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Detection of *Leishmania* species by using nested PCR in Al-Kut city

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Abstract
The present study was intended to detect the cutaneous *Leishmaniasis* from a total of suspected cases that were gathered from dermatology center of AL-Zahra'a and AL-Karamah Teaching Hospital in Al-Kut city from October 2015 to the end of February 2016. Totally 70 patients aged 1<45 year were included 30 male, 40 female. The collected wounds specimen were looked at with microscope examination of smears stained with Giemsa staine and genospecific Nested PCR, Of 70 patients microscopy uncovered amastigotes in 21(30%), while Nested PCR identified *Leishmania* in 55(79%) positive samples. Nested PCR revealed a total of 55 very much archived cases in the present study 49(70%) were brought on by *Leishmania major* and 6(9%) by *Leishmania tropica*. There was a critical relationship between the conveyances of cutaneous *Leishmaniasis* among patients as indicated by sex, age and habitation. Settled PCR offers fast ID of *Leishmania* to the perplexing level.

Keywords: *Leishmania*, Nested PCR, *Leishmania tropica*, Giemsa stain

1. Introduction
Leishmaniasis is protozoan disease caused by a hemoflagellate of the genus *Leishmania* and transmitted by sand flies, The pathogenesis of *Leishmaniasis* range from fatal infection of the visceral organs to mild cutaneous infection [1]. Cutaneous *Leishmaniasis* (CL) is endemic in 88 nations, its predominant all through the tropical and subtropical locales of Africa, Asia, Mediterranean, Southern Europe (old world) and South and Central America (New world) [2]. Two millions of new cases –"1.5 million of cutaneous *Leishmaniasis* is more than 90% happen in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil and Peru, About 500 000 of the instinctive type of the sickness happen every year, except announcement of the ailment is obligatory in just 32 nations and a generous number of cases are never recorded [3, 4]. Human infection is brought about by more than 20 distinct species that taint mammals. A single species can create more than one clinical type of the ailment, the skin, mucosa, and mononuclear Phagocytic framework might be influenced by giving three types of *Leishmaniasis*: cutaneous *Leishmaniasis*, mucocutaneous *Leishmaniasis* and instinctive *Leishmaniasis* [5]. *Leishmania* parasite are transmitted by the nibble of the contaminated female of *Phlebotomus*, sand fly [6]. The sand fly gets to be distinctly tainted when sucking blood from repository have, which incorporate man, or local and wild creatures, Immunity of host, against CL assume an imperative part in disposal of intracellular parasites [7]. The established strategies that utilized for determination *Leishmania* species, (for example, coordinate spread examination and cultures) that required long time, encounter and it's not effective and might be prompt to misdiagnosis [8]. More up to date strategies for distinguishing proof that could conceivably arrange *Leishmania* straightforwardly from patient examples (annulling the necessity for culture) incorporate monoclonal antibodies and various DNA-based examines including multiplex polymerase chain response (PCR) and Nested (PCR) [9]. Identification of the contaminating parasite to the complex or species level is essential for prognostic, epidemiologic, and remedial reasons [10]. This study means to give a quick distinguishing proof of the tainting *Leishmania* species in Al-Kut city, which accordingly offers the potential for focused treatment procedures.

2. Materials and Methods
2.1 Samples collections
A total of 70 (30 male, 40 female) specimens of patients in age (1 < 49 year) was considered from dermatology center of AL-Zahra'a and AL-Karamah Teaching Hospital in AL-Kut city

from October 2015 to the end of February 2016. The patients were suspected with cutaneous *Leishmaniasis*; these cases were affirmed as CL patients in light of clinical side effects. Seventy (70) wound specimens were gathered from patients and put in two sterile test tubes, one of these test tube utilized for direct Giemsa-recolored spread [11]. The other test tube put away in Deep-solidifying until genomic DNA extraction step.

2.2 Genomic DNA Extraction

Genomic DNA was separated from solidified injury sore wounds by utilizing (Genomic DNA Mini Kit, Geneaid. USA) [12]. The extraction was done by directions of utilizing Proteinase K for cell lysis. After that, the removed g DNA was checked by Nano drop spectrophotometer, and afterward put away in deep freezer at – 20 C° until utilized as a part of PCR intensification.

2.3 Nested PCR amplification

nPCR assay was carried out by using specific primer for Kinetoplast DNA (kDNA) in genus *Leishmania* that include External primers CSB2XF (CGAGTAGCAGAAACTCCCGTTCA), CSB1XR (ATTTTTCGCGATTTTCGCAGAACG) and Internal primers 13Z(ACTGGGGGTTGGTGTAAAATAG), LiR (TCGCAGAACGCCC CT) were using to amplify 750bp PCR product for *L. tropica* and 560bp PCR product *L. major* [12]. These primers were provided by (Bioneer Company. Korea). The first round PCR master mix that includes CSB2XF and CSB1XR" were set up by utilizing (Accu Power® PCR PreMix unit. Bioneer. Korea). The PCR premix tube contains solidify dried pellet of (Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl2 1.5mM, stabilizer, and following color) and the PCR ace blend response was set up as indicated by unit guidelines in 20µl aggregate volume by included 5µl of purged genomic DNA and 1.5µl of 10pmole of forward groundwork and 1.5µl of 10pmole of invert preliminary, then entire the PCR premix tube by deionizer PCR water into 20µl and quickly blended by Exispin vortex axis (Bioneer. Korea). The response was performed in a thermo cycler (Techne TC-3000. USA) by setting up the accompanying thermo cycler conditions; introductory denaturation temperature of 94 °C for 5 min; trailed by 30 cycles at denaturation 94 °C for 30 s, strengthening 55 °C for 1 min, and augmentation 72 °C for min and after that last expansion at 72 °C for ″ 7 min. the second round of nested PCR was including 13Z and LiR primers and the same PCR master mix except 3µL of template PCR product. After that PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV Trans illuminator.

2.4 Statistical Analysis

All the measurable examination was finished by utilizing T-test, the appropriate factual technique keeping in mind the end goal to investigate and evaluate by utilizing this test.

3. Results

In this study, the results showed that the rate of contamination with *Leishmania* species was overwhelming as there were 21 positive cases from aggregate specimens which constitute (30%) by utilizing the direct recolored spread, while the atomic system Nested PCR reported 55 positive examples constitute (79%) from aggregate examples (Table 1, Fig.1). Factually there is acritical relationship between the appropriations of CL among patients as indicated by demonstrative techniques.

Table 1: Prevalence of CL by using direct smear and Nested PCR technique

Diagnostic method	Positive (%)	Negative (%)
Direct smear	21(30%)	49(70%)
Nested PCR	55(79%)	15(21%)

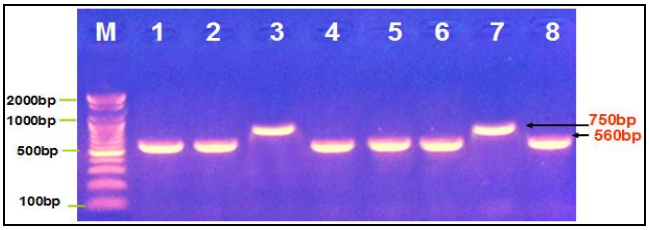

Fig 1: Agarose gel electrophoresis image that show the Nested PCR product analysis of kDNA in *Leishmania* positive isolates. Where M: marker (2000-100bp), lane (1, 2, 4, 5, 6, and 8) positive *L. major* at (560bp) PCR product size and lane (3 and 7) positive *L. tropica* at (750bp) PCR product size.

As indicated by consequences of this study contingent upon Nested PCR, from the aggregate number examples, the rate of disease with *Leishmania major* was (89%) and *Leishmania tropica* recorded rate was (11%)(Fig 1)(Table 2). Measurably there is critical contrast between the circulations of two types of *Leishmania* among patients as indicated by Nested PCR.

Table 2: Identification of *Leishmania* species by using Nested PCR technique

***Leishmania* species**	**Positive (%)**
L.major	49(89%)
L.tropica	6(11%)
total	55(100%)

As per the sexual orientation the outcomes showed that the occurrence of CL contaminations in male were sure in 17 tests that constitute (30.90%) while female specimens were certain in 38 tests and that constitute (69.09%) (Table 3).Statistically there was a critical relationship between the conveyances of CL among patients as indicated by sex.

Table 3: Distribution of CL cases in relation of Age, gender and Residence

Age (year)	Gender	Residence		Subtotal	Total	Total%
		Urban	Rural			
1-12	M	2	7	9	26	47%
	F	5	12	17		
13-24	M	2	4	6	16	29%
	F	4	6	10		
25-36	M	0	2	2	7	13%
	F	1	4	5		
37-48	M	0	1	1	4	7%
	F	1	2	3		
49 and more	M	0	0	0	2	4%
	F	1	1	2		
Subtotal	M	4	14	18	55	100%
	F	12	25	37		
Total%	-	16(29%)	39(71%)			

In this study the information is demonstrated that all age gatherings were influenced, the most noteworthy diseases of CL accounted for in (1-12)year 33(47%) while the least contamination were 5(4%) and 5(7%) in (49 and that's only the tip of the iceberg) year and (37-48)year separately, in (13-24) year age a mass the predominance of disease was 20(29%), while 9(13%) in the age bunch (25-36) year. Table (3). Statistically there is a huge relationship between the conveyances of CL among patients as per age.

Cutaneous *Leishmaniasis* with two types of rustic and urban is the endemic infections and as a medical issue in our nation. ID of parasite species and kind of sickness are essential for treatment of ailment and for arranging of control program [13].The rate of disease as per the home demonstrated that the occurrence of CL in provincial locales 39 (71%) was higher than the frequency in urban districts 16 (29%) (Table 3). Factually there is a critical relationship between the appropriations of CL among patients as indicated by habitation.

4. Discussion

There are many diagnostic tests used to detect the *Leishmania* parasite, microscope examination is the most reliable and conventional method, the parasite is demonstrated in direct smear stained with Giemsa stain or leishman stain to detect the presence of amastigotes [14], several reports have shown the high rates of infection with *Leishmania* species and that similar with our results, our finding is steady with the discoveries of the study conducted by others [13]. Al Samarai and Al Obaidi [15] reported that 73% of the cases were certain to Giemsa stain and 43% were sure in societies for Al Hawija locale of Kirkuk region in Iraq. Israa *et al*. [16] that exhibited Parasites in 80% of CL smears against 44% of positive societies for Baghdad area in Iraq, and Rahi [17] was reporting that the rate of contamination was 97.8% by utilizing Gimsa recolored technique for Wasit territory in Iraq, Rahi [17] recording the pervasiveness of positive instances of CL was 94% by utilizing smear strategy for Wasit region in Iraq and Microscope examination of smears is quick and simple to use for conclusion of CL. In an endemic territory, CL is to a great extent analyzed by its clinical appearance and the symptomatic difficulties emerge when cases show up in non-endemic zones, when the clinical picture is bended, or when any atypical variations are seen even in endemic regions. In expansion, optional contamination or abuse can modify the clinical picture of CL and cause trouble in determination and postponement in treatment [13].

Distinguishing proof of *Leishmania* species is fundamental to decide the clinical forecast and an animal groups particular remedial approach [13]. Most regularly utilized strategies for the immediate identification of the parasite (e.g., minute examination of Giemsa-recolored smears and in vitro development) need affectability as a result of the shortage of *Leishmania* parasites in a few examples or the parasites might be inadequate and are generally extracellular in the slide arrangements, or are hampered by the issue of defilement [11]. These techniques have restricted sensitivities since they require coordinate perception of the parasites and the scarcity of parasites inside the injury is a sign of sores with old age. Our result were about like the outcome reported in Iraq [13]. At present, an assortment of biochemical and sub-atomic strategies, based on intrinsic elements, have been created for exact distinguishing proof of *Leishmania* species. Among these, currently, the most ordinarily utilized strategy is DNA-based systems, utilizing PCR and particular ground works for species and even strains portrayal [18, 19].The most sub-atomic demonstrative strategies created for genotyping *Leishmania* species depended on the polymorphic kDNA minicircle, which is viewed as a prime possibility for a touchy test in light of the nearness of 10000 to 20000 minicircles per cell [19]. The settled PCR gives a quick, delicate, and particular contrasting option to customary procedures. In addition, analysis of *Leishmania* contamination and species identifications are done at the same time [20].

In this study [20] the frequency of *Leishmania major* was higher than *Leishmania tropica* this outcomes are in concurrence with the aftereffects of Maraghi *et al*. [20] which reported (90%) *Leishmania major* and (10%) *Leishmania tropica* for Khuzestan area in Iran, and Mahmoodi *et al*. [21] that demonstrated the outcomes (90.5%) were *L. tropica* and *L. major* (9.5%) by utilizing Polymerase Chain Reaction (PCR) for Mashhad territory in Iran, Maraghi *et al*. [12] reported (94.5%) *Leishmania* noteworthy and (5.5%) *Leishmania tropica* for Khuzestan area in Iran, and Rahi [17]. recording the rates of disease in *Leishmania major* and *Leishmania tropica* were (60%) and (40%) respectively for Wasit region in Iraq, Azizi *et al.* [22] demonstrated that (89.51%) of cases were tainted as *L. major* and 12 (9.67%) cases were contaminated by *L. tropica* for Isfahan region in Iran, while the finding of this study was contradiction with Azizi *et al*. [23] which recorded the rate of *L.* significant contamination (40%) and (90%) by utilizing smears and Nested PCR individually for Hormozgan area in Iran, Kheirandish *et al*. [24] reported that the rates of disease in *L. tropica* is (72.6%) and *L. major* is (274.%) by utilizing PCR method for Lorestan territory in Iran.

In atomic indicative techniques both kinetoplast DNA and chromosomal DNA can be utilized as layout for ID of

Leishmania species by PCR and Nested PCR grants both exceptionally touchy location and distinguishing proof of *Leishmania* species [20].

Depending on gender, the consequences of the present study showed the infections of female were higher than male, this results not agree with some past examinations of Arroub *et al*. [25] for Yazd territory in Iran, AlSamarai and AlObaidi [15]. For Al Hawija area of Kirkuk region in Iraq. Maraghi *et al*. [20] recording that the male disease was upper than female for Kuzestan region in Iran, Azizi *et al*. for Esfahan region in Iran, while Mahmoodi *et al.* [21] reported no critical contrast between the genders and the male and female were similarly contaminated for Mashhad region in Iran, However Rahi [26] reported that the commonness of disease in male was more than female for Wasit region in Iraq, the after effect of Arroub *et al*. [25] uncovered that no noteworthy affiliation was seen between sexual orientation for Azilal region in Iran, the separation between the aftereffects of research because of that the contamination happen with the presentation to the chomp of sand fly [15]. The outcome of our study in age group concurrence with Arroub *et al*. [25] that recorded the most very age gathering was (5-9) year with a rate of (1.8%) in *Leishmania tropica* for Yazd territory in Iran, Maraghi *et al*. [20] reported that the most noteworthy contamination in age gathering was under 10 years with rate of(42%) and the least rate was(4%) at the age gathering of over 40 years for Khuzestan region in Iran, while the most astounding rate of disease reported by Azizi *et al*. [22] was in (10-19) age assemble for Esfahan region in Iran, the investigation of Arroub et al. [25] uncovered that the most noteworthy rate of positive sores was found in the age gathering of 9 years or under (86.67%) for Azilal area in Iran. The frequency of not exactly the more noteworthy age and can be clarified by the improvement of insusceptibility with the progression of time for the general population or the consequence of past presentation to disease, and that gives individuals a perpetual resistance [27].

We have recently reported the significant prevalence of CL by habitation, the rate of infection in urban region was lower than rural, and this finding was reliable with Wisam [28] for Baghdad territory in Iraq, Qader *et al*. [13] for Al-Qadisia region in Iraq, Rahi [26] for Wasit region in Iraq and Al-Difaie [14] for Al-Qadisia province in Iraq, The higher frequency of the infection in rustic locales might be disclosed by presentation to vector and store, this makes them more subject for introduction to causative specialists in this endemic zone, So, the most patient of CL originated from country regions this might be connected with vector pervasiveness [16].

5. Conclusion

Present Nested PCR assay was more touchy than parasitological technique in recognition of *Leishmania* parasite in skin biopsy tests, and the direct smear could be considered a good test for diagnosis the cutaneous *Leishmaniasis* the high rate of infection with *Leishmania* species in Al-Kut city was attributed to the connected with vector.

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