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Reemergence of zoonotic cutaneous leishmaniasis in an endemic focus, northeastern Iran

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ABSTRACT

Zoonotic cutaneous leishmaniasis (ZCL) is a form of disease, which is mostly caused by *Leishmania major* (*L.major*) in the Old World with high incidence through early life. Recently, the high incidence of CL has been reported in Jajarm County, northeastern Iran. The aim of the present study was to investigate the epidemiological aspects of CL and to identify *Leishmania* spp. in the area. Between 2012 and 2018, patients with active lesions suspected of CL were examined. Diagnosis was performed by methods of clinical examination and direct smears. Conventional kDNA polymerase chain reaction (PCR) was used to identify species of *Leishmania*. During the seven-year study period, 629 subjects were diagnosed as CL cases by clinical and microscopic examination. Considering the population of the study area, the average incidence rate was 237.8 per 100,000 population. Most (29.4%) of the subjects with ZCL were aged <10 years. Most of the patients (58.7%) were male and the rest were female. Most ulcers were observed on the face (32.2%) and hands (23.7%), respectively. Most patients (90%) had two or more ulcers on their body. The highest incidence was in the autumn in October (32%). Our data revealed that an ongoing reemergence of ZCL focus caused by *L.major* has arisen in the study area. Moreover, direct PCR on the smears for identifying *Leishmania* showed 100% specificity, and the species was identified as *L. major* using species -specific kDNA PCR. The use of conventional PCR on skin smears seems to provide a valuable method for confirming the clinical diagnosis of ZCL, which is very specific and sensitive particularly for clinical correlative studies. Further investigations are needed to improve planning strategies of vectors and reservoirs populations.

1. Introduction

Leishmaniasis is a widespread tropical infection caused by different species of *Leishmania* protozoa (Reithinger et al., 2007). This protozoon is transmitted by various species of sandflies. There are three main clinical forms of the disease with varying severity: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL) (Reithinger et al.,

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2007). Cutaneous form is the most conventional form of the disease in the world with an incidence of 600,000 to 1 million new cases per year. CL is endemic in about 88 countries, and in 2018 about 85% of new cases occurred in 10 countries of Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran (Islamic Republic of), Iraq, Pakistan, the Syrian Arab Republic and Tunisia (World Health Organization, 2020). Iran is one of the countries where most CL cases occur. Almost more than 20,000 new cases of it are reported annually (Knapp and Alpern, 2020). Two forms of CL are reported in Iran, including zoonotic cutaneous leishmaniasis (ZCL) caused by *Leishmania major* (*L. major*) and anthroponotic cutaneous leishmaniasis (ACL) caused by *Leishmania tropica* (*L. tropica*) (Shirzadi et al., 2015). ZCL and ACL are observed in rural and urban areas, respectively, and cause wet and dry lesions at the wound site, respectively. (Firooz et al., 2020) In Iran, the rural type is prevalent in about 17 provinces of Iran while the urban type is affecting almost all of the country's urban areas (Shirzadi et al., 2015; Norouzinezhad et al., 2016). North Khorasan is one of the provinces in which ZCL is endemic. The average incidence rate in this province, during 2011–2017, has been reported as 81.25 /100,000. The records of the provincial health center in this province indicate a recent worrying increase in the incidence of CL in Jajarm County, located in the southwest of the province (Shirzadi et al., 2019; Alavinia et al., 2009). Parasitological confirmation via microscopy remains the gold standard in CL diagnosis and is the most affordable approach, but relies heavily on the parasite yield, sampling, quality of stained slides from skin biopsies, and the skills and experience of personnel (World Health Organization, 2014). The low density of amastigotes in direct smears and tissue samples may lead to delayed diagnosis or clinically and histologically misdiagnosis (World Health Organization, 2010). The polymerase chain reaction (PCR) technique is starting to be used as an extra diagnostic tool for CL and has been proved to be sensitive for typing of its causative agent (de Vries et al., 2015). Hitherto the diagnosis of CL in Jajarm was based on the clinical signs of the disease and microscopic observation of parasites in stained skin biopsies. Specific and sensitive molecular diagnostic tools have not yet been implemented in this area. Therefore, the present study was conducted to provide a comprehensive overview of the epidemiology of the disease, including examination of patients, the rate and nature of the disease, determining the causative agents in development of the disease and molecular identification of causative *Leishmania* spp. during 2012–2018 in Jajarm County, northeastern Iran. To our knowledge, this is the first molecular typing of CL causative agents in Jajarm County. Thus, the aim of the present study was to determine the status of CL burden and also molecular typing of *Leishmania* spp. in the area throughout 2012–2018.

2. Patients and methods

2.1. Study area

This study was conducted from June 2012 to September 2018 in Jajarm County, North Khorasan Province (56° 12′ to 57° 4′ N, 36° 36′ to 37° 24′ E), in an area of 7650 km^2. The population of Jajarm is reported as 36,898 based on the latest population census. The province is one of the 31 provinces of Iran placed in the northeastern of the country, bordering Turkmenistan as a main focus of ZCL (Fig. 1).

2.2. Sample collection and examination

This descriptive study was performed on 629 patients with suspected CL lesions who referred to health centers of Jajarm County during 2012–2018. Smears were prepared from all of the patients, were fixed in methanol for 5 min and were stained with Giemsa stain for 30 min. The prepared smears were examined microscopically for the presence of the leishman bodies (amastigote stage) of *Leishmania*. After being diagnosed with the disease, the patients' epidemiological information was recorded in a patient-specific form and the patients were subsequently treated according to the national guideline for CL case management. Required demographic and clinical data were registered and the relevance of these factors to disease incidence was analyzed. These factors included age, sex, nationality, place of residence, type and number of lesions, location of the lesion on the body and month of infection. The disease incidence rate in the County was calculated based on the number of CL diagnosed patients in the 7-year period.

Before the beginning of the study, the approval was obtained from the Ethics Committee of Mazandaran University of Medical Sciences (MAZUMS), Mazandaran, Iran (IR.MAZUMS.REC.1397.2728). The patients' data were collected, analyzed and reported unanimously. Thereafter, the data were analyzed in SPSS software (version 20). The differences were considered statistically significant when $P \leq 0.05$.

2.3. Species-specific kDNA-PCR assay

2.3.1. DNA extraction

DNA extracted from all positive microscopic smears was used for PCR. For DNA extraction using a modified salting-out procedure, samples were scraped from each slide with a clean blade and transferred to 2 mL microtubes. The smear scrapings were added to 200 μL lysis buffer containing 50 mM Tris-HCl, pH 7.5; 1 mM EDTA; 1% Tween 20; and 10 μL proteinase K solution (20 mg/mL). The tubes were incubated for 3 h at 60 °C, and then 60 μL of 6 M NaCl was added. After being shacked slowly, the tubes were centrifuged at 12,000 ×*g* for 12 min. Then, a supernatant solution containing DNA was precipitated with 400 μL cold absolute ethanol. After precipitating the DNA and drying its ethanol, we added 100 μL sterile distilled water and stored at 4 °C until it could be tested for *Leishmania* kinetoplast DNA(kDNA) (Aljanabi and Martinez, 1997).

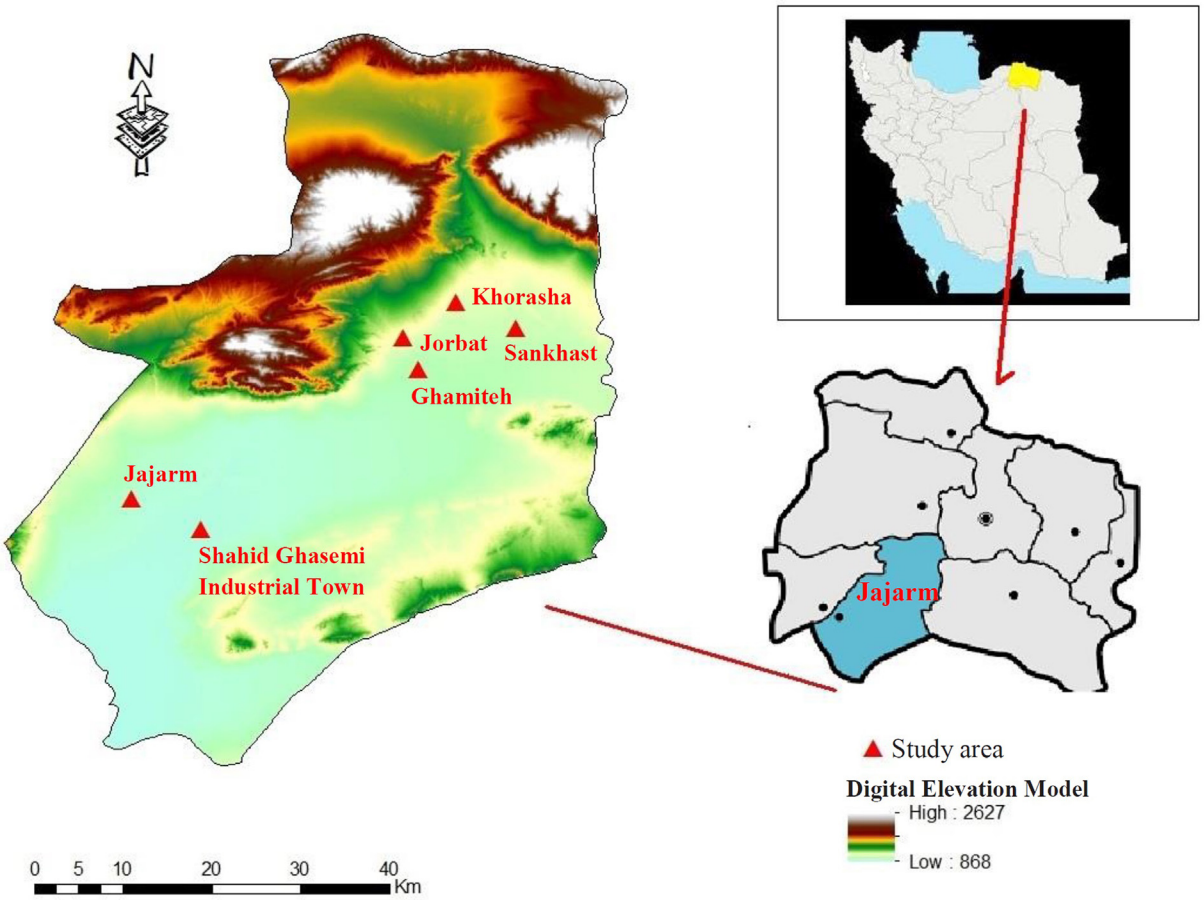

Fig. 1. Map of study area in Jajarm County, North Khorasan Province, northeastern Iran, 2012–2018.

2.3.2. kDNA amplification

Species-specific PCR assay that amplifies a variable region of kDNA minicircle was performed as described by Karamian et al., with slight modification (Karamian et al., 2008) in a reaction volume of 25 μl using primers, LINR4 (forward, GGG GTT GGT GTA AAA TAG GG) and LIN17 (reverse, TTT GAA CGG GAT TTC TG). Next, the samples were amplified as follows: initial denaturing at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min followed by final extension cycle at 72 °C for 5 min (Karamian et al., 2008). Finally, 10 μl of each amplified product was analyzed by electrophoresis on 1.5% agarose gel in Tris/boric acid/EDTA buffer (pH 8.5). The gel was stained with SYBR green, visualized under ultraviolet light, and sized by comparison with a 100 bp DNA ladder as a molecular size marker. The length of amplified DNA segments varies in different reference *Leishmania* species. It is 650 and 760 bp for *L. major* and *L. tropica*, respectively. The reference strains of *L. major* (MHOM/SU/73/5ASKH) and *L. tropica* (MHOM/SU/71/K27) were used as standard DNA.

Also, *Leishmania* DNA extracted from smears belonging to 5 cases of CL (parasitologically confirmed), were used as positive controls and 5 smears of samples from lesions with different skin diseases other than CL, those which referred to the regional Leishmaniasis Diagnostic Referral Lab (LDRL), MAZUMS, were used as negative controls.

3. Results

Between 2012 and 2018, a total of 629 patients with active CL lesions were diagnosed positive by microscopic examination for the presence of *Leishmania* amastigote. Of 629 subjects with lesions, 369 were male (58.7%) and 260 were female (41.3%). The average age of the subjects was 18.2 years and the ages ranged from 3 month to 83 years. A male subject with CL was 1.4 times more likely than a female subject, the difference was statistically significant ($P = 0.0001$) (Table 1).

Most (29.4%) of the subjects with CL were aged <10 years with significantly higher frequency than other age groups ($P = 0.00001$) (Table 1). The number of lesions on the patient's body varied from 1 to >3 lesions. The majority (54.7%) of them had 3 or more lesions, which were significantly more than patients with one lesion ($P = 0.0001$) as shown in Table 1. Most lesions (70.4%) were on the face (32.2%), hands (23.7%) and head and neck (14.5%), while 29.6% of lesions were on other parts of the

Table 1
Distribution of zoonotic cutaneous leishmaniasis (ZCL) among patients in Jajarm County, Northern Khorasan Province, 2012–2018, using PCR.

Variable	Frequency	Percent (%)		P-value (Chi-square test)
Sex				
Male	369	58.7		0.0001
Female	260	41.3		
Number of lesion				
1	61	9.7		0.0001
2	224	35.6		
≥ 3	344	54.7		
Age group (years)				
0–9	185	29.4		0.0001
10–19	141	22.4		
20–29	109	17.3		
30–39	92	14.6		
40–49	34	5.4		
>50	68	10.8		
Location of lesion				
Face	202	32.2		0.0001
Head and neck	91	14.5		
Hands	149	23.7		
Forearm	29	4.6		
Arm	23	3.7		
Trunk	21	3.3		
Feet	39	6.2		
Leg	40	6.4		
Tigh	34	5.4		
District				
Jajarm	71	11.3		0.0001
Shahid Ghasemi industrial town	112	17.8		
Khorasha	138	21.9		
Ghamiteh	117	18.6		
Sankhast	98	15.6		
Jorbat	93	14.8		
Year		*Percent (%)*	*Incidence/100000*	
2012	103	16.4	289	0.0001
2013	158	25.1	412	
2014	81	12.9	212	
2015	145	23.1	378	
2016	38	6	102	
2017	55	8.7	146	
2018	49	7.8	126	

body including legs, feet, thighs, forearms, arms and trunk (Table 1). Most of the patients were successfully treated *with intralesional and or intramuscular Glucantime® (containing 100 mg antimony/mL)* at 20 mg/kg/day for 4–5 weeks.

The CL cases were observed in all months of the year except May; however the frequency of cases varied in different months. The highest frequency (93.2%) was between September to December, which was significantly higher than other months ($P = 0.0001$).

In addition, CL cases belonged to 6 districts of Jajarm County (Table 1). Most (58.3%) of them were inhabitant in the three districts of Khorasha, Ghamiteh and Shahid Ghasemi industrial town, as CL endemic areas in the south of North Khorasan Province, in which their frequency was significantly higher than the other districts. ($P = 0.0001$). The incidence rate of the disease in the study area was estimated 102–412/100000 population throughout current study periods (2012–2018); consequently a reemerged focus of ZCL caused by *L.major* occurred in Jajarm County.

For all positive samples with leishman bodies observed in the collected smears, species-specific PCR was performed to identify *Leishmania* species. The size of the amplified target was 650 bp, and kDNA of *L.major* was identified in 100% of positive microscopic smears and also positive controls using species-specific PCR, while all negative controls were negative (specificity, 100%; Fig. 2).

4. Discussion

The results showed that the annual incidence rate in Jajarm County during the study period of 2012–2018, was in average 237.8/1,00,000 people, which was more than 7 times higher than the incidence rate of CL in the country. The average annual incidence of CL over 30 year period was reported 32/1,00,000 people of Iran (Shirzadi et al., 2015). Based on our study the incidence rate is high comparing to ZCL endemic areas in other provinces of Iran. Our data clearly indicates an establishing reemergence of a

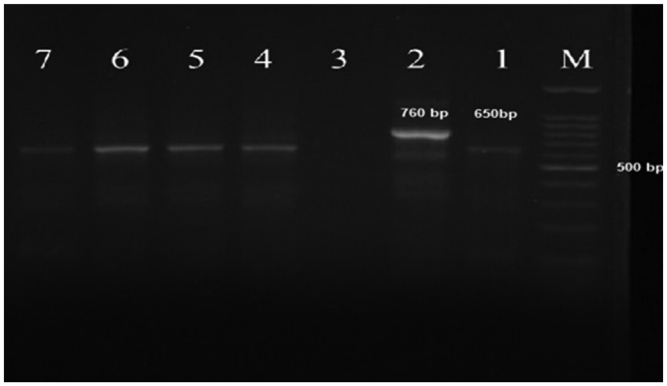

Fig. 2. Species-specific PCR assay of leishmaniasis patients in Jajarm County, North Khorasan Province, 2012–2018; Lane1: Positive control for *L. major*, Lane 2: Positive Control for *L. tropica*, Lane 3: Negative Control, Lane 4–7: Positive Samples for *L. major*. M: 100 bp Molecular Marker.

ZCL focus in the Iranian northeastern province; however, the real burden of ZCL among general population living in the area is thought to be significantly underestimated. Firstly, because our study was designed as passive case finding and the patients mostly not referred to the health centers and also because of low sensitivity of direct smears many of cases typically were not diagnosed.

The followings are several possible evidences concerning reemerging ZCL in the study area. Proximity of North Khorasan Province with Turkmenistan neighboring country as a highly ZCL endemic zone (Ghatee et al., 2020), lack of continuation of control programs against sandflies and rodent populations, low socio-economic status of inhabitants, development of agricultural projects, climate and ecological changes.

In view of the fact that North Khorasan Province has a close border with Turkmenistan, our reports are of particular importance for the proper monitoring of tourists from Turkmenistan at the border. Moreover, these data provide a warning message for the health systems in both countries and may affect ecotourism. Based on WHO report, between 2000 and 2009, 1562 cases of CL were reported from Turkmenistan, mostly from the southern and eastern parts of the country, especially from Mary Province close to Herat, Western Afghanistan, in neighboring Iran. About one-third of these cases were in children under 14 years of age and most of the patients were male (Razakov and May, 2009).

In the present study, most of the patients were male, which is in accord with the results of other studies in Golestan (Jorjani et al., 2019), Mazandaran (Ghavibazou et al., 2019), Northkhorasan (Alavinia et al., 2009) and Qom (Rostami et al., 2013) provinces, and also the study carried out over four decades in Iran (Piroozi et al., 2019). The higher leishmaniasis cases in men seems to be due to more outdoor activity and less coverage compared with women wearing Hijab in Muslim countries, leading to being bitten more by sand flies.

Most of the patients were aged <10 years. They are the active age group who might be exposed to sand fly bites due to the existing sand fly breeding places and reservoir host colony near human dwellings in the study area. This finding is in accordance with studies conducted in Golestan (Jorjani et al., 2019) and Kerman (Khosravi et al., 2017) provinces, in northern and southeastern Iran, respectively. However, contradict with studies conducted in the areas with low endemicity of CL such as Mazandaran (Ghavibazou et al., 2019), in northern and Kashan (Moein et al., 2018), in central Iran, which reported the highest incidence in the age group of 20–29 years. It is worth noting that when the endemicity of a certain disease is high (as in the current study), the disease is more common in younger age groups.

The occurrence of disease in lower age groups is showing the higher endemicity of disease in the area and would be because of some of the following reasons: higher frequency of sand flies and higher exposure to sand fly bites in the area, higher abundance of rodent nests and other sand fly breeding sites near human dwellings. In our previous study in this area, the fauna, ecology and biodiversity of sand flies was studied. Rodent hosts were abundant around human dwellings and *Phlebotomus papatasi* (*P. papatasi*) was the most predomestic species in the area (Unpublished data).

According to our unpublished study in the study area and the previous study on the composition and diversity of sand fly species in North Khorasan Province, *P. papatasi* was the dominants species and constituted about 80% of the sand fly community in Jajarm County (Arzamani et al., 2018). This species is known as the main vector of disease in ZCL endemic and non-endemic foci of Iran and the neighboring countries (Ghatee et al., 2020; Yaghoobi-Ershadi, 2012). There would be direct correlation between *P. papatasi* frequency in the study area and the occurrence of ZCL in human cases. Accordingly, in another study in Golestan Province the number of collected *P. papatasi* and the incidence rate of ZCL were significantly correlated in different foci of disease ($r = 0.837$, $P = 0.019$) (Sofizadeh et al., 2018).

In the present study, the peak of disease distribution was observed from September to December with the highest frequency in October. This finding is because of the activity season of sand flies in the study area. Due to the fact that most of the sand flies are parous at the end of sand fly season, their highest rate of infection and in turn, more disease transmission and more disease occurs after the incubation period from sand fly bites to nodule development (Yaghoobi-Ershadi et al., 2005; Yaghoobi-Ershadi and

Akhavan, 1999). This monthly distribution is due to the duration of disease and reflects the rural type of disease in the present study. In other studies, for example in Golestan (Jorjani et al., 2019) and Mazandaran (Ghavibazou et al., 2019), the peak of disease distribution was in November, in North Khorasan Province (Alavinia et al., 2009) in September and in Kashan (Moein et al., 2018) and Qom (Rostami et al., 2013) in central Iran, in Autumn.

As far as the anatomical location of the lesions on the patients' bodies is concerned, most of the lesions were found on the face and then on the hands. Since face and hands are mostly uncovered and are likely to be bitten by sand flies, they suffer higher frequency of lesions. This result agrees with the study carried out in North Khorasan Province, which the lesions were observed more frequently on the face. In the studies in Kashan (Moein et al., 2018), Kerman (Khosravi et al., 2017) and Mazandaran (Ghavibazou et al., 2019) provinces most of the lesion were reported on the hands. In CL, dermal lesions are commonly seen on bare areas of the body. Therefore, depending on the traditions and the manner of dressing in different areas, the anatomical location of the lesions may be different in different parts of the world and even in one country.

The results of this study showed that in most cases, there were three or more lesions on their bodies. In the study conducted in Isfahan Province, in most cases, the number of lesions has been reported more than three (Nilforoushzadeh et al., 2014). Due to the discordance feeding behavior of *P. papatasi*, as the main vector in the study area, multiple lesions are likely to occur on the body.

In our study, *Leishmania* DNA was identified by PCR (based on kDNA) in all samples of ZCL cases, which is in agreement with the findings of other researchers (Rostami et al., 2013; Andrade et al., 2011; Akhoundi et al., 2013). In a study in rural areas of 11 provinces of Iran that are endemic for ZCL, the causative agent was mainly (95%) *L. major* (Mahmoudzadeh-Niknam et al., 2012). The kDNA PCR was the most sensitive PCR-based test that was examined in several investigations (Karamian et al., 2008; Andrade et al., 2011; Bensoussan et al., 2006; Aflatoonian et al., 2019).

According to our results and previous studies, PCR seems to be a very sensitive and reliable test for *Leishmania* typing, particularly when other methods had failed to identify the parasite species (Marques et al., 2006).

5. Conclusion

In conclusion, our findings show that the incidence of ZCL caused by *L.major* is relatively high and worrying in the study area, north-eastern Iran; hence reemergence of ZCL focus was occurred. Moreover, our results showed that the use of conventional PCR on smears of clinically suspected ZCL cases is a valuable and reliable tool particularly for retrospective studies. On the other hand, PCR test could recognize the *Leishmania* spp. for accurate intervention. Our data provide valuable information regarding the epidemiology of reemerged ZCL in Jajarm, northeastern Iran, which will likely be paid more attention to improve public surveillance, increasing community awareness as well as developing control planning of vectors and reservoir populations.

Declaration of Competing Interest

None.

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