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Original Article

Molecular Detection of Virulence Factor Glycoprotein (*Gp63*) of *Leishmania* spp. in Phlebotomus Sand Flies

Hadi M. Hamza Al-Mayali[1*], Ali Jawad Abdulaali Alyasiri[2]

[1]Department of Biology, Education College, University of Al-Qadisiyah, Iraq
[2]Department of Biology, Science College, Al-Muthanna University, Iraq

Abstract

Introduction: Gp63 is the major surface glycoprotein of *Leishmania* which is prevalent in the promastigote stage of *Phlebotomus sergenti*. Glycoprotein 63 (gp63) or leishmanolysin is a zinc-dependent metalloprotease found on the surface of *Leishmania*. It was initially discovered in 1980and described biochemically and genetically as a surface antigen expressed in promastigotes of *Leishmania* species, having a range of substrates including casein, albumin, fibrinogen, haemoglobin, and gelatin. On the surface of amastigote, GP63 is present at a very low level. Glycoprotein 63 is a zinc-dependent metalloprotease that is active in the pH range of 7–10, which is neutral to alkaline.

Materials and Methods: During the study, which was carried out in Al-Muthanna province from July 2017 to August 2018, about 2550 sand fly samples including 719 males and 1633 females were collected. Three species of sand flies were recorded according to morphological features which included *Phlebotomus papatasi*, *Phlebotomus sergenti*, and *Sergentomyia sintoni*. The caught specimens showed that *P. papatasi* was the most prevalent species. Two primers were used for the diagnosis of *Leishmania* spp. using a nested PCR technique, which was designed in a previous study for 1250 samples of sand flies from 25 locations in the study area.

Results: Results showed that 13 samples were positive, containing DNA for the *Leishmania* parasite, and 12 samples were negative. Positive samples include 11 samples of *L. major* species and only 2 samples of *L. tropica* species. Additionally, the results showed that the *L. major* species was the dominant species in the study area. Positive samples (13) of sand flies had *Leishmania* parasite based on molecular diagnosis and virulence factor gene (*Gp63*) of *Leishmania* parasite was detected in sand flies using PCR method The results showed that virulence factor gene (*Gp63*) was detected on the surface of promastigote. This refers to the presence of *Leishmania* parasite in sand fly. The study is the first one which detected the presence of the virulence factors gene (GP63) of *Leishmania* in the body of sand flies.

Conclusion: This study revealed the possibility of diagnosing leishmaniasis by virulence factor gene (*Gp63*) in *leishmania* promastigotes.

Keywords: *Leishmaniasis*, Virulence factor, Gp63, Al-Muthanna province.

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Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are hematophagous insects and vectors of leishmaniasis

and other bacterial and viral diseases, for several species of Sandflies, are minor in size, with a linear measure of about 3 mm. They are known for long bodies and wings that are covered with dense hair as well as long legs (1). They act as vectors of human and animal infections (2). There are over 800 species of sandflies in the world, 98 of which are vectors of human leishmaniasis. They include 42 *Phlebotomus* species in the Old World and 56 *Lutzomyia* species in the New World (3).

Phlebotomines, typically mentioned as *Leishmania* spp. vectors, are protozoan parasites that cause kala-azar and various kinds of human leishmaniasis (4).

Proteinases play significant roles in parasite invasion of host cells and immune system, movement over tissue, and inflammation stimulation inside the host (5). When it comes to infecting their own host for survival and prevalence, parasites always employ the most basic skill. Many different surface proteins, including glycoinositolphospholipids (GIPLs), can be used by *Leishmania* as possible virulence factors (6). Actually, it is known that *Leishmania* can utilize various surface proteins, recognized as potential virulence factors (e.g., GIPLs, lipophosphoglycan, cysteine protease, *glycoprotein* 63), to thwart the host macrophage defense system and therefore, permitting its survival and progression within the harsh environment of the phagolysosome (7).

The gp63 is the main surface glycoprotein of *Leishmania*, which is prevalent in promastigotes of *Leishmania*. Glycoprotein 63 (GP63), also called leishmanolysin, is

*Corresponding Author: Hadi M. Hamza Al-Mayali, Email: Hadi.Hamza@qu.edu.iq

a zinc-dependent metalloprotease which is present on the surface of *Leishmania*. It was discovered for the first time in 1980, as a significant surface antigen presented in promastigotes of *Leishmania* species. GP63 contains albumin, gelatin, casein, fibrinogen, and *haemoglobin*. GP63 is present on the amastigote. GP63 as a zinc-dependent metalloprotease could even be active in pH range of 7–10 (8).

In *L. major*, there are seven *gp63* genes and they exhibit the stage-specific differences in their expression: five homologous tenderly repeated *gp63* genes 1–5 are expressed in promastigotes only, a separated gene 6 is expressed in both promastigotes and amastigotes, while a gene 7 is expressed in stationary phase promastigotes and amastigotes. *gp63* is abundantly expressed on the surface of the promastigote form, upregulated in infectious metacyclic promastigotes, and have a low but detectable expression level in the intracellular amastigote stage (9).

Materials and Methods

Collection Sites

This study was conducted in Al-Muthanna province from July 2017 to August 2018. Sand fly traps were placed in fields around their resting places in large numbers eight times a month (twice a week). The most visible sand break sites are animal pens, human houses, bird houses, leaf scraps, tree holes, or under rocks, and animal burrows, rock cracks, and between vegetation.

Collection Methods

Sticky Paper Traps

Sticky paper traps represent a technique for sand fly sampling by intercepting them instead of the attraction process. These traps are usually economical and easy to use in large numbers. Sticky traps are usually preferred to light traps to collect sand fly from indoors because they are unattractive. Traps were placed in the evening one hour before sunset and were collected after sunshine in next morning. The sandflies were collected from the traps using small forceps, washed with diluted detergent, and kept in 70% alcohol in the Eppendorf tubes with appropriate labeling until treatment and subsequent laboratory identification (10). They were selected to study their morphology and perform molecular diagnosis.

Light Traps

These traps are frequently utilized in investigations of sandfly fields. Sandflies are caught with tiny light traps. Sandflies were sampled from peri-domestic and agricultural fields using these traps. Traps should be placed at a height of 1.5–2 m from the ground level, preferably at least 10 m away from any external light source (6).

As a result, areas near other light sources, as well as spots exposed to high winds, sites near buildings containing animals, and areas exposed to industrial odors and smoke should be avoided. Furthermore, no light traps were utilized at home since light traps may attract flies from outside and may not represent real endophagic/endophilic species (2,11,12).

Laboratory Examination

The sand flies that have been caught were removed using a small brush or forceps. The sand flies were processed in a conventional way. The sandflies were thoroughly washed twice with distilled water to remove castor oil and excess hairs. Sandfly samples were preserved in 1.5 mL Eppendorf tubes containing 70% ethanol and drops of glycerin to prevent stiffen of sample and to avoid samples dry from alcohol. Then, labels indicating the site and day of sampling were attached. They were processed according to the methods described before (13). Samples shall be kept for a short period to prevent decomposition of samples. In the laboratory, the samples are washed thoroughly with distilled water. The samples were then placed in alcohol for an hour for removing water. The samples were then placed on glass slides. The samples should be well positioned to allow observing their taxonomic and morphological characteristics. External morphological analyses of the different species were carried out based on the sand fly mounted on each slide. Drops of mounting medium (DPX mountant) were added to avoid the occurrence of air bubbles. Then, the sample was left for a period of time to dry, and the cover slip was gently put on the specimen. These slides of sand fly were prepared for morphological identification using classification keys as described in a previous study (14).

Internal morphological analyses of different organ structures were performed using a light and dissecting microscope. Identification of sand flies was based on morphological structures; therefore, specimens were required to be prepared on microscopic slides (15).

Molecular Study

Primers

Two types of primers (Bioneer, Korea) were used for the diagnosis of *Leishmania* species using a nested PCR technique, which were designed in a previous study (2) as follows:

First primer F CGAGTAGCAGAAACTCCCGTTCA
KDNA R ATTTTTCGCGATTTTCGCAGAACG 750bp

Second primer F ACTGGGGGTTGGTGTAAAATAG
KDNA R TCGCAGAACGCCCCT 560bp

Virulence Factors Primers (PCR Primers)

The *GP63* primers of *leishmania* spp. were used for the diagnosis of glycoprotein 63 gene in *Leishmania spp*. These primers are designed in this study by using a gene bank database NCBI and Primer3Plus software.

F GTACGTTACTCTCGACGCCG
R ATCACGAAGTCGGTGTTGCT 405bp

Molecular Diagnosis

The PCR technique was used to detect *Leishmania* spp. and distinguish it from DNA samples of the insect. This method was implemented according to a previous study (16).

DNA Extraction

The gSYNC™ DNA Extraction Kit is enhanced for genomic .The purified DNA is suitable for use in PCR or other enzymatic reactions. Genomic DNA from tissue samples were extracted by using gSYAN DNA mini kit extraction kit (Insect Protocol Procedure) Geneaid, USA, and done according to manufacturer's instructions.

The quality of the isolated genomic DNA was determined using a Nanodrop spectrophotometer (Thermo, USA), which assessed DNA concentration (ng/L) and DNA purity by reading the absorbance at 260/280 nm.

Preparation of Nested PCR Master Mix

The Nested PCR master mix was prepared using Maxime PCR PreMix (iNtRON, Korea) according to the company instructions.

First-Round PCR

Nested PCR master mix for the first-round PCR was prepared according to the manufacturer's instructions by adding 5 µL of genomic DNA, 1 µL of first-round PCR product (10 pmol), 1 µL of reverse primers (10 pmol), and 13 µL of PCR water in a total volume of 20 µL.

In addition, the components of the above-mentioned PCR master mix reaction were lyophilized and stored in special PCR preMix tubes containing all additional PCR reaction components such as Taq polymerase DNA, Tris-HCl (pH: 9.0), dNTPs, MgCl2, KCl, stabilizer, and tracking dye. Then, the tubes were centrifuged in an ExiSpin vortex centrifuge for 3 minutes and transferred to the PCR thermocycler.

Primary Thermocycler Reaction Conditions

PCR thermocycler conditions were set as follows (2): 1 cycle of initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, 30 cycles of annealing at 55°C for 30 seconds, 30 cycles of extension at 72°C for 30 seconds, and 1 cycle of final extension at 72°C for 5 minutes), then left at 4C° for indefinite time.

Second-Round Nested PCR

Nested PCR master mix was prepared by adding 3 µL of first-round PCR product, 1.5 µL of forward primers (10 pmol), 1.5 µL of reverse primers (10 pmol), and 14 µL of PCR water in a total volume of 20 µL. After the preparation of the polymerase chain reaction mixture was completed, the tubes were closed and mixed carefully for 10 seconds. Then, the tubes were transferred to a PCR thermocycler.

Secondary Thermocycler Reaction Conditions

PCR thermocycler conditions were set as follows:
One cycle of initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, 30 cycles of annealing at 55°C for 30 seconds, 30 cycles of extension at 72°C for 30 seconds, and 1 cycle of final extension at 72°C for 5 minutes). then left at 4C° for indefinite time.

Electrophoresis

Nested PCR products at last steps were analyzed by loading in 1% Agarose to determine the presence or absence of PCR products as in the previous steps.

Detecting of the Virulence Factor GP63 of Leishmania in Sandfly

PCR technique was performed to detect virulence factor gene (*gp63*) of *Leishmania* spp. in DNA of positive cutaneous leishmaniasis samples of sand flies in the previous steps (nested PCR technique).

Preparation of PCR Master Mix

PCR master mix was prepared by adding 5 µL of genomic DNA, 1 µL of forward primers (10 pmol), 1 µL of reverse primers (10 pmol), and 13 µL of PCR water in a total volume of 20 µL. After preparation, the above-mentioned PCR master mix reaction components were placed in standard PCR tubes containing the PCR PreMix as lyophilized material containing all additional PCR reaction components such as Taq DNA polymerase, Tris-HCl (pH: 9.0), dNTPs, MgCl2, KCl, stabilizer, and tracking dye.

Then, the tubes were centrifuged for 3 minutes in an ExiSpin vortex centrifuge and put in PCR thermocycler.

PCR Thermocycler Reaction Conditions

PCR thermocycler reaction conditions were as follows: 1 cycle of initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, 30 cycles of annealing at 55°C for 30 seconds, 30 cycles of extension at 72°C for 1 minute, and 1 cycle of final extension at 72°C for 5 minutes. Then left at 4°C for indefinite time.

Electrophoresis

In the last step, Nested PCR products were analyzed by loading in 1% Agarose gel to determine the presence or absence of PCR products as in previous steps.

Results and Discussion

Molecular Diagnostics of Leishmania Inside the Insect Using Nested PCR

Twenty-five samples were used to detect the parasite causing the disease in the study area. The results of the gel

electrophoresis indicated that the DNA samples extracted from the insects collected from the study areas infected with *Leishmania*. Special primers for the *L. major* and *L. tropica* species were used for kinetoplast DNA gene (kDNA), with Nested PCR product of 560 bp length for *L. major* as well as 750 bp for *L. tropica*.

Based on the results of the gel electrophoresis, 13 samples were positive, containing DNA for the parasite including *L. major* and *L. tropica* species (52%), and 12 samples were negative for both species (48%). Positive samples included 11 samples of *L. major* species (84.6%) and only 2 samples of *L. tropica* species (15.3%). The results show that rural species of *L. major* was the dominant species in the study area. Figure 1 shows the appearance of DNA bundles of *Leishmania* species found in the study area.

A nested-PCR-based approach allows the detection of *Leishmania* directly from samples (12). It was reported that nested PCR is a reliable method for diagnosing and identifying *Leishmania* species that can be used in epidemiologic studies. With the correct primers, it can be used to distinguish between species (13).

It was mentioned that nested PCR entails the use of two sets of primers in two separate cycles. A secondary target within the first product is amplified by the second pair of primers. This eliminates contamination from primer dimers, hairpins, and alternate primer targets in the second PCR result. This study employed an approach similar to that utilized by Akhavan et a1 (13) in Iran.

The PCR methodology was utilized to diagnose *L. major* (560 bp) gene as well as *L. tropica* (750 bp) gene.

Molecular Diagnosis of the Virulence Factor Glycoprotein 63 (gp63) Gene in Promastigote Form of Leishmania Samples from Sandflies Using PCR

The DNA of positive samples of sand flies including 11 samples of *L. major* species and 2 samples of *L. tropica* species was used for diagnosis of virulence gene (*GP63*) on the surface of promastigote form of *Leishmania* from insect using PCR technique. The results show that the parasite contains virulence factor gene (*Gp63*) on the surface of promastigote. Figure 2 shows the appearance of bundles of virulence factor gene (*Gp63*) in cutaneous leishmaniasis positive samples in the study region.

The *Leishmania* genus has developed several ways for inhibiting the innate immune response, allowing infection and spread within the macrophages of the mammalian host.

Several virulence factors of *Leishmania* have been discovered to have a role in the progression of leishmaniasis.

Recent discoveries, on the other hand, highlight the significance of GP63 as an avirulence factor that has a significant impact on signaling processes and activities of host cells. The results corroborate previous findings.

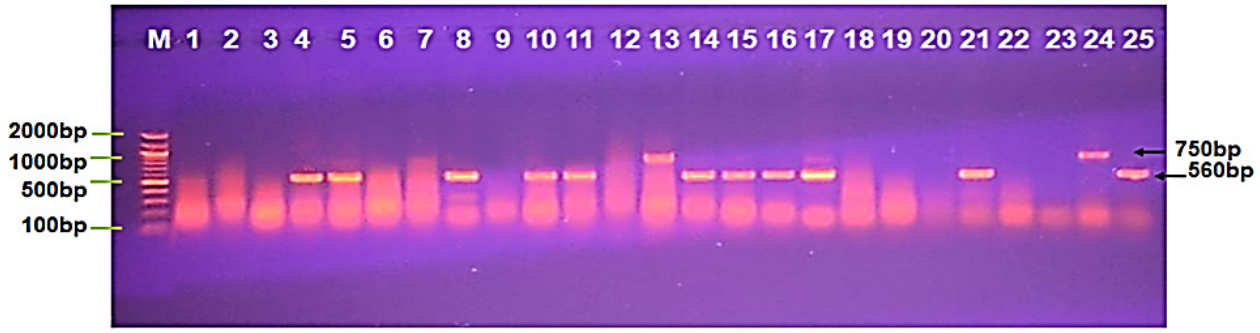

Figure 1. Image of Agarose Gel Electrophoresis of DNA fragment Stained with Ethidium Bromide.

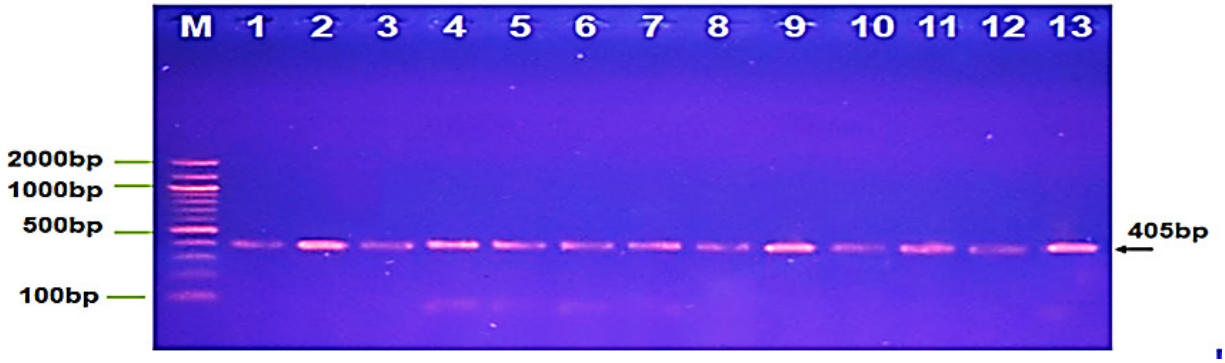

Figure 2. Image of Agarose Gel Electrophoresis showing the PCR Product Analysis of Virulence Factor Gene (Gp63) in Cutaneous Leishmaniasis Positive Samples.

Glycoprotein 63, known as leishmanolysin, is a zinc-dependent metalloprotease that has been demonstrated to promote phagocytosis and macrophage survival by mediating entry into macrophages (15).

The biological activity of parasites is linked to their ability to shield themselves from host enzymes in insect vector midguts and macrophage phagolysosomes (16). The GP63 genes are found in both promastigotes and amastigotes, and their products play a role in parasite attachment and macrophage internalization. GP63 is also used by *Leishmania* species to move across the extracellular matrix, avoid lysis by inactivating complement system components, and hydrolyze intracellular macrophage targets. *Leishmania* uses this method to survive and spread within mammalian host cells. A greater understanding of the processes by which this virus is able to evade the innate immune response may aid in the development of new anti-*Leishmania* therapies (17).

Glycoprotein 63 (gp63) or leishmanolysin, which is a 63 kDa protein and a metalloprotease, is a virulence factor that interacts directly with host macrophage receptors as well as with the complement system. The role of GP63 in a vector is yet unknown.

The enzyme could help with nutrition acquisition and protect promastigotes from digestive enzymes in the midgut. Sand is a substance that can be used in a variety of ways. The midgut protease activity of sandflies appears to peak one to three days afterward.

Gp63 is a virulence factor that plays a range of roles in host-parasite interactions. Expression of a cloned leishmanolysin gene, which has been proposed as a virulence factor, could considerably amplify the phenotypic differences.

These results suggest that leishmanolysin is involved in *Leishmania* pathogenesis (18). *Leishmania* spp., which reside as extracellular promastigotes in the Sand Fly and internal amastigotes in macrophages' phagolysosome compartment, have been discovered to include (gp63). The importance of gp63 in the *Leishmania* vector is less well understood.

Gp63 is a virulence factor that plays a range of roles in host-parasite interactions. The importance of gp63 in *Leishmania* vector is less well understood. Gp63 is thought to break down hemoglobin and other proteins of the blood in the meals, providing nourishment for promastigotes to reproduce. It also interacts with the complement system, which helps amastigote form of *Leishmania* spp. survive inside macrophages (8,19).

This is the first study conducted in Iraq which used the virulence factors gene Leishmanolysin (glycoprotein 63) or *Leishmania* metalloproteinase to detect *Leishmania* in sand flies.

Conclusion

Three species of sand flies are found in Al-Muthanna province, two species of *Phlebotomus* and one species of *Sergentomyia (Phlebotomus papatasi, Phlebotomus sergenti,* and *Sergentomyia sintoni*, respectively). *P. papatasi* is the most abundant species in the province and possibly the main carrier of *Leishmania.*

This study revealed the possibility of diagnosing leishmaniasis by virulence gene (*gp63*) in *Leishmania* promastigotes.

Conflict of Interests

The authors declared that no competing interests exist.

Ethical Issues

In this research, ethical considerations have been fully observed.

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Authors' Contributions

We did writing, editing of the manuscript, statistical analysis, and data collection.

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