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EPIDEMIOLOGICAL STUDY OF CUTANEOUS LEISHMANIASIS IN SOME IRAQI PROVINCES

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ABSTRACT

Background

Cutaneous leishmaniasis (CL) remains a serious public health concern in some parts of Iraq. The aims of this study to report of CL in some parts of Iraq, by different parasitological, cultural, and molecular methods and evaluate sex differences among infected patients. This is the first study conducted to characterize *Leishmania* species causing CL among Iraqi patients using the sequence analysis of Internal Transcribed Spacer1(ITS1) at Wasit Province.

Methods

A total of 700 cases of suspected CL were referred to the Iraqi clinics and health centres and they checked for *Leishmania* amastigote using a Giemsa-stain; however, the Novy Macneal Nicolle (NNN) culture led to the growth of promastigotes in all samples, then the samples were examined using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP)-PCR methods.

Results

The present study indicated that the prevalence of CL as follow: AL-Diwaniyah 88(15.1%), Wasit 85 (14.5%), Najaf 79 (13.6%), Thi-Qar76 (13.1%), Basrah 67 (11.5%), Baghdad 65 (11.2%), Diyala 63(10.8%), and Salah-Edin province which recorded lower infection 60 (10.3%), and it appeared in 83.3% by using Giemsa-smeared stain. The highest infection rate (100%) appeared using PCR while the lowest infection (68%) appeared by culture on NNN media. The present study was revealed that the highest infection (60%) was caused by *L.major* rather than *L.tropica* (40%). Our results showed that 368 (52.6%) of CL patients were had single lesion and 215 (30.7%) had multiple lesions, and the ulcerative wet type lesions were present in 49.6%, while the nodule dry type lesions were present in 33.7%. The overall prevalence of cutaneous leishmaniasis in the study area was very high (83.3%) having a statistically significant association with sex; males are more prone (56.4%) to CL as compared to females (43.6%).

Conclusions

A clear and reliable bias toward males exists in some tropical diseases, such as leishmaniasis. CL is a major health problem in Iraq and CL caused by many countries including Iraq. Health authorities should be aware of the fact that war and terrorist activities induce expansion of the disease and increase the incidence rate in the situation that access to medical treatment is not easy especially in poor conditions in leishmaniasis endemic areas.

Key words: *Cutaneous leishmaniasis, Gender, Molecular, Human, Iraq*

Leishmaniasis is an antique vector-borne neglected disease and a major public health problem in some endemic regions caused by protozoan parasite belong to genus *Leishmania*. The World Health Organization (WHO) estimated that currently around 10th of the world population are at risk of getting one form of leishmaniasis, the disease is reported from 98 countries on 4 continents and the disability-adjusted life years (DALYs) of about 2.4 million.[1,2]

In parasitic diseases, males are affected more frequently and more severely than females.[3,4] To identify a clear gender bias during infection and disease, several critical factors have to be taken into account. For example, the prevalence of parasitic diseases is high in developing countries; however, in such countries, traditional differences in sociocultural behaviour between males and females are more pronounced than in industrialized countries. Therefore, external factors such as task sharing, access to infection sources, and motility might falsely create sex bias during infection and disease.[5,6]

The clinical picture of the disease depends upon both the causative species and the host immune response and includes cutaneous leishmaniasis (CL) which is the most common form of the disease, the potentially lethal form of visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL). The disease is caused by more than 20 species of *Leishmania* and transmitted by more than 30 different sand fly species. Leishmaniasis is a major health problem in EMRO (Eastern Mediterranean Region Office) countries. Leishmaniasis is endemic to 14 of 22 EMRO/WHO countries, and the causative agent of VL is mostly *L. infantum*, and CL is either anthroponotic (ACL) caused by *L. tropica*, transmitted by *P. sergenti* sand flies or is zoonotic caused by *L. major*, with small rodents serving as the animal reservoir of ZCL and transmitted by *P. papatasi.*[1,2,7] Old World cutaneous leishmaniasis (OWCL) is most often associated with the species *Leishmania major* and *Leishmania tropica.* CL can self-heal without drug treatment after a period of 7–12 months.[8]

Every year about 500,000 cases of ZCL caused by *L. major* appear in Central Asia, the Middle East, North Africa, and some sub-Saharan countries and outbreaks in other rural areas depending on fluctuations in the rodent population. As well, ACL caused by *L. tropica* is transmitted in urban zones and affects around 400 000 patients annually. It was found that 90% of VL cases occur in India, Bangladesh, Sudan, Brazil, Nepal, and Ethiopia, and 90%of CL cases occur in Afghanistan, Algeria, Ethiopia, Sudan, Iran, Iraq, Saudi Arabia, Syria, Brazil. and Peru.[9]

VL and CL are endemic in different parts of Iraq, VL is caused by *L. infantum* and *L. donovani* according to Avar *et al*. in recent years annually 1,800 VL cases reported in Iraq and estimated nearly 4,000–5,000 cases, Iraq will be added to the list of countries with 90% of VL and CL in Iraq is caused by *L. major* and *L.tropica.*[9] Previous reports indicate that CL was mainly endemic in the northern part of the country such as Diyala, Kirkuk, Salah-Eldin, Wasit, and Missan. Thousands of CL cases were also reported from US armies in Iraq and Afghanistan.[10] Due to various changes such as war, terrorist activities, etc. leishmaniasis cases are increasing during the last decade. The aim of this study is to report the current status of CL in Iraq and evaluate sex differences among infected patients.

METHODOLOGY

Ethical Consideration

The proposal of the study was approved by the Ethical Committee of Wasit University, the patients were interviewed and the ones who were willing to

participate and sign an informed consent were recruited. A questionnaire(s) form including duration of the lesion(s), number, type and location of the lesion(s), history of travel, etc. was completed for each patient.

Area of Study

The study areas consisted of 8 Iraqi provinces of Baghdad, Basrah, Wasit, Diyala, Salah-Edin, Najaf, Diwaniyah and Thi-qar. The study population was the patients with suspected CL lesion who were referred to the Iraqi's clinics and health centres during for a period of one year from January 1, 2015 to the end of December 2015.

Specimens Collection

The referred patient was physically examined by a physician and her/his lesion was checked by a dermatologist. One of the patient's lesions was selected and samples were collected from around the edges of lesion. The samples were collected from 8 Iraqi provinces as follows: Diwaniyah 100, Wasit 99, Najaf 94, Thi-Qar 91, Basrah 82, Baghdad 80, Diyala 78, and Salah-Edin 76 samples. Three samples were collected from the lesion, one was used for direct smear and stained using Giemsa and checked for amastigotes under high-power microscope with immersion oil. The second sample was transferred into biphasic culture media NNN (Novy, Mc Neal & Nicole) and was incubated at 26 ± 1°C and checked for promastigote growth after 2 days and at 4 days intervals for up to 4 weeks. The liquid phase of NNN medium was harvested by centrifugation and washed 3 times with sterile phosphate buffer saline (PBS). The pellet was re-suspended in 1000µl of sterile PBS and stored at −70°C for later evaluation by polymerase chain reaction (PCR). The third skin samples was spotted onto Whatman paper (FTA Cards No: WB 120210) and was air-dried and kept for molecular tests.

Sample Application from Direct Smear

The tissue on stained slides known to contain amastigotes was scraped off with a sterile scalpel and re-suspended into 100 µl of sterile PBS for DNA extraction.

DNA extraction

One-hundred µl of culture suspension which was stored at –70°C or slide scraped suspension was added into 200 µl of lysis buffer (100 mM Tris; 1% SDS; 10 mM EDTA; 100 mM NaCl) and 20 µl Proteinase K and incubated at 56°C for 60 minutes. Three-hundred µl phenol-chloroform (50:50 v/v) was added to the lysate's micro tube and centrifuged for 5 minutes at 5000 rpm. The upper layer was collected and added to an equal volume of phenol and centrifuged for 5 minutes at 5000 rpm. The supernatant was added to an equal volume of isopropanol and 1/10 volume sodium acetate. Following incubation at −20°C for 10 minutes, the sample was centrifuged at 12000 rpm for 15 minutes. The pellet was washed with 300 µl 70% ethanol and centrifuged at 5000rpm for 5 minutes. The residue was re-suspended in 20 µl of sterile distilled water and stored at −20°C.

PCR Amplification

PCR assay was carried out by using specific primer(s) for small subunit (SSU) ribosomal RNA (rRNA) and 5.8S rRNA regions that are related to ribosomal ITS1, the primers forward primer (CTGGATCATTTTCCGATG) and reverse primer (TGATACCACT TATCGCACTT) were used to amplify approximately (350 bp PCR product) in *L. major* and *L. tropica*. The primers were provided by (Bioneer company, Korea). The PCR premix tube contains freeze-dried pellet of [Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10 mM, KCl 30 mM, MgCl2 1.5 mM, stabilizer, and tracking dye] and the PCR master mix reaction was prepared according to kit instructions in 20 µl total volume by added 5 µl of purified genomic DNA and 1.5µl of 10 pmol of forward primer and 1.5 µl of 10 pmol of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20 µl and briefly mixed by Exispin vortex centrifuge (Bioneer) to sequence analysis.

RESULTS

The present study indicated that the infection of CL in 8 Iraqi provinces as follow: Diwaniyah 88(15.1%), Wasit 85 (14.5%), Najaf 79 (13.6%), Thi-Qar 76 (13.1%), Basrah 67 (11.5%), Baghdad 65 (11.2%), Diyala 63 (10.8%), and Salah-Edin province which recorded the lower infection 60(10.3%), and it appeared 83.3% by using Giemsa-smeared with 2 types of lesions; wet 347 (49.6%) and dry 236 (33.7%). Infection

in males 329 (56.4%) was more predominant than in females 254 (43.6%) and that also applies on single lesion 368 (52.6%) to multiple lesion 215 (30.7%), as outlined in the following tables:

TABLE 1 Distribution of CL Cases by Parasitological Diagnostic Methods

Result	Giemsa-smeared No. (%)	Culture on NNN No. (%)
Positive	583/700 (83.3%)	476/700 (68%)
Negative	117/700 (16.7%)	224/700(32%)
Total	700/700(100%)	700/700 (100%)

TABLE 2 Distribution of CL Cases According to the Age and Gender

Age Group / Year	Positive Cases	Male	Female
1–10	308	200(34.3%)	108(18.5%)
11–20	93	54(9.3%)	39(6.7%)
21–30	74	23(4%)	51(8.7%)
31–40	51	23(4%)	28(4.8%)
41–50	36	19(3.2%)	17(2.9%)
>50	21	10(1.7%)	11(1.9%)
Total	583	329(56.4%)	254(43.6%)

TABLE 3 Distribution and Frequency of CL in Some Iraqi Provinces

Province	Number	Male	Female
Wasit	85(14.5%)	68(11.6%)	17(2.9%)
Baghdad	65(11.1%)	35(6%)	30(5.1%)
Basrah	67(11.5%)	36(6.2%)	31(5.3%)
Diyala	63(10.8%)	33(5.7%)	30(5.1%)
Thi-qar	76(13.1%)	39(6.7%)	37(6.4%)
Diwaniyah	88(15.1%)	45(7.7%)	43(7.4%)
Najaf	79(13.6%)	43(7.4%)	36(6.2%)
Salah-Edin	60(10.3%)	30(5.1%)	30(5.1%)
Total	583(100%)	329(56.4%)	254(43.6%)

TABLE 4 Distribution of Leishmania Species in Iraqi CL Patients using PCR –RFLP Assay

Leishmania sp.	RFLP-PCR result	Total %
L.major	60	60 (60%)
L.tropica	40	40 (40%)

DISCUSSION

Sex affects susceptibility to several infectious diseases, including some clinical signs of leishmaniasis. The disease is caused by protozoan parasites that enter to the skin and can spread to the lymph nodes, spleen, liver, bone marrow, and sometimes lungs. Parasites induce host defenses including cell infiltration, resulting in protective or ineffective inflammation. These responses are often affected by host genotype and sex.[11]

The results of the current study were revealed that the prevalence of cutaneous leishmaniasis in the study area was very high (83.3%) having a statistical significant association with sex; males are more prone (56.4%) CL as compared to females (43.6%).

Sex differences in some parasitic diseases like CL appear as a distinct age pattern. Its incidence reveals a significant male dominance during infancy that decreases as age progresses.[12]Therefore, the sex bias appears to be elevated at the stages of life characterized by increased levels of sex hormones. Protective immunity towards cutaneous leishmaniasis is controlled by Th1 responses, while Th2 responses have been related to susceptibility and disease progression.[13] Because testosterone activates production of Th2 cytokines and estrogen stimulates pro-inflammatory Th1 responses,[14] differential levels of sex hormones may cause this sexual dimorphism in disease outcome.

Correct diagnosis of *Leishmania* species is essential to determine the clinical prognosis and a species-specific therapeutic approach. Specification of different species of genus *Leishmania* depends on several factors such as the geographical distribution of an isolate, the clinical finding of the disease and the epidemiology of the vector and the animal reservoir.[15]

A round shape *Leishmania* parasite without flagellum was grown in culture media with biphasic NNN media as demonstrated by light microscopy examination. The change of promastigotes to amastigotes did take place completely in culture. These finding again emphasize that optimal conditions for propagation of axenic amastigotes vary and have to be determined for each *Leishmania* species isolates. DNA isolated from promastigote forms obtained from in vitro culture of *Leishmania* allowed for optimization of PCR reaction.[16]

FIG. 1 Iraq map with provinces where the study was carried out (United Nations, 2014).

The diagnosis of CL classically relies on microscopic examination and in vitro cultivation. These classical methods require the presence of a relatively high number of viable or morphologically intact parasites. This may pose a problem particularly in the chronic phase of CL where parasite levels in skin lesions are very low. In contrast, the molecular approach is both sensitive and specific.[17] In this study we set up a well-documented, genus-specific PCR to detect *Leishmania* species in clinical cutaneous samples and compared this method with classical methods.

Ulcerative wet type lesions were present in 71.9%, while the nodule dry type lesions were present in 28.1%. These observations are in agreement with those reported from Iraq,[18] Iran,[19] Colombia,[20] Pakistan,[21] and Afghanistan.[22] The present study indicated that the incidence rate of multiple lesions in CL patients was 32.8%. This result could be due to long periods of exposure to plebotomine sandflies and the high population density of sandflies in this area.

PCR methods using either genomic or kinetoplast DNA (kDNA) are now frequently cast in this role. When the amplicon is digested with restriction enzymes, it is possible to identify almost all pathogenic *Leishmania* species by RFLP, allowing direct, rapid characterization and identification of the infecting parasite.[23]

Several DNA targets were used for DNA amplification, such as the SSU rRNA gene , the ITS regions , the microsatellite DNA or extra-chromosomal DNA, such

as the repetitive kinetoplast DNA (kDNA) minicircles. Related to the sequence variation in the first part of the spacer, the ITS1-DNA target allows the identification of almost all medically relevant *Leishmania* parasites with the use of only one restriction enzyme (HaeIII) for amplicon digestion.

As a result of digestion with HaeIII, ITS1-PCR products yielded 140-bp and 220-bp fragments corresponding with *L. major*, and 60-bp and 200-bp fragments corresponding with *L. tropica*. In this study, we applied ITS1-RFLP as a tool for identification of *Leishmania* species. For a further characterization of DNA polymorphisms within *L. major* and *L. tropica* isolates from different areas of Iraq, we used sequencing of the amplified ITS1 region of representative strains of each RFLP pattern. Through PCR-RFLP, a genetic polymorphism was determined for *L. major* as LmA and LmB and for *L. tropica* as LtA and LtB for a number of samples. This may be related to either strain heterozygosity or mixed strains, as isolates were not cloned. Also, the Giemsa-stained slides were examined by both microscopy and ITS1-PCR. Most of the slides that were high scored amastigote numbers as microscopy positive were also positive by PCR-RFLP. Although the costs for PCR-RFLP diagnosis are higher and its concordance is lower than microscopic examination, but this method can identify *Leishmania* species without the need for cultivating them.

Identification of the causative *Leishmania* agent of CL is an important issue since choosing treatment strategy and prognosis of the disease depend on the causative species. Characteristics of the lesion and epidemiological information are not enough to define *Leishmania* species especially in endemic areas with mixed ACL and ZCL infections.[24,25]

Cutaneous leishmaniasis is the most severe form of leishmaniasis that is caused by the species of *L.major* and *L.tropica*. Although differentiation between these 2 species is important because of the differences in their epidemiology and pathology, this had been a difficult task. Typing this parasite using isoenzyme analysis is difficult and requires a large amount of the parasite or may sometimes be unreliable. The advent of molecular tools provided methods which are robust in discriminating these 2 species and to study their phylogenetic relationships.[26]

CONCLUSIONS

A clear and reliable bias toward males exists in some tropical diseases, such as leishmaniasis. Cutaneous leishmaniasis is a major health problem in Iraq and CL caused by many countries including Iraq. Health authorities should be aware of the fact that war and terrorist activities induce expansion of the disease and increase incidence rate in the situation that access to medical treatment is not easy especially in poor conditions of leishmaniasis endemic areas.

RECOMMENDATIONS

The current study revealed that sex-specific physiological factors were associated with CL infection in Iraq and null hypothesis for both populations, males tend to be more susceptible to CL than females, no previous study has focused to analyze this relationship.

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