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 predisposing 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 (Ilgoutz 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 speiec 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(ACTGGGGGT GGAGTGAATAG), LiR (TGGCAGAAGCCCT) 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 10p mole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20µl and briefly mixed by Exisip 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 Leishmania 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.

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
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 GPIls 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.
The present study showed presence the virulence factor GPI-Ls 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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