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Investigating *Crithidia spp.* in ulcer smear of patients suspected of leishmaniasis in Aq-Qala, Golestan province, Northern Iran, 2019-2020

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Abstract

Background: Cutaneous leishmaniasis (CL) is a parasitic infection that causes significant health problems. This study aimed to survey the prevalence of *Leishmania* and *Crithidia spp.* co-infection in the wounds of patients with suspected Leishmaniasis in Golestan Province, northern Iran.

Methods: This retrospective descriptive study included 140 smears of patients suspected of CL who were referred to the Leishmaniasis Diagnostic Laboratory in Aq-Qala Health Center, Golestan Province, northern Iran, in 2019-2020. The sore smears, along with demographic and epidemiological data of patients, were obtained from the laboratory archives. DNA was extracted from Giemsa-stained smears, and PCR was performed on the ITS1 and GPDH genes for *Leishmania* and *Crithidia spp.*, respectively.

Results: In our research, 140 patients suspected of CL were studied. PCR results showed that 117 patients (83.5%) were positive for *L. major*, three patients (2.1%) were positive for *L. tropica*, and 20 patients (14.2%) were negative for the *Leishmania* parasite. One patient's smear was positive for *Crithidia* species. Five patients (3.5%) had a history of treated leishmaniasis. The results indicated that 74.7% of the patients with one wound, 89.5% of the patients with two wounds, 96.4% of the patients with three wounds, 90.9% of the patients with four wounds, and 100% of the patients with five or more wounds were positive for *L. major*.

Conclusion: More studies should be conducted in the field of simultaneous infection of *Leishmania* and *Crithidia* in humans. In addition, research on the effect of leishmaniasis treatments on *Crithidia spp.* can be useful.

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Highlights

What is current knowledge?

Leishmaniasis is a parasitic disease found in over 99 countries. Leishmaniasis is usually observed in several forms, such as cutaneous, visceral, and mucocutaneous. In Iran, cutaneous leishmaniasis and visceral leishmaniasis have been reported. Golestan Province is located in northern Iran and is an endemic region for rural cutaneous leishmaniasis.

What is new here?

- Recent studies have shown that *Crithidia* parasites were found in the lesions of patients with cutaneous leishmaniasis, and drug resistance has been observed in the treatment of patients with cutaneous leishmaniasis in Golestan Province.
- The presence of co-infections, *Crithidia* and *Leishmania*, can indeed lead to inadequate treatment and potentially result in severe complications for patients. With the simultaneous infection of these two parasites, preventive measures can be implemented concerning epidemiology and treatment strategies.

Introduction

Leishmaniasis is a parasitic disease found in over 99 countries (1,2). Annually, 1.5 to 2 million individuals become infected with this disease (3). Leishmaniasis is usually observed in several forms, such as cutaneous, visceral, and mucocutaneous. Visceral leishmaniasis can even lead to death. Every year, about 350,000,000 people worldwide are exposed to this disease, and twelve million people are infected with it (4). For this reason, it is one of the six important diseases of tropical regions that the World Health Organization has recommended and supports for studying and conducting research on its various aspects (5). In Iran, cutaneous leishmaniasis (CL) and visceral leishmaniasis have been reported. CL is the predominant form (6). Recent studies have shown that *Crithidia* parasites have been found in the lesions of patients with CL (7,8). Many studies have shown that *Crithidia* can also infect the *Leishmania* culture medium (9). In addition, *Crithidia* has been identified in CL lesions in humans (10) and animals, such as rodents (11) and dogs (12). Interestingly, the gene sequence of ribosomal RNA (rRNA) in *Crithidia* species shows a high degree of similarity to *Leishmania*. *Crithidia* is a parasite transmitted by the bites of infected insects, such as sandflies or fleas (9,13). Golestan Province is located in northern Iran and is an endemic region for rural CL. One of the endemic areas of CL in this province is Aq-Qala City, which is located in the eastern part of Golestan Province. Recently, drug resistance has been observed in the treatment of patients with CL (14). The presence of co-infections of *Crithidia* and *Leishmania* can indeed lead to inadequate treatment and potentially result in severe complications for patients (15,16). This research aims to investigate the slides of patients with CL for the concurrent presence of *Crithidia* and *Leishmania* parasites.

Methods

In this retrospective descriptive study, 140 Giemsa-stained smears from patients suspected of CL, along with their demographic and epidemiological data, were collected from the archives of the laboratory at the Leishmaniasis Diagnostic Laboratory, Aq-Qala Health Center, Golestan Province, Iran, during 2019-2020. DNA was extracted from the Giemsa-stained smears, and PCR was used for the amplification of the ITS1 and GPDH genes in *Leishmania* and *Crithidia* spp., respectively.

DNA extraction and PCR

We extracted the DNA from the Giemsa-stained smears of patients suspected of CL using a DNA extraction kit (DENAzist, Mashhad, Iran) according to the manufacturer's protocol. CRF: 5′-TCCATGTGCGAGGACAACGTGCT-3′ and CRR: 3′-CGCGTCGTTGATGAAGTCGCT-5′ primers, based on the sequence of the GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) gene, were used to identify *Crithidia* spp. PCR was performed in a 25 µl volume, with 5 µl of DNA, 2 µl of 10 pmol primers, 12 µl of 2× master mix, and 6 µl of deionized water, following the PCR program: an initial step at 94°C for 5 min, 30 cycles at 94°C for 30 sec, annealing at 55°C for 1 min, extension at 72°C for 90 sec, and final extension at 72°C for 5 min to amplify an 800 bp fragment of the GPDH genes in *Crithidia* spp. For the detection of *Leishmania* and differentiation of *L. major* and *L. tropica*, primers LIN4R (F): 5′-GGGGTTGGTGTAAAATAGGG-3′ and LIN17(R): 5′-TTTGAACGGGATTTCTG-3′ were used to amplify the partial ITS1 gene. The PCR program was as follows: 95°C for 5 min, 35 cycles at 94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec, and 72°C for 8 min to amplify fragments of 650 bp for *L. major* and 760 bp for *L. tropica*. In these experiments, a mastermix solution, primers, and distilled water without DNA were used as negative controls, while *Leishmania major* and *Leishmania tropica* DNA were used as positive controls.

Statistical analysis

The results of the questionnaires were analyzed using SPSS version 25.0. Chi-square and Fisher's exact tests were used. P-values < 0.05 were considered significant.

Results

The result of the *Leishmania*

In our research, 140 patients suspected of CL were studied. PCR results showed that 117 patients (83.5%) were positive for *L. major*, three patients (2.1%) were positive for *L. tropica*, and 20 patients (14.2%) were negative for *Leishmania* parasite. Of the 140 patients, 44 (31.5%) were women, and 96 (68.5%) were men. The results showed that in the 20-40 age group, 33 patients (91.7%) were positive for *L. major*, while among those over 40 years old, 42 patients (87.5%) were positive for *L. major*. Three patients (One from each group) under six years old (5.9%), 7-19 years old (2.9%), and over 40 years old (2.1%) were infected with *L. tropica*. Our results showed that 84.6% of patients living in the city and 83.1% of those in the village were positive for *L. major*. Meanwhile, 5.1% of patients in the city and 0.9% of patients in the village were positive for *L. tropica*. Five patients (3.5%) had a history of treated leishmaniasis, and 51 patients (36.5%) had an unknown history of *Leishmania* infection. There was no significant relationship between *Leishmania* infection and the history of CL (P-value > 0.05). The appearance of wounds was wet (Ulcers with cerusite) in 137 people (97.8%) and dry (Ulcers without cerusite) in three people (2.2%). The results showed that 117 patients (85.4%) with wet wounds were infected with *L. major*. In addition, all three patients (100%) with dry wounds were infected with *L. tropica*. Eleven patients (7.8%) had cutaneous ulcers on the head and face, three patients (2.1%) had neck ulcers, 10 patients (7.1%) had trunk ulcers, 67 patients (47.8%) had upper limb ulcers, and 49 patients (35%) had lower limb ulcers. Chi-square test showed that PCR results of *L. major* are the most frequent. The result indicated that 74.7% of patients with one wound, 89.5% of patients with two wounds, 96.4% of patients with three wounds, 90.9% of patients with four wounds, and 100% of those with five or more wounds were positive for *L. major* (Figure 1).

The result of the *Crithidia* spp.

In evaluating the PCR results for *Crithidia*, among the 140 studied samples, only one female patient (0.7%) tested positive for *Crithidia* species. She was aged 7-19 (2.9%), had one wound (1.3%) on her foot (2%), and the wound was wet (0.7%), with no previous history of infection. This patient was a student and a city resident. The results showed that this patient was positive for *Crithidia* PCR and was also infected with L. major (Figure 2).

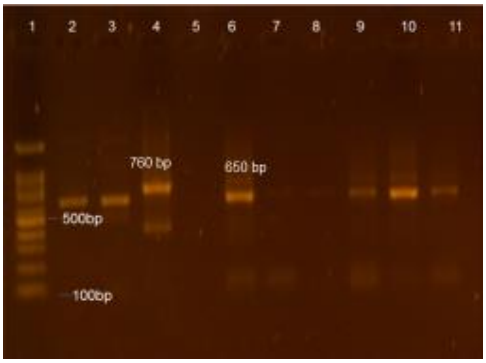

Figure 1. PCR product electrophoresis from sample of patients
Lane 1: DNA ladder marker 100bp
Lane 2: *Leishmania major* positive control
Lane 3, 6-11: *Leishmania* major positive samples
Lane 4: *Leishmania tropica* positive control
Lane 5: Negative control

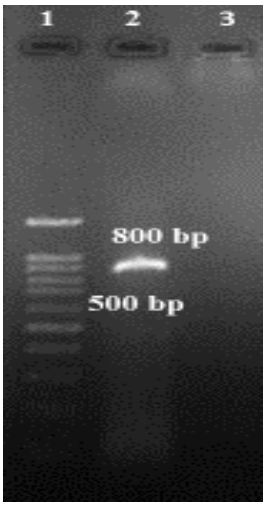

Figure 2. Gel electrophoresis of sample from the CL patient was *Crithidia* positive
Lane 1: DNA ladder marker 100bp
Lane 2: Positive sample (*Crithidia spp.*) 800bp
Lane 3: Negative control

Discussion

Cutaneous leishmaniasis is one of the health and research priorities of the World Health Organization, especially in relation to developing countries. It has received special attention in Iran, with a focus on its various aspects. The disease is one of the most important infectious diseases in Golestan Province, northern Iran, and has caused significant health problems in society. In the present study, 117 of the smears (83.6%) were positive for *Leishmania major* parasite, three of the smears (2.1%) were positive for *Leishmania tropica*, and one of the smears (0.7%) was positive for both *Crithidia* and *Leishmania major* parasites. In other words, there is a significant relationship between *Leishmania* PCR and the type of ulcer, such as wet or dry (P-value < 0.05). Therefore, people with wet wounds are infected with *Leishmania major*, and people with dry wounds are infected with *Leishmania tropica*. In the study by Ghobakhloo et al., 92.8% of patients suspected of CL had only *Leishmania major*, 1.8% of patients had only *Crithidia spp.*, and 5.4% of patients were infected with both *Leishmania major* and *Crithidia spp.* (15). Co-infection of *Crithidia spp.* in clinical cases of CL has been frequently reported in Iran. In Kalantari's study, *Crithidia* was detected in two CL patients who were chronically infected with L. major. In this study, there was one patient with concomitant *Crithidia spp.* and L. major infection. Another study by Ghobakhloo in 2019 showed that 50% of patients had *Leishmania* spp. and *Crithidia spp.* and were resistant to Glucantime (4). The increasing prevalence of Glucantime resistance and severity of cases, particularly in Iran, may be associated with the co-infection of *Leishmania* spp. and *Crithidia spp.* This concomitant infection could potentially explain the drug resistance observed in CL patients (17,18). In the study by Kalantari et al., 61.2% of Tatera indica were infected with *Leishmania*, and 2.4% were reported positive for *Crithidia* (16). In Doudi et al.'s study, 201 samples were isolated from suspected patients with CL who referred to the Leishmaniasis Research Center or other health centers in the vicinity of Isfahan. Of these, 33.3% of the samples were infected with *Crithidia spp.*, and 58.3% were infected with *Leishmania* spp. (7). In the study by Brazesh et al., out of 66 patients with CL in Shiraz, 60 samples were infected with *Leishmania major*, and six samples were infected with *Leishmania tropica*. Furthermore, two samples were infected with both *Leishmania* and *Crithidia spp.* (19). In the study by Mirzapour et al., out of 70 patients with CL in Shiraz, 46 samples (65.7%), 17 samples (24.2%), and seven samples (10%) were infected with *Leishmania major*, *Leishmania tropica*, and *Crithidia* fasciculata, respectively (14). In some previous studies, *Crithidia spp.* have been frequently reported, likely because these studies were mostly conducted on rodents, where this parasite is commonly found in animals. In the present study, we found that 12.1% of the participants under 6 years old, 25.2% of the participants aged 7-19 years, 26.7% of the participants aged 20-40 years, and 35.6% of the participants over 40 years old had leishmaniasis. The Chi-square test showed that the frequency of L. major was the highest in all age groups; however, there was no statistically significant relationship between *Leishmania* and age groups (P-value > 0.05). In the study by Axmedovich et al., 20% of leishmaniasis patients were under 20 years old, 46.4% were 20-40 years old, and 32.8% were over 40 years old (1). In the present study, 68.5% of individuals were men, and 31.5% were women. Similarly, in the study by Axmedovich et al., 42.1% of leishmaniasis patients were women, and 57.9% were men (1). In the study by Khazaei et al., 55.1% of the patients were men, and 44.8% were women (2). In the study by Al-Khayat et al., 56.7% of *Leishmania*-positive samples were male, and 43.3% were female (3). In the same study by Al-Khayat et al., 57.6% of patients with dry wounds were men, and 48.6% were women. In addition, 51.4% of patients with wet wounds were men, and 42.4% were women (3). In this study, out of the 140 patient samples, 137 (97.8%) had wet ulcers, and three (2.2%) had dry ulcers. In addition, 67.9% of the patients with wet wounds were men, and 32.1% were women, while 100% of the patients with dry wounds were men. Al-Khayat, in 2019, reported that case severity features, based on characteristics such as the number, size, site, and type of lesions, were more pronounced in males compared to females (3). In this study, 85.7% of patients were positive for the *Leishmania* parasite, with 61.4% of the positive cases being males. In this respect, the results of our study conform to those of Al-Khayat. These differences may be attributed to immunological variations between genders, with males potentially experiencing increased parasitism. Females typically exhibit stronger immune responses, and the hormone testosterone has been found to have a disease-promoting effect (20,21). Another reason for the higher incidence in men compared to women could be the full coverage typically worn by women, especially in Iran. Furthermore, the existence of habits such as sleeping outdoors among men and their greater contact with environmental factors due to work reasons can be considered as factors contributing to the increase in their incidence (22). In the study by Khazaei et al., 62.7% of patients had upper limb ulcers, 24.8% had neck ulcers, 2.7% had trunk ulcers, and 22.7% had lower limb ulcers (2). In the present study, 63.6% of patients with facial wounds were men, and 36.4% were women. Among those with trunk wounds, 50% were men and 50% were women. In addition, 68.7% of patients with hand ulcers were male, while 31.3% were female. Similarly, 71.4% of patients with foot ulcers were male, and 28.6% were female. In Al-Khayat et al.'s study, 59.3% of patients with limb ulcers were male, and 40.7% were female. Among those with facial wounds, 48.5% were men, while 51.5% were women. In addition, 53.8% of those with trunk wounds were men, and 46.2% were women (3). The Chi-square test showed that PCR results for *Leishmania major* were the most frequent; however, no

statistically significant correlation was found between PCR results and wound site (P-value > 0.05). Lesions of CL usually appear on uncovered and exposed parts of the body. Therefore, depending on the customs and clothing of the residents of different regions and the blood-feeding habits of sandflies, the organs that have the most wastes vary across different parts of the world and even within countries. In the present study, 60% of the patients were new cases, and 40% were other cases. In the study by Khazaei et al., 96.91% of cases were new cases (2). Moreover, in the present study, 27.8% of people with ulcers were urban residents, and 72.1% were rural residents. In the study by Khazaei et al., 62.6% of people with CL lived in cities, and 37.4% lived in villages (2). A limitation of this study is that, due to the low rate of *Crithidia spp.* positive samples, the potential interference of this parasite in response to patient treatment is unclear. This study was retrospective, and we had no discretion in selecting patients.

Conclusion

More studies should be conducted in the field of simultaneous infection of *Leishmania* and *Crithidia* in humans. In addition, research on the effect of leishmaniasis treatments on *Crithidia spp.* could be useful.

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Ethical statement

Approval was granted by the ethical committee of Golestan University of Medical Sciences, Gorgan, Iran, with ethics code IR.GOUMS.REC.1400.393.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

AKh collected the samples and conducted the experiments. ZM performed the molecular experiments. FT was responsible for conceptualization, methodology, analysis, writing the manuscript, and review and editing.

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