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The Jordanian Mid Jordan Valley is a classic focus of *Leishmania major* as revealed by RFLP of 56 isolates and 173 ITS-1-PCR-positive clinical samples

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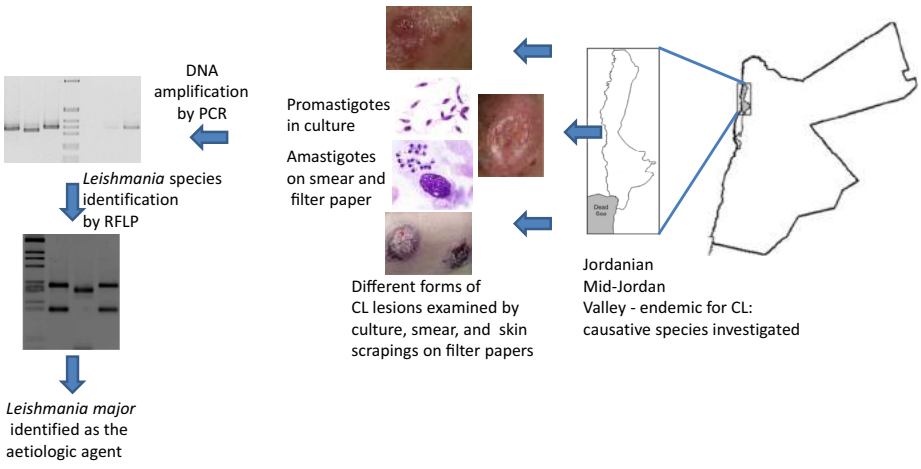
HIGHLIGHTS

- A molecular diagnostic analysis on high number of CL patients.
- A better epidemiological assurance of CL distribution and causative species in JMidJV.
- ITS-1 PCR is a good technique for diagnosis of typical and atypical lesions of CL.
- RFLP is confirmed useful for species identification of *Leishmania*.
- The JMidJV is a classic focus of *L. major*.

GRAPHICAL ABSTRACT



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ABSTRACT

The identity of the causative species of cutaneous leishmaniasis (CL) in the endemic Jordanian Mid Jordan Valley (JMidJV) was investigated using the polymerase chain reaction (PCR) amplifying the ribosomal internal transcribed spacer 1 (ITS-1) followed by the restriction fragment length polymorphism (RFLP). The geographical distribution of CL and the usefulness of ITS1 PCR in diagnosis of suspected CL in the study area were also addressed. Over the period from 2004 to 2009, 56 clinical isolates of *Leishmania* promastigotes and 185 lesion scrapings spotted on filter papers were obtained from suspected CL patients living in the JMidJV, which is divided into northern and southern districts. The majority (67.1%) of patients occurred in the populated eastern part of the southern district. Of the 185 suspected CL patients, 173 (93.5%) were confirmed positive using PCR. Leishmanial DNA was detected in 27 (90%) of 30 patients having clinically atypical lesions of CL and in 60 (92%) of 65 smear- and culture-negative cases having typical lesions of CL. The parasites in all of the 56 isolates and the 173 PCR-positive scrapings

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were classified as *Leishmania major*. In conclusion, PCR is useful in diagnosis of CL especially when smear and culture are negative. It is also recommended as a differential diagnostic tool of atypical lesions when CL is endemic. The identification of *L. major* as the causative species in such a considerable number of CL cases, representative of all mini foci of CL in the study area, shows that the JMidJV is a classic focus of *L. major*.

1. Introduction

The Jordanian Mid Jordan Valley (JMidJV, Fig 1) is endemic for cutaneous leishmaniasis (Arbaji et al., 1993; Khoury et al., 1996) and the disease is underreported (Mosleh et al., 2008a). The actual annual incidence of the disease is, however, known to be quite high (Khoury et al., 1996; Mosleh et al., 2008a; Oumeish, 1991). Khoury et al. (1996), for example, reported 987 cases from the Jordanian side of the Jordan Valley between 1983 and 1992. In the same region, the highest incidence, of more than 313/100 000 cases per year, was reported in 2004/2005 (Mosleh et al., 2008a). The desert rodent (*Psammomys obesus*) was confirmed as the main reservoir host (Saliba et al., 1994) and *Phlebotomus papatasi* as the vector of the disease caused by *L. major* (Janini et al., 1995b). In Jordan, the disease is diagnosed clinically, and when parasitological investigation is performed, it is restricted, in most of the cases, to the smears of lesion scrapings stained with Giemsa.

Species identification of the causative agent is vital to determine the best ways of control measures and treatment regimens (Schönian et al., 2003). A well-established PCR method, targeting the internal transcribed spacer 1 (ITS1) region between the SSU and 5.8S rRNA genes, is useful for the direct diagnosis of different forms of leishmaniasis. The method is highly specific and sensitive, able to detect approximately 0.2 parasites per sample (Schönian et al., 2003) and identify all medically relevant *Leishmania* species which are distinguished by DNA sequencing or restriction fragment length polymorphism (RFLP) of the PCR product (Dávila and Momen, 2000; Schönian et al., 2000, 2001, 2003).

Few studies have investigated the identity of the causative species of cutaneous leishmaniasis (CL) in the endemic JMidJV (Mosleh et al., 2008b, 2009; Nimri et al., 2002; Saliba et al., 1988, 2004). These studies either performed on a limited number of cases, not designed to cover all of the foci in the region, or do not precisely record the origin of the reported *Leishmania* species. The present study is a wide scale

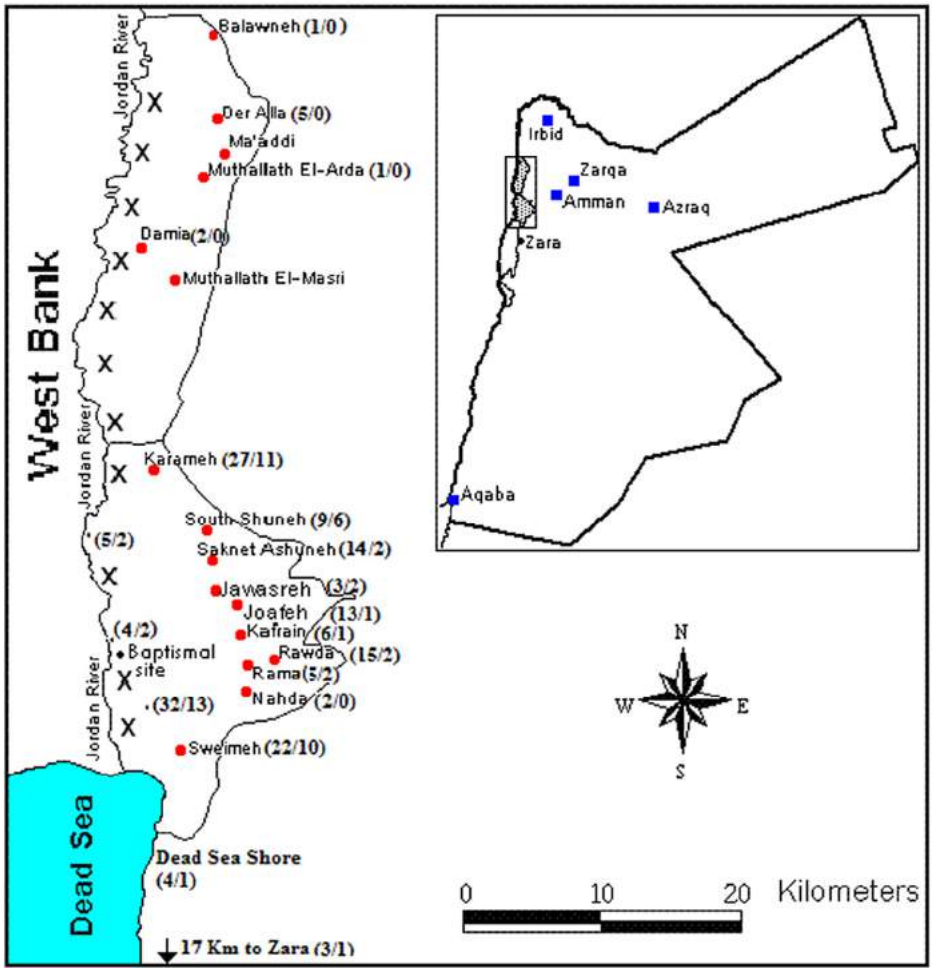

Fig. 1. Map of Jordan with emphasis on the study area (enclosed by the rectangle). The study area is enlarged on the left showing the origin of CL samples from the mini foci of CL represented by the numbers in parentheses. The numbers on the left of the slash represent the origin of the 173 PCR-positive spotted filter papers; whereas the numbers on the right of the slash indicate the origin of the clinical isolates of *Leishmania* promastigotes in each mini focus within the JMidJV. The 'X' represents military-troop zones.

investigation of the identity and the geographical distribution of *Leishmania* along the JMidJV. It also investigates the usefulness of ITS1 PCR in diagnosis of *Leishmania* in lesion scrapings of Jordanian patients.

2. Materials and methods

2.1. Study area

The JMidJV is divided into two districts (Fig. 1) with a total population of around 87 000 (Department of Statistics, Jordan). The majority of the population is distributed in 24 towns and villages, which are mainly located in the eastern part of the two districts. The strip of the valley that is located along the eastern bank of the Jordan River ('X's' in Fig. 1) is a poorly inhabited military zone. There are 28 governmental health centers distributed in the villages and towns of the two districts. CL patients, primarily diagnosed by the general practitioners of the health centers, are referred to the attending dermatologist in the main hospital, South Shuneh Hospital (SSH), where the treatment is centrally stored.

2.2. CL patients

The passive approach was the main mode of case detection. We also used an active case-finding strategy focused in specific locations that were identified as mini foci. The passively detected group of CL cases is comprised of patients who sought treatment for skin lesions at the 28 health centers of the JMidJV (Fig. 1) and then were referred to the SSH, and those who came to the SSH directly seeking medical care for skin lesions. Registration of the residence of the first few dozens of self-referral cases 'index cases' revealed that a number of them were clustering at specific locations in the study area. These locations were identified as mini foci of CL. The active detection was conducted by visiting these mini foci to detect CL cases who did not seek medical care. During these visits the dermatologist investigated the inhabitants of the mini foci for CL lesions and the detected cases were invited to the SSH for further investigations.

2.3. Clinical samples and CL inclusion/exclusion criteria

Passively and actively detected patients were examined at the SSH. Smears, cultures, and lesion scrapings spotted on filter papers were taken from all patients who had (1) clinically typical or atypical lesions and (2) were residents of the CL endemic JMidJV or remained overnight in this area during the previous 6 months. However, the patients with parasitologically negative lesions that were cured after a short-course of antibiotic were excluded. The clinically typical lesion is the characteristic lesion or nodule which is considered by the attending dermatologist as "highly suspected oriental sore". The characteristic lesion is a nodule of 1–5 cm in diameter with a thin golden superficial crust which might be thickened to replace the nodule or fallen away leaving an ulcer (Fig. 2) with a raised edge (Bryceson, 1987). In contrast, other forms of lesions that do not fit in the category of typical lesions stated earlier and do not respond to a short term of antibiotics, but presented in patients who fulfill the criteria 1–2 described earlier are considered suspected atypical CL lesions. Patients were included only when they were in agreement with the procedure of the study and were able to give an informed consent. The general protocol of the study was approved by the Ethical Review Board of the SSH (Approval # K-9-1-1SSS dated 20.08.2004).

Specimens were obtained from all patients by slit-skin scrapings from one lesion for each patient. A 3-mm slit at the lesion edge was made with a sterile scalpel while the lesion was strongly held between fingertips. Portions of scrapings from the lesion as well as the fluid oozing from the slit were cultured in Schneider's Drosophila medium with 20% fetal calf serum and antibiotics, smeared on a glass slide, and spotted on filter papers (Mosleh et al., 2008b). Smears were fixed with methanol, stained with Giemsa, and microscopically investigated. Positive cultures were preserved in liquid nitrogen. The spotted filter papers were dried and kept frozen. The clinical specimens from CL patients were collected over the period from 2004 to 2009.

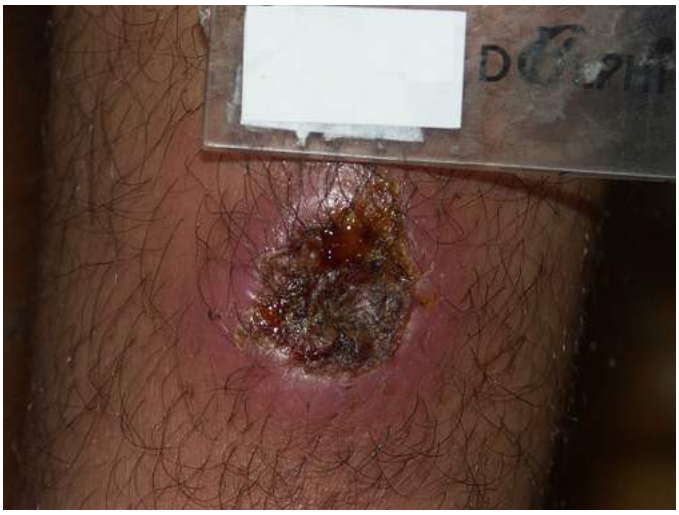

Fig. 2. A typical lesion of cutaneous leishmaniasis. Note the crust in the middle of the lesion and the raised edge.

2.4. PCR–RFLP analysis

Phenol–chloroform was used to extract DNA from the lesion scrapings of CL patients (Meredith et al., 1993) or from the cultured promastigotes (Schönian et al., 1996) of any clinical isolate and the following reference strains: *L. major* (MHOM/TM/82/Lev), *L. tropica* (MHOM/SU/80/K28), and *L. infantum* (MHOM/TN/80/IPT1). The samples of leishmanial DNA so obtained were investigated by PCR–RFLP, so that the parasites in the new isolates and scrapings could be identified to species (Dweik et al., 2007). In the PCR, the sequence corresponding to the internal transcribed spacer (ITS1) of *Leishmania* was amplified using the LITSR and L5.8S primers (El Tai et al., 2000), as described elsewhere (Schönian et al., 2003); the primers were synthesized commercially (TIB-MOLBIOL, Berlin, Germany). The amplicons from the PCR-positive samples (of 300–350 bp, depending on the species) were digested with HaeIII to give smaller fragments that, by comparison with the corresponding fragments produced from reference strains of *Leishmania*, allowed the species of *Leishmania* causing the sampled lesion to be identified (Dweik et al., 2007; Schönian et al., 2003).

3. Results

3.1. PCR results and geographical distribution of CL patients

DNA extracts from the lesion scrapings of the suspected CL patients ($n = 185$, one lesion/patient), the cultured promastigotes of the clinical isolates ($n = 56$), and the reference strains were investigated by PCR. All the (56) clinical isolates and 173 out of the 185 lesion scrapings gave an amplicon similar to that shown in Fig. 3a.

PCR results of the 185 patients, categorized into groups 1–5, are shown in Table 1. PCR detected leishmanial DNA in at least 90% of the patients in all of the five groups. When the total number of the suspected CL patients (185) were considered, regardless of the lesion type or the parasitological results, 173 (93.5%) of the cases had PCR positive results. Smear and culture together, on the other hand, detected *Leishmania* only in 60% of the 162 (97 + 65) parasitologically examined patients (Table 1). The geographical distribution of the 173 PCR-positive CL patients, along with the clinical isolates obtained from 56 of them, is shown in Fig. 1. The majority (67.1%) of the CL cases were

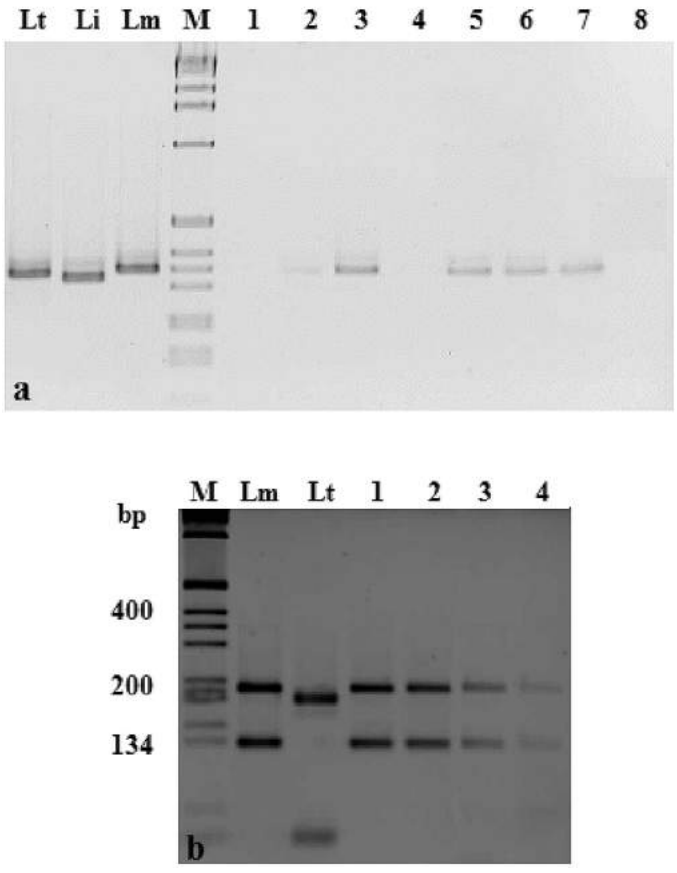

Fig. 3. Typical results of the amplification and identification of leishmanial DNA in lesion scrapings or culture by PCR–RFLP. (a) When the products of the PCR were subjected to electrophoresis on 1.5% agarose gel, the clinical samples positive for *Leishmania* (lanes 2, 3, 5, 6, and 7) gave a 350-bp band. A clinical sample negative for *Leishmania* is demonstrated on lane 4. Also run on the same gel were negative controls for the PCR (lane 1) and DNA extraction (lane 8), reference samples of Lt, *L. tropica* (MHOM/SU/80/K28), Li, *L. infantum* (MHOM/TN/80/IPT1), and Lm, *L. major* (MHOM/TM/82/Lev) and a 1 kb 'ladder' (M). (b) The amplicons from the PCR-positive samples and new isolates in culture were then digested with HaeIII to give restriction fragments that allowed all the parasites (lanes 1–4) to be matched with a reference strain of Lm, *L. major* (MHOM/TM/82/Lev), each giving fragments of about 130 and 200 bp. Also run on the same gel were the corresponding fragments of Lt, *L. tropica* (MHOM/SU/80/K28) and a 1-kb 'ladder' (M).

distributed in the populated eastern part of the southern district of the JMidJV. Only 5.2% came from the northern district of the valley. The rest (23.7%) were distributed in the poorly inhabited military strip of the valley that is located along the eastern bank of the Jordan River ('X's' in Fig. 1) and along the eastern shore of the Dead Sea extending from the northern shore to Zara. The latter CL patients were temporary residents (farmers, soldiers, and construction workers) deployed in the strip or along the Dead Sea shore.

Table 1
PCR results of lesion scrapings of 185 suspected CL patients from the study area (JMidJV) categorized into five groups.

Group of CL patients	Description	Number	PCR positive	%
Group 1	Clinically typical lesions	155	146	94.2
Group 2	Clinically atypical lesions	30	27	90.0
Group 3	Parasitologically positive lesions (positive smear or culture)	97	92	94.8
Group 4	Parasitologically negative lesions (negative smear and culture)	65	60	92.0
Group 5	No parasitological examination (no smear or culture)	23	21	91.3
Total	Clinically typical and atypical lesions regardless of parasitological results	185	173	93.5

3.2. Parasite identification

The amplicons from the 173 PCR-positive lesion scrapings and the 56 clinical isolates from CL lesions were investigated using RFLP by digestion with HaeIII to give smaller fragments and compared with the corresponding fragments produced likewise from the reference strains of *Leishmania*. After doing so, all investigated samples gave restriction fragments that indicated that *L. major* was the infectious agent (see Fig. 3b).

4. Discussion

ITS1 PCR followed by RFLP was used to identify *Leishmania* species found in the JMidJV. This technique worked well regardless of the type of the clinical sample investigated. The investigation on a considerable number of cultured promastigotes and lesion scrapings spotted on filter papers revealed that only one species, *L. major*, is the causative species of the endemic CL in the JMidJV. Earlier studies performed in the study area using the isoenzyme characterization (Nimri et al., 2002; Saliba et al., 1988, 2004) identified *L. major* in most of the isolates. However, in few cases assumed to be infected in the JMidJV *L. tropica* was identified. Saliba et al. (2004) reported *L. tropica* only in one soldier who was not a resident of the JMidJV and had slept in many other localities. In the other study (Nimri et al., 2002) the number, the origin, and the mobility of the detected *L. tropica* CL cases were never published. It was only denoted that the CL patients were from "the central region of Jordan (e.g. South Shuneh)" who were referred during 1992–2000 to the Central Laboratories in Amman, around 60 km far from the JMidJV (Nimri et al., 2002).

The controversy regarding the existence of *L. tropica* in the area under study is difficult to be resolved. Although the transient existence of *L. tropica* in the JMidJV is possible, the probability that these *L. tropica* cases were originating from the surrounding areas outside the JMidJV is valid. The latter possibility is based on the policy adopted by the health authorities for the treatment of CL cases in the endemic region. In this regard, CL suspected patients who attend to the primary health centers of the JMidJV or the surroundings are referred to the attending dermatologist in the main hospital – South Shuneh Hospital (SSH) – where the treatment is centrally stored (Mosleh et al., 2008a). It is, therefore, reasonable to assume that patients originating in the surroundings of the JMidJV, where *L. tropica* occurred during 1990–1995 (Kamhawi et al., 1995; Saliba et al., 1993, 1997), were referred from the SSH together with those originating in the JMidJV to the Central Laboratories in Amman. This assumption is supported by the similarity in the isoenzyme profile of the *L. tropica* isolates from patients assumed to be infected in the JMidJV (Nimri et al., 2002) and those variants isolated, in the same period of time, from the high altitude surroundings of the JMidJV (Kamhawi et al., 1995; Saliba et al., 1993, 1997). In more details, Mon-137 reported by Nimri et al. (2002) was isolated by Saliba et al. (1993, 1997) from the mountainous Eira-Yarqa, only 10 km east to the JMidJV, during 1990–1995 which overlaps with the time of sampling by Nimri et al. (2002) that extended through 1992–2000. The other *L. tropica* zymodeme (ZMON-54 var PGD96-97) identified by Nimri et al. (2002) was also isolated from about 40 km far high altitude regions in the same study by Nimri et al. (2002) in Um Qais and by Kamhawi et al. (1995, a strain close to Mon-54) in Malka. Both villages are of the Irbid governorate located north to the Jordan Valley. In addition, the two zymodemes of *L. tropica* reported by Nimri et al. (2002), which are similar to that from the high altitude surroundings, are different from the Mon-7 isolated, as early as 1990, by Saliba et al. (2004) in South Shuneh, JMidJV. In the present study, *L. tropica* was not the causative agent of any of the 173 PCR-positive cases of CL detected in different

localities representative of the JMidJV (Fig. 1), although *Phlebotomus sergenti*, a common vector of *L. tropica*, was found to be present in some of the CL foci of the JMidJV (Janini et al., 1995a) but was never incriminated in CL transmission there. If the *L. tropica* cases reported previously (Nimri et al., 2002; Saliba et al., 2004) were really infected in the JMidJV, it is logic then to speculate that *L. tropica* could transiently have existed but did not establish in the JMidJV. Thus, the situation in the Jordanian side of the MidJV is different from that in the side of the Palestinian authority where *L. tropica* has become established in Jericho and its environs, a classical focus of *L. major* (Al-Jawabreh et al., 2004).

To date, the routine diagnosis of Jordanian cases of CL is restricted to microscopical examination of smears stained with Giemsa. The use of culture is limited and never become a routine. Smear and culture together failed to detect *Leishmania* in 40% of the clinically diagnosed CL patients tested using both techniques (see Table 1). This is the first study to use PCR to diagnose CL on a large number of Jordanian patients. Our previous works that used PCR to diagnose CL in Jordan (Dweik et al., 2007; Mosleh et al., 2008b, 2009) were involving a limited number of CL cases. The ITS1-PCR detected leishmanial DNA in lesion scrapings of 93.5% of 185 clinically diagnosed CL patients in the JMidJV. It efficiently diagnosed *Leishmania* in 92% of the patients that had no parasitological confirmation by any of the direct smear or culture and in 90% of the patients presenting with atypical lesions of CL (see Table 1). This confirms the usefulness of this technique in diagnosis of leishmaniasis in Jordan especially the CL patients missed by conventional techniques or those clinically confused to meet CL diagnosis. The ITS1-PCR of lesion scrapings spotted on filter papers has been shown to be a useful means for diagnosing suspected CL with varying – 52% (Al-Jawabreh et al., 2004), 71% (El-Beshbishy et al., 2013), 84% (Dweik et al., 2007), 91% (Bensoussan et al., 2006), and 100% (Toz et al., 2009) – rates of positivity. The high rate of positivity reported in the present study (93.5%) could be partially attributed to the inclusion/exclusion criteria of the CL patients adopted. In this context, many patients were presented with bacterially infected lesions, unknown to be a primary or secondary infection. Once they responded to a short antibiotic treatment, they were excluded from the study. Likewise, patients who appeared with lesions similar to that of CL, but never slept in any endemic region of CL, they were excluded.

In conclusion, the JMidJV is a classic focus of *L. major* CL as it was the only species of *Leishmania* identified in a large number of CL cases representative of all of the detected mini foci and locations in the endemic area under investigation. PCR is confirmed to be a powerful technique for the diagnosis of CL from clinical lesion scrapings spotted on filter papers and it is recommended to be used on Jordanian patients especially when the results of the less sensitive conventional methods are negative. RFLP can be added for further identification of the species and in special cases, such as confirmation and identification of the causative agent of atypical lesions and when false positive PCR amplicons – same size but different DNA sequence – are to be ruled out.

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