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Published online: 09 Mar 2021.

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ABSTRACT

Cutaneous leishmaniasis (CL) is endemic in Sri Lanka. Giemsa-stained slit-skin-smears (SSS-Giemsa) and histology are routinely used in diagnosis with a sensitivity of 40–70%. PCR currently has limited accessibility. Therefore, we assessed the sensitivity and specificity of a previously described fluorescence *in situ* hybridization assay, on skin smears and biopsy samples to overcome the limitations encountered with routine diagnostic methods.

Samples from a total of 123 suspected CL patients were collected and subjected to SSS-Giemsa, fluorescence *in situ* hybridization (FISH) on slit skin smears (SSS-FISH), formalin-fixed-paraffin-embedded-tissues stained with Hematoxylin & Eosin staining (FFPE-H&E) and FISH on formalin-fixed-paraffin-embedded-tissues (FFPE-FISH). Negative controls of 61 patient samples were collected from a CL non-endemic area and subjected to the same procedures. The gold standard PCR was used as a comparator. For FISH, two previously described cyanine 3 tagged *Leihsmania* genus-specific probes were used.

Compared to PCR, SSS-Giemsa, SSS-FISH, FFPE-H&E, and FFPE-FISH had sensitivities of 76.5%, 79.1%, 50.4% and 80.9%, respectively. Routine diagnostic tests (SSS-Giemsa and FFPE-H&E) had a specificity of 100%. SSS-FISH and FFPE-FISH had specificities of 96.7% and 93.4%, respectively. FFPE-FISH had a statistically significant higher diagnostic performance than FFPE-H&E ($p < 0.001$). The relative performance of SSS-Giemsa, SSS-FISH and FFPE-FISH was similar ($p > 0.05$ for all comparisons).

We conclude that FFPE-FISH is a more accurate diagnostic tool than FFPE–H&E. SSS-FISH did not have an additional advantage over SSS-Giemsa in diagnosis. However, SSS-FISH could be recommended as a minimally invasive method in studies assessing wound healing where immunological probes are used.

Introduction

Leishmaniasis, caused by *Leishmania* spp. and transmitted by the bite of an infected Sandfly, is endemic in 98 countries worldwide. Of the three clinical forms (cutaneous, mucocutaneous and visceral), cutaneous leishmaniasis (CL) is the most common manifestation (WHO 2018). Cutaneous leishmaniasis caused by *Leishmania donovani* MON 37 is endemic in Sri Lanka [1], a lower middle-income country [2]. Cutaneous leishmaniasis is endemic in 7 out of the 25 districts reporting more than 100 new cases a month [3]. In Sri Lanka, the first CL case was reported in 1992 [4] and, by 2019, the case incidence of CL had reached around 4,000 [3]. Whilst the infection is rarely life-threatening,

the resulting disfiguring scar of CL causes a significant psychosocial impact on patients' lives [5]. Scar formation could be minimized by early diagnosis and prompt treatment [6]. The currently available tests have both advantages and disadvantages [7]. In Sri Lanka, Giemsa stained slit skin smears (SSS-Giemsa) and formalin-fixed paraffin embedded tissues stained with Hematoxylin & Eosin staining (FFPE-H&E) are used for routine diagnosis of clinically suspected CL patients reporting to government hospitals. PCR is as an option available only at a few semi-government higher educational centers where it is performed less frequently on a referral basis for diagnosis and research.

Fluorescence *in situ* Hybridization (FISH) (reportedly having a specificity of 100%) in diagnosing CL on canine tissue has been successfully demonstrated [8]. However, very little information is available on the use of FISH in diagnosing human CL. One study conducted in Germany used FISH as a supplementary assay in diagnosing human CL where 16 FFPE samples were tested (FFPE-FISH) [9]. Due to the previously reported high sensitivity and specificity of FISH in the diagnosis of infectious diseases [10,11] and due to limited availability of data on FISH in the diagnosis of human CL to date, this study was designed to evaluate the sensitivity and specificity of previously designed FISH probes in the diagnosis of CL. Furthermore, we applied these FISH probes on skin smears (SSS-FISH) for the first time to assess their performance in comparison to routine SSS-Giemsa. An advantage of performing a highly sensitive and specific SSS-FISH is its potential application in assessing wound healing, immunological studies and in druggability pipelines with minimal invasion in sample collection.

Methods

Study design and sampling

A descriptive cross-sectional study was carried out over an 18-month period (2018 October-2020 March). Samples from highly suspicious CL lesions were collected from dermatology clinics conducted at District General Hospital-Matara [12], and Base Hospital-Tangalle [13], areas which are highly endemic for CL in Sri Lanka. Cutaneous leishmaniasis patient selection was carried out by a consultant dermatologist based on the guidelines defined by the Sri Lankan College of Dermatologists [14]. Patients over 18 years of age and who provided written informed consent were recruited into the study. Any person with a debilitating illness/immunosuppression, who had previously received standard treatment for CL for the same presenting lesion, with lesions at sites from which punch biopsies could not be taken (i.e. eyelid, pinna of the ear) and having a history of foreign travel to any leishmaniasis-endemic country were excluded. From each patient, three SSS and two punch biopsies from adjacent sites of the lesion's active edge were taken. The clinical profile of the lesions was recorded. Sampling was taken by adhering to the WHO recommended bench aids for CL [15].

As negative controls, discarded skin samples and impression smears of wounds were collected from patients admitted to the surgical casualty theater at Base Hospital-Panadura, Sri Lanka [16], covering a catchment area which is not endemic for CL. All patients were over the age of 18 years, and written informed consent was obtained prior to sample collection.

Reference standard

A previously optimized PCR using LITSR/L5.8S primers targeting the ITS1 region of Genus *Leishmania* with a reported high sensitivity (92.1%) and specificity (100%) for detecting *L. donovani* was used as the gold standard test in our study to confirm CL and to compare the performance of different tests [17].

Sample size

Sample size calculation was done as described in literature [18]. A minimum of 115 samples were required to detect a sensitivity of 95% with an alpha error of 0.05 and an acceptable margin of error of 4%. For negative controls, a sample size of 52 was required to estimate a specificity of 95% with an alpha error of 0.1 and an acceptable margin of error of 5%. Therefore, we screened and obtained samples from 123 patients with highly suspected CL lesions. Further, samples were obtained from 61 patients who fell under negative control criteria.

Routine tests- (SSS-Giemsa & FFPE- H&E)

Skin smears were air-dried, fixed in methanol, stained with 10% Giemsa and examined under oil immersion for the presence of *Leishmania* amastigotes [15]. The positive SSS-Giemsa were graded to assess parasite abundance in the lesions [19].

H&E staining was performed on thin (3 μm) sections of the FFPE tissues (FFPE-H&E) as routine. These were examined using an Olympus FSX100 inverted fluorescence microscope under bright light (x60).

Experimental tests – (SSS-FISH & FFPE-FISH)

FISH probes

Two previously described *Leishmania* genus-specific probes (Cyanine 3 tagged 18SrRNA probes – Leish18S 651: 5′-Cy3-GGC-GCC-ACA-CAC-CGA-ACC-3′ and Leish18S 840: 5′-Cy3-AAAGCG-GGC-GCG-GTG-CTG3′) [9] were selected and purchased from IDT, USA. The probe specificity was assessed on *Leishmania donovani* cultures established in Schneider's Insect media supplemented with 10% FBS (Sigma Aldrich). Nuclei staining was carried out using DAPI. This was then compared with Giemsa staining and scanning electron microscopy (SEM) performed on similar smears prepared from the same *in vitro* culture (Figure 1).

A macrophage marker was included in selected samples to benchmark the *in situ* assembly of the amastigotes within a macrophage. Macrophage membrane-specific Anti-CD163 antibody produced in rabbit and Anti-Rabbit IgGF(ab′)2 fragment – Atto 488 (Sigma-Aldrich) were used as primary and secondary antibodies at dilutions of 1:1000. 10% fetal bovine
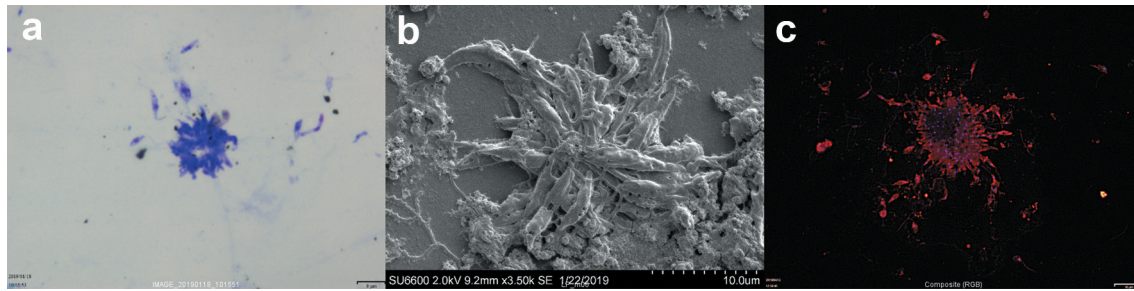
Figure 1. FISH probe specificity assessment on cultures. *L. donovani* promastigotes stained with Giemsa (A); under scanning electron microscopy (B); under FISH in which the promastigote nuclei are stained in blue (DAPI staining) and cytoplasm in red (Cyanine 3 tagged Leish18S 651 and Leish18S 840 probes) x400 magnification of Olympus FSX 100 microscope.

serum in Phosphate buffer saline was used as the blocking buffer. Atto 488 Immunofluorescence steps were done following hybridization with *Leishmania* probes (Leish18S 651 and Leish18S 840). The FISH procedure was carried out according to a published protocol [20] with modifications to match the selected probes and the size of the tissue [9,21]. A summary of the modified protocols is described below.

FFPE-FISH

For FFPE-FISH test, the tissue section was heat fixed (at 55°C for 12 h) and deparaffinized in two changes (10 min each) of Roti®-Histol. Tissue was dehydrated in two changes of 100% Ethanol (5 min each). Pretreatment was done in 0.2 N HCl and 1 N NaSCN and washed in 2X Saline Sodium Citrate (SSC). Enzymatic digestion was done with Proteinase K and 5 ng of Leish18S 651 and Leish18S 840 probes were used for hybridization at 46°C in a humid chamber for 1 h. Washing was done in SSC with Tween 20. Slides were counterstained and mounted in 10 μl of VECTASHIELD® with DAPI.

SSS-FISH

For SSS-FISH test, the SSS was fixed in 100% methanol for 10 min. Hybridization, washing and counter-staining were done as described above.

Imaging

All FISH samples were examined and images were captured with Olympus FSX100 inverted fluorescence microscope (x60 objective) under UV excitation. Image enhancement was done using Image J software (Fuji version) [22].

Data analysis

In all cases, samples were assessed independently by two individuals. Frequency distributions and cross-tabulations of results of different tests were generated

with percentages. Sensitivities, specificities, and positive and negative predictive values of each of the tests were calculated using PCR as the gold standard. The relative performances of the routine and experimental tests were compared using the McNemar's test. The performance of SSS-FISH, FFPE-H&E and FFPE-FISH were assessed against the parasite load estimated in positive SSS-Giemsa samples. Statistical analysis was done using R Version 4.0.1.

Ethics approval

Ethics approval for the study was obtained from the Ethics Review Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka (approval number 69/17).

Results

Clinical profile of the selected samples

Out of the 123 clinically suspected patients that were screened from endemic areas,115 were PCR positive (93.5%) for CL. The CL positives included a spectrum of clinical presentations, ranging from small papules to ulcers and wide plaques. This group included almost equal numbers of male and female participants (males n = 62, females n = 53) and had a mean age of 46.2 years (SD ±16.2, range = 18–77). Majority of the CL lesions were single (88.7%, n = 102), located in the upper limb (58.3%, n = 67) and were <4 months of duration (66.1%, n = 76).

In the negative controls from a non-endemic area (n = 61), 39.3% (n = 24) of the lesions were due to diabetes mellitus.

Comparison of diagnostic tests

In the PCR positive group (n = 115) from the endemic area, 42 samples became positive by all 4 methods (SSS-Giemsa, SSS-FISH, FFPE-H&E and FFPE-FISH) and 6 PCR positive samples were negative by all the other 4 tests.

Table 1. The diagnostic performance of SSS-Giemsa, SSS-FISH test, FFPE-H&E and FFPE-FISH test compared to PCR[a] (*n* = 115).

Diagnostic test	TP	FP	TN	FN	Sensitivity	Specificity	PPV	NPV
SSS-Giemsa	88	00	61	27	76.5%	100.0%	100%	69.3%
SSS-FISH	91	02	59	24	79.1%	96.7%	97.8%	71.1%
FFPE-H&E	58	00	61	57	50.4%	100%	100%	51.7%
FFPE-FISH	93	04	57	22	80.9%	93.4%	95.9%	72.2%

[a]ITS1-PCR was taken as the standard test to compare (LITSR/L5.8S primers – with a known sensitivity of 92.1% and specificity 100% [13]). TP: true positives; FP: false positives; TN: true negatives; FN: false negative; PPV: positive predictive value; NPV: negative predictive value.

The results of routine and experimental tests compared to PCR are summarized in Table 1. The routine tests had 100% specificity with sensitivities of 76.5% and 50.4% for SSS-Giemsa and FFPE-H&E, respectively. Of the experimental tests, SSS-FISH had a sensitivity of 79.1% and a specificity of 96.7%, and FFPE-FISH had a sensitivity of 80.9% and specificity of 93.4%, compared to PCR.

The routine and experimental test results were compared with each other using McNemar's test to compare the performances of the tests (Table 2). The performance of SSS-Giemsa was similar to SSS-FISH and FFPE-FISH; among SSS-Giemsa positives, 43% (n = 38) tested negative with FFPE-H&E. There was no difference in the performance of SSS-FISH and FFPE-FISH. Among SSS-FISH positives, 45% (n = 41) tested negative with FFPE-H&E; among FFPE-FISH positives, 40% (n = 37) tested negative with FFPE-H&E. Therefore, SSS-Giemsa, SSS-FISH and FFPE-FISH performed significantly better than FFPE-H&E (p < 0.001).

Parasite load of the wounds was determined by SSS-Giemsa. Parasitic grade 4+ and above were grouped due to small sample sizes in each group. Table 3 summarizes the performance of routine/experimental tests by parasite load based on results of SSS-Giemsa.

Compared to PCR, among the false negatives of SSS-Giemsa, FFPE-FISH was able to detect the most number of positives (19/27) followed by SSS-FISH (15/27) and FFPE-H&E (8/27) (Tables 2 and 3). Twelve out of the 24 samples (50%), 14 out of 22 (63.6%) and 38 out of 57 samples (63.8%) that were false negatives based on PCR by SSS-FISH, FFPE-FISH and FFPE-H&E, respectively, were positive by SSS-Giemsa (Table 1–3). In general, the percentage of SSS-Giemsa positives which became positive with the other tests increased

with parasite load but there was no clear trend in either of the routine or experimental tests (Table 3).

Figures 2 and 3 demonstrate smear and tissue results of different tests. We also demonstrated Macrophage-specific CD163 antibodies in amastigote filled macrophages by combining SSS-FISH with an immunofluorescence protocol (Figure 4)

Discussion

We evaluated a previously described FFPE-FISH method [9] which was performed on a smaller number of human samples (n = 16). Here, we used the same *Leishmania* probes with a slightly modified protocol and reproduced similar results in a larger sample. In addition, we demonstrated a sensitive SSS-FISH for the first time to diagnose CL using the same probes but with a slightly modified protocol.

Although FISH is widely used for the diagnosis of infectious diseases, studies using FISH for diagnosis of leishmaniasis are limited [7]. Application of FISH on Malaria positive blood smears had shown a sensitivity of 85.6% and a specificity of 90.6% [23]. When a species-specific digoxigenin labeled probe was used to diagnose canine CL on tissue, the sensitivity of the *in situ* hybridization assay was 70.6% with a specificity of 100% [8]. A German study which used the exact FISH probes that we used in our study reported positive results in 15 out of the 16 samples, with no false positives in detecting human CL [9]. Our SSS-FISH had a specificity of 96.7% and a sensitivity of 79.1%. For FFPE-FISH, we observed a higher sensitivity of 80.9% but a lower specificity of 93.4% than that reported in the German study. The lower specificity may be due to the background noise of the FISH assay. Background noise is a common

Table 2. The relative performance of routine and experimental tests.

Test	Result	SSS-FISH		FFPE-H&E		FFPE-FISH	
		Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)
SSS-Giemsa	Positive	76 (86.3)	12 (13.6)	50 (56.8)	38 (43.1)	74 (84.1)	14 (15.9)
	Negative	15 (55.6)	12 (44.4)	08 (29.6)	19 (70.4)	19 (70.4)	08 (29.6)
	McNemar's test (*p*-value)	0.46		<0.001		0.14	
SSS-FISH	Positive			50 (54.9)	41 (45.1)	77 (84.6)	14 (15.4)
	Negative			08 (33.3)	16 (66.7)	16 (66.7)	08 (33.3)
	McNemar's test (*p*-value)			<0.001		0.51	
FFPE-H&E	Positive					56 (77.8)	02 (22.2)
	Negative					37 (64.9)	20 (35.1)
	McNemar's test (*p*-value)					<0.001	
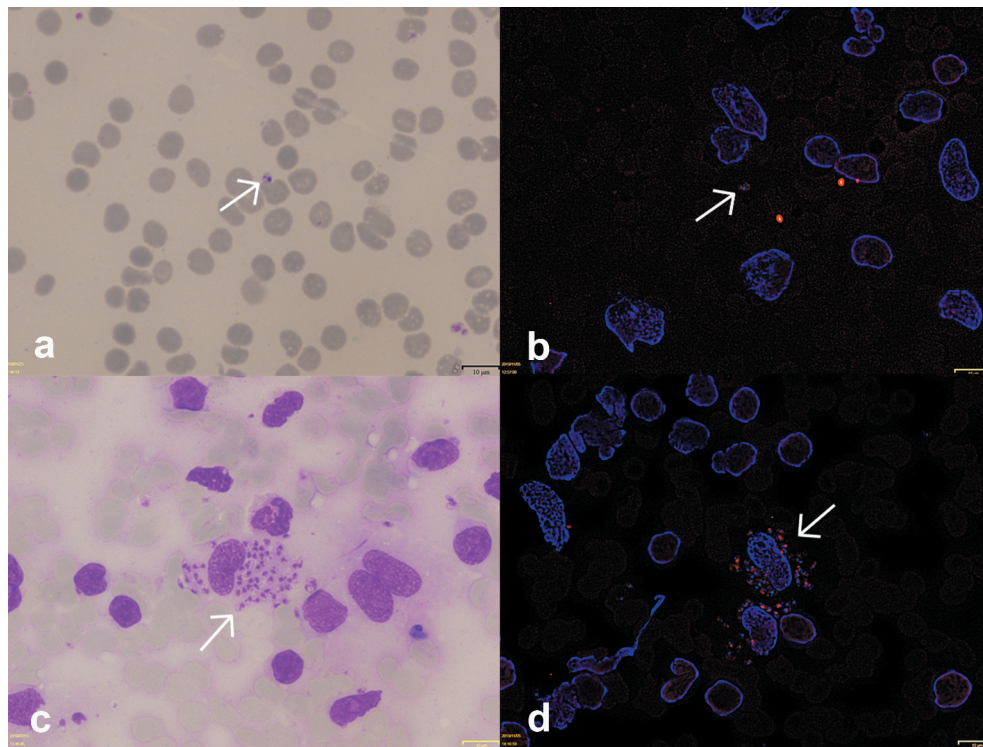
Figure 2. Positive SSS results. SSS-Giemsa in low parasite load (A); SSS-FISH in low parasite load (B); SSS-Giemsa in high parasite load (C); SSS-FISH in high parasite load (D).
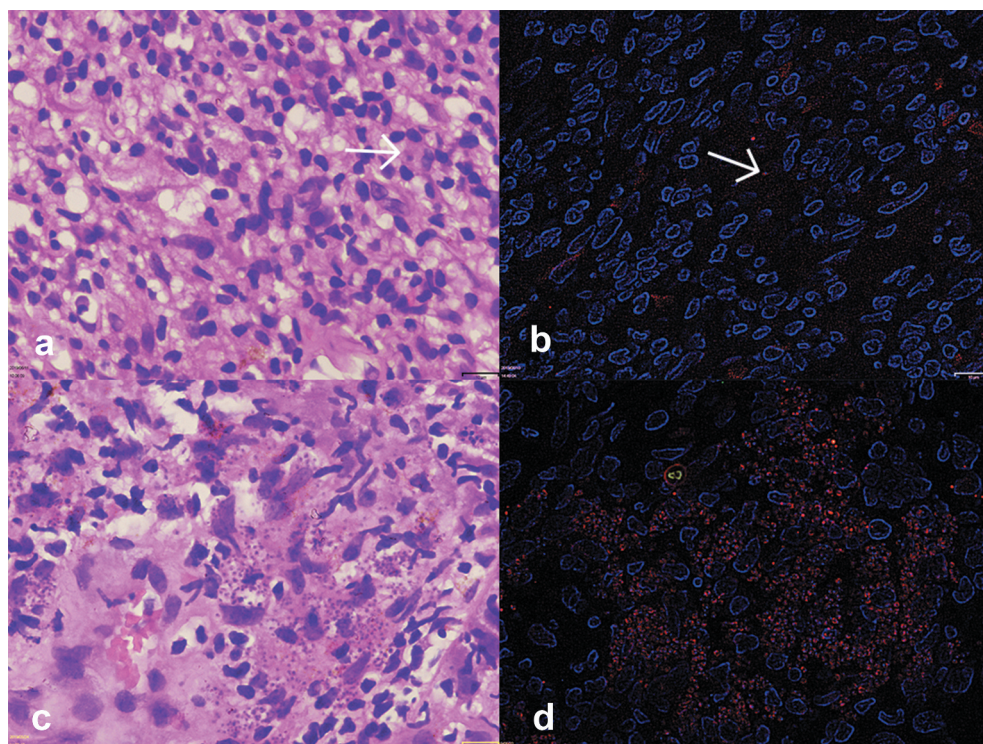

Figure 3. Positive FFPE results. FFPE-H&E in low parasite load (A); FFPE-FISH in low parasite load (B); FFPE-H&E in high parasite load (C); FFPE-FISH in high parasite load (D).

drawback of FISH on FFPE tissue. Different protocols, models and optimizing steps for FISH on FFPE sections are available in literature [21,24–26]. The discussed protocol could be experimented with and improved to reduce the background noise. Optimizing fixation; adjusting incubating durations and chemical concentrations of immersion solutions and Proteinase K pretreatment; varying probe concentrations; and changing hybridization and post-hybridization washing conditions might help in achieving better results.

Table 3. Performance of routine/experimental tests by parasite load based on results of SSS-Giemsa.

Parasite load in SSS-Giemsa	SSS-FISH		FFPE-H&E		FFPE-FISH	
	Positive N (%)	Negative N (%)	Positive N (%)	NegativeN (%)	Positive N (%)	NegativeN (%)
0	15 (55.6)	12 (44.4)	8 (29.6)	19 (70.4)	19 (70.4)	8 (29.6)
1+	16 (72.7)	6 (27.3)	12 (54.5)	10 (45.5)	19 (86.4)	3 (13.6)
2+	13 (86.7)	2 (13.3)	8 (53.3)	7 (46.7)	12 (80.0)	3 (20.0)
3+	15 (83.3)	3 (16.7)	7 (38.9)	11 (61.1)	14 (77.8)	4 (22.2)
≥4+	32 (96.7)	1 (3.3)	23 (69.7)	10 (30.3)	29 (87.9)	4 (12.1)
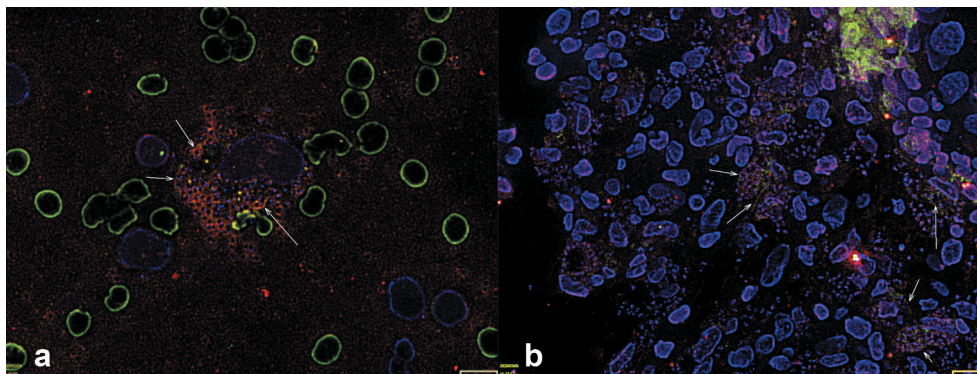
Figure 4. *In situ* **assembly of amastigotes demonstrated with macrophage marker**. Slit skin smear (A); FFPE section (B). Parasite and eukaryotic nuclei in blue (DAPI staining). Parasite cytoplasm in red (Leishmania genus-specific CY3 tagged probe). Macrophage membrane in green (Atto 488 tagged macrophage membrane marker).

Cryosections have been introduced superior to FFPE tissue in terms of better probe permeability [24]. Also, a specific molecular beacon could be tested with instead of the regular linear DNA probes to minimize the background noise [26].

The FISH test that we applied on an SSS is minimally invasive and could be performed on air-dried smears fixed in methanol; the whole test can be performed within 2 hours compared to about 7 hours if PCR is done on invasive punch biopsy samples. The SSS-FISH method is, therefore, a major advantage for a diagnostic center. However, FFPE-FISH would still require long processing times (~48 h) and invasive biopsies although they showed a significantly higher sensitivity than the FFPE-H&E method. This highlights the advantage of SSS-FISH over FFPE-FISH as there was no significant difference in the relative performance between the two tests (Table 2). However, the performance of SSS-Giemsa, SSS-FISH and FFPE-FISH was significantly better than FFPE-H&E ($p < 0.001$).

Another novel advantage of SSS-FISH we identified in our study is the ability to demonstrate amastigote-infected macrophages in a skin smear using immunofluorescent antibodies and FISH probes. Since SSS is a minimally invasive method of sample collection, the possibility of using SSS-FISH in studies following up wound healing where immunological probes are required is an area that needs to be pursued further. SSS-FISH may replace invasive FFPE-FISH, FFPE-H&E in such studies without causing any disruption to wound healing, causing any iatrogenic secondary bacterial infections or scar formation.

A limitation of FISH is that it requires basic knowledge and skills of staining using fluorescent dyes and afluorescence microscope. There are published reports on less costly equipment which could be attached to light microscopes in generating fluorescence signals [10]. Therefore, this may be considered in low-resource centers.

PCR may still be considered the standard diagnostic test in CL as it is highly sensitive and specific in diagnosing chronic lesions of CL and lesions with low parasite loads [27]. In the current study, PCR detected CL in 93.5% (n = 115) of clinically suspected samples (n = 123).

Conclusion

We demonstrated the successful application of the FISH technique on SSS and FFPE sections for the diagnosis of human CL in a large sample. PCR with a biopsy sample is still the most sensitive method to diagnose CL. SSS-Giemsa, which is cheaper, would still be good for routine assessments in resource-poor laboratories. SSS-FISH may be used in studies requiring immunological probes as it is a minimally invasive method of sample collection. FFPE-FISH is a more accurate diagnostic tool than FFPE–H&E.

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