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DETECTION OF *LEISHMANIA* SPECIES BY NESTED-PCR AND VIRULENCE FACTORS GIPLS, GP63 IN *L. MAJOR* BY CONVENTIONAL-PCR

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ABSTRACT : The present study aimed to molecular detection of *Leishmania* spp.by Nested- PCR assay and virulence factors glicoinositolphospholipids(GIPLs) and the major surface glycoprotein(GP63) for *Leishmania major* by using polymerase chain reaction assay. 50 samples were collected from patients infected with cutaneous leishmaniasis reviewers AL-Diwaniyah Teaching hospital in AL-Qadisiyah province at the period from the beginning of November 2017 to the end of February 2018. The results showed that the highest rate of infection was recorded among the age groups 1-10 years was (26 cases) percentage (52%) and about(15 cases) percentage(30%) of the age groups 10-20 years and the lowest of the age groups 40-50 years(1 case) percentage(2%). Also the number of infected males reached 26 percentage (52%) and females 24 (48%). The results showed that 35 positive samples were *L.major* (560bp) and percentage (70%), *L.tropica* were 7 samples (750bp) and percentage (14%) and 8 negative samples (16%) by using Nested-PCR assay,to investigate the virulence factors GIPLs (354bp) and GP63(885bp) that were found in all positive samples for *L.major* in percentage (100%) by using polymerase chain reaction PCR.

Key words : *Leishmania major*, PCR, nested PCR, GIPLs, GP63.

INTRODUCTION

Leishmania is an intracellular protozoan parasites, causes parasitic disease in the world (Bates, 2007). This disease spread in more than 80 countries, Leishmaniasis prevalence was estimated to be around 12 million cases and each year this number increases by 1.5-2 million (Ameen, 2010), Clinical manifestation of this disease include skin ulcers to fatal visceral forms (Ameen, 2010; Croft and Coombs, 2003). The WHO considers the disease to be one of the most serious parasitic diseases globally (González *et al*, 2010; Piscopo and Azzopardi, 2007).

Leishmania infection have been reported in the Americans, estern Europe, Africa, western and central Asia, Australia and India (Allison, 1993; Stark *et al*, 2008). All the predispoding factors of occurrence the disease exist in Iraq like malnutrition and possible immunodeficiency of patients have distorted the epidemiological aspects of the disease (Mohebali *et al*, 2004). There are two morphological phases in the life cycle of these parasite: promastigote form with flagella that inhabit in sand fly gut and deposits during the blood meal in the skin of the host and amastigote form without flagella that inhabit in mammalian cells (Bates and Rogers, 2004). These forms considered virulent form of *Leishmania* and the beginning of infection (Marzochi and Marzochi, 1994), the host may remain without symptomatic for long time and this play important role in transmission leishmaniasis in their region (Reithinger *et al*, 2007).

Virulence factors were characterized as some parasite's components that enable the parasite to infect and invade the mammalian host (McNeely *et al*, 1989). These virulence factors include glicoinositolphospholipids (GIPLs) and the major surface glycoprotein (GP63).

GIPLs are glycoconjugates of the intracellular amastigote form and might play important role to invade macrophage and parasite survival inside phagocytes (llgoutz *et al*, 1999; Tachado *et al*, 1997).

GIPLs help *Leishmania major* survival within macrophages by inhibiting nitric oxide synthase (Proudfoot *et al*, 1995) and protein kinase C (Zufferey *et al*, 2003).

Another important virulence factors is a metalloproteinase (MP) that called the major surface protein (MSP or gp63) these factors present on promastigotes and amastigotes (Bouvier *et al*, 1985; Schlagenhauf *et al*, 1998). It protects the parasites from the host enzymes in the midgut of sand fly and the phagocytosis of macrophages and protect promastigotes

from complement- mediated lysis in the mammalian host and this proteinase decrease the fixation of the complement components on parasites (Yao *et al*, 2003; Yao, 2010). Effect of gp63 on immune modulation occurs by the activation of protein tyrosine phosphatases (PTPs) in macrophages leading to reduce NO production and innate inflammatory responses therefore increase the parasites survival (Gomez *et al*, 2009).

MATERIALS AND METHODS

Collection of samples

50 samples were collected from patients in AL-Diwaniyah Teaching hospital in AL-Qadisiyah province and placed in sterile test tubes, then transported to laboratory and stored in refrigerator until genomic DNA extraction.

Extraction of DNA

The extraction process of DNA from frozen samples by using (Genomic DNA Mini Kit, Geneaid, USA) depending on company instructions using Proteinase K for cell lysis then, the extracted gDNA was examined by Nanodrop spectrophotometer and storage at -20ºC in refrigerator until used in PCR amplification.

Nested PCR amplification

nPCR assay was performed to detect speice of Leishmania by using specific primer for Kinetoplast DNA (kDNA) in genus *Leishmania* that include External primers CSB2XF (CGAGTAGCAGAAACTCCCGTTCA), CSB1XR (ATTTTTCGCG ATTTTCGCAGAACG) and Internal primers 13Z (ACTGGGGG TTGGTGTAAAATAG), LiR (TCGCAGAACGCCC CT) were using to amplify 560bp PCR product *L. major*. These primers were provided by (Bioneer Company, Korea). The first round PCR master mix that include CSB2XF and CSB1XR were prepared by using (AccuPower® PCR PreMix kit. Bioneer, Korea), The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl2 1.5mM, stabilizer, and tracking dye) and the PCR master mix reaction was prepared according to kit instructions in 20µl total volume by added 5µl of purified genomic DNA and 1.5µl of 10p mole of forward primer and 1.5µl of 10pmole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20µl and briefly mixed by Exispin vortex centrifuge (Bioneer, Korea). The reaction was performed in a special thermocycler conditions (Techne TC-3000. USA), the second round of nested PCR was including 13Z and LiR primers and the same PCR master mix except 3µL of template PCR product ,then gel electrophoresis was carried out using the agarose gel 1%, add DNA radioactive dye Ethidium bromide and determine PCR product using Ultraviolet light source.

PCR amplification

PCR assay was performed to detect Virulence genes (GIPLS-GPI12 and gp63 gene) in positive *Leshmania major* by using specific primer as in Table 1.

In this study, the primers were design GenBank: AY157268.1 and GenBank: Y00647.1 respectively and provided by (Bioneer Company, Korea). The PCR master mix were prepared by using (AccuPower® PCR PreMix kit. Bioneer. Korea). And same reaction mix of first round in nested PCR. The reaction was performed in a special thermocycler conditions (Techne TC-3000. USA) then gel electrophoresis was carried out using the agarose gel 1%, add DNA radioactive dye Ethidium bromide and determine PCR product using Ultraviolet light source.

Table 1 :

Primer	Sequence		Amplicon
GIPLS-GPI12 gene	F	AAAGGAGCGTGAGAAGGAGC	354bp
	R	GACCACAGGGCATAAGGGAG	
gp63 gene	F	AGCAACACCGACTTCGTGAT	885bp
	R	GTCAGTTGCCTTCGGTCTGA	

RESULTS

Prevalence of cutaneous leishmaniasis to the age groups

Table 2 shows that the highest rate of infection in the age group (1-10) years old and about 26 cases percentage (52%) and the lowest was between 40-50 years old about 1 cases (2%) with a significant difference at p>0.05.

Prevalence of cutaneous leishmaniasis according to the gender

The present study showed that the number of infected male were 26 cases (52%) and the number of infected female were 24 cases(48%) for the total number as shown in Table 3 without significance difference at p> 0.05.

Nested-PCR technique

DNA extraction results showed for 50 samples, there are 35 positive samples were *Leishmania major* at (560bp) PCR product size (70%) by using nested-PCR method which used to identify leishmania and its species when electrolyted on the agarose gel and examined under ultraviolet light as shown in the Fig. 1. And 8 negative samples were (16%) while *L. tropica* were 7 positive samples (14%) at (750bp) PCR product size.

PCR technique

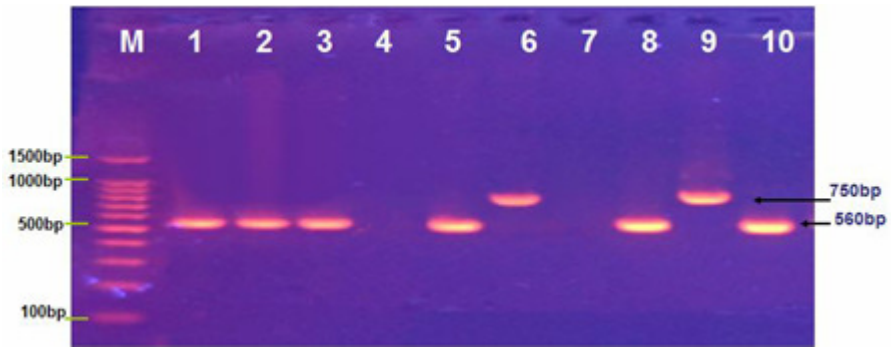

Fig. 1 : Agarose gel electrophoresis image that show the Nested PCR product analysis of kDNA in Leishmania positive isolates. Where M: marker (2000-100bp), lane (1, 2, 3, 5, 8 and 10) positive *L. major* at (560bp) PCR product size, lane(6 and 9) positive *L. tropica* at (750bp) PCR product, and lane (4 and 7) negative samples.

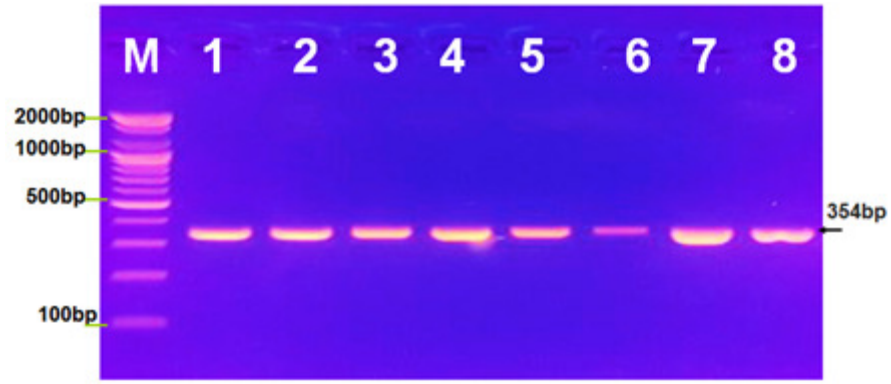

Fig. 2 : Agarose gel electrophoresis image that show the PCR product analysis of Virulence gene (GIPLS-GPI12) in some positive *Leshmania major* isolates. Where M: marker (2000-100bp), lane (1- 8) positive *L. major* at (354bp) PCR product size.

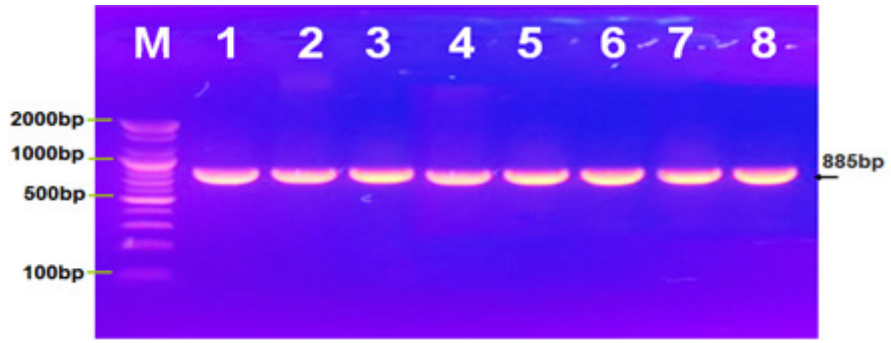

Fig. 3 : Agarose gel electrophoresis image that show the PCR product analysis of Virulence gene (gp63) in some positive *Leshmania major* isolates. Where M: marker (2000-100bp), lane (1- 8) positive *L. major* at (885bp) PCR product size.

The results of the current study were indicated using polymerase chain reaction for *L.major* in 35 samples, all these samples (100%) were contain the virulence factors GIPLs at (354bp) PCR product size as shown in Fig. 2 and gp63 at (885bp) PCR product size as shown in Fig. 3 when electrolyted on the agarose gel and examined under ultraviolet light.

DISSCUSSION

Cutaneous leishmaniasis was an endemic disease around the world, about 20 species of leishmania have the ability to infect humans, and cutaneous leishmaniasis was closely related to geographical distribution. In addition some species of leishmania are associated with human presence and therefore spread in cities such as *L. tropica* while some other species are usually associated with species of animals and hence are considered of zoonotic type cutaneous leishmaniasis such as *L.major* (Vergel *et al*, 2006).

Prevalence of cutaneous leishmaniasis according to the age groups

The results of the present study showed that the most affected group was less than 10 years and percentage (52%), followed the age group 10-20 years percentage (30%) then followed the age group (20-30) years (10%). Its noted that the infection decreases with the age increases and can be explained by the evolution of immunity with time or people from the previous exposure to infection

Table 2 : The prevalence of cutaneous leishmaniasis according to the age groups.

Age groups	The number of infected patients	%
0-10	26	52
10-20	15	30
20-30	5	10
30-40	3	6
40-50	1	2
χ^2	48.37	
P value	0	

Table 3 : The prevalence of cutaneous leishmaniasis according to the gender.

Gender	The number of patients	%
Males	26	52
Females	24	48
χ^2	0.160	
P value	0.689(NS)	

NS : Non-significant difference.

which gives people a permanent immunity.

Prevalence of cutaneous leishmaniasis according to the gender

Its noticed that the number of males infected was slightly higher than females, it may be due to that most males play without adequate clothing, therefore exposing them to insect bites compared with females according to costoms and traditions (Mahmood, 2006).

Diagnosis of the parasite using Nested-PCR method

Nested PCR method was used as a basic method in the diagnosis of parasite. The traditional methods for diagnosing leishmaniasis were not sensitive and high quality that they can be relied upon (Luz *et al*, 2001), for diagnosis of cutaneous leishmaniasis has been used for diagnostic techniques based on DNA and kinetoplast that contain about 10,000 DNAs, the kinetoplast DNA minicircle is one of the best parts of the parasite genome for sequencing to identify different leishmania species (Rodriguez *et al*, 1995). The present study showed that ratio *L.major* in positive samples (70%) while percentage *L.tropica* (14%), the negative samples of *L.major* were (16%) This doesn't agree with Abdullah *et al* (2009) that all positive samples were *L.major* while this the present study agree with Mirzaei and Sharifi (2011), Maraghi *et al* (2013) in Iran which confirmed that nested PCR technique is a sensitive and accurate method to investigate and differentiate between species of leishmania parasite.

Diagnosis of virulence factors using PCR method

Leishmania has many virulence factors, which can be defined as a set of parasite components that work to keep them in mammals, that promote the parasite survival and immune modification of the mammalian host. These factors play an important role in the parasitic ability of these parasites (Beverley and Turco, 1998).

The present study showed presence the virulence factor GIPLs percentage (100%) of positive samples and this agree with Silva-Almeida *et al* (2012).

Results showed presence the virulence factors GP63 percentage(100%) of positive samples and this agree with Silva-Almeida *et al* (2012), Elmin *et al* (2005).

CONCLUSION

Our report revealed that patients reviewers to AL-Diwaniyah Teaching Hospital were infected with *Leishmania* species after examined them by Nested-pcr technique.

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