

Molecular Investigation of Heat Shock Protein (HSP 70) among Persons Infected with the Parasite *Giardia lamblia* in Najaf Province

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Abstract

The current study was conducted in Najaf Province for the period from the beginning of September 2020 to the end of May 2021 to investigate the parasite *Giardia lamblia* Among the patients admitted to hospitals and health centers located in Najaf Province, a questionnaire was prepared for each patient to study the factors that may affect the prevalence of infection such as age, gender, treatment of injury, place of residence and occupation. The study included the collection of 350 stool samples from people with diarrhea, whose ages ranged from less than five years to 68 years, and a microscope was used to detect parasites. The results of the current study showed that the number of samples infected with intestinal parasites was 163, with a rate of 46.57%. The percentage of non-infected people was 187, or 53.43%, and it appeared that the symptoms were caused by other factors. The results of the extraction of 57 positive stool samples for patients, whose microscopic examination showed the presence of a parasite In *Giardia lamblia*, 40 samples of them contained the genetic material of the parasite DNA at a rate of 41%. The results of the PCR examination that were statistically analyzed showed that there were significant differences at the probability level of $P < 0.05$, as the highest percentage of infection with genotype (B) compared to genotype (A) which amounted to 61.54% and 38.46%, respectively. The current study showed that the amount of heat shock protein HSP70 by using the (ELIZA) test in infected persons and comparing them with healthy samples resulted in an increase in the concentration of HSP70 protein in the serum of persons infected with *Giardia lamblia* compared with the percentage of its presence in the control group with a significant difference of $0.01 < p. 28.04 \text{ ng / ml } \%$, while the percentage in healthy samples was $3.98\% \text{ ng / ml}$.

Key-words: Eliza, Microscopic, Infection.

1. Introduction

Infectious parasites, including primary parasites, are among the oldest parasites that cause diarrhea, and among them, which infect the digestive system is a parasite. *G. lamblia* Which affects

humans and animals all over the world (Adam et al., 2016). The clinical manifestation of giardiasis is fatty stool, flatulence, diarrhea and abdominal cramps, although the majority of cases are asymptomatic (Einarsson et al., 2016). The parasite belongs to the protozoan flagella and is unicellular, transmitted through contaminated food, which leads to serious economic repercussions at the global level (Reyan et al., 2019).

Giardia lamblia is a proto-intestinal protozoan parasite, and it is one of the most prevalent intestinal parasites in the world, and it is estimated that about 280 million people suffer from this parasite every year (WHO, 1996). The parasite has been observed since 1681 and given its name by Giard in 1895. Although *G. intestinalis* was the first parasite described, its role as a pathogen was not recognized until the 1970s, after the outbreak of the disease in travelers returning from infested areas, where it was believed before that time that this parasite is a harmless organism. (Leder, 2021).

Giardia lamblia appear as pear shaped and has one or two claw-shaped transverse average body. *G. agilis* is tall and slender, with a medium teardrop-shaped body; While the *G. muris* is shorter and more rounded and has a medium, small, round body. *G. lamblia* is found in humans and a variety of other mammals, *G. muris* is found in rodents, and *G. agilis* is found in amphibians (Feely, 1985).

The following classification of *Giardia lamblia* was adopted for the world (1915, Stiles) and given by the world (Despommier & Karapelou, 2012).

Kingdom: Protista

Phylum: sarcomastigophora

Sub phylum: Mastigophora

Class: Zoomastigophora

Order: Diplomonadina

Family: Hexamitidae

Genus: *Giardia*

Species: *G. lamblia*

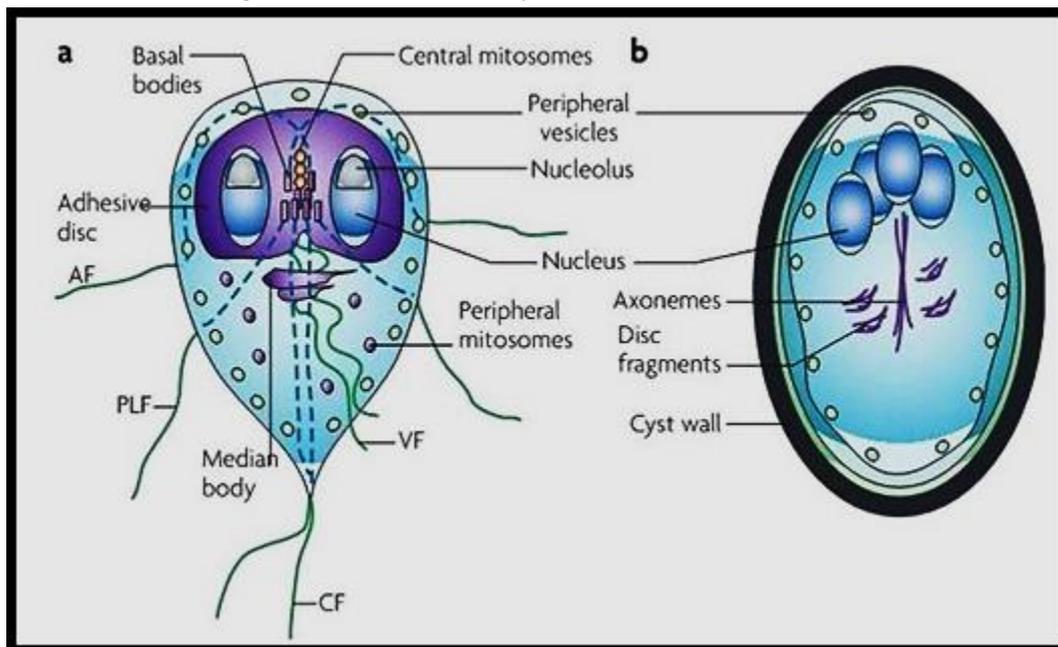
2. Parasite phenotype General Morphology of Parasite

G. lamblia is one of the most important intestinal parasitic protozoa, which is characterized by two phases: active Trophozoite, also called the vegetative stage, which parasitizes in the upper duodenum, and the cyst, which is the infective stage of the parasite and is usually excreted with faeces (Wernery & kaaden, 2002; Nester et al., 2001).

Cyst Phase

The cyst is oval in shape, ranging in length from 8 to 12 micrometers in length and 6 to 10 micrometers in width. The immature cyst contains two nuclei and two medial bodies, while the mature cyst contains four nuclei and four medial bodies. The cytoplasm slightly departs from the cyst wall, leaving a clear void in the cyst wall. Electron microscopy consists of a layer of fibrils and chitin-like materials that help it to resist unfavorable environmental conditions, as it is the contagious and causative phase (1991, Adam), Figure (1).

Figure 1 - The Active and Cystic Phase of *Giardia Lamblia*



Active Phase

The active phase has (12 - 15 x 5 - 9 μm) in dimension (Fig. 1) has a rounded front end and a tapered back. Its dorsal surface is convex, while its ventral side is flat, containing concave oval absorbent discs that are used to attach the parasite to the intestinal mucosa and resist its successive undulating movement. Its facial appearance resembles a tennis racket without the handle. It contains two nuclei, in each of which a large central nuclear body for activators, in addition to the above, two axial anvils, two basophils, basal flank bodies, and four pairs of flagella that move the animal in a fast, twisting movement similar to the movement of fallen leaves. The parasite cannot be developed in agricultural circles. The cyst is ellipsoidal in shape (8-14 x 7-10 μm) with a smooth smooth wall

containing four nuclei gathered at one of its poles. It can survive for a year at room temperature, while the high temperature (72°C) kills the phases engulfed by it. (Al-Hadithi and Awad, 2000).

Heat Shock Protein (70 HSP Heat Sock Proteins (HSP70))

The heat shock protein is 70 kDa (HSP70) is a stress-causing protein that has been shown to protect the brain from various nervous system injuries. It allows cells to withstand potentially fatal insults through its flanking functions. Its properties can help with protein folding and prevent protein build-up after many of these insults. Although its neuroprotective properties are largely attributable to its chaperone functions, HSP70 may interact directly with proteins involved in cell death and inflammatory pathways after injury. Through the use of mutant animal models, gene transfer, or heat stress, a number of studies have now reported positive results for HSP70 induction. However, these methods are not practical for clinical translation. Thus, pharmaceutical compounds that can induce HSP70, mostly by inhibiting HSP90, have been investigated as potential therapeutics to alleviate neurodegenerative diseases and lead to neuroprotection (Kim, et.al, 2020).

Heat shock proteins (HSPs) are the most comprehensively studied stress proteins. They were originally observed when sublethal heat stress was applied to cells. Autopsy studies have also documented the induction of HSP in the human brain after various types of heat stress, such as hyperthermia or fire-related deaths (Wenger et al., 2021). When core body temperature exceeded 40 °C, increased HSPS transcripts were detected in postmortem brain samples. The study of brain samples autopsied from hyperthermic patients led to the conclusion that HSP70 induction could be a biomarker of death in the brain (Mash, 2009). And the HSPs are chaperones that normally function in the cytosolic space, and are involved in helping with protein folding, degradation, complex assembly, and translocation. They have demonstrated the ability to prevent the accumulation of damaged proteins as well as facilitate the synthesis of polypeptides for newly synthesized proteins. The diverse roles by which HSP70 and HSP90 regulate aggregation proteins appear to be involved in neuroprotection such as models demonstrated by brain injury. HSP70 induction also represents an intrinsic protective mechanism that occurs in the para-hippocampal region, but not in other core regions, in the stroke model. (Weinstein, 2004) More than two decades of research involving such models have shown that HSP70 has the ability to protect against multiple types of necrosis. Specifically, HSP70 interferes with multiple cell death, including apoptosis and II death pathways (Frebel, 2006).

Heat shock proteins (70 HSP) molecular chaperones that are stimulated when the body is exposed to stress and that these proteins have an important role in the immune response against pathogens, including the parasite Giardia, as these proteins work to preserve the functions of proteins by helping amino acid chains to fold in their correct protein shape and remove damaged proteins, and they also display the antigen by delivering antigens Peptide to class 1 histocompatibility complex (MHC), which is found on the surface of cells (Ma and Luo, 2020). The defense against infection with the Giardia parasite is localized because the parasite does not attack the epithelial layer and is characterized by relatively little or no mucosal infection (Li, 2004), as the immune response is important to eliminate the parasite during the infection stage, because interleukins are proteins produced by lymphocytes and monocytes, and some of them are produced by Peyerspatches CD4 + cells or by lymphatic tissues associated with the mucous layer, which leads to Stimulation of long-acting antigen or cystic stage G parasite. lamblia (Scott et al., 2004), and in turn, consists of chemical kinetics that respond when the inflammatory response occurs after infection with parasitic diseases. It has been seen through multiple studies that the concentration of IL-6, IL-2, and L-10 or TNF-a increases after Examination of the serum of persons infected with giardiasis after comparing these concentrations with those of the control, the quantity and type of these cytokinetics affected by the infection of the parasite in terms of whether it is invasive or non-invasive (Zhou et al., 2007).

Aim of the Study

1. Determination of the heat shock protein concentration (HSP70) in the serum of people with the parasite and compared with the control group using ELISA technique.
2. Use technique PCR-Nested in the identification of a heat shock protein gene.
3. Conducting some immunological tests for acute and chronic infection.

3. Material and Method

Blood samples were collected by drawing (5 ml) of venous blood using a wine syringe. After sterilizing the area with ethanol at a concentration of 70%, (3 ml) of blood is placed in a test tube and left to clot for half an hour, then the samples were centrifuged using a centrifuge. (Centerfuge) at 3000 rpm for 10 minutes, separate the serum from other blood components using a micro pipette and place it in 10ml tubes. Then the tubes containing the serum were kept at -20°C until tests were performed on them. Serum is used to estimate the concentration of the heat shock protein Hsp70.

Detection of Heat Shock Protein 70 (HSP70) by ELISA

In this study, the ELISA test was used to detect heat shock protein (HSP70) and a test kit (HSP70 Bioassay ELISA Kit, USA) was used, and the method of work is Sandwich enzyme-linked immune-sorbent assay technology. The basis of the work includes adding samples and standards to the plate pits that contain monoclonal antibodies to the protein HSP70 (Monoclonal antibody specific for HSP 70). These antibodies bind to HSP 70 protein, After incubation and washing, a solution containing polyclonal antibodies to HSP 70 polyclonal (antibody specific for 70 anti HSP), which is yellow in color, which binds to HSP 70 protein that adheres to the walls of the pits of the lamina, is added. Incubation and washing A solution containing (HRP conjugate), which is blue in color, is added to each hole in the plate, which binds with antibodies. After incubating and washing this plate, the substrate (TMB substrate) is added to each hole.

1. Contents of the Test Kit Components

- Plate of 96 flat-bottomed holes covered with monoclonal antibodies. HSP70 monoclonal antibody).
- Standard Calibrator Antibody Dilution Buffer 1 Pack (10 ml/ μ g)
- Buffer solution Sample Dilution Buffer One 20 ml bottle consisting of a saline buffer containing detergents.
- Washing solution(X20) wash buffer One 30 ml packet consisting of a saline buffer containing detergents.
- HSP 70 PAB (Biotin-Anti- Humam- HSP 70 Antibody) One packet (120 ul) is a yellow liquid and is a polyclonal Rbbit antibody specific for HSP 70.
- HRP-Streptavidin conjunction (SABC) is a 10 ml bottle. It is a blue liquid consisting of rabbit IgG antibodies conjugated with peroxidase enzyme from horseradish (anti-rabbit IgG conjugated to horseradish peroxidase).
- Base material TMB substrate (one 10ml bottle) contains TMB tetramethyl benzidine,3,3,5,5 and peroxide hydrogen).
- STOP solution Stop solution 1 bottle (10 ml) of HCl aqueous (IN).

2. Working Method Procedure

Balanced TMB of the substrate before adding the reagent to the Wells pit for 30 minutes at 37°C. When the samples and reagent are dissolved, they must be mixed completely and parallel.

1. Zero all of the standard drill Standard, Sample and zero (control) samples on the previously covered plate, respectively, and then record their location respectively.
2. Add 0.1 ml of ml /pg 2000, 1000 ml /pg, ml /pg 500, ml /pg 250, ml /pg 125, ml /pg 62.5, ml /pg 31.25 of the standard solutions to the standard pits and numbered (1-7) which represents (S7, S6, S5, S4, S 3, S 2, S1) which is the standard calibration for the rest of the readings in the plate.
3. Add 0.1 ml of Dilution Buffer to samble and standerd pits for (yellowish) test. 4- 0.1 ml of the diluted sample of (serum) was added to the test pits.
4. Close the plate with a lid and incubate at 27. °C for 90 minutes.
5. Remove the cap and discard the contents of the plate plate content Wash the plate twice with Wash Buffer and do not let the pits dry completely at any time.
6. Added 0.1 ml of (HSP 70 PAB) solution to pits (stander, control, sample) and these solutions were added to the bottom of the pits Well without touching the wall
7. The plate was placed with the lid and incubated at 37°C °C for 60 seconds
8. Remove the cover and wash the plate 3 times with Wash Buffer Let the wash solution sit on the pits for one minute at a time.
9. 10 - add 0.1 ml of HRP - conjunction to the pits, cover the plate and incubate at 37 °C for 30 minutes
10. Remove the cover and wash the plate five times in the wash solution, leaving the wash solution in the pits for 1-2 seconds each time.
11. Added90 ml of TMB substrate to all pits, cover the plate and incubate at 37 °C in the dark for 15-30 seconds.
12. Added 50ml Stop Salution in all the holes and mix them well.
13. I read the absorbance at 450 nm in a device Micro plate Reder immediately after adding stop solution.

3. Calculate the Results Calculation of Results

The ELISA device gives a reading of the optical density (Optical Density (OD), which represents the concentration of (HSP70) and give a standard curve, or the results are calculated according to the following equation:

$$\text{Relative OD450 (OD .450 of each Well) - (OD 450 of zero well)}.$$

4. Results

Serological tests were conducted on 90 samples, and the number of samples that confirmed infection with *Giardia* parasite was 65 samples in some areas of Najaf Province and 25 healthy samples without parasitic infections for the purpose of comparison between the two groups and for the detection of immune antibodies.

1. Determination of Heat Shock Protein Concentration HSP70 using the ELIZA Test

The current study showed that the amount of heat shock protein HSP70 in infected persons and comparing them with healthy samples resulted in an increase in the concentration of HSP70 protein in the serum of persons infected with *Giardia lamblia* compared with the percentage of its presence in the control group with a significant difference of $0.01 < p$. After conducting the examination of the positive samples, it was found that the average protein concentration is 28.04 ng/ml% While the percentage of healthy samples was ng/ml 3.98%.

Table 1 - Heat Shock Protein HSP70 in Serum of Infected & Compared with Healthy Samples

| Groups | Number of sample | Mean (SD±) | Values calculated t | Values tabular t | P value |
|---------------|------------------|-------------|---------------------|--------------------------|---------|
| Study group | 65 | 28.04(4.94) | 67.34 | 2,633 df=88 P≤0.01 | <0.01 |
| control group | 25 | 3.98(0.74) | | | |
| Total | 90 | | | | |

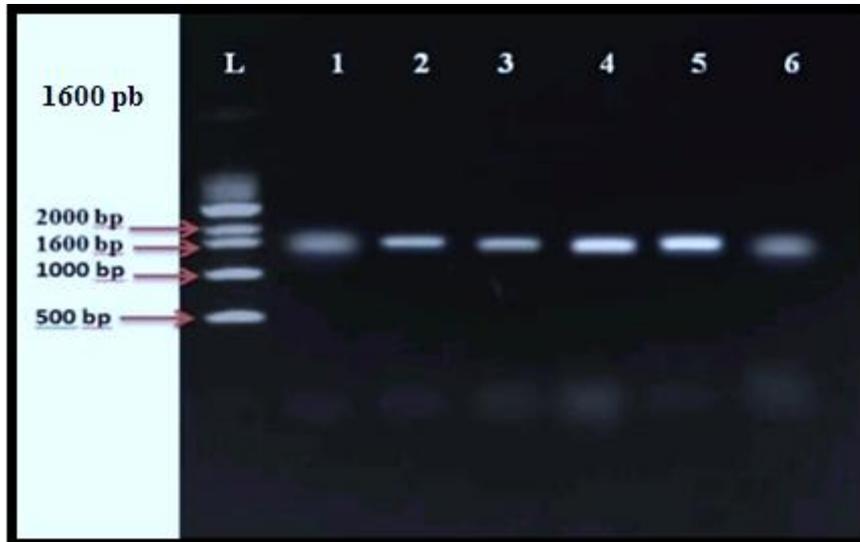
PCR Molecular Diagnostics (Polymerase Chain Reaction)

1. DNA Extraction from Stool

The current study showed the examination of 90 samples by PCR technique, including samples that were confirmed to be infected with *Giardia lamblia* parasite by light microscopy to

confirm infection, as well as to obtain the resulting DNA for use as a template in PCR technology through direct DNA extraction of stool samples as shown in Figure (2).

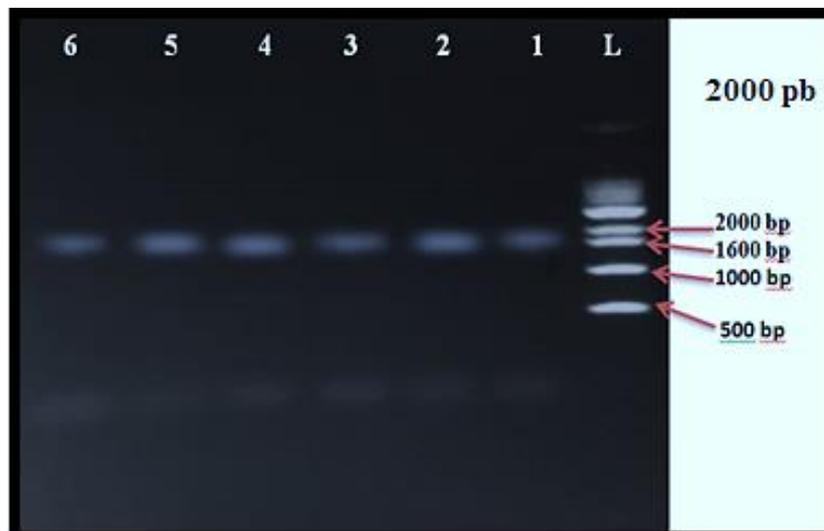
Figure 2 - Electrophoresis Product of *G. lamblia* by Diagnostic PCR on Agarose Gel (1.5%, 100V for 1 Hour) for 16S rRNA Gene Amplifiers (1600bp), Column L: The Volume Index Represents 1Kbp. Column 1 - 6: represents Samples Positive for Amplification



2. Diagnosis of Shock Protein by PCR Technique of the Heat Shock Protein Gene

The current study relied on diagnosing the heat shock protein HSP70 gene in stool samples of people infected with Giardia Lamblia parasite as shown in Figure (3).

Figure 3 - Agarose Gel Electrophoresis Image of the Product of PCR Analysis of the HSP 70 Gene in some Isolates of *G. Lamblia* Parasite, Column L: The Volume Index Represents 1Kbp. Column 1 - 6: Represents the Positive Sample Numbers and Pb 2000 Yield



5. Discussion

Serological Study

1. Heat Shock Protein Relationship HSP70 in *Giardia Lamblia*

The current study showing the presence of a high concentration of heat shock protein HSP70 in infected people and comparison with healthy samples which resulted in an increase in protein concentration HSP70 in the serum of people infected with the parasite *Giardia lamblia* compared to the percentage of its presence in the control group, Where It was found that the average protein concentration is 28.04 ng / ml %, while the percentage in healthy samples was 3.98% ng / ml.

The current study also revealed the presence of the heat shock protein gene HSP70 in stool of people infected with Giardia parasite, which is the first study to diagnose protein in stool samples of people infected with Giardia parasite in Najaf and the second in Iraq. In this study, a high level of HSP70 so that this concentration has an important role in the regulation of monocytes. Many of TLR2 legends and endogenous TLR4 such as HSP60 and HSP70 have been identified as a melancholic alarm signal that triggers an immune response when released (Curtin et al: 2011; Abdollahi-Roodsaz et al., 2009), studies have confirmed the role of heat shock protein HSP70 in the immune response against the Giardia parasite, as it works to resist and inhibit it through multiple mechanisms that bind with the parasite antigens and present them to the exhibiting cells. MHC antigens help in this to be presented to the T-cells, which work to resist the parasite. (Williams and Goombs 1995; DeNagel and Pierce, 1992). Several studies have shown the importance of these proteins in defending the body and maintaining its stability against many chronic diseases, cancers and pathogens, as shown between (Bendz et al., 2007). HSP70 has the ability to display antigens specific to diseased cells affected by tumors on MHC-1 antigens in addition to its role as a cell guard by acting as a molecular chaperon.

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