ORIGINAL ARTICLE

Leishmania major cutaneous leishmaniasis outbreak in the Jordanian side of the Northern Jordan Valley

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ABSTRACT

Cutaneous leishmaniasis (CL) is endemic in many foci of Jordan and the Jordanian Mid Jordan Valley (JMJV) is the most affected and the incidence is quite high. The situation in the northern part of the Jordanian side of the Jordan Valley (NJJV) was different; before 2008, CL has rarely been reported from this area. From April 2008 to May 2009, passive detection followed by active detection was used to trace cases of CL from the NJJV. DNA was extracted from seven clinical isolates of *Leishmania* promastigotes and lesion scrapings spotted on filter papers obtained from 51 suspected CL patients living in the NJJV. The identity of the causative species of CL in the NJJV was investigated using ITS1-PCR followed by RFLP. In 2008/2009, 183 cases were clinically diagnosed of having CL in the NJJV. The parasites in five of the isolates and in 48 PCR-positive scrapings were classified as *Leishmania major*. In two isolates and in one PCR-positive scraping *Leishmania tropica* was identified. Investigations on the origin of CL cases revealed that the *L. tropica* cases were residents of two towns outside the NJJV. Herein, we report the clinical features, parasitological diagnosis, etiology, and the geographical distribution of CL cases from NJJV with the aim of documenting, for the first time, an outbreak in this area.

KEYWORDS

Cutaneous leishmaniasis; PCR diagnosis; species identification; ITS1-PCR; *Leishmania major*; RFLP; CL focus; CL outbreak; Jordan

Introduction

Cutaneous leishmaniasis (CL) is a protozoal infection of the skin caused by several species of *Leishmania* (Kinetoplastida: Trypanosomatida). It is mainly transmitted by phlebotomine sand flies. *Phlebotomus papatasi* (Diptera: Psychodidae) is the most common vector for CL in Jordan [1,2] and its neighboring countries [3–6]. Most of the CL cases all over the world reported in 2015 were from 10 countries: Afghanistan, Algeria, Brazil, Colombia, Iran, Iraq, Peru, the Syrian Arab Republic, Tunisia, and Yemen which together account for 87% of global reported CL incidence [7]. In Jordan, the Ministry of Health reported 2560 CL cases in the period between 1994 and 2014 [8]. The disease is endemic in many foci of Jordan and the Jordanian Mid Jordan Valley (JMJV) is the most affected. On the Jordanian side, the valley is divided into northern, mid and southern regions. In the JMJV, although endemic, the disease is underreported [9]. The actual annual incidence of the CL in this area is known to be quite high [9–11] with the highest incidence, of more than 313/100 000 cases per year, was reported in 2004/2005 [9]. The JMJV is a classic focus of the zoonotic CL caused by *Leishmania*

major [12] and the fat sand rat (*Psammomys obesus*, Rodentia: Muridae) was confirmed as the main reservoir host [13].

In the northern part of the Jordanian side of the Jordan Valley (NJJV) the situation is different. Before 2008, to the best of our knowledge, CL has rarely been reported from there [11]. Herein, we report the clinical features, laboratory diagnosis, geographical distribution, and the etiologic parasite of an outbreak of CL occurred in the NJJV in 2008/2009.

Materials and methods

Study area

The NJJV (Figure 1) has a total population of around 106,000 (Department of Statistics, Jordan). The majority of the population is distributed in towns and villages, which are mainly located in the eastern part of the district. The strip of the valley that is located along the eastern bank of the Jordan River is a poorly inhabited military zone. There are 14 governmental health and village centers distributed in the villages and towns of the district (Department of Statistics, Jordan). CL patients, primarily diagnosed by the general practitioners of

Figure 1. Map of Jordan with emphasis on the study area (enclosed by the rectangle). The study area is enlarged on the left showing the geographical distribution of CL cases in the study area represented by the numbers in parentheses. Also shown are the military troop zones represented by 'X' and the location of the Sede Eliyahu new focus on the western side of the Jordan River. Source: Authors.

the health centers, are referred to the attending dermatologist in the main hospital, Abu Obeida Hospital (AOH, Wadi Arrayan, Jordan Valley), where the treatment is centrally stored.

CL patients

The passive approach followed by the active case-finding strategy was used in CL case detection. The passively detected group of CL cases is comprised of patients who sought treatment for skin lesions at the health centers of the NJJV and then were referred to the AOH, and those who came to the AOH 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 AOH for further investigations.

Laboratory diagnosis

Passively and actively detected patients were clinically examined at the AOH. Laboratory diagnosis included conventional and molecular diagnostic methods. Smears, cultures, and/or lesion scrapings spotted on filter papers for the polymerase chain reaction (PCR) analysis were taken from the majority of patients. Specimens were obtained by slit-skin scrapings from one lesion for each examined 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 as previously described [14]. 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.

CL inclusion/exclusion criteria

Subjects were considered as CL patients if they (1) had clinically typical lesions described elsewhere [12], (2) were residents of the NJJV or remained overnight in this area during the previous 6 months, and (3) responded to treatment or had simultaneous or recent history of parasitologically positive lesions in the household. Patients with suspected (atypical) lesions were considered CL if they were parasitologically positive. Patients with parasitologically negative suspected lesions were subjects for differential clinical diagnosis by the dermatologist, who decided if they had to receive treatment for CL or not. According to the Ministry of health regulations clinically diagnosed cases are treated regardless of the results of parasitological tests. However, patients without any parasitological test or those with parasitologically negative lesions that were cured after a short-course of antibiotic were excluded. Patients were included only when they were in agreement with the procedure of the study and were able to give an informed consent. The study followed the principles of the Helsinki Declaration. The Review Board of the South Shuneh Hospital, Jordan Valley, approved the study on 'The investigation of the causative species of cutaneous leishmaniasis in patients from the Jordan Valley' under which medical data were obtained (Approval # K-g-1-1555). The clinical specimens from CL patients were collected over the period from April 2008 to May 2009.

DNA Extraction and PCR-RFLP Analysis

DNA from cultured promastigotes of any clinical isolate and reference strains was extracted as described before [15,16]. Filter papers with CL lesion scrapings were cut small with scissors into 3–5 mm pieces. To avoid DNA contamination, the scissors were alcohol flamed before cutting out the next clinical sample. For each specimen few pieces were placed in 250 µl lysis buffer (50 mM NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA, pH 7.4), 1% Triton X-100 and 200 µg proteinase K/ml) and incubated for three hours or overnight at 60 °C. DNA in lysates of filter papers or promastigotes was extracted by phenol–chloroform extraction as previously described

[17]. Briefly, lysates were mixed with equal volumes of a phenol:chloroform (1:1) mixture. After centrifugation two distinct phases were formed. The aqueous phase on top of the organic phase (phenol:chloroform) with the nucleic acids in it was pipetted off. The DNA in the aqueous phase was then mixed with isoamylalcohol:chloroform (1:24) mixture and centrifuged to get the aqueous phase on top that was pipetted off. The DNA in the aqueous phase was precipitated with ice cold 96% ethanol. After centrifugation, the DNA pellet is washed with 70% ethanol. The pellet is then centrifuged under vacuum to dry ethanol. The DNA in the pellet is dissolved in 20 µl TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0), quantified, aliquoted and stored at –20 °C. The samples of leishmanial DNA obtained were investigated by PCR–RFLP. After amplification with PCR and endonuclease restriction analysis the parasites in cultures and in lesion scrapings could be identified to species [18]. In the PCR, the sequence corresponding to the internal transcribed spacer (ITS1) of *Leishmania* was amplified using the LITSR and L5.8S primers [19], as described elsewhere [20]; 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 identification of *Leishmania* species causing the sampled lesion [18,20].

Results

CL patients in 2008/2009: diagnosis, geographical distribution, and clinical data

Using the passive and active case detection approaches, a total of 183-NJJV individuals were diagnosed of having CL from April 2008 to May 2009 and treated with cryotherapy and/or sodium stibogluconate. Of them, 137 patients were parasitologically tested with one or more of the: smear, culture, and PCR and 114 (62.3%) cases were accordingly confirmed positive for CL. The diagnosis of the 183 patients, categorized into groups 1–4, is shown in the Table 1. The geographical distribution

Table 1. Distribution of 183-NJJV patients detected in 2008/2009 among four groups defined as CL patients.

Group of CL patients	Description	Number	%
Group 1	Clinically typical lesions No parasitological examination Response to CL treatment and/or had simultaneous or recent history of parasitologically positive lesions in the household	46	25.1
Group 2	Clinically typical lesions Parasitologically negative patients (tested in one or more technique) Response to CL treatment and/or had simultaneous or recent history of parasitologically positive lesions in the household	23	12.6
Group 3	Clinically typical lesions Parasitologically positive patients (positive PCR, smear, or culture)	105	57.4
Group 4	Clinically atypical lesions Parasitologically positive patients (positive PCR, smear, or culture)	9	4.9
Total		183	100

of the 183 clinically diagnosed CL patients is shown in Figure 1. The CL cases were distributed in the area from Kraymeh south to Waqqas north, clustering more in Al Mashareh, Tabakat Fahl, and Sayl El Hemmeh. A fraction (35%) was also clustering in the military strip that is located along the eastern bank of the Jordan River and in the surrounding villages, namely Wadii Arryan and Al Qarn. This area includes the military units and farms. At the time of the clinical examinations, the cases had had their skin lesions for between 1 week and 3.5 months. The number of lesions on each case ranged from one (seen in 24% of the cases) to 15 (seen in 2% of the cases), with 57% of the cases having three or more lesions each. Most of the cases had lesions on their upper (53%) or lower (35%) limbs. Some patients had lesions on their head (18%) or trunk (4%).

PCR results

DNA extracts from the lesion scrapings of 51 of 183 clinically diagnosed CL patients (1 lesion/patient) or from the cultured promastigotes of 7 clinical isolates and the reference strains were investigated by PCR. All the 7 clinical isolates and 49 (96%) lesion scrapings gave an amplicon similar to that shown in Figure 2(a).

Parasite identification

The amplicons from the 49 PCR-positive lesion scrapings and the 7 clinical isolates from CL lesions were investigated using RFLP by digestion with *Hae*III to give smaller fragments and compared with the corresponding fragments produced likewise from the reference strains of *Leishmania* (*L. tropica* (MHOM/SU/80/K28), *L. infantum* (MHOM/TN/80/IPT1), and *L. major* (MHOM/TM/82/Lev). After doing so, all investigated samples except 3 of them

gave restriction fragments that indicated that *L. major* was the infectious agent [see Figure 2(b)]. In these three samples, two isolates and one filter paper, the restriction fragments indicated that *L. tropica* is the causative species of the lesions in the three patients [see Figure 2(b)].

Discussion

This study reports an outbreak of CL in the Jordanian side of the Northern Jordan Valley (NJJV). To the best of our knowledge, the 2008/2009 outbreak is the first outbreak reported from this area. In contrast to the endemic MJJV, the disease was, in fact, rarely reported from the neighboring NJJV [8,11]. The rarity of CL before the 2009 outbreak in the NJJV was ascertained by the Ministry of Health (MOH) reports and the attending dermatologist serving in the main hospital (AOH) of the NJJV. According to the 2014-MOH report [8] only one case of CL was reported during 2006 and 2007. Collectively, only a dozen of CL cases was reported by the health authorities of the NJJV during the 25 years from 1983 to 2007. In the present study, in the season from April 2008 to May 2009, 183 CL cases were detected in the study area. This high rate could not be attributed to the active case detection approach adopted here together with the passive approach as most of the CL cases were detected by the passive approach, which in the years previous to the outbreak rarely detected CL cases in NJJV. Our focused index case-active detection approach only detected additional cases who were invited to the hospital for treatment and was useful to discover the mini foci of CL in the outbreak. It is unclear whether we are dealing with a new focus of CL or an old focus with 'uncertain' incidence. The passive surveillance of the MOH, which rarely detected CL in the NJJV in the decades [8,11] before the 2008/2009 outbreak, continued, in contrast, to detect considerable numbers of CL in

Figure 2. 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 run on 1.5% agarose gel electrophoresis, the clinical samples positive for *Leishmania* (lanes 5, 6, and 7) gave a 350-bp band. A clinical sample negative for *Leishmania* is demonstrated on lane 2. Also run on the same gel were negative controls for the PCR (lane 1 and 3) and DNA extraction (lane 4), 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 isolates in culture were then digested with *Hae*III to give restriction fragments that allowed all the parasites in lanes 4–9 to be matched with a reference strain of Lm, *L. major* (MHOM/TM/82/Lev), and the *Leishmania* in lane 2 to be matched with a reference strain of Lt, *L. tropica* (MHOM/SU/80/K28). Also run on the same gel were the corresponding fragments of a reference strain of Li, *L. infantum* (MHOM/TN/80/IPT1), undigested amplicon from a PCR-positive sample (lane 1), and a 1-kb 'ladder' (M).

the years after the outbreak. According to the 2014-MOH report [8] and the 2015 records 34, 10, and 20 CL cases were reported from the NJJV in the seasons of 2010/2011, 2012/2013, and 2014/2015, respectively. These relatively high numbers may, in one hand, support the idea of an emerging new focus or may be due to awareness for CL accumulated after the outbreak that made the passive surveillance detect more CL cases. However, further investigations of the incidence of CL over the next years are needed. The NJJV may also be an old focus with 'uncertain' incidence (probably due to underreporting [9]), but this seems not to be supported by the fact that the same passive surveillance of the MOH, with the underreporting defect in it [9], has identified the Jordanian Mid Jordan Valley (JMJV) as a known CL endemic area since a long time ago [10,11] but not the neighboring NJJV [11]. In addition, the origin of the rare cases registered in the NJJV prior to the outbreak is uncertain and it is possible that they had contracted CL in the neighboring JMJV. The numbers reported after the outbreak may be even higher and may not reflect the actual incidence of CL in the NJJV as the disease is known to be underreported in Jordan and in general. In a previous study done in the CL-endemic JMJV the disease was estimated to be underreported by a factor of 1:47 [9]. Similar underreporting in the NJJV cannot be excluded. The possibility of higher incidence is supported by observations collected in 2017 from three of the mini foci that were primarily identified in 2008/2009. These mini foci were military troop caserns where soldiers with CL-attributable scarring advocated they were infected either in 2012, 2013, or 2014 and treated outside the Jordan Valley or did not seek medical care. Few mentioned they and some of their comrades (not interviewed by authors) treated their lesions using local plant extracts, or topical antibiotics. Others used lightened cigarettes to destroy their lesions (cautery).

The NJJV outbreak is one in a series of outbreaks that occurred in Jordan and the neighboring countries. The expansion of the *Leishmania* parasite into areas where CL cases were minimal seems to be a phenomenon in the region. Several outbreaks of *L. tropica* and *L. major* have occurred in endemic or non endemic areas of Jordan and the neighboring countries over the last 15 years [5,21–28]. These outbreaks and the emergence of new foci increased the incidence of CL in the region [26,28,29]. According to MOH records in Jordan, the trend of incidence rate of CL shows an evident and remarkable increase from an average of 0.5/100 000 during the 5-year period between 1999 and 2003 to 4.2/100 000 during the following 5 years from 2004 to 2008.

Parallel to the NJJV CL outbreak also an *L. major*-CL focus has emerged between 2007 and 2011 in Sede Eliyahu village [6] that is located in the Beisan valley on the immediate western side of the Jordan River (about 7 km westwards from the NJJV outbreak, Figure 1). This indicates a regional outbreak of CL that was temporally caused by the same *Leishmania* species. In contrast to the

Sede Eliyahu CL focus which was limited to the 650-population village, the NJJV outbreak extended along about 20-km area, but the clustering of most of the cases was in the villages that are located directly on the same line eastwards from the Sede Eliyahu CL focus, namely Al Mashareh and Sayl El Hemme (Figure 1).

In this study ITS1-PCR detected leishmanial DNA in lesion scrapings of 96% of the tested 51 clinically diagnosed CL cases. This confirms the usefulness of this technique in diagnosis of leishmaniasis in Jordan especially the CL patients missed by conventional techniques. These results confirm previous investigations using ITS1-PCR to detect leishmanial DNA in scrapings spotted on filter papers [12,18,30–33]. The high sensitivity of PCR reported here can be attributed to the inclusion/exclusion criteria (Materials and Methods).

ITS1-PCR followed by RFLP was used to identify *Leishmania* species found in the NJJV. In the present study and in others [12,23,34] this technique worked well regardless of the type of the clinical sample investigated. The investigation on cultured promastigotes and lesion scrapings spotted on filter papers revealed that one species, *L. major*, is the causative agent of CL in the NJJV. The clinical picture recorded in NJJV – duration and multiple lesions found predominantly on the limbs – are typical of those recorded in foci of zoonotic CL [35,36]. However, specimens from three patients collected in Abu Obeida Hospital in the NJJV were identified as *L. tropica*. When the origin and history of CL of those patients was followed up, two of them, a mother and her son came from Halawah, and the other patient, a 3 years old girl, lives in Osarah. The two towns are located in the high altitude region few kilometers east to the Jordan Valley (Figure 1). When the patients and their relatives were interviewed they advocated they never slept in the Jordan Valley, which indicates that the three patients were infected outside the NJJV. These results show that detection of CL cases of different etiologies visiting a central hospital does not necessarily mean that these etiologies co-occur. Such a conflict about the co-occurrence of *L. major* and *L. tropica* has been reported in the JMJV [37]. The co-occurrence of the two *Leishmania* species in this region has long been debated as few *L. tropica* isolates of uncertain origin were collected in the JMJV [37,38]. A wide scale investigation of the etiology of CL in the JMJV, however, revealed that the area is a classic focus of *L. major* and based on the isoenzyme data of these *L. tropica* isolates of uncertain origin, it has been proposed that those *L. tropica* cases originated from the surrounding high altitude regions [12]. The follow up and determination of the origin of the *L. tropica* cases in the present study to be the surrounding high altitude region support this argument. The policy adopted by the health authorities for the treatment of CL cases is to refer the CL suspected patients who attend to the primary health centers in the Jordan Valley and the surroundings to the attending dermatologist in the main hospitals in the Jordan Valley where the treatment is centrally stored [9].

In conclusion, the NJJV CL outbreak was caused by *L. major* as it was the only species of *Leishmania* identified in all detected patients residing or spend nights in the NJJV. The *L. tropica* cases were found to be originating from high altitude regions east to the NJJV. ITS1-PCR is confirmed to be a powerful technique for the diagnosis of CL from clinical lesion and its combination with *HaeIII* digestion is an effective method to identify the *Leishmania* species. The series of CL outbreaks that repeatedly occur in the whole region and the emergence of temporal foci and outbreaks such as those on both sides of the Jordan River indicate that leishmaniasis is a regional issue that requires international awareness.

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Disclosure statement

The authors report no conflicts of interest.

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