to participate in the study through the signing of informed consent. All patients were subjected to the homemade intradermal reaction of Montenegro (IDRM) prepared at the Gorgas Memorial Institute of Health Studies with reference strains of *Leishmania (V.) panamensis* (MHOM/PA/98/WR2306). Skin lesion scrapings were analysed by conventional and molecular tests at the Gorgas Memorial Institute of Health Studies (ICGES), Panama, skin lesion biopsy samples were analysed by histopathology at the Pathology Laboratory of the Hospital Santo Tomás, Panama, and immunohistochemistry was performed at the Laboratory of Pathology of Infectious Diseases of the Medical School, University of São Paulo, Brazil. The biopsy and skin scraping samples were taken from the same lesion to avoid alterations in the comparison test results. After CL diagnosis, all patients were treated with intramuscular glucantime according to the Panamanian guidelines for leishmaniasis control [5].

2.2. Sample collection

2.2.1. Scraping of the cutaneous lesions

To obtain the skin lesion sample, the area surrounding the lesion was cleaned with surgical liquid soap, cleaned with 70% alcohol, and the outer edge of the lesion was scraped with a sterile lancet. A local injectable epinephrine anaesthetic with lidocaine was applied to the subcutaneous tissue with a # 22 hypodermic needle in a volume no > 3 mL. Then, the area was scraped with the blunt edge of a sterile scalpel [3].

2.2.2. Cutaneous biopsy samples

To obtain skin lesion biopsy samples, the area surrounding the lesion was cleaned with surgical liquid soap and then with 70% alcohol. While the lesion was still under an anaesthetic effect, the biopsy was taken from the outer edge of the ulcer with a punch of 4 mm in diameter, within no > 48 h, the sample was fixed in 10% buffered formalin and placed in paraffin [17–20] to be processed through histopathology and immunohistochemistry. The biopsy and scraping samples were taken by the same person to avoid individual variations.

2.3. Diagnostic tests

2.3.1. Intradermal reaction of montenegro (IDRM).

A total of 0.1 mL of Montenegro antigen solution was inoculated intradermally in the suprascapular region at a concentration of 10^6 promastigotes of *Leishmania (V.) panamensis* lysed by ultrasound (MHOM/PA/98/WR2306). The appearance of an induration area (≥ 5 mm) at the point of antigen inoculation after 48 h was considered positive [1].

2.3.2. Conventional tests (smears and culture)

For the smear test, scraping samples were taken with a sterile lancet, and a total of 4 smears were prepared (2 per slide). Then, the smears were stained by Giemsa to search for *Leishmania* amastigotes. For parasite isolation and culture, a scraping was taken and placed in a sterile saline solution with antibiotic (penicillin, 100 U and streptomycin, 0.1 mg) at 10% [3] in duplicate for transport. Under sterile conditions, scrapings were transferred to a solution of biphasic Senekjie's medium, Schneider's medium, M-199 Special medium with glutamine, and Hank's Salt. The culture tubes were stored at 26 °C and checked daily for 1 month to verify the presence of *Leishmania* promastigotes [1,7].

2.3.3. Histopathology

All samples were fixed in 10% buffered formalin and processed within no > 48 h from sampling by a standard histology technique [14,20] in the Laboratory of Pathology of Santo Tomas Hospital, Panama city. All tissue samples were dehydrated, cleared, and embedded in paraffin, and 4-μm sections were stained with haematoxylin-eosin (HE) [15]. The lesion sections were evaluated by the presence of amastigote forms, and crosses were attributed according to the Ridley modified parasitic index where 0 amastigotes per standard section is (−) negative, ≥1 amastigotes per standard section is 1+, ≥10 amastigotes per standard section is 2+, ≥100 amastigotes per standard section is 3+ and ≥ 1000 amastigotes per standard section is 4+ [16].

2.3.4. Immunohistochemistry

Paraffin-embedded tissue was submitted to IHC according to Moreira et al. [21]. The procedure was carried out in the following stages: inhibition of the endogenous peroxidase with H_2O_2, recovery of the antigen with 10 mM citrate buffer pH 6.0, blocking of the non-specific sites with 6% milk powder (Molico®, Nestle) with PBS and incubation with 10% foetal bovine serum. Samples were incubated overnight with the primary antibody diluted 1:1000 at 4 °C. Polyclonal hyperimmune serum produced in the Laboratory of Pathology of Infectious Diseases at the Medical School of the University of São Paulo Brazil from mice chronically infected with *Leishmania (L.) amazonensis* was used as the primary antibody.

The reaction with the secondary antibody and streptavidin solution was developed using the universal detection system with the LSAB Kit, Dako, USA (Catalogue number K0690, DAKO Corporation, Carpinteria, CA, USA) with diaminobenzidine (DAB) as the chromogen. The sections were evaluated by the presence of amastigote forms, attributing crosses according to the Ridley modified parasitic index, where 0 amastigotes per standard section is (−) negative, ≥1 amastigote per standard section is 1+, ≥10 amastigotes per standard section is 2+, ≥100 amastigotes per standard section is 3+ and ≥ 1000 amastigotes per standard section is 4 [16].

2.3.5. Molecular diagnosis

2.3.5.1. Sample collection and DNA extraction. For molecular analysis, skin scrapings were placed in 1.5 mL tubes containing 100 μL of TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). DNA extraction was performed using the Qiagen QIamp® DNA Blood Mini Kit according

to the manufacturer's instructions (Qiagen, CA, USA). *Leishmania* DNA from culture-isolated parasites was purified using the Wizard™ Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.3.5.2. PCR diagnosis for leishmania. Primers B1 (5′-GGGGTTGG-TGTAATATAGTGG-3′) and LV (5´-ATTTTTGAACGGGGTTTCTG-3′) were used to specifically amplify the entire 750-bp minicircle of *Leishmania (Viannia)* [1,22]. Amplification reactions were performed in a final volume of 50 µL containing Master Mix (Promega, Madison, WI, USA), 0.6 µmol/L of each primer and 5 µL of DNA extracted from the TE buffer.

2.3.5.3. PCR Hsp-70. PCR was performed with the oligonucleotides F25 (5´-GGACGCCGGCACGATTKCT-3′) and R1310 (5´-CCTGGTTG-TTGTTCAGCCACTC-3′), which amplify a 1286 bp product from the repeated gene heat shock protein 70 (Hsp70) as previously described [23]. Amplification reactions were performed in a final volume of 50 µL containing 25 µL of Go Taq Green Master Mix 2× (Promega), 0.6 µmol/L of each primer and 5 µL of DNA from the clinical samples and 1 ng from the reference strains. Thermal cycling was performed in an Applied Biosystems® 2720 Thermal Cycler.

2.4. Leishmania characterization

Additionally, positive culture samples were characterized by PCR Hsp-70 / RFLP using *Hae*III enzymes, and subsequently by *Bcc*I or *Rsa*I, to observe the differentiation patterns of the *Leishmania* species described in the literature [24].

2.5. Data analysis

A positive CL laboratory diagnosis was based on the tests routinely used at the Gorgas Institute for leishmaniasis diagnosis, which included parasitological tests using Giemsa-stained smears, *in vitro* cultures and/or positive PCR tests (Diagnostic criteria). Sensitivity was calculated by comparing the results of the different evaluated tests (IDRM, smears, culture, PCR *Viannia* and Hsp-70, HP and IHC) with the established positive criteria. Sensitivity calculations were estimated at a 95% CI. The results obtained from the different diagnostic tests were compared in 2 × 2 contingency tables, calculating the precision parameters, such as the degree of general agreement, sensitivity, specificity, estimated efficiency, positive predictive value (PPV) and negative predictive value (NPV), using SPSS statistics 23 and Prism 5.0 software. The Kappa index was used to determine the concordance index between tests and the percentage of general agreement.

2.6. Ethical considerations

This work was approved by the Research Bioethics Committee in the Gorgas Institute of Health Studies (Panama) and by the Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de São Paulo (Brazil) under protocol number 141/13. All participants signed an informed consent and agreed to participate in the study freely and voluntarily.

3. Results

3.1. Performance of the diagnostic tests

A total of 48 samples from patients with clinically and epidemiologically suspected CL diagnoses were analysed, of which 70.8% (34/48) corresponded to males and 29.2% (14/48) corresponded to females. The average age of the patients was 39 years, varying between 21 and 73 years. The average number of lesions was 2 (1 to 8 lesions) with an average time of evolution of 35 days (10 to 90 days); lesions were mostly distributed in the upper extremities (63%), and the rest were distributed in the lower extremities (18%), face/neck (12%), back (5%) and abdomen (4%). The majority of patients (62.5%) had lesions with an evolution time ≤ 30 days. The patients were from the province of Panama (85.4%: 41/48), Darién (10.4%: 5/48) and Coclé (4.2%: 2/48).

From the total samples analysed, 95.8% (46/48) were positive for *Leishmania* sp. by at least one of the diagnostic tests (diagnostic criteria); from the positive samples, 78.3% (36/46) were characterized as *Leishmania (V.) panamensis* by PCR-Hsp70 / RFLP.

In order to demonstrate that the IHC could be a useful tool for the diagnosis of cutaneous leishmaniasis improving the sensitivity of the HP, different diagnostic tests were compared with the IHC. From the samples positive according to the diagnostic criteria, 100% (46/46) were characterized as positive by IDRM, 60% (28/46) by direct search for parasites in the smears stained with Giemsa, 93.3% (42/46) by parasite isolation in the biphasic culture medium, 100% (46/46) by subgenus Viannia PCR, 78.3% (36/46) by Hsp70-PCR, 50% (23/46) by histopathology (HP) and 76.1% (35/46) by immunohistochemistry (IHC).

To calculate the different diagnostic parameters, the result of each test was compared with the diagnostic criteria used in the National Reference Center of Leishmaniasis in Panama, in which a sample is considered positive for CL when a positive result is obtained by at least one of the routine diagnostic tests (smear, culture or PCR Viannia). Based on this consideration, IDRM showed 100% sensitivity, 66.7% specificity, 97.8% PPV, 100% NPV and 97.9% efficiency; skin lesion smears stained with Giemsa presented 60% sensitivity, 100% specificity, 100% PPV, 14.4% NPV and 62.5% efficiency; and cultures showed a high sensitivity (91.3%) and specificity (100%), with a PPV of 100%, an NPV of 50% and an efficiency of 93.8% (Table 1).

Regarding the molecular tests, subgenus Viannia PCR obtained 100% for all parameters, including sensitivity, specificity, PPV, NPV and efficiency, while Hsp-70 PCR showed 78.3% sensitivity, 100% specificity, 100% PPV, 25% NPV and 8.3% efficiency (Table 1).

For histopathology, a low sensitivity (50%) with 100% specificity, 100% PPV, 8.0% NPV and 52.1% efficiency was obtained, while IHC reflected a better sensitivity (76.1%) and efficiency (77.1%), an equal specificity (100%), a PPV of 100% and an NPV of 15.4% (Table 1).

3.2. Agreement among the diagnostic tests

The level of concordance between the tests was calculated using the Kappa index, comparing each methodology against the diagnostic criteria mentioned above. IDRM and subgenus Viannia PCR showed

Table 1
Sensitivity, specificity, efficiency, positive predictive value (PPV) and negative predictive value (NPV) compared with the positive criteria[a] of IDRM, smear, culture, PCR *Viannia*, PCR Hsp-70, histopathology (HP) and immunohistochemistry (IHC).

Parameter	IDRM (IC 95%)	SMEAR (IC 95%)	Culture (IC 95%)	PCR viannia (IC 95%)	PCR hsp70 (IC 95%)	HP (IC 95%)	IHC (IC 95%)
Sensitivity	100% (92.1–100)	60% (44.3–74.3)	91.3% (81.7–98.6)	100% (92.3–100)	78.3% (65.4–90.4)	50% (35–65)	76.1% (62.1–86.1)
Specificity	56.7% (20.8–93.9)	100% (44–100)	100% (44–100)	100% (34.2–100)	100% (43.9–100)	100% (85–100)	100% (34.2–100)
PPV	97.8% (88.5–99.9)	100% (87.2–100)	100% (92–100)	100% (92.3–100)	100% (90.3–100)	100% (34–100)	100% (89.7–100)
NPV	100% (15.8–100)	14.4% (3.1–36.3)	50% (11.8–88.1)	100% (34.2–100)	25% (5.5–57.2)	8.0% (1–26)	15.4% (1.8–42.8)
Efficiency	97.9%	62.5%	93.8%	100%	81.3%	52.1%	77.1%

[a] Diagnosis criteria: A positive cutaneous leishmaniasis laboratory diagnosis was based on a positive parasitological test (Giemsa stained smears or *in vitro* culture) and/or PCR assay.

Table 2
KAPPA index for IDRM, smear, culture, PCR *Viannia*, PCR Hsp-70, histopathology (HP) and immunohistochemistry (IHC) according to diagnosis criteria[a]. The kappa index has a range from 0 to 1.00, in which k < 0.4 indicates poor concordance, k of 0.4–0.7 indicates good concordance and k > 0.70 indicates excellent concordance.

Parameter	IDRM	SMEAR	Culture	PCR Viannia	PCR Hsp70	HP	IHC
KAPPA index (IC 95%)	0.789	0.158	0.636	1.000	0.333	0.077	0.210
Agreement	Excellent	Poor	Good	Excellent	Poor	Poor	Poor
Overall Percent Agreement	98%	63%	94%	100%	81%	52%	75%

[a] Diagnosis criteria: A positive cutaneous leishmaniasis laboratory diagnosis was based on a positive parasitological test (Giemsa stained smears or *in vitro* culture) and/or PCR assay.

excellent concordance levels (IDRM, k = 0.789 and subgenus Viannia PCR, k = 1.000). Culturing presented good concordance levels (k = 0.636), and the other tests showed poor concordance levels in the following order: Hsp-70 PCR (k = 0.333), IHC (k = 0.210), smears (k = 0.158), and histopathology (k = 0.077) (Table 2).

When calculating the percentage of agreement between IHC and HP, 75% of the total percentage concordance (confidence interval of 27% - 66%) was obtained. The concordance between the positive results was 65.71% (95% CI: 49.15% - 79.17%), and that between the negative results was 100% (95% CI: 77.19% - 100%). The kappa index between the two tests showed a moderate agreement (k = 0.509). All positive results for HP were positive for IHC.

The number of amastigotes observed in the tissue sections was analysed by HP and IHC. For HP, 34.8% of the samples (16/46) had 1+, 6.5% (3/46) had 2+, and 8.7% (4/46) had 3+; for IHC, 21.7% of the samples (10/46) had 1+, 17.4% (8/46) had 2+, 28.3% (13/46) had 3+, and 8.7% (4/46) had 4 + .

3.3. Combinations among the diagnostic testes

The use of combined methodologies is recommended to obtain a better diagnosis [11,25,26]. Based on this, the combined sensitivity of the different diagnostic tests performed in this study was calculated. The diagnostic sensitivity of HP increased when combined with IHC, detecting 76.1% (35/46) of the positive cases. In this way, the sensitivity of culture/IHC and the smears/IHC was calculated, and these combinations detected 97.8% (45/46) and 89.1% (41/46) of the positive cases, respectively (Table 3). It was also observed that the combination of PCR or IDRM with any test increased the sensitivity to 100%. In addition, the combination of culture, smears and histopathology reached 100% sensitivity, as well as did the combination of smears with IDRM.

Table 3
Sensitivity of the combined tests used for the diagnosis of CL in Panama.

Procedures	Number of positives cases	Percentage of positives cases[b]
smear + culture	43	93.5%
smear + hp	37	80.4%
smear + ihc	41	89.1%
smear + pcr viannia	46	100.0%
smear + hsp70	40	86.9%
IHC + hp	35	76.1%
ihc + pcr viannia	46	100%
hp + pcr viannia	46	100%
culture + hsp70	43	93.5%
culture + hp	45	97.8%
culture + ihc	45	97.8%
idrm + smear	46	100%
idrm + smear + culture	46	100%
smear + culture + pcr viannia	46	100%
smear + culture + hp	46	100%
smear + hp. + ihc	41	89.1%
smear + culture + ihc	45	97.8%
idrm + smear + culture + pcr viannia	46	100%
smear + culture + pcr viannia + hp. + ihc	46	100%
idrm + smear + culture + pcr viannia + hp. + ihq	46	100%

[a] Diagnosis criteria: A positive cutaneous leishmaniasis laboratory diagnosis was based on a positive parasitological test (Giemsa stained smears or *in vitro* culture) and/or PCR assay.

[b] The percentage of positive cases was calculated based on the number of positive samples according to the diagnosis criteria[a] (n = 46).

4. Discussion

An accurate diagnosis of the *Leishmania* sp. parasite is important for adequate treatment of the patient and for the epidemiological surveillance of CL [3]. According to the WHO, new and better diagnostic approaches and alternative methodologies are necessary for the control of CL [2]. In Panama, only parasitological methods, such as lesion smears stained by Giemsa, immunological methods, as IDRM and histopathology using paraffin section stained by haematoxylin-eosin and Giemsa are performed by the country's health institutions for the diagnosis of CL [3,12]. Culture and molecular tests, such as polymerase chain reaction (PCR), are not performed in diagnostic routine, it is used only in reference centres [7, 10]. So, in order to demonstrate that the IHC is able to improve the sensitivity of HP to detect amastigotes of *Leishmania* sp., we compared the performance of IHC against other routine tests, including the HP. In this sense, we compared the IHC test against the IDRM, smears, culture, HP, Viannia PCR and Hsp-70 PCR tests which have been used in Panama to diagnose CL. A total of 48 samples from patients with suspected CL were analysed, of which 95.8% were positive for *Leishmania* sp., and characterized as *L.(V.) panamensis* by PCR-Hsp70/RFLP.

IHC tests have become one of the most important techniques in the diagnosis of human infections, such as leishmaniasis. IHC allows us to identify the antigen in tissues and evaluate the immune response of patients [25,27]. In the present study, IHC showed a sensitivity of 76.1%, similar to other studies where the sensitivity varied from 58% to 91.8% [25,28,29]. In 2009, Quintella et al. [25] reported 80% sensitivity for IHC in skin biopsies infected with *Leishmania (V.) braziliensis*. Among the tests analysed in this study, IHC had the fifth highest sensitivity (76.1%) (Table 1). PCR *Viannia* (100%), IDRM (100%), *in vitro* culture (91.3%) and Hsp-70 PCR (78.3%) were the tests with superior sensitivity; but unfortunately, only IDRM is used currently in the health centres. It is important to mention, that IDRM is an immunological test that indicates the patient's cellular immune response against *Leishmania* sp. parasite; it is an indirect diagnosis test. The disadvantage of IDRM is that it does not differentiate between past and current infections [7,30], as it has been reported that patients older than 19 months presented a positive test after treatment [31,32].

Immunostaining techniques are used to increase histopathological tests sensitivity [10,25]. These tests are based on the observation of *Leishmania* parasite amastigotes in paraffin tissue sections. It has been reported that the sensitivity of histopathological analysis is usually low [12,33,34]. In this sense, IHC is a valuable tool for the diagnosis of CL, facilitating the observation of amastigotes in tissue and avoiding confusion in the identification of the parasite, therefore improving the quality of diagnosis [15,34] (Fig. 1). Sotto et al. [29] worked with 40 biopsies of human patients with cutaneous or mucocutaneous leishmaniasis in South

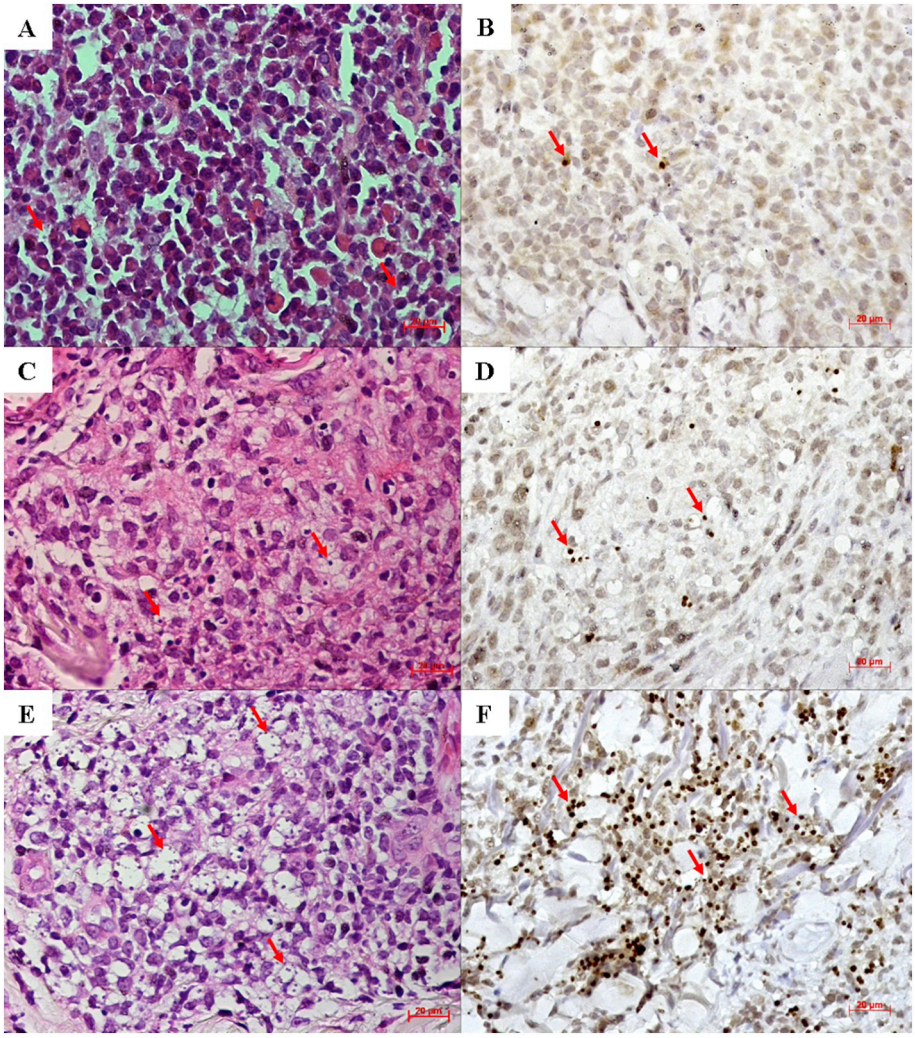

Fig. 1. Histological section of the cutaneous lesion by *L. (V.) panamensis* stained by Hematoxilin-Eosin and immunohistochemistry, respectively showing mild (A and B), moderate (C and D) and intense (E and F) parasitism in the skin tissue.

America and showed the presence of parasites in 20% of the biopsies by the usual techniques performed in histological sections stained by HE, while it was possible to visualize the parasite in 64.51% of the biopsies by immunoperoxidase reaction. In our study, the amounts of amastigotes detected by HP and IHC in the tissue were compared, and it was observed that 56% of the positive samples detected by IHC presented higher amounts of parasite amastigote forms when compared with HP. A study conducted by Quintella et al. [25] reported an improvement of sensitivity of HP (53.3%) when IHC was used (80%) in the testing of 30 skin biopsies of patients a by *Leishmania (V.) braziliensis* infection. In our study, the sensitivity of HP (50%) improved by combining it with IHC (76.1%). When comparing HP with IHC, a moderate correlation was obtained (k = 0.509), and all samples confirmed positive by HP were also confirmed positive by IHC. Although IHC had a poor correlation to with the diagnostic criteria, it increased the sensitivity from 60% to 89.1% in smear tests and from 50% to 76.1% in histopathological tests. It has been demonstrated in the literature that IHC improves the diagnostic sensitivity of conventional tests, such as smears [11].

Conventional tests have shown an unsatisfactory sensitivity for the parasitological diagnosis of CL, with a sensitivity of approximately 70% when combined methods are used [25]. The results obtained in this study demonstrate that IHC is a useful and sensitive diagnostic method for the detection of amastigotes in the lesions of patients with active CL when used in correctly processed samples, thus contributing to the improvement of the parasitological diagnosis.

The use of combined methodologies is recommended to obtain a better diagnosis [11,25,26]. In Panama, the routine diagnosis involves the performance of IDRM, lesion scraping smears stained with Giemsa in local health centres; culture in biphasic medium, PCR *Viannia* in reference centres; and histopathology in local hospitals. The PCR used for the diagnosis of CL by the Gorgas Memorial Institute of Health Studies (ICGES), showed a high sensitivity and specificity to be able to detect the positive cases, agreeing with other studies that use kDNA PCR [1,3,17,18]. In contrast, Hsp-70 presents less sensitivity, but shows high specificity and can be used for the characterization of *Leishmania* species by PCR-Hsp70/RFLP [23,24], which allows the determination of the presence of the different species of parasites that circulate in the country and can cause more severe forms of disease. The low sensitivity of Hsp-70 can probably be because of the copy number of target DNA and it can be improved by performing a Nested PCR [13,35]. Concerning culture in biphasic medium, a high sensitivity (93.7%) was observed in our study compared to others. In a study conducted in Panama with the same methodology, the culture sensitivity was 44.6% [1], and studies conducted in Brazil obtained sensitivities of 69.7% [36] and 52% [37]. The combined sensitivity of the routine tests used in the CL diagnosis reaches 100% in reference centre (ICGES), while hospitals only have low-sensitivity methods such as HP [3,12]. Therefore, the use of smears and HP increased the sensitivity from 50%–60% to 80% (Table 3), and both tests can be performed in hospitals that have a pathology service.

Based on the results shown in Table 3 regarding the diagnostic sensitivity of the combined methods, in rural areas of difficult access that have a primary health care center, the use of diagnostic tests such as smears and/or IDRM is recommended because it is easy to perform with a microscope and Giemsa stain; and they do not require special equipment or laboratories [10,19]. In regions that have hospitals with a pathology service, the use of smears, HP and IHC is recommended, providing greater autonomy in diagnosis to health centers without having to depend on the diagnosis provided by the reference center located in capital of country, Panama City, where it is often far from the primary care centers located throughout the country. In areas that have infrastructure, equipment and trained personnel, the use of molecular tests, culture and/or smear is recommended. And to carry out studies with research purposes to evaluate the immune response in patients with CL, the use of IHC tests is recommended. Based on the results, it is possible to observe that IHC is able to improve the CL diagnosis in conjunction with other tests. Currently, in Panama, we have the equipment and infrastructure necessary to perform IHC analysis in hospitals that perform routine histopathological diagnosis. In addition, the implementation of IHC is important to conduct future studies that evaluate the immune response of patients in Panama with leishmaniasis caused by *Leishmania* spp.

5. Conclusion

In conclusion, IHC was able to improve the detection of *Leishmania* parasites in histopathological sections, supporting its implementation as a tool for CL diagnosis in the regional hospitals with available pathology service in Panama. In addition, immunohistochemical techniques allow the evaluation of the patient's immune response and thus provide new guidelines for the treatment and control of CL in Panama.

Conflict of interest

The authors declare no potential conflict of interest.

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Authors' contribution

Hector Paz and Salomon Puga helped with patient sampling and care. Victor García and Rosendo Diaz performed the histopathological processing of the samples. Kadir González, Aracelis Miranda and Thaise Tomokane performed the conventional, molecular and immunehistochemical tests. José Calzada, Marcia Dalastra Laurenti, Azael Saldaña assisted in drafting the manuscript and in coordinating the study. Kadir González is the main author. Marcia Dalastra Laurenti is the corresponding author.

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