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Diagnosing Cutaneous leishmaniasis using Fluorescence *in Situ* Hybridization: the Sri Lankan Perspective

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ABSTRACT

Cutaneous leishmaniasis (CL) caused by *Leishmania donovani* MON-37 is becoming a major public health problem in Sri Lanka, with 100 new cases per month being reported in endemic regions. Diagnosis of CL is challenging for several reasons. Due to relative specificity and rapidity we propose Fluorescence *in Situ* Hybridization as a diagnostic tool for CL.

The objective was to evaluate the potential of Fluorescence in Situ Hybridization as a diagnostic method for Cutaneous leishmaniasis in Sri Lanka.

Literature on current laboratory tests used to diagnose Cutaneous leishmaniasis in Sri Lanka and globally was reviewed. Sri Lankan data were reviewed systematically following the PRISMA guidelines. A narrative of the results is presented.

There is currently no gold standard diagnostic method for Cutaneous leishmaniasis. Fluorescence *in Situ* Hybridization has been previously applied to detect dermal pathologies including those involving infectious agents, and its use to detect the *Leishmania* parasite in human cutaneous lesions reported in small number of studies, generally with limited numbers of subjects. Advantages of FISH has been specificity, cost and ease-of-use compared to the alternatives.

Based on the available literature and our current work, FISH has potential for diagnosing CL and should now be evaluated in larger cohorts in endemic regions. FISH for CL diagnosis could find application in countries such as Sri Lanka, where laboratory facilities may be limited in rural areas where the disease burden is highest.

KEYWORDS

Leishmania; Cutaneous leishmaniasis; Fluorescence *in Situ* Hybridization; diagnosis; Leishmania donovani; PCR; Slit skin smear; Sri Lanka

1. Introduction – cutaneous leishmaniasis

Cutaneous leishmaniasis (CL), caused by the protozoan *Leishmania*, has been described in historical texts by medieval writers as 'Balkh sore' [1]. It is a vector-borne disease transmitted by the Sand fly [2]. Once inoculated, metacyclic stages of the *Leishmania* promastigotes are taken up by dermal macrophages where they transform into obligatory intracellular amastigotes [3]. The World Health Organization lists CL as the commonest type of the three clinical forms of Leishmaniasis, which are visceral, cutaneous and mucocutaneous [2]. Cutaneous leishmaiasis involves papules, nodules, ulcers (wet or dry) and plaques on exposed areas of the skin and leaves disfiguring scars following healing [4,5]. Leishmaniasis represents the 9th largest global infectious disease burden, with an annual incidence of 0.7 to1.2 million CL cases [6]. The global disability adjusted life years lost by CL is 0.58/100,000 [7].

The first indigenous case of Cutaneous leishmaiasis in Sri Lanka was reported in 1992 although other forms of leishmaniasis are uncommon [8]. Sri Lankan CL is unique since the only causative strain identified so far; *Leishmania donovani* MON-37, is generally a visceralizing zymodeme elsewhere in the world [9]. *L. donovani* has also been associated with CL in some other locations including parts of India [10], Lebanon [11], Turkey [12] and Sudan [13]. According to the Sri Lankan Weekly Epidemiological Reports, the incidence of CL is increasing in Sri Lanka, with an annual case incidence of 1508 reported from 1st January to 29 December 2017, rising from 428 in 2010, 1216 in 2012 and 1253 in 2016 [14].

2. Method

A literature search was conducted between March 2018 and December 2018. Data of journal articles and abstracts written in English, if the full text/abstract was available online were included. Manuscripts lacking required data were excluded from the review. The first 100 results generated using relevant phrases on Google Scholar were explored. We also wrote to authors to

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receive data on Sri Lankan studies that were not published online. In addition, a systematic literature review was conducted following the 'Preferred Reporting Items for Systematic Reviews and Meta-Analyses' (PRISMA) guidelines to search for Sri Lankan publications [15]. We searched PubMed database using the search term '(Sri Lanka) AND Cutaneous leishmaniasis [Medical Subject HeadingsTerms (MeSH)]' and searched manually with non-MeSH terms such as 'diagnosis' with a filter restricting to studies on humans, in the advanced search system of PubMed. The target population and the exposure of interest were patients with Cutaneous leishmaniasis, diagnosed either clinically or by laboratory methods. There was no control group because no comparison with non-Cutaneous leishmaniasis group was necessary for the research aim. Studies that described sensitivity and specificity of laboratory tests used to diagnose CL were included. Case reports, if full text/abstract was not available and manuscripts lacking required data were excluded from the systematic review. The summary of the literature search of Sri Lankan studies is outlined in flowchart 1.

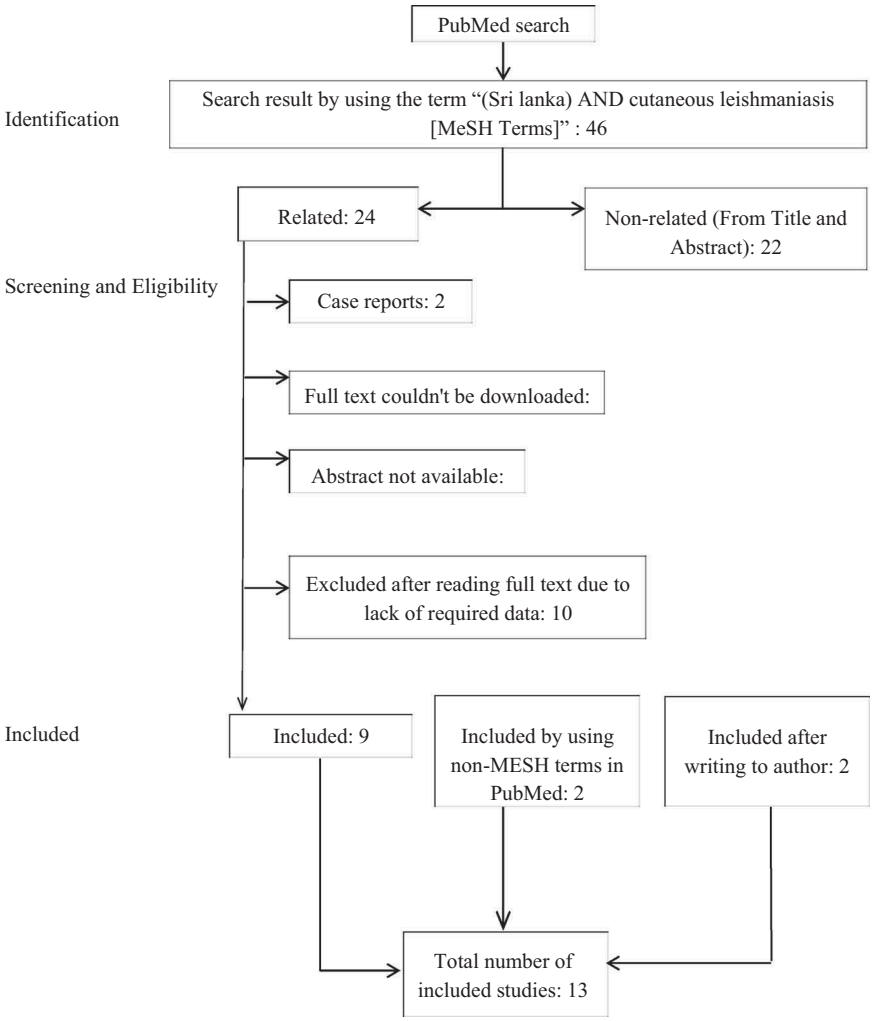
2.1. Data extraction and heterogeneity analysis of the sri lankan data

When summarizing the sensitivity and specificity of the tests (Table 4), ranges of PCR test performance are given by grouping studies that have used different primers according to their primer target. Also DNA extraction done by any commercial kit was considered similar. Ranges of culture test performance are given by grouping studies according to the culture technique and media used.

3. Findings

3.1. Diagnosis of cutaneous leishmaniasis

The differential diagnosis of Cutaneous leishmaniasis (CL) is challenging [16,17]. Accurate early diagnosis is important to prevent unnecessary patient exposure to toxic drugs and to minimize scar formation [18]. A recent World Health Organization report highlights the need for an effective diagnostic tool [19]. CL is currently diagnosed by direct (microscopy, histopathology and



Flowchart 1. Summary of the systematic literature search of Sri Lankan studies on cutaneous leishmaniasis and diagnosis.

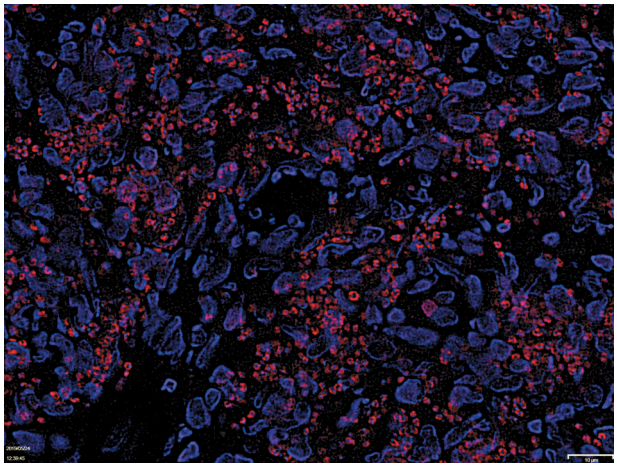


Figure 1. Leishmania donovani amastigotes in formalin fixed paraffin embedded tissue section. Amastigote nuclei, tissue cell nuclei stained with 4', 6-diamidino-2-phenylindole and amastigote cytoplasm stained with a 18s rRNA targeted, Cyanine-3 tagged Leishmania genus specific DNA probe.

culture) and indirect methods (serology and molecular assays) [20]. Routine blood tests, i.e. full blood count and blood picture, have no value in diagnosing CL [21].

3.2. Sample collection methods

As recommended by WHO, cutaneous samples for diagnosing Cutaneous leishmaniasis (CL) should be collected from the indurated margin of the lesion [22]. However, according to a Columbian group, which has studied on *L. panamensis* and *L. braziliensis* in a group of 115 CL patients, sample collection from the base of the lesion resulted in better diagnosis sensitivity due to high parasitic load at the centre of the lesion and helped in preservation of the morphology of the amastigotes [23].

For microscopy, samples are normally collected as skin scrapings, impression smears, slit skin smears, and aspiration smears, by using filter papers and as sequential tape stripping [24] [25]. 'Press impression smears' are invasive, requiring a biopsy [26]. Histopathological diagnosis of CL is performed on invasive biopsy samples. Cultures are performed with fine needle aspirations or cutaneous biopsies. However one study, which has been performed on 7 CL patients with lesions in extremities and no mucosal lesions, reports the isolation of the parasite in saliva, which has promising implications on disease surveillance if further tested on a larger cohort [27]. In Sri Lanka, polymerase chain reaction (PCR) is usually performed on biopsy samples. However, wound swabs and aspiration samples have been used, with the former being more sensitive [28]. Also PCR-restriction fragment length polymorphism analysis has been performed on skin smears of CL patients as a less invasive approach [29]. Serological techniques require drawing of blood.

3.3. Diagnostic methods

The choice of the diagnostic test for CL depends on feasibility and accuracy. There is no single test currently considered as the 'gold standard' [30]. Parasitological diagnosis by direct methods such as PCR and isoenzyme analysis due to high specificity and indirect methods, due to high sensitivity have been considered as reference tests by various research groups [31–33]. Since the majority of Sri Lankan patients are reported from remote areas where minimum laboratory facilities are available, the microscopic method is commonly used and the rest are performed only at reference centres on a referral and research basis. Details of the characteristics of the included Sri Lankan studies, properties of the direct and indirect tests and sensitivity and specificity ranges of the tests are given in Tables 1–4, respectively.

3.3.1 Microscopic examination

Microscopic examination is both quick and easy to perform with relatively high specificity but low sensitivity. In a Turkish study conducted on 1,104 patients, microscopy had a sensitivity of 61.9% compared to a standard PCR [42]. According to the majority of publications, the sensitivity of microscopy on slit skin smears done in Sri Lanka is approximately 73% with a specificity of 100% compared to PCR [43]. The sensitivity of microscopic examination drops further with the duration of the lesion and if the amastigotes load is low [44]. Sensitivity depends on the sample collection method, where it has been reported that slit skin smears have a higher sensitivity (63.5%) compared to aspirate and filter paper smears [42]. It has been reported that the use of aspirate smears results in higher sensitivity (89%) compared to the scraping method [45]. Drop scrapings have shown a greater sensitivity (63.6%) than smear scrapings (38.6%) [46].

3.3.2. Histopathological diagnosis

Histopathological diagnosis helps in classifying the stage of CL as early and late based on dermal and epidermal changes [47]. Microscopic and histopathological examination of amastigotes can be

Table 1. Characteristics of the included Sri Lankan studies.

Reference	Journal	Cases included	Samples	Tests done for parasitic confirmation
[34]	Ceylon Medical Journal	Clinical	Slit skin smears, Biopsy	Microscopy, Histopathology
[35]	Annals of Tropical Medicine & Parasitology	Clinical	Lesion aspirates, slit skin biopsies	Microscopy, Histopathology, Culture
[54]	Scientific Electronic Library Online	Smear (direct) positives	Biopsy	Polymerase chain reaction
[36]	Annals of Tropical Medicine & Parasitology	Clinical	Lesion aspirates	Microscopy, Culture
[59]	Ceylon Medical Journal	Smear (direct/culture) positives	Lesion aspirates	Loop-mediated isothermal amplification assay
[37]	Annals of Tropical Medicine & Parasitology	Clinical	Lesion aspirates	Microscopy, Culture
[38]	The Korean Journal of Parasitology	Clinical	Slit skin smears, Biopsy	Microscopy, Polymerase chain reaction
[43]	PLOS ONE	Clinical	Slit skin smears, Biopsy	Microscopy, Polymerase chain reaction, Rapid diagnostic immunochromatographic strip
[51]	Annals of Tropical Medicine & Parasitology	Clinical	Lesion aspirates	Microscopy, Culture
[39]	BMC Infectious Diseases	Clinical	Slit skin smears	Microscopy
[63]	The American Journal of Tropical Medicine and Hygiene	Clinical	Biopsy	Microscopy, Histopathology
[40]	Proceedings of the annual academic sessions of Sri Lanka College of Microbiologists – Abstract	Laboratory confirmed – method not defined	Slit skin smears, Lesion aspirates	Microscopy, Culture
[41]	Proceedings of the annual academic sessions of Sri Lanka College of Microbiologists – Abstract	Clinical	Slit skin smears, Lesion aspirates, Biopsy	Microscopy, Polymerase chain reaction

Table 2. Properties of the direct tests discussed in the included Sri Lankan studies.

	Microscopy		Histopathology	Cultures		
Reference	Type of smear	Stain	Stain	Technique	Culture medium	Day of detection of most number of amasitgotes
[34]	Slit skin	Giemsa	Haematoxylin-eosin			
[35]	Aspirate	Giemsa	Not specified	Not specified	Not specified	Not specified
[36]	Aspirate	Giemsa		Micro culture	RPMI 1640	3–6
				Test tube culture	EMTb	3–6
[37]	Aspirate	Giemsa		Micro culture	RPMI 1640	3
				Micro culture	M 199	7
[38]	Slit skin	Giemsa				
[43]	Slit skin	Giemsa				
[51]	Aspirate	Giemsa		Micro culture	RPMI 1640	3
				Modified Micro culture	RPMI 1640	3
				Test tube culture	EMTb	Not specified
[39]	Slit skin	Giemsa				
[63]	Tissue impression	Giemsa	Not specified			
[40]	Slit skin Aspirate	Not specified		Not specified	Not specified	Not specified
[41]	Slit skin	Not specified				

EMTb – Evans' modified Tobie's medium.

Table 3. Properties of the indirect tests discussed in the included studies.

	PCR					LAMP		
Reference	Method of DNA extraction	Type of PCR	Primer	Target of the parasitic DNA	Amplification	Primer	Target	IC-RDT
[54]	Commercial kit	Conventional PCR	JW11/12	KDNA	120bp			
			LITSR/L5.8S	ITS1 region	320bp			
			LdF/LdR	KDNA	600bp			
[59]	Commercial kit	Nested PCR	R221/R332	KDNA	603bp	FIP/BIP	KDNA	
			R223/R333		358bp	F3/B3C		
[38]	Commercial kit	Conventional PCR	T2/B4	KDNA	260 bp			
[43]	Commercial kit	Conventional PCR	LITSR/L 5.8S	ITS1 region	320bp			Cell lysate tested on a strip
[41]	Not specified	Conventional PCR	Not specified	18s rRNA	Not specified			

PCR – Polymerase chain reaction; LAMP – Loop-mediated isothermal amplification assay; IC-RDT – Rapid diagnostic immunochromatographic strip.

confused with other parasites i.e. *Trypanasoma* species, resulting in false positives, unless examined by an expert [48]. If the parasitic load is low, the process could be long and cumbersome. According to a Brazilian study, the sensitivity and specificity of histopathological diagnosis has been improved from 14–50% up to 83.3% with 100% specificity by coupling with immunohistochemistry and immunocytochemistry [49].

3.3.3. Cultures

Compared to PCR, culture has low sensitivity (29%-50.7%) and compared to microscopic examination sensitivity is approximately 76% [42]. Cultures are

Table 4. Sensitivity and Specificity ranges of the tests.

Reference	Test	Gold standard	Sensitivity range	Specificity range	Range of negative predictive value
[43]	Microscopic examination of slit skin smears	Conventional PCR targeting ITS1 region	73%	100%	58%
[36,37,51]	Micro culture using RPMI 1640 medium	Microscopic examination of lesion aspirate smears	50%-77.3%	64%-83.3%	-
[51]	Modified micro culture using RPMI 1640 medium	Microscopic examination of lesion aspirate smears	67%	82%	-
[37]	Micro culture using M199 medium	Microscopic examination of lesion aspirate smears	61.9%	72.2%	-
[36,51]	Test tube culture using EMTb medium	Microscopic examination of lesion aspirate smears	40%-54.8%	46%-50%	-
[54]	Conventional PCR, primers targeting kDNA	Microscopic examination of slit skin smears	71.1%-92.1%	100%	-
[54]	Conventional PCR, primers targeting ITS1 region	Microscopic examination of slit skin smears	92.1%	100%	-
[59]	Nested PCR	Microscopy (direct/cultures)	100%	100%	100%
[59]	4 primer LAMP	Microscopy (direct/cultures)	82.6%	100%	66%
[43]	IC-RDT	Conventional PCR targeting ITS1 region	37%	36%	-

PCR – Polymerase chain reaction; LAMP – Loop-mediated isothermal amplification assay; IC-RDT – Rapid diagnostic immunochromatographic strip.

prone to contamination, especially during sample collection and bedside inoculation, resulting in low diagnostic accuracy. Establishing cultures on artificial media is time consuming and requires technical expertise. The diagnostic accuracy of *Leishmania* culture varies with the technique and culture medium used. The micro culture technique has been reported to be more accurate than routine test tube methods [50]. Sri Lankan data shows that microculture using RPMI1640 medium has a negative predictive value of 67% and sensitivity of 82% compared to microscopic examination of lesion aspirate smear [51]. However, cultures are useful in strain typing via Multilocus Enzyme Electrophoresis (MLST) for example, which is considered to be the gold standard for genus typing by WHO [52], but is only applicable on cultured *Leishmania* species [53].

3.3.4. Molecular methods

Both globally and in Sri Lanka, PCR shows high sensitivity and high specificity in detecting *Leishmania* parasite compared to conventional diagnostic methods [28,42,54]. PCR results differ with the sample, the DNA extraction method, the thermal cycle, the copy number of the amplified gene and the primer used [55]. For example, LITSR/L5.8S and 13A/13B primers have a sensitivity of 100% whereas Lmj4/Uni21 primer pair has a sensitivity of 79% [56]; KDNA is more sensitive than other primer targets in diagnosing CL using PCR [57]. Real time PCR is preferred over conventional PCR as it has a sensitivity of 96.8% as compared to the latter, which is about 92.4% [42]. Fluorescence Resonant Energy Transfer based real time PCR is said to be highly sensitive and specific for species differentiation and rapid compared to other real time PCR assays [58]. Based on Sri Lankan data, nested PCR has been reported to have a sensitivity of 100% as compared to microscopy [59].

Isothermal Recombinase Polymerase Amplification combined with Lateral Flow Immunochromatographic strip has been tested for diagnosis of CL; the authors claim it to be highly sensitive and specific and recommend that it could be used as a point of care diagnostic test [60]. Quantitative Nucleic acid Sequence-based Amplification, which is useful in diagnosing live *Leishmania* parasites and quantification, has shown a sensitivity of 97.5% and specificity of 100% compared to conventional PCR in a study conducted in Netherlands involving 55 CL positive skin biopsies [61]. Loop-mediated Isothermal Amplification (LAMP) follows a simpler procedure compared to PCR in diagnosing CL; it is a rapid test with high sensitivity [62]. In a Sri Lankan study, '4 primers LAMP' had a sensitivity of 82.6% and a specificity of 100% as compared to microscopy [59].

An improved rapid method of DNA extraction and Recombinase Polymerase Amplification has been described in a recent study using a mobile suitcase method with a reported sensitivity of 65.5% and specificity of 100% in Sri Lanka. This has potential for application as a point of care diagnostic test [63]. However, positivity of molecular assays does not necessarily confirm the presence of live parasites. Molecular assays detect DNA which may have been released from nearby sites; the entire parasite need not be present for its detection [48]. Molecular techniques are relatively expensive, should be placed at tertiary care laboratories, demand sophisticated equipment and require well trained staff to perform. Few standardized protocols are available for these tests which are liable for contamination.

3.3.5. Serology and direct antigen detection

Indirect Immunofluorescence Assay (IFA), Enzyme Linked Immunosorbent Assay (ELISA), Enzyme Immune Assay (EIA), Flow Cytometry and Immunoblotting (Western

blot) are the current methods available for serological diagnosis of CL [64]. Some studies have shown Western blot as the most sensitive (100%) [65]. EIAs have a reported sensitivity of 93.3% and a specificity of 90.8% [64].However, these tests are expensive, require laboratory infra-structure and are time consuming. Flow cytometry has been shown to be more sensitive than ELISA but less specific [66]. In these tests low specificity is due to cross reactivity of antibodies [65] and may produce false interpretations in immunosuppressed patients. K39 ELISA is reportedly less effective in diagnosing CL compared to Immunofluorescence titres due to low antibody load [67]. rK39 dipstick test has shown to be specific in detecting visceral leishmaniasis with no cross reaction with species causing CL in Brazil where both stains coexist [68]. However, serological assays are useful in seroepidemiological surveys and follow up of patients [69].

In a Sri Lankan study, rapid diagnostic immunochromatographic strip targeting the peroxidoxin antigen of *Leishmania* species, had a very low sensitivity and specificity (37% and 36%, respectively) compared to PCR [43].

3.3.6. Montenegro skin test

The Montenegro skin test, which detects skin hypersensitivity to *Leishmania* antigens, is not frequently effective in detecting acute infection in the disseminated form of CL, and in immunocompromised patients. It has a low specificity due to false positives. However due to its high sensitivity and non-invasive nature, it has been suggested as a complementary test to clinical diagnosis of CL [44,70].

3.4. Fluorescence in Situ Hybridization and cutaneous leishmaniasis

3.4.1 Fluorescence in Situ Hybridization applications in general

Fluorescence *in situ* Hybridization (FISH) was first introduced in the 1980s for mapping genes on Drosophila polytene chromosomes [71]. Since then, the method has advanced a great deal. In FISH, a probe with highly conserved DNA sequence tagged with a fluorophore, which can hybridize to target complementary DNA sequences in samples, is used. It is considered as a reference method for molecular investigations in malignancies, prenatal diagnosis and microbial and parasitological diseases [72–76]. The method has been applied successfully to diagnose dermal malignant conditions such as melanomas, especially to discriminate ambiguous lesions [77–80]. It can be performed on formalin fixed, paraffin embedded (FFPE) tissues and requires less technical expertise as compared to other molecular cytogenetic methods such as comparative genomic hybridization [81]. FISH has also been applied characterizing culture negative bacterial skin lesions by a Danish research group [82]. It has also been tested on tissues to diagnose fungal infections where PCR could not be applied due to the possibility of contamination, especially when targeting pervasive fungi [83]. FISH has been successfully applied in diagnosing Varicella zoster skin lesions in formalin fixed paraffin embedded samples [84].There are several studies where FISH has been performed to identify obligatory intracellular organisms in situ [85,86, 92].

3.4.2. Fluorescence in Situ Hybridization applications in parasitology

Filariasis [87], Cryptosporidiosis [88], Giardiasis [88], Amoebiasis [89], Trypanosomiasis [90], Malaria [91] Schistosomiasis [93] and Trichomoniasis [94] are examples of parasitological diseases investigated by FISH to date. As explained in a South East Asian regional study on Malaria and FlSH, the testcould be implemented in laboratories with limited facilities [91]. However, thus far, FISH is not being used as a diagnostic test for parasitic infections in Sri Lanka. As Section 4.1 highlights, FISH could successfully be used on skin tissue and in identifying obligatory intracellular organisms. It is therefore justifiable to use FISH as a diagnostic method in CL in which dermal tissue harbouring the intracellular parasite is used. To date, FISH has been commonly used for genetic studies of *Leishmania* parasite [73,75]. There are however limited studies that have investigated the use of *In Situ* Hybridization in diagnosing Leishmaniasis [48,74,95]. The characteristics of these three studies are tabulated in Table 5.

3.4.3. Sample collection for fluorescence in Situ Hybridization to diagnose cutaneous leishmaniasis

The successful application of FISH on formalin fixed paraffin embedded human tissues to diagnose CL has been reported in a single study [95]. The requirement for a biopsy sample of at least 3mm makes the technique invasive. However, there are protocols published for performing FISH on smears for microbiology and parasitological diagnoses [91,96,97]. These methods may be adapted and optimized to establish 'FISH on smears' enabling it to be used as a less invasive test to diagnose CL.

3.4.4. Advantages of fluorescence in Situ Hybridization in diagnosing cutaneous leishmaniasis

Due to rapid identification of the parasite in formalin fixed paraffin embedded tissue (1.5h without deparaffinization and 2.5 h with deparaffinization), low material cost, and high contrasting staining of the amastigotes which enable less experienced individuals to diagnose with high specificity even at low microscopic magnifications, some researchers have suggested using FISH as a routine diagnostic procedure for CL [95]. The limit of detecting *Leishmania*

Table 5. Characteristics of the studies that investigated In Situ Hybridization in diagnosing Leishmaniasis.

Reference	Type of probe	Target	Sequence	Specimen	Gold Standard	Sample size	Results
[48]	Digoxigenin labelled Genus specific	5.8S rRNA	5′ ACG-GGG-ATG- ACA-CAA-TAG- AGC-TTC-TCC3′	Formalin fixed paraffin embedded canine tissues	Histology and PCR	3 histologically confirmed 10 clinically suspected	All ISH positive All ISH negative All Histology negative 7 PCR negatives and 3 PCR positives
[74]	Digoxigenin labelled Genus specific	5.8S rRNA	5′ ACG-GGG-ATG- ACA-CAA-TAG- AGC-TTC-TCC3′	Formalin fixed paraffin embedded canine skin tissues	Culture	51 culture positives	Sensitivit y – 70.6% Specificit y – 100% Sensitivit y – 74.5% Specificit y – 100%
	Digoxigenin labelled Species specific (*L. infentum*)	kDNA	5′ GCC-CCT-ACC- CGG-AGG-ACC- AGA-AAA-GTT3′				
[95]	Cy3 labelled Genus specific	18s rRNA	5′ Cy3-GGC-GCC- ACA-CAC-CGA-ACC3′ 5′ Cy3-AAA-GCG- GGC-GCG-GTG-CTG3′	Formalin fixed paraffin embedded human tissues	Microscop y	16 Microscopy positives	15 FISH positives

amastigotes by FISH in blood is 100 times greater than in ordinary microscopic method [97]. In a study where FISH was applied to canine skin tissue to diagnose CL due to *L. infentum*, a specificity of 100% was reported for genus and species level probes which was higher than that for immunohistochemistry and histopathology (70.6% and 74.5%, respectively) [74].

Unlike culture, FISH does not rely on live cells and organisms [84], and in contrast to PCR, FISH detects intact amastigotes within tissue, enabling morphological and distributional evaluation to quantify the parasitic load in relation to the lesion which could help in staging the severity of the disease [98]. Compared to PCR and standard multilocus enzyme electrophoresis, FISH requires general facilities needed for histopathology and can be used to diagnose at species level without the need of culture facilities, which is time consuming to produce results [99]. This ability of FISH to visualize the distribution of different species in the skin lesion using differently tagged FISH probes [100] should be performed with control probes as recommended to minimize the non-specific binding of the probe, and careful interpretation of the signals is advised for a reliable diagnosis [84]. Compared to serological tests, FISH is not prone to produce false positive results due to possible cross-reactivity and also indicates the duration of the disease [30,101].

3.4.5. Disadvantages of fluorescence in Situ Hybridization in diagnosing cutaneous leishmaniasis

Fluorescence *in situ* Hybridization (FISH) can produce false positive results due to auto fluorescence and lack of specificity of the probe; it can also give false negatives due to low penetration, low target material (i.e. RNA) and photo bleaching [102]. There are models and different protocols designed to limit these errors [103]. Other limitations include using only a limited number of target probes at a time to minimize cross hybridization and overlapping of signals, and the need of a previously specified DNA sequence [81].

Degradation of parasitic traget nucleic acid with storage time, false negatives in low parasitic loads and fewer publications on reliable species-specific probes are problems identified in this method. Using FISH as a diagnostic method in CL should further be tested on a larger sample in an endemic area.

3.4.6. Research team's experience in fluorescence in Situ Hybridization and cutaneous leishmaniasis

Promising results have been obtained from an ongoing study in our laboratory on FISH and CL. Figure 1 , shows the presence of *Leishmania donovani* amastigotes in tissue. Amastigotes' nuclei are stained with 4', 6-diamidino-2-phenylindole and cytoplasm is

stained with a 18s rRNA targeted, Cyanine-3 tagged *Leishmania* genus specific DNA probe. Investigations are in progress to assess test sensitivity and specificity.

4. Discussion

More than 100 new cases of CL per month have been reported in Sri Lanka between 2009 and 2016, mainly from the Central, North Western, Southern, Sabaragamuwa, Eastern, Northern and Uva Provinces, sparing inland regions. These are remote areas of the country where agricultural-based livelihoods predominate.

CL is currently diagnosed by direct (microscopy, histopathology and culture) and indirect methods (serology and molecular methods); there is no gold standard to date. Each test has its advantages and disadvantages in terms of accuracy and feasibility.

Since the introduction of FISH, the methodology has evolved and is now being used as a molecular diagnostic tool which, could be performed by less experienced operatives in laboratories with basic histopathology facilities. As indicated by regional studies, FISH could be implemented as a comparatively lower cost test provided that a fluorescence microscope is available. Various protocols have been published addressing how to minimize the drawbacks of FISH such as background noise.

Considering the successful application of FISH in diagnosing dermal pathologies such as Melanomas and in visualizing pathogens *in situ*, combined with the ability of the method to rapidly identify organisms to species level and to detect intact organisms in their natural environments, FISH could be optimized to be used as a routine diagnostic method of CL. The little available evidence in literature and our team's preliminary findings, in detecting *Leishmania* parasites in skin by FISH shows promising results such as higher accuracy compared to conventional diagnostic methods. Currently FISH has been introduced as a supplementary test in diagnosing CL [95], and more testing should be done in cohorts living in endemic regions. There is a need to develop and validate a non-invasive method for sample collection from CL lesions for FISH analyses which is currently being investigated by us.

5. Conclusion

Considering both global and Sri Lankan data on CL diagnosis, each test has inherent advantages and disadvantages. FISH could be a new way forward. There is currently an unmet need for a sensitive, specific, and less invasive diagnostic test for CL for use in settings with minimal laboratory facilities; FISH may be by which it could be achieved. Such a test would be beneficial to the patients and would also open up new pathways in research for Sri Lankan CL.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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