ORIGINAL ARTICLE

Geographical distribution and molecular characterization for cutaneous leishmaniasis species by sequencing and phylogenetic analyses of kDNA and ITS1 loci markers in south-eastern Iran

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ABSTRACT

This study aimed to explore geographic distribution and molecular characterization of cutaneous leishmaniasis (CL) species by amplifying two popular markers in kinetoplast DNA and internal transcribed spacer 1 loci by nested-PCR, and characterized by sequencing and phylogenetic analyses. Findings demonstrated that two species co-existed in the province: *L. tropica* (88.5%) and *L. major* (11.5%). All gender and age groups were equally infected, although males, 21–30 years old, exhibited a significantly higher infection. Sequencing and phylogenetic analyses of 34 randomly selected samples showed that *L. tropica* isolates exhibited some degree of heterogeneity. Both anthroponotic CL and zoonotic CL are present in south-eastern Iran with predominance of *L. tropica* species. Some level of heterogeneity was observed in *L. tropica* isolates which possibly reflects different colonies in the area. Implementation of diagnostic tools directly from clinical samples could be an important strategic approach for exploration of spatial distribution, molecular characterization and phylogenetic analyses.

KEYWORDS

Cutaneous leishmaniasis; geographical distribution; molecular characterization; sequencing; phylogenetic analysis; Iran

Introduction

Leishmaniasis has become a social and medical challenge in 98 tropical and subtropical endemic countries worldwide [1]. An estimated prevalence of 12 million people and 1.5–2 million annual incidences have been reported globally [2]. Cutaneous leishmaniasis (CL) is the most common form of the disease which encompasses about three-fourths of the total cases. This disease is one of the few infectious diseases recently rising in incidence globally [1,3].

Current revision of the formal documents indicates that CL is a poverty stricken neglected disease. If the global burden of disease (GBD) in terms of prevalence of scared patients (nearly 40 million cases) instead of only those with active lesions (approximately 4 million cases) is being considered, then the new estimated burden would be drastically increased by a factor of 10 [4]. This finding poses serious impacts with respect to prioritizing

CL for global research and development (R&D) and control strategy needs, such as diagnostic tools, therapeutic drugs and efficacious vaccines [5]. Therefore, revision of current CL burden due to continued stigma and psychological depression, shows that this disease represents a major large-scale global health problem. Iran is among the seven most common affected frontier countries which are Brazil, Peru, Afghanistan, Syria, Saudi Arabia, Iran and Algeria [2].

Two epidemiological CL forms are present in 58% (18/31 provinces) of the provinces of Iran [6]. Anthroponotic CL (ACL) caused by *Leishmania tropica* is reported primarily in Kerman and Bam counties and to a lesser extent, in other localities [7]. Humans are the main reservoir and *Phlebotomus sergenti* is the principal female sand fly vector [8], while zoonotic CL (ZCL) due to *L. major* harbours in small gerbils as the major reservoir and *Ph. papatasi* as the main vector. Yearly, 20,000 new cases of both ACL and ZCL are reported; although, the

actual figure is assumed to be 4 to 5-fold higher than routinely recorded [9,10].

Currently, CL has remarkably increased and extensively expanded to new geographical zones as documented in Iran and abroad [9,11]. The unexpected rise of CL patients in endemic countries is multifactorial and mainly attributed to population movements, ecological disturbances, changing pattern of international trade and travel, political unrest, natural disasters and drug resistance [6,9,11–14].

Traditional diagnosis of *Leishmania* species is achieved by direct smear microscopy and culturing of clinical specimens [15]. However, such conventional methods do not discriminate the causative agents to the species level, as they exhibit identical morphometric organisms in slide smear preparations (amastigote or Leishman body) or culture media (promastigote). Recently, highly sensitive intrinsic methods were introduced. Among which, multilocus enzyme electrophoresis (MLEE) has been used for characterization of the *Leishmania* species, over the last few decades [15–19], but it has currently been shown not to be as discriminatory as many DNA-based molecular methods. However, this method is highly sophisticated, requires live organisms and is rather laborious, that is why it is restricted to highly elaborate research laboratories such as WHO reference centers [16]. Enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT) using monoclonal antibodies have also been used to identify leishmanial infections [17]. Over the past two decades, polymerase chain reaction (PCR) based methods targeting different genes have been successfully used to detect and characterize *Leishmania* species directly in clinical specimens and also for taxonomic, epidemiologic, population genetic and phylogenetic analyses [18–22]. In addition, the refined characterization of a gene is carried out through sequencing for spatial distribution and molecular identification [23–27].

This study aimed to explore geographical distribution, molecular characterization and phylogenetic analyses by evaluating two popular markers in kDNA and ITS1 loci, directly from clinical samples with polymerase chain reaction and sequencing in south-eastern Iran. This large scale study was performed due to recent earthquakes in south-eastern Iran and resulting environmental modifications, labourers' migration, development of various agricultural projects, and for molecular-epidemiological assessment and also planning control strategies against CL. Moreover, this work is unique and relatively different from that were previously carried out in the areas in regard to phylogenetic analyses of kDNA and ITS1 loci markers throughout the major focus of CL in south-eastern Iran. Additionally, new areas which were not previously explored for species identification, were sampled.

Methods

Ethical consideration

The protocol (no. 94/261) was reviewed and consequently approved by the Steering Committee and the Ethical Committee of Kerman University of Medical Sciences (Ethics no. IR. KMU. REC. 1394. 739). A written informed consent was obtained from each participant and personal data were kept confidential. All CL patients were treated with proper drugs, accordingly.

Geographical area

Kerman is the largest province of Iran (Figure 1) and consists of 20 main counties and encompasses 11% of the total land with an area of 180,726 km². The population of the province is approximately 3 million people and is located in 30.2907° N 57.0679° E. The climatic conditions are unique and vary across regions. The height differs significantly between 300 m in the south close to the Persian Gulf and 4500 m above sea level in the Hazar Mountain. The north, northwest and central parts experience a dry climate, whereas in the south and southeast, the weather is warm and relatively humid. CL has been endemic in Kerman province, mainly of ACL form in the counties of Bam and Kerman [28,29].

Kerman province is prone to earthquake. A recent devastating earthquake struck Bam at a magnitude of 6.6 RS, killing about 40,000 inhabitants, 100,000 individuals were left homeless and, as a result, 60,000 individuals mostly from other provinces arrived in the area and a considerable number left the Bam county for Kerman city, the center of Kerman province. A massive CL epidemic occurred four years following the earthquake, and cases increased 4-fold, the epidemic was sustained for five years and, since then, a change in the epidemiological trend of CL has been observed in the province. Initially, the mean number of CL cases was assessed for the years 2014–2016. Then, based on the number of affected counties and population, each case was chosen randomly from a larger set, with respect to the population to provide the pre-determined sample size. However, in some cases due to change in incidence of CL cases, the corresponding numbers were not as exact as previously determined.

Data collection

The present study was basically performed in two series. Initially, the present status of the CL cases was evaluated by passive case-detection approaches through the health surveillance system from 2014 to 2016. At this preliminary stage, all the CL cases were detected microscopically to the genus level (*Leishmania* species). Then, a descriptive cross-sectional survey was started with an active case-detection manner. Overall, 174 cases were

Figure 1. Geographic distribution of patients with cutaneous leishmaniasis by *Leishmania tropica* (●) and *Leishmania major* (★) in Kerman Province, south-eastern Iran. Source: https://en.wikipedia.org/wiki/Kerman_Province.

randomly selected in 2016 in the health centers across the province. Initially, the mean number of the CL cases was assessed for the years 2014–2016. Then, based on the number of affected counties and population, each case was sequentially chosen randomly from a larger set, in respect to population to provide the pre-determined sample size. However, in some cases due to change in incidence of CL cases and also change in the affected areas the corresponding numbers were not as exact as previously determined.

Clinical smear preparation

A scalpel and blade (no. 15) was used to prepare the clinical samples. The skin lesions were cleaned with alcohol (70%) and tissue smears were obtained from the periphery of each papule or nodule. Simultaneously, for each patient, two slide smears were prepared. All the dried microscopic slides were fixed with methyl alcohol, stained by routine Giemsa, precisely observed and screened systematically under a light microscope (Nikon, Japan). Case diagnosis was done by direct observation of the amastigote stage or Leishman body. For each case, a questionnaire consisting of demographic and clinical status was filled.

DNA extraction

The genomic extraction method was carried out according to the instructions of the High Pure PCR Template Preparation Kit (Roche, Germany) with some modifications. In brief, the smears were thoroughly cleaned by xylene to remove oil and extraneous materials. Sterile blades were used to scrape smears into 1.5 ml micro tubes and gently centrifuged at 8000 rpm for 1 min and

washed three times in sterile physiological saline. The deposited plate was used for DNA extraction.

Molecular characterization

Nested PCR assay

This method is a modification of PCR which is performed according to the Noyes's technique [21]. In brief, the procedures were carried out in two steps. The extracted target DNA underwent the first run with the first set of external primers: CSB1XR (ATTTTCGCGATTTCGAGAACG) and CSB2XF (CGAGTAGCAGAACTCCCGTTCA), and with the original described thermal conditions. Then, the product from the first round underwent a second run with the second pair of specific primers: 13Z (ACTGGGGGTTGGTGAAATAG) and liR (TCGAGAACGCCCCCT). The amplicons were visualized by 1.5% agarose gel electrophoresis (Uvitech, Cambridge, England) at 260 nm wavelength. The nested-PCR method amplifies a variable portion of kinetoplast DNA (kDNA). Known positive and negative *Leishmania* isolates together with a DNA size marker were used in each run of nested-PCR and gel electrophoresis.

Sequencing and phylogenetic analysis

For further phylogenetic analysis, specific ITS1 amplification was carried out for *Leishmania* isolates by [30] primer set. Depending on the leishmanial species, a 265–288 bp fragment of ITS1 was amplified by using the upstream 5'-AGCTGGATCATTTTCCGATG-3' and downstream 5'-ATCGCGACACGTTATGTGAG-3' primers. The PCR reaction was performed in a total volume of 25 µl containing 12.5 µL PCR Master Mix (Ampliqon, Denmark), 5 µL DNA, 40

nM of each primer and sterile DNase/RNase-free, using a Flexcycler 2 PCR machine (Analytic Jena, Germany). Initial denaturation for 3 min at 95 °C was followed by 40 cycles of denaturation at 95 °C for 25 s, annealing 30 s at 57 °C, extension for 1 min at 72 °C per cycle, and final extension for 1 min at 72 °C.

PCR products were purified and sequenced by Macrogen Company (Korea). DNA sequences were trimmed by Sequence Scanner version 1.0 (Applied Biosystems, U.S.A.) and compared for similarity with sequences in GenBank using the BLAST, National Institutes of Health, U.S.A. (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to prepare consensus sequences for each sample. Phylogenetic study was done by multiple sequence alignments with BioEdit version 6 [19] and MEGA version 6 [20] software. The nucleotide substitution model with the best fit to the data-set was evaluated in MEGA6. The Kimura 2 parameter model with gamma distribution (K2 + G) was used to build the distance matrix and the tree was inferred using the Maximum Likelihood (ML) approach. The phylogeny was confirmed by the bootstrap method using 1000 bootstrap replications.

Statistical analysis

Data were entered into a computer using a SPSS statistical package software version 17.0 (Chicago, IL, U.S.A.). Differences between infection rates and demographic or clinical status were analyzed by Chi-square (χ^2) test. Significant level was set at $p < 0.05$.

Results

Epidemiological data

Altogether, 2487 CL cases were detected as *Leishmania* species by direct smear microscopic examination; of

which, 174 clinical samples were randomly selected for further confirmation by molecular identification, and are representative of 34 randomly selected samples that were sequenced for phylogenetic analyses. The results indicated that Kerman (48%) was the most infected county, followed by Bam (28.7%), Rafsanjan (6.2%), Orzoiieh (4%), Jiroft (3.6%) and to a lesser extent, other counties (Table 1). All sex and age groups were infected (Table 2); although the male individuals, 21–30 years old, exhibited a significantly higher infection rate ($p < 0.05$) when compared with other age groups. Both diseases, ACL and ZCL, displayed similar pattern of extrinsic characteristics. Hands (45.5%) were the most infected anatomical sites, followed by face (17.8%), legs (15.5%), trunk (2.3%) and the remaining (18.9%) were found as multiple locations (Figure 2). Majority of (Figure 3) the cases showed single lesion (62.6%), double lesions (23.6%) and ≥ 3 lesions (13.8%).

Molecular characterization

Nested-PCR amplified 750 and 560 bp of *L. tropica* and *L. major* minicircle fragments of kDNA, respectively (Figure 4); this could be distinguished by two species co-existing in the area. *L. tropica* (88.5%), which was the most common species, caused ACL while *L. major* (11.5%) was found in low level (Figure 5). All of the CL cases in Kerman, Bam, Jiroft and Anar were purely *L. tropica*, whereas other counties exhibited either single or mixed species (Figure 6). Molecular identification of the *Leishmania* species was performed on the 174 positive slide smear preparations which were previously confirmed by direct microscopic examination.

Overall, of 174 identified specimens (Figure 6), Kerman was the most infected county (43.7%), followed by Bam (21.9%), Jiroft (10.9%), Anar (6.9%), Sirjan (5.7%), Rafsanjan (5.2%) and to lesser extent other counties (5.7%). The majority of species in Kerman, Bam, Jiroft, Anar, Zarand and Kuhbanan exhibited predominantly *L. tropica*, whilst patients in other counties, particularly in Sirjan, Rafsanjan, Shahr-e-Babak, Shahdad, Ravar and Baft were detected to be *L. major* as the main species.

Phylogenic analysis

Nucleotide sequences of 34 randomly selected clinical samples (Figure 7) were clustered in two groups:

Table 1. Geographical distribution of the routinely diagnosed clinical samples in patients with cutaneous leishmaniasis in Kerman Province, south-eastern Iran.

County/Year	2014	2015	2016	Total
	No (%)	No (%)	No (%)	No (%)
Kerman	409 (43.0)	319 (43.0)	466 (58.7)	1194 (48.0)
Bam	328 (34.4)	204 (28.0)	182 (22.0)	714 (28.7)
Rafsanjan	63 (6.6)	59 (8.0)	32 (4.0)	154 (6.2)
Orzoiieh	27 (2.8)	42 (5.6)	30 (4.0)	99 (4.0)
Jiroft	21 (2.2)	37 (5.0)	31 (4.0)	89 (3.6)
Anar	27 (2.8)	18 (2.4)	10 (1.2)	55 (2.2)
Sirjan	28 (3.0)	13 (2.0)	12 (2.0)	53 (2.1)
Shahr-e-Babak	10 (1.0)	10 (1.0)	13 (2.0)	33 (1.3)
Rodbar	10 (1.0)	16 (2.0)	4 (0.5)	30 (1.2)
Baft	9 (1.0)	4 (0.5)	9 (1.1)	22 (0.9)
Anbarabad	8 (0.8)	3 (0.4)	1 (0.1)	12 (0.5)
Ghaleh Ganj	5 (0.6)	2 (0.3)	2 (0.2)	9 (0.3)
Kahnouj	4 (0.4)	4 (0.5)	0 (0.0)	8 (0.3)
Zarand	1 (0.1)	6 (1.0)	1 (0.1)	8 (0.3)
Kuhbanan	1 (0.1)	1 (0.1)	1 (0.1)	3 (0.1)
Bardsir	1 (0.1)	1 (0.1)	0 (0.0)	2 (0.1)
Ravar	0 (0.0)	1 (0.1)	0 (0.0)	1 (0.1)
Rabor	1 (0.1)	0 (0.0)	0 (0.0)	1 (0.1)
Total	953 (100.0)	740 (100.0)	794 (100.0)	2487 (100.0)

Table 2. Age and sex distribution of the patients with cutaneous leishmaniasis in Kerman Province, south-eastern Iran.

Age groups (years)	Female	Male	Total
	No (%)	No (%)	No (%)
<10	13 (18.0)	14 (13.7)	27 (15.5)
11–20	13 (18.0)	24 (23.6)	37 (21.3)
21–30	20 (28.0)	36 (35.3)	56 (32.2)
31–40	20 (28.0)	20 (19.6)	40 (23.0)
>41	6 (8.0)	8 (7.8)	14 (8.0)
Total	72 (100.0)	102 (100.0)	174 (100.0)

Figure 2. Anatomical location of lesions patients with cutaneous leishmaniasis in Kerman Province, south-eastern Iran.

Figure 3. Number of skin lesion in patients with anthroponotic cutaneous leishmaniasis and zoonotic cutaneous leishmaniasis in Kerman Province, south-eastern Iran.

L. tropica (19/34) and *L. major* (15/34). Also, these sequences demonstrated variable degrees of nucleotide changes in *L. tropica* as compared to *L. major* isolates. All the individual *Leishmania* samples ($n = 13$) reported in this article have been submitted in the GenBank database. Based on the phylogeny tree, 11 *L. tropica* isolates from Zaraned (RMZAR86), Anar (RMAN78), Sirjan (RMSIR73, and RMSIR7), Kerman (RMKR61 and RMKR158), Jiroft (RMJI41, and RMJI42), Shahr-e-Babak (RMSHK147), Rafsanjan (RMRAF156) and Ravar (RMRAV80) showed relatively more similar nucleotide sequences and were closely related to *L. tropica* MHOM/MA/88/LEM1314. Two other *L. tropica* samples: one from Kerman (RMKR22) and the other from Anar (RMAN76) displayed higher degrees of nucleotide variations.

On the other hand, 4 individual *L. major* samples, although indicating slight nucleotide variation, were similar to other global isolates [31]. These clinical samples

belonged to Sirjan (RM5IR72), Shahdad (RMSHD97), Kerman (RMKR23) and Rafsanjan (RMRAF19).

Discussion

Routine diagnosis of CL is based on direct microscopic examination of skin scrapings of Giemsa-stained slides and culturing of the clinical samples. These conventional methods, regardless of low sensitivity, differentiate the organism to the genus level [32,33]. Diagnosis of CL by the feature of the clinical lesion is usually done as only complementary in endemic areas, but for definitive diagnosis, this requires considerable familiarity with the disease. One of the primary aims of the present study is to identify and analyze the clinical samples in vast and spatially distributed areas of mixed foci (ACL and ZCL). Implementation of highly sensitive, precise and powerful diagnostic tools, directly from clinical specimens

Figure 4. Agarose gel electrophoresis of *Leishmania* species as analyzed by nested-PCR. Lane 1, DNA size marker 100 bp; lane 2, negative control (distilled water); lane 6, *L. tropica* (positive control 750 bp); lane 10, *L. major* (positive control 560 bp); lanes 3, 4, 5, 7 *L. tropica* and lanes 8, 9 *L. major* obtained from skin lesions of the patients in Kerman Province, south-eastern Iran.

Figure 5. Representative clinical presentations of anthroponotic cutaneous leishmaniasis (ACL) and zoonotic cutaneous leishmaniasis (ZCL) cases in south-eastern Iran. A & B; Ulcerated plaque with lymphedema (ZCL), C; Lupoid (leishmaniasis recidivans), D; Multiple lesions, E; Plaque (ACL), F; Nodule (ACL).

Figure 6. Geographical distribution of patients with anthroponotic cutaneous leishmaniasis and zoonotic cutaneous leishmaniasis in Kerman Province, South-eastern Iran.

in combination with bioinformatic tools could be an important strategic approach, as different *Leishmania* species generally require distinct treatment modality [24]. Furthermore, spatial distribution of the disease and exploration of the epidemiological data is a critical approach for designing future control measures [1].

Findings from this study clearly indicate that two species: *L. tropica* and *L. major* co-exist in the province of Kerman [28]. *L. tropica* was the main species which comprised 88.5% of the CL cases, while *L. major* included 11.5% of the detected cases. These results are consistent with previous report in this province [29]. Cases in Kerman, Bam, Jiroft and Anar counties were purely ACL due to *L. tropica*, whereas other counties exhibited either single or mixed species of *L. tropica* or *L. major*, although in low level. Few of the CL cases that were detected in Kerman and Jiroft were non-residents. In an inquiry into the origin of these patients, they declared that they were either native to ZCL areas within the province [34] or inhabitant of other endemic provinces in the country and came to Kerman province for various reasons [20,35]. The predominance of ACL over ZCL has been confirmed by several studies for incriminating major vectors implicated in the parasite cycles. Consistent with previous findings in the province, *Phlebotomus sergenti* is the principal vector, notably in Kerman and Bam counties [36], while *P. papatasi* is the main sand fly in Orzoieh county in the south-western part of the province [34]. In ACL areas, two distinct *L. tropica* strains by isoenzyme electrophoresis have already been reported from Bam: Mon-39 and Mon-55 [28].

In the current study, there is a significant difference ($p < 0.05$) among the age group, 21–30 years old, when compared with the others, more frequently among males than females. This finding is consistent with previous studies from south-eastern Iran. Most of these patients were male labourers who were exposed to the bite of female sand flies in high risk areas, where they slept outdoors with poor living and improper hygienic conditions [37].

Comparison of the extrinsic characteristics in terms of anatomical location and number of lesions between *L. tropica* and *L. major* shows that both species displayed similar pattern of lesion characteristics. Hands were the most infected part and most of them showed single lesion. These findings are also consistent with a previous report from the province [38]. Similarity between *L. tropica* and *L. major* extrinsic factors might be due to low number of *L. major* (11.5%) cases, although such comparison might be statistically inconclusive.

Overall, the amplification of kDNA gene is a suitable target for discriminating medically relevant CL species [18,20,21,29,39,40]. The kinetoplast contains about 10,000 min convoluted and circular kDNA minicircles. Such abundance makes the kDNA an appropriate and unique target for numerous diagnostic tests [24]. However, since the species can be associated with certain forms of the disease, parasite identification should not be limited to species determination. In these cases, additional typing techniques are recommended [41]. Since the minixon sequencing process is cumbersome, ITS1 was amplified and sequenced as a popular and effective marker in *Leishmania* species typing [40,41]. This kind of information could help in determining strain heterogeneity link to geographical origin. Moreover, the technique has the advantage of detecting genetic variability directly from clinical samples without culturing the causative organism [42].

Analysis of nucleotide sequences of the representative samples following ITS1-PCR should prove valuable for use in genetic analysis and consequently for management of CL patients where the disease is endemic [23,40]. In the present study, the reason for selecting disproportionately *L. major* isolates relative to *L. tropica* isolates was limited nucleotide variation of the former species [43–45]. The constructed phylogeny tree exhibited more heterogeneity in *L. tropica* samples as compared to *L. major*, possibly due to circulation of different clones within the study area in the province. This finding is in agreement

Figure 7. Phylogenetic analysis by Maximum Likelihood method.

Notes: The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 34 nucleotide sequences. All positions containing gaps and missing data were eliminated.

with the results obtained as they suggested that genetic variability of the complex fingerprints might be due to the spread of multiple clones of *L. tropica* [21].

L. tropica is reported to be an insidious and perplexing organism which has the ability to spread to a wide geographical area by anthroponotic cycle ranging from Europe to Middle East countries where the ongoing conflicts are persistent [46]. It also extended its range to African countries and the Indian-subcontinent as well [47]. Furthermore, *L. tropica* seems to have an exceptionally high intraspecific diversity and due to its genetic variability potential, it causes various clinical presentations [23,48]. Sequencing analysis of a desired gene as a method of choice for use in epidemiological, clinical, genetic and taxonomic studies, and also for management

of CL patients with unknown origins, is highly emphasized [23,26].

Conclusion

CL in Kerman province is predominantly of ACL form due to *L. tropica* (85.5%). Kerman (43.7%) is the most infected county, followed by Bam (21.9%), Jiroft (10.9%), Anar (6.9%), Sirjan (5.7%), Rafsanjan (5.2%), and to lesser levels other counties. Nested-PCR with high sensitivity power is a suitable method for identification of *Leishmania* species extracted from clinical samples. Phylogenetic analyses targeting ITS1 gene are valuable tools in genetic analytical characterization and management of CL patients where the disease is endemic. Moreover, this study

indicated that *L. tropica* has some degree of intraspecific diversity relative to *L. major*. Due to recent earthquakes and resultant ecological changes, labourers' migration and development of various agricultural projects, molecular-epidemiological assessment of the causative agents of CL is crucial for planning control strategies against the disease in the endemic areas.

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Disclosure statement

The authors declare that they have no conflict of interest.

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