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A comparison of the efficiency of three sampling methods for use in the molecular and conventional diagnosis of cutaneous leishmaniasis

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ABSTRACT

In human cutaneous leishmaniasis (CL), the success of positive diagnoses and species identifications depends, primarily, on how biopsies are taken and then processed and examined. The efficiency of three methods of taking skin biopsies from suspect cases of CL was compared using the classical methods of microscopy of stained smears, *in vitro* culture of tissue aspirate, and internal transcribed spacer region 1 (ITS1)-polymerase chain reaction in diagnosing positive cases and identifying the species of *Leishmania* causing them. From 1994–2014, biopsy samples from the skin lesions of 2232 CL-suspected patients were collected as unstained smears, as smears stained with Giemsa's stain and on filter paper, and compared in the diagnostic tests employed. Matched comparison based on testing biopsy samples from 100 patients, microscopy, *in vitro* culture and ITS1-PCR were also conducted to assess the most suitable combination of methods for diagnosing leishmaniases. In the 100-case-matched comparison, the three different types of sample proved to be equally good with no significant difference ($P > 0.05$). However, skin tissue imprints on filter paper revealed most cases of CL. The kappa statistic for measuring the degree of agreement among the three samples was 89%, which is considered good. Agreement was highest between imprints on filter paper and unstained smears, and lowest was for stained smears. In the overall comparison between the ITS1-PCR and conventional methods, the ITS1-PCR using samples from filter papers was the most sensitive method but the difference was insignificant ($P = 0.32$). The combination of microscopy together with ITS1-PCR on samples from filter papers increased the sensitivity significantly to 46%, compared to using the methods individually ($P = 0.003–0.0008$). On comparing the results of the tests done on the samples from the 2232 patients after applying ITS1-PCRs to their samples from filter papers, unstained smears, *in vitro* culture, microscopy, and stained smears showed, respectively, test sensitivities of 81, 69, 64, 57 and 48%. Of the tests and samples adjudicated, ITS1-PCRs run on skin tissue samples from filter papers proved best for the routine laboratory diagnosis of CL. Adding microscopy of stained smears to it, improved its diagnostic value significantly.

1. Introduction

The various cutaneous leishmaniases (CL) appear in different clinical guises that can be confused with other dermatological diseases and conditions, therefore, diagnosis based solely on clinical signs and symptoms can be misleading. Since CL are caused by different species of *Leishmania,* the diagnosis of CL should include the detection of leishmanial parasites in lesions as either whole organisms or by the presence of elements from them, e. g., leishmanial DNA; and when possible, the identification at the species level. This is especially important in areas where more than one species of *Leishmania* are circulating. This is particularly important situation in foci where the species *L. infantum* is circulating which is one of the species causing visceral leishmaniasis (VL) but also CL without the obvious signs and symptoms of VL (Frank et al., 1993; Gramiccia et al., 1987; Rhajaoui et al., 2007; Rioux and Lanotte, 1990). Cases of CL caused by *L. infantum* have occurred in Palestine (Azmi et al., 2012). Leishmanial species identification is also necessary for deciding the need, type and regime of therapy as this can

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differ depending on the species. Similarly, the different leishmanial species have different animal hosts and sand fly vectors that require different control measures (Al-Jawabreh et al., 2017; Alvar et al., 2012; Koarashi et al., 2016).

In many endemic areas, microscopy of stained smears is used to diagnose cases of CL, as recommended by the World Health Organization (WHO, 2010), because of its relative simplicity and cost effectiveness (Al-Jawabreh et al., 2006; Herwaldt, 1999; Isaza et al., 2002). Culture is used less frequently, as it requires good sterile conditions, a supply of fresh normal rabbit blood to make NNN and semisolid media and is prone to contamination. However, it is necessary for obtaining living organisms for leishmanial species identification unless one has access to molecular biological methods that can circumvent it. The introduction of molecular biology techniques and polymerase chain reactions (PCRs), has added greater sensitivity and discrimination to laboratory diagnosis and been a great benefit to conventional epidemiology, adding the discipline of molecular epidemiology to it (Al-Jawabreh et al., 2004; Schallig and Oskam, 2002; Suarez et al., 2015).

Various DNA targets have been used in the molecular biological diagnosis of leishmaniases for the detection, identification and genotyping of their causative agents. Among these target are kinetoplast DNA (kDNA), the mini-exon gene, the glucose 6 phosphate dehydrogenase (G6PD) gene, the mannose phosphate isomerase (mpi) gene, the heat-shock protein 70 gene (hsp70), and ribosomal DNA (Al-Jawabreh et al., 2017; Castilho et al., 2003; Kato et al., 2008; Marfurt et al., 2003; Montalvo et al., 2010; Reale et al., 1999; Rodgers et al., 1990; Schonian et al., 2003). In the case of ribosomal DNA, amplification of the ribosomal internal transcribed spacer 1 (ITS1) region, which is located between the genes coding for ssurRNA and L5.8S rRNA) gives an amplification product that is used, firstly, to determine if the causative agents are leishmanial organisms and, secondly, after restriction of the product by the enzyme *Hae*III, to identify the species of *Leishmania* (el Tai et al., 2000; Schonian et al., 2003).

Several studies have compared and evaluated the performance of various parasitological diagnostic methods employing microbiological technology, comparing either the methods (Al-Jawabreh et al., 2006; Rodriguez et al., 2002; Xavier et al., 2006), or the types of sample used (Boni et al., 2017; Manna et al., 2004)or both (Strauss-Ayali et al., 2004; Taslimi et al., 2017; Weigle et al., 2002).

PCR-based techniques for diagnosing leishmaniases require an optimal sampling method. Many types of sample have been employed: formalin-fixed-paraffin-embedded skin tissue biopsies (de Lima et al., 2011; Muller et al., 2015; Xavier et al., 2006); culture; skin tissue smears stained with Giemsa's stain (Al-Jawabreh et al., 2006; Motazedian et al., 2002); skin and mucous membrane biopsies; skin scrapings; whole blood and lymph node samples (Al-Jawabreh et al., 2004; Manna et al., 2004; Medeiros et al., 2002; Suarez et al., 2015); tear drops collected on swabs (Strauss-Ayali et al., 2004); samples collected with cytology brushes (Boggild et al., 2011; Suarez et al., 2015); tissue samples collected with cotton swabs (Boggild et al., 2011; Mimori et al., 2002), samples collected with dental broach (Sharquie et al., 2002); samples collected on stripping tape (Taslimi et al., 2017). All of these sought a sensitive, accurate, and where possible, least to non-invasive and economic diagnostic method.

This study evaluated the use of samples taken from dermal blood and tissue scrapings spotted onto filter paper and used ITS1-PCR for the diagnosis of CL and compared this to the use of tissue scraped from unstained and stained tissue touch smear preparations. The ITS1-PCR results were also compared to those obtained by the conventional diagnostic methods of microscopy of smears stained with Giemsa's stain and *in vitro* culture.

2. Materials and methods

2.1. Human cases and study area

During the 22 years from June 1994 to September 2016, patients of both genders and various ages presenting either single or multiple skin lesions were referred to the Leishmaniases Research Unit in Jericho as suspect cases of CL. Lesions were at different body sites and of varying durations. Patients came from all the Palestinian districts. Positive control DNA from *L. turanica* (MRHO/MN/83/MNR-6) was used. The same species was used for inhibition control to check for the integrity of DNA (Al-Jawabreh et al., 2006). Master mix alone was used as negative control. Negative control samples were obtained from people from the Gaza Strip, a closed region considered free of CL.

2.2. Ethical clearance

CL-suspected patients were referred from Ministry of health clinics for routine laboratory diagnosis. Study design was approved by the ethics committee of Al-Quds Public Health Society (184/2014).

2.3. Sample collection and diagnostic procedures

Patient datasheets were filled into record demographic and epidemiological information. Diagnostic procedures included microscopical examination of Giemsa-stained smears of tissue from dermal lesions, *in vitro* culture of aspirates from the dermal lesions in NNN medium and ITS1-PCR assays applied to skin tissue spotted on filter papers (FP, Whatman no 4) and stained (SS) and/or unstained smears (US). Sampling, DNA extraction, and the diagnostic methodology were described (Al-Jawabreh et al., 2003; Al-Jawabreh et al., 2004; Al-Jawabreh et al., 2006; Schonian et al., 2003).

2.4. Case definition of CL

A multiple simultaneous testing strategy was adopted for determining and defining cases of CL. A positive result from any one the five tests used was considered sufficient to confirm a case.

2.5. Statistical analysis

Epi Info 7 (Centers for Disease Control and Prevention, USA), SPSS version 24, and online quick calcs from Graph pad http://www.graphpad.com/quickcalcs/CatMenu.cfm were used for the statistical analyses. Cochrane's Q test was used to compare three or more methods, followed by a McNemar post hoc test. Kappa statistic was used to measure the degree of agreement between any two methods exceeding that would be expected by chance alone.

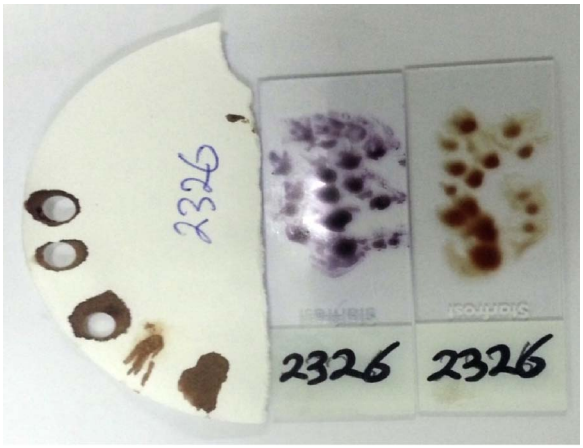
3. Results

3.1. Optimal sample for ITS1-PCR

One hundred suspect cases of CL were tested by the same ITS1-PCR method, which was applied to samples taken from FP, US and SS. In addition, 59 samples from people from the Gaza Strip were used as negative controls. All 100 cases and 59 negative control samples were matched for comparison. The Cochrane Q test and its subsequent McNemar's post hoc test showed no significant difference between the three types of sample ($P = 0.11$). However, more cases positive of CL (35) were identified using samples from FP (Table 1). Still, disagreement among the three types of sample by ITS1-PCR was noticed (Fig. 2). With kappa statistic as a measure of agreement between methods, the degree of strength between ITS1-PCR using the three types of samples, FP, US and SS was considered good (Kappa coefficient = 0.7). Agreement of the three types of sample was beyond

Table 1
Matched comparison results of 100 suspected cases of CL and 59 negative controls identified by ITS1-PCR using three types of samples, FP, US, and SS.

		US			SS		
		Positive	Negative	Total	Positive	Negative	Total
FP	Positive	26	9	35	23	12	35
	Negative	8	116	124	5	119	124
		34	125	159	28	131	159
US	Positive	–	–	–	23	11	34
	Negative	–	–	–	5	120	125
		–	–	–	28	131	159



(a) (b) (c)

Fig. 1. The three types of sample used in ITS1-PCRs: (a) Filter paper using Whatman™ no. 4 with tissue scrapings also containing blood from a suspected lesion, showing three punctured sites per DNA extraction; (b) Giemsa-stained smear; (c) Unstained smear.

chance for 142 (89%) results, as the sum of the negative and positive agreements, with disagreement for 17 (11%) results (Table 1).

3.2. Positivity rate of diagnostic methods

Five diagnostic methods were used to screen 2232 Palestinian suspect cases of CL (Fig. 1 and Table 2). However, not all suspect cases were tested with all methods. About 56.3% (504/895) of the cases were male and 43.7% (391/895) were female, and 60.5% (514/849) were children under 14 years old as described elsewhere (Al-Jawabreh et al., 2017). The overall positivity rate was 43% (964/2232). The highest positivity rate was recorded by the ITS1-PCR used on unstained smears, the sample of choice. Microscopy of stained smears and ITS1-PCR used on stained smears gave equally low positivity. The cultures of skin tissues from 65 patients (9.8%) were contaminated. Depending on how many of these would have been positive, the success of this method might have been somewhat higher.

Table 2
Positivity rates of the five diagnostic methods used on the 2232 suspect cases of CL.

	Positive (%)	Negative	Total
Microscopy	496 (24)	1540	2036
In vitro culture	219 (37)	375	594[a]
ITS1-PCR-FP	595 (37)	1031	1626
ITS1-PCR-US	136 (44)	172	308
ITS1-PCR-SS	77 (26)	225	302

[a] Excluding 65 contaminated cultures. FP = Filter paper; US = unstained smear; SS = stained smears.

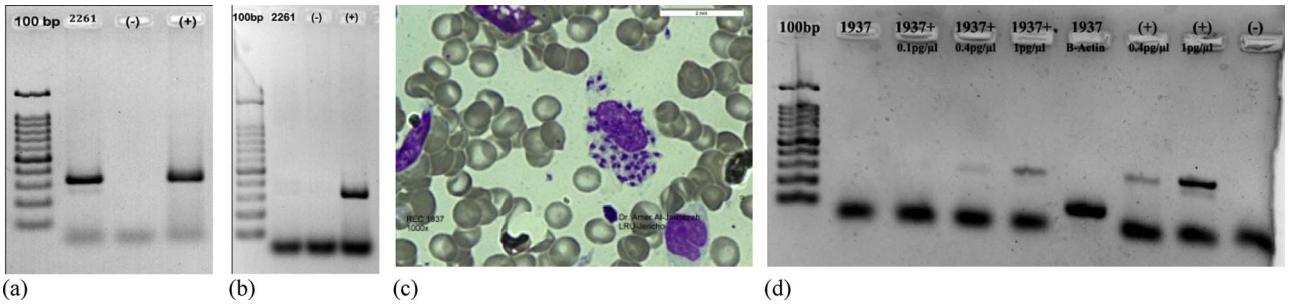
Table 3
Comparison of the sensitivities of the five diagnostic methods in identifying cases of CL, taking the simultaneous multiple testing strategy as the gold standard to confirm a case of CL.

	Stained smear	*In vitro* culture[a]	ITS1-PCR-FP	ITS1-PCR-US	ITS1-PCR-SS
Positive	494	219	595	136	75
Negative	372	121	143	61	81
Total	866	340	738	197	156
Sensitivity,%	57	64	81	69	48

[a] Excluding 65 contaminated cultures.

3.3. Sensitivity and positivity rates of the methods employed

Taking all of the 2232 patients' samples into account, tested by any given method, as the base for an unmatched comparison, Table 3 shows that performing an ITS1-PCR using samples taken from FP was the method with highest sensitivity, resulting in 595 (81%) positive results among 738 confirmed cases of CL compared to an ITS1-PCR using samples taken from SS, which recorded the lowest sensitivity of 48% (75/156). In the matched comparison of the three types of sample taken from 100 suspect cases of CL, that were evaluated by all the diagnostic methods, which, again, depended on simultaneous multiple tests for exposing case of CL, the degree of positivity of the methods increased incrementally from 28% for the ITS1-PCR done on samples from SS, to 29% for *in vitro* culture, to 31% for microscopy, to 32% for the ITS1-PCR done on samples from US, to 35% for the ITS1-PCR done on samples from FP. Discrepancies were seen among the results generated by each method (Fig. 2). The difference is not statistically significant (P = 0.32). However, adding culture to microscopy of SS raised positivity to 39% and using the three methods based on the ITS1-PCR



(a) (b) (c) (d)

Fig. 2. Discrepancy of results: (a) Electrophoresis, run in a 1.5% agarose gel, of the ITS1-PCR amplification product of a sample from a stained smear showing a 340 bp band, indicating the presence of a species of *Leishmania*; (b) a negative result on using a sample from a filter paper from the same patient REC 2161; (c) intracellular and extracellular amastigotes seen by microscopy of a smear stained with Giemsa's stain from patient REC1937; however, no amplification product was generated by an ITS1-PCR for samples taken from a filter paper, lane 2 in (d). A series of inhibition controls, lanes 3–5, using different DNA concentrations from *L. turanica*. Lane 6 shows the housekeeping gene (120 bp). Positive (lanes 7 and 8) and negative (lane 9) controls of leishmanial DNA were used.

Table 4
The effect of storage time in years on the positivity of stained smears from 76 cases of CL exposed by microscopy and negativity of the SS, US and FP samples after their amplification by ITS1-PCRs.

	0–4	5–9	10–14	> 15	Total
SS	5	10	1	18	34
US	8	2	2	1	13
FP	11	17	11	1	40
Total	24	29	14	20	87[a]

[a] 11 cases were tested more than once using different samples.

increased positivity to 43%. By using all five methods, positivity increased to 51%. Using a combination of one conventional method, e. g., microscopy of SS, and one PCR method, e. g., an ITS1-PCR on FP samples, increased positivity to 46%. This application of two methods, microscopy and ITS1-PCR using FP samples raised positivity significantly from very to extremely statistically significant (P = 0.0002–0.0088) when compared to each method applied individually. Using a combination of microscopy and culture, the two conventional methods, together, did not significantly improve positivity in exposing cases of CL (P > 0.05).

3.4. Anomaly between methods

Out of 1645 suspected cases of CL examined by microscopy of stained smears (SS) and ITS1-PCRs on SS, US and FP, 76 were positive by microscopy, but negative for their ITS1-PCR (4.6%). This unexpected anomalous result increased when using SSs as the samples for ITS1-PCRs to 11%. Furthermore, storage time of SSs increased discrepancy between results reaching 85% (29/34) among samples older than five years. Samples from FPs were least exposed to this anomaly (Fig. 2, Table 4).

3.5. Species identification

Unlike the conventional methods, ITS1-PCR aided with *Hae*III restriction enzyme revealed that 41% (295/723) were *L. major*, 56% (404/723) were *L. tropica*, and the rest (3%) were undetermined.

4. Discussion

Microscopical examination of stained tissue smears remains the main way of diagnosing cases of leishmaniasis. It is quick and technically less demanding. Here, it was the most widely applied diagnostic method and 2030 of the 2232 patients were checked by it. Scrapings from stained tissue smears can be and were used here in a diagnostic PCR system but showed low sensitivity (57%), also reported by others (43%) (Koarashi et al., 2016). However, microscopical examination requires expertise and optimal staining of smears as stain particles and stained tissue debris can be misread as amastigotes, giving false positive cases of leishmaniasis and distorting negative results in statistical analysis. When amastigotes are few to very scanty, many fields have to be screened before a smear should be declared negative. Insufficient scanning can also lead to false negative results. *In vitro* culture using NNN medium is a very useful adjunct to diagnosis if suitable culture medium is available but is a more demanding procedure requiring a sterile facility and access to sterile fresh defibrinated normal rabbit blood, which, in many countries, requires animal ethical clearance. Contamination, which was 9.8% here, and length of time to acquire results (1–4 weeks) are limitation to *in vitro* culture. In this study, only in extremely rare instances were cultures positive after a period as short as 18 h, on this study, and *in vitro* culture was, compared to microscopy, of low sensitivity (64%). Rodriguez et al. reported even lower sensitivity at 42%. Therefore, culture cannot be the method of choice for diagnosis (Rodriguez et al., 2002), but should, where possible, be used in conjunction with microscopy where it could expose cases when amastigotes are too few to be seen in stained smears.

Multiple simultaneous testing for the diagnosis of CL as was used here raised the number of positive cases from the 595–964 by using ITS1-PCRs done on FP samples, the most discerning single method of the five methods employed. It is unfeasible carrying out five tests on each patient as a routine medical diagnostic policy. However, the adoption of two methods, microscopy and an ITS1-PCR done on FP samples for the diagnosis of CL is technically and economically feasible and would significantly increase diagnostic sensitivity as shown here. The World Health Organization (WHO) has adopted the conventional combination of microscopy of stained smears and culture for identifying cases of CL (WHO, 2010), but in this study they did not perform significantly (P > 0.05) better compared to the molecular biological-based methods or the combination of both.

The three types of samples used for ITS1-PCR performed equally well statistically with a good level of agreement, however, discrepancy was seen. This could be owing to: uneven distribution of either amastigotes or parasite DNA among samples (Mathis and Deplazes, 1995); the stage of development and self-cure of the lesion, *i. e.,* whether new and active with increasing numbers of amastigotes or old and almost healed with no amastigotes remaining (Weigle et al., 2002). Furthermore, Suarez et al. found variation in parasite load on comparing biopsies from the border, base, and centre of the same ulcer (Suarez et al., 2015). The presence of inhibitors such as haemoglobin, stain components and artefacts caught by immersion oil, bacterial DNA from secondary infection caused by *Staphylococcus aureus* or *Streptococcus pyogenes* is another source of ITS1-PCR false negative discrepancy, particularly when the concentration of the target DNA is very low. This was overcome by using PCR inhibition control and found to be minimal. A probable cause of such discrepancy is DNA extraction failure, which was detected by DNA extraction control (Al-Jawabreh et al., 2004, 2006) (Fig. 2). The false negative discrepancy is one reason for using more than one method of diagnosis, a conventional one and a molecular-based one.

The PCR-based assay used here proved superiority to traditional methods in sensitivity. However, some studies have given conflicting results, showing higher sensitivity of smears than sample generated by PCRs (Ramirez et al., 2000; Robinson et al., 2002). A major source of discrepancy is the use of different 'gold standards' to define a case of CL. Romero et al., used *in vitro* culture as their 'gold standard' that led to a sensitivity of 95% for stained smears (Romero et al., 2001). Belli et al. used microscopy as their 'gold standard', obtaining a 100% sensitivity and specificity for samples generated by PCR (Belli, 1998). Others, owing to the lack of a good 'gold standard', used laboratory and clinical criteria to define cases of CL, and showed that diagnosis employing PCRs gave higher sensitivity (Weigle et al., 2002). Another factor affecting the performance of a diagnostic method is the biopsy sampling technique. The sensitivity of ITS1-PCRs increased to 94% when the skin tissue samples were collected using tape strip discs, which also increased the sensitivity of *in vitro* culture to 100% from the 51% when the skin tissue samples were collected using classical sampling (Taslimi et al., 2017). A study found that biopsies taken with cotton swabs showed 93% sensitivity on using the presence of leishmania kDNA as a positive diagnosis for CL (Boni et al., 2017). The target gene also plays a role in the sensitivity of molecular biological diagnostic methods. In this study, routine molecular biological diagnosis using an ITS1-PCR reached a sensitivity of up to 81% compared to 78% in other studies targeting the ITS1 gene (Beldi et al., 2017; Boni et al., 2017; Taslimi et al., 2017). When the mini-exon gene was targeted, the sensitivity decreased to 68% (Beldi et al., 2017). One study targeted the HSP70 gene and kDNA where the sensitivity was 41%; and 92%, respectively (Boni et al., 2017). An indirect source of discrepancy among diagnostic methods used in this study was the high prevalence of CL in the study area, which increased the positive predicative value of all types of tests.

In conclusion: Where possible, a molecular biological-based method

should be added to the conventional routine diagnosis of CL by microscopy of smears stained with Giemsa's stain. An ITS1-PCR applied to tissue biopsies from suspects lesion spotted on filter paper is recommended because of its very high sensitivity and ability to identify the species of *Leishmania* causing the leishmaniasis.

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