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COMPARATIVE STUDY BETWEEN CLASSICAL AND MOLECULAR METHODS IN DIAGNOSIS OF *LEISHMANIA* SPECIES IN IRAQ

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ABSTRACT

Background and Objective

Cutaneous leishmaniasis (CL) remains a serious public health concern in some parts of Iraq. The aims of this study to diagnose the *Leishmania* sp. causative agent of CL in some parts of Iraq, by different parasitological, cultural, and molecular methods. It was carried during the period October 2014 to February 2015.

Materials and Methods

One-hundred sixty-one skin samples were examined by direct Giemsa-smeared culture on NNN medium and Nested – PCR methods in different age groups.

Results

The results of our study showed that 110 (68.3%) gave positives by Giemsa-smeared, 104 (64.6%) culture and 67 (100%) by Nested – PCR. Our results appeared that there was slight gender predilection; CL cases were more frequent in males (62.1%) than females (37.9%). Also, the type of infection showed that disease was in wet type 133(82.6%) more than in dry type 28 (17.4%). It was found that 98 (60.9%) of CL cases had previous contact with rodents while 63(39.1%) of cases had not.

Conclusions

CL disease is endemic in many parts of Iraq with high incidence and expanding to new foci that is considered a public health threat which needs special attention. Women appeared to be better equipped than men to contain the infection and its clinical consequences, but the sex factor tended to lessen at higher levels of exposure.

Keywords: *Cutaneous Leishmaniasis, Giemsa, Culture, Human, N-PCR*

Leishmaniasis are parasitic diseases transmitted by sandflies via bitting, affecting largely the poorest of the poor, mainly in developing countries; 350 million people are considered at risk of contracting leishmaniasis and some 2 million new cases occur yearly in 88 countries.[1,2] The two most common clinical forms of the disease; cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) are mainly seen in 14 of the 22 countries of Eastern Mediterranean Region (EMRO).[3] Leishmaniasis depends upon a causative agent and the host's genetic background presents various manifestations ranging from a self-healing lesion to a lethal systemic form of the disease. According to WHO estimates, 90% of CL cases occur in six countries.[4] The disease is endemic in Iraq and in neighbouring countries such as Kuwait, Saudi Arabia, and the Islamic Republic of Iran . In Iraq there are 2 main species of the genus *Leishmania* causing the CL infection: *Leishmania major* and *Leishmania tropica.* It seems that the majority of CL cases reported in Iraq are caused by *L.major*. Two epidemic outbreaks of CL have been reported in Diwania Province in 2008 with about 300 cases and in Baghdad / Rahmania in 2009 with about 400 cases.[5]

The main insect vector for transmission of *L. major* is the sand fly species *Phlebotomus papatasi*. In some urban centers of Middle East and Asia exist completely anthroponotic life cycles of the parasites, i.e. human beings are the main or only reservoir host. In such places cutaneous leishmaniasis caused by *L. tropica* can be highly endemic, but no animal reservoir is to be recognized.[6]

Identification of the infected *Leishmania* species based on clinical signs and symptoms can be problematic because several species cause both visceral and cutaneous involvement. With the advent of the PCR technology, several PCR-based assays such as the SSU rRNA gene, repetitive sequences, the gp63 gene locus, kinetoplast minicircle sequences, mini-exon gene sequences for *Leishmania* species differentiation, were developed. Culture techniques require a sophisticated laboratory setup, are time-consuming and carry an increased risk of contamination.[7,8] The present study aimed to diagnose the *Leishmania* sp. causative agent of CL in some parts of Iraq, by different parasitological, cultural, and molecular method by Nested PCR.

MATERIALS AND METHODS

Study Area

A total of 161 skin samples from patients of different ages with clinically suggestive CL by dermatologists whom attended to hospitals from different areas of Iraq from October 2014 to February 2015.

Specimens Collection

Skin lesions were cleaned and sterilized with disinfectant. Sterile saline (0.1 - 0.2 mL) was drawn into a syringe (1-mL, 25-gauge needle), and the needle was inserted into the nodule or ulcer's margin and rotated gently several times. A small amount of saline was expressed into the tissue, the needle was rotated, and some tissue aspirate and freed tissue were withdrawn.

Giemsa-Smear

Skin lesion was smeared on glass slides, air dried and fixed with methanol for a few seconds. Giemsa stain was filtered and diluted 1:20 with phosphate buffer (pH 7.2). The slide was covered with Giemsa stain for 5 minutes and washed with tap water then air dried. The Giemsa smear was examined under a microscope with 40× lens and 100× oil immersion lens. If at least one intra- or extra-cellular amastigote with a distinctive kinetoplast was found, the smear was declared positive. When no amastigotes

were seen after 15 minutes the smear was declared negative.

Primers

Nested – PCR was performed as follows: in the first stage two external primers CSB1XR (CGAG-TAGCAGAAACTCCCGTTCA) and CSB2XF (ATTTTTCGCGATTTTCGCAGAA CG) and in the second step, two internal specific primers 13Z (ACTGGGGGTT GGTGTAAA ATAG) and LiR (TCGCAGAACGCCCCT) were used for amplification of variable minicircles of *Leishmania* kDNA. All primers provided from (Bioneer, Korea) company.

RESULTS

Giemsa Smear in Relation to the Age and Gender

There were high statistically significant differences between age groups and the gender of patients. This higher rate of infection was apparent in the 21–30 years old age group in favour of the males by Giemsa smear of CL (Table 1).

Distribution of CL Culture in Relation to Age and Gender

The study showed that 104 cases (64.6%) produced a positive culture while 57 (35.4%) of cases gave a negative culture (Table 2).

Nested-PCR in Relation to Age and Gender

Table 3 shows that the higher rate of infection appeared in the 21–30 year old age group and the male was more predominant among males.

The Relationship between Leishmania Species and Site of Infection

Hands were the most common region of ulcer (38.8%) and the legs were the lowest (14.9%) (Table 4).

Distribution of CL According to the Types of Infection and Lesions

Table 5 shows that 28 patients (17.4%) had dry lesions, 133 (82.6%) had wet lesions, and 110 cases (68.3%) had multiple lesions and 51 cases (31.7%) had a single lesion.

TABLE 1 Distribution of CL Cases Examined by Giemsa-Smear in Relation to Age and Gender

Age/ year	Gender	Culture		Subtotal	Total
		Positive	Negative		
10 or less	**M**	**9**	**5**	**14**	**22**
	F	**5**	**3**	**8**	
11-20	**M**	**12**	**6**	**18**	**28**
	F	**7**	**3**	**10**	
21-30	M	18	8	26	42
	F	11	5	16	
31-40	M	16	6	22	34
	F	8	4	12	
41 or more	M	14	6	20	35
	F	10	5	15	
Subtotal	M	69	31	100 (62.1%)	161
	F	41	20	61 (37.9%)	
Total	-	110 (68.3%)	51 (31.7%)	161 (100%)	-

TABLE 2 Distribution of CL Cases Examined by Culture in Relation to Age and Gender

Age/ year	Gender	Culture		Subtotal	Total
		Positive	Negative		
10 OR LESS	**M**	**11**	**4**	**15**	**22**
	F	**3**	**4**	**7**	
11-20	**M**	**12**	**6**	**18**	**28**
	F	**7**	**3**	**10**	
21-30	M	18	7	25	42
	F	8	9	17	
31-40	M	16	6	22	34
	F	7	5	12	
41 or more	M	12	7	19	35
	F	10	6	16	
Subtotal	M	69	30	99 (62%)	161
	F	35	27	62 (38%)	
Total	-	104 (64.6%)	57 (35.4%)	161 (100%)	-

TABLE 3 Distribution of CL Cases Examined by Nested-PCR in Relation to Age and Gender

Age / year	Gender	Nested-PCR		Subtotal	Total
		L.major	*L.tropica*		
10 or less	M	5	1	6	8
	F	2	0	2	
11-20	M	6	2	8	13
	F	4	1	5	
21-30	M	11	1	12	21
	F	7	2	9	
31-40	M	5	2	7	12
	F	5	0	5	
41 or more	M	5	2	7	13
	F	6	0	6	
Subtotal	M	32	8	40 (59.7%)	67
	F	24	3	27(40.3%)	
Total		56(83.6%)	11(16.4%)	67(100%)	

TABLE 4 Molecular Identification of *Leishmania* Species by Location of Lesion

Location of lesion	*L.major* %	*L.tropica* %	Total %
Face	11(16.4%)	4(6%)	15(22.4%)
Hand	22(32.8%)	4(6%)	26(38.8%)
Legs	7(10.5%)	3(4.4%)	10(14.9%)
More than 2 locations	16(23.9%)	0(0)	16(23.9%)
Total	56(83.6%)	11(16.4%)	67(100%)

TABLE 5 Distribution of the Type of Infection According To Type of Lesions and Age Group of Patients

Age/ year	Type of Lesions	Type of Infection		Subtotal	Total
		Dry	Wet		
10 or less	S	2	3	5	22
	M	2	15	17	
11-20	S	5	3	8	28
	M	2	18	20	
21-30	S	4	10	14	42
	M	2	26	28	
31-40	S	4	8	12	34
	M	2	20	22	
41 or more	S	4	8	12	35
	M	1	22	23	
Subtotal	S	19	32	51 (31.7%)	161
	M	9	101	110(68.3%)	
Total	-	28 (17.4%)	133(82.6%)	161 (100%)	-

S = single; M = multiple.

Distribution of Patients in Relation to Gender, Rodent Finding, and Age Group

There were highly statistically significant differences between age groups and patient gender. This difference was higher in the 21-30 year-old age group with a predominance of males without the rodent finding (Table 6).

DISCUSSION

CL disease is endemic in many parts of Iraq with a high incidence and it is expanding to new foci. The diagnosis of several species of CL is necessary for treatment and control strategies. Most of the previous differentiations were based on extrinsic factors such

TABLE 6 The Distribution of Patients in Relation to Gender, Rodent Finding, and Age Group

Age / year	Gender	Rodent Finding		Subtotal	Total
		Found	Not Found		
10 or less	M	5	9	14	22
	F	2	6	8	
11-20	M	4	14	18	28
	F	2	8	10	
21-30	M	4	20	24	42
	F	3	15	18	
31-40	M	3	19	22	34
	F	4	8	12	
41 or more	M	6	14	20	35
	F	3	12	15	
Subtotal	M	22	76	98(60.9%)	161(100%)
	F	14	49	63(39.1%)	

S = single; M = multiple.

as clinical, epidemiological, and biological characteristics of host – parasite features. Lack of strict vector-parasite specificity in different localities and clinical manifestations and different epidemiological forms constitute major limitations for definitive identifications of the causative parasite.[9]

The correct diagnosis of *Leishmania* species is essential to determine the clinical prognosis and a species-specific therapeutic approach.[10] Specification of various species of genus *Leishmania* depends on many factors such as the clinical finding of the disease, geographical distribution of an isolate and the epidemiology of the vector and the animal reservoir.[11]

Gender is a relevant infectious disease risk-factor, as males and females can have variant probabilities of acquiring an infection and expressing immune response of a disease.[12] Previous studies in humans show that infectious disease incidence, as well as parasite-induced mortality is higher among men than women.[13,14]

The results of recent study investigated sex-related effect on clinical CL and determined that CL cases were more frequent in males (62.1%) than females (37.9%). These results agreed with those reported earlier,[7,12] but disagreed with some authors who reported higher rates in females.[13,14] Also, our results confirmed those of Nimri et al. who confirmed the relationship between the CL and gender.[15] Also Magill, observed that the infection rate of 72.4% in males compared to a rate of 27.6% in females.[16] This difference does not necessarily mean that males are more susceptible to infection or females are immune to it, but mostly is attributed to the existence of some contributing factors such as study design, population, climatic variations, and culture. Also, this difference could be due to more frequent exposure of male to sandfly bites due to time spent outdoors, military training, work or sleep in open areas, as well as men's clothing being less covering than women's.[17,18] Additionally, androgens can partially suppress the immune response in males.[19,20] Although it is believed that sex hormones may influence the establishment and the course of parasitic diseases, behavioural factors also make males more likely to

be exposed to vectors in fields and other transmission environments, which is probably equally if not more important.[21,22] The current study was found that male: female ratio was 1.6:1; which may indicate that both males and females are equally exposed to the risk of transmission of the disease.

The present study showed that the highest rate in both genders was in the 21- to 30-year-old age group which might be due to more exposure to the sandfly bites during their travel and activities in the endemic areas. Our result agreed with Sharifi[23] and Maraghi et al.,[24] but disagreed with UL-bari et al.,[25] who recorded that children seem to be more prone to infection than other age-groups.

The results of current study showed that a higher incidence of multiple lesions were observed higher 110 (68.3%) cases while there were 51 (31.7%) cases with single lesions. These results were similar with other studies, like Al-Samarai and AlObaidi,[7] Al-Mashhadany in Iraq,[26] and in contrast with AL-Hucheimi,[27] who reported that most of the cases were with single lesions. The exposure of humans to the sandfly bites plays an important role in the number of lesions.

The current study showed that the wet type lesion was more predominant at 133 (82.6%) cases than the dry type with 28 (17.4%) cases. This result was in agreement with other studies[7,27] in Iraq, Also, it agreed with other studies,[24,28,31] that recorded that wet type lesions were more frequent than the dry type, this is due to causative agents and sometimes secondary infection. The lesions were found to be of both dry and wet types indicating the presence of different strains within the country.

Classical methods like Giemsa - smears and culture are routinely used in the laboratory for diagnosing leishmaniasis and do not differentiate *Leishmania* species and their sensitivity less than molecular methods.[30] It is possible that infections of *Leishmania* parasites will be missed due to disparate growth rates of different parasites in the cultures.[31] The molecular approach is both sensitive and specific.[24,31] In this study we set up a well-documented, Nested PCR to detect *Leishmania* species in clinical cutaneous samples and compared this method with classical methods.

CONCLUSIONS

CL disease is endemic in many parts of Iraq with a high incidence and expanding to new foci that is considered a public health threat which needs special attention. Women appeared to be better equipped than men to contain the infection and its clinical consequences, but this factor tended to shrink at higher levels of exposure.

RECOMMENDATIONS

The present study indicated that sex-specific physiological factors were involved in the clinical epidemiology of CL in Iraq and null hypothesis for both populations, male–female differences in incidence are solely due to differences in exposure.

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