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Human Leukocyte Antigen Class II Alleles in Children with Giardiasis

Thesis Submitted For Partial Fulfillment of the Requirements for M.D. Degree in Basic Medical Science (Medical Parasitology)

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To My Parents

My Brother & Sisters

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Chapter I

Introduction & Aim of the work

Introduction & Aim of the work

1.1. Introduction:

Giardia lamblia (*G. lamblia* synonymous *Giardia intestinalis* and *Giardia duodenalis*) is an intestinal flagellated protozoa causing giardiasis, a major gastrointestinal disease, mainly in developing countries due to inadequate sanitation and insufficient water treatment **(McInally and Dawson, 2016)**. The parasite is responsible for a great proportion of diarrheal outbreaks all over the world and persistent travellers' diarrhea **(Nabarro *et al*., 2015)**. Therefore, *G. lamblia* was classified by World Health Organization (WHO) in 2004 as one of the neglected diseases initiative **(Wegayehu *et al.,* 2016)**.

The main feature of *G. lamblia* infection is varying severity of clinical manifestations from asymptomatic cases that account for about half of infections, to loss of appetite, abdominal pain, loose watery stool, nausea, vomiting and severe malabsorption syndrome **(Fletcher *et al.,* 2012)**. This may be attributed to the variability in virulence of different *Giardia* strains, the number of infecting *Giardia* cysts, and the quality of the host's immune system **(Fenollar *et al*., 2003)**.

Solaymani-Mohammadi and Singer (2010) demonstrated the development of both innate and adaptive host immune mechanisms against *G. lamblia* infection, but the exact defense mechanisms are still poorly understood. For example, despite what was expected, *Giardia* trophozoites liberate chemokines that attract dendritic cells to act as antigen-presenting cells and in turn initiate T- and B-cell responses **(Roxstrom-Lindquist *et al*., 2005)**. Also, the parasite stimulates major histocompatibility complex class-II (MHC-II) cells other than dendritic cells and B-cells which are essential to present *Giardia* antigens to naive

CD4+T cells and initiate cellular immunity, but which type of MHC-II cell is still unknown **(Grit *et al.,* 2014a)**.

Major histocompatibility class-II molecules are surface heterodimers expressed by antigen-presenting cells and are important for presenting exogenous antigenic peptides to T-cell receptors on CD4+T cells **(Tsai and Santamaria, 2013)**. It is worth noted that, MHC class-II molecules are encoded by three main human leukocyte antigen (HLA) polymorphic genes; HLA-DR, -DQ, and -DP **(Volpi *et al*., 2000)**.

Major histocompatibility region encodes HLA that participates in antigen presentation, inflammation regulation and complement system function as well as innate and adaptive immune mechanisms **(Shiina *et al*., 2009)**. Moreover, polymorphisms in the MHC locus can influence host susceptibility to different infectious diseases **(Hill-Burns *et al.,* 2011)**. It has been reported that, MHC loci polymorphism is associated with protection against some major infectious diseases **(Blackwell *et al*., 2009)**. With regard to giardiasis, MHC class-II molecule expression is increased on the surface of bovine dendritic cells, when a mixture of *Giardia* assemblage A and E trophozoites was used (**Grit *et al.*, 2014b**).

Giardiasis was also associated with increased neutrophils infiltrating the epithelial layer in 16% of infected patients **(Koot *et al.*, 2009)**. In fact, neutrophils play a critical role in inflammation by phagocytosing and killing the invading microorganisms, therefore they are considered as the first line of defense against infectious diseases **(Faurschou and Borregaard, 2003)**.

When neutrophils are stimulated during inflammation, they release a variety of granule proteins and soluble proteins such as calprotectin **(Srinivasan, 2013)**. Accordingly, calprotectin level in fecal samples is a result of neutrophils' migration into the gastrointestinal tissue **(Damms**

and Bischoff, 2008) and can be used as a non-specific marker for neutrophils activation as well as gastrointestinal inflammation **(Hestvik *et al.,* 2011)**.

1.2. Aim of the work:

This study was conducted aiming to:

1- Detect any correlation between host genetics; HLA-DRB1 alleles and the development of susceptibility or resistance to *Giardia* infection.

2- Identify the influence of HLA-DRB1 alleles on the clinical manifestations of giardiasis.

3- Asses fecal level of calprotectin in children with giardiasis.

4- Report any association between fecal calprotectin level and the development of symptomatic or asymptomatic giardiasis.

Chapter II

Review of literature

2.1. *Giardia lamblia*

2.1.1. History of *Giardia* discovery:

In 1681, *Giardia* was initially described by Antony Van Leeuwenhoek during examination of his diarrheic stools **(Adam, 2001)**. In 1888, Blanchard steered the name *Lamblia intestinalis*, and in 1915, Charles Wardell Stiles introduced the name of *Giardia lamblia* **(Ford, 2005)**.

2.1.2. Taxonomy of *Giardia*:

Concerning the widely used 1980 methodical scientific categorization which bolsters the morphology, *Giardia* has a place with:

Kingdom: Protista

Subkingdom: Protozoa

Phylum: Sarcomastigophora

Subphylum: Mastigophora

Class: Zoomastigophora

Order: Diplomonadida

Family: Hexamitidae

Genus: *Giardia*

Species: *lamblia* **(Corliss, 2001)**.

With respect to the scientific classification that depends on atomic sequence studies and gathering them with different hereditary, basic and natural science, *Giardia* is under the subdivision of:

Phylum: Metamonada

Taxon: Trichozoa

Category: Eopharyngia

Category: Trepomonadea

Taxon: Diplozoa

Order: Giardiida

Family: Giardiidae (**Cavalier-Smith, 2003**).

2.1.3. *Giardia* assemblages and sub-assemblages:

Among the six known *Giardia* species, exclusively *G. lamblia* has been found to infect humans and most mammals (**Liu *et al*., 2015**). *G. lamblia* has eight genotypes named A-H that take issue in host dissemination, among them genotypes A and B are the most common causes of human contaminations (**Jerez Puebla *et al*., 2015**). Genotype A has 2 subtypes; AI a fundamental animal illness subtype and AII concerned with anthroponotic infections, and it has been scarcely detected in animals. Assemblage B is the most frequent worldwide genotype that has been isolated into two subtypes named BIII and BIV; that is considered to be specific for humans (**Pestechian *et al*., 2014**).

Each of the remaining assemblages from C to G is mainly linked to a particular animal host (**Caccio *et al*., 2005**).

2.1.4. Prevalence:

Giardiasis is usually related to serious episodes of human diarrheal diseases, which are the commonest cause of the overall mortality and morbidity following pulmonary diseases (**Cama and Mathison, 2015**). The prevalence of *Giardia* is more common in children than adults **(Feng and Xiao, 2011).** The estimated prevalence of giardiasis ranges from 3% to 7% in developed countries and from 20% to 30% in developing countries, with reports of 100% prevalence in some populations (**De Lucio *et al*., 2015**).

Giardia is the most widely recognized intestinal parasite in developing countries as it is responsible for approximately 2.0 million cases annually (**Asher *et al*., 2016**). It is also estimated that about

500,000 new cases are detected annually with about 200 million cases suffering from symptomatic giardiasis are Asians, Africans and Latin Americans (**Sanchez *et al*., 2017**).

In Egypt, *G. lamblia* is the most common parasitic infection with a prevalence of 18 % to 38 %, respectively **(Elswaifi *et al*., 2016; Ghieth *et al*., 2016; Hussein *et al*., 2017a**).

The prevalence of each assemblage differs significantly from nation to another, with assemblage B appears to be the most widely distributed **(Feng and Xiao 2011)**. This coincides with the incidence of assemblage B in Egypt that is higher in symptomatic giardiasis children (40%) than asymptomatic cases (20%), while assemblage AI is the common among asymptomatic children (66.6%) compared to (53.3%) symptomatic children **(Hussein *et al*., 2017b)**. Nonetheless, **Foronda *et al.* (2008) & Abdel-Moein and Saeed (2016)** demonstrated assemblage E for the first time in human cases in Egypt.

2.1.5. <u>Morphology (Figure 1):</u>

Giardia is a fascinating single eukaryotic cell (protist). On the basis of structural, functional and morphological characters, *G. lamblia* has two distinct forms, the motile trophozoite and the cyst **(Nino *et al*., 2013**).

Giardia trophozoite is pear-shaped, about 12-15 µm long, 5-7 µm wide, and 1-2 µm thick (**Solari *et al*., 2003**). The trophozoite contains two nuclei and is outfitted with eight flagella emerging from the cell body, an adhesive disc and a noticeable heap of microtubules called the median body **(Carpenter *et al*., 2012**).

While, *Giardia* cyst is oval in shape, measuring 6-10 µm and characterized by the presence of a highly defensive wall; 0.3-0.5 µm in thickness that opposes brutal natural conditions outside the host's

digestive tract; hence the cyst can live for months in water at 4°C **(Birkeland *et al*. 2010)**. The cyst cytoplasm contains two to four nuclei, contracted flagella and fragmented segments of the ventral disk **(Palm *et al.,* 2005)**.

Despite the fact that *Giardia* assemblages have no differences morphologically, they can be distinguished essentially in their biology, virulence and hereditary qualities, as higher rate of cyst shedding occurs with assemblage B comparative with assemblage A **(Sarkari *et al*., 2012)**. Also, assemblage B can easily infect mice, while assemblage A is cleared before the infection established. Moreover, assemblage A has faster growth rate and can efficiently be encysted *in vitro*, in comparison with assemblage B that is difficult to encyst **(Ankarklev *et al.,* 2010)**.

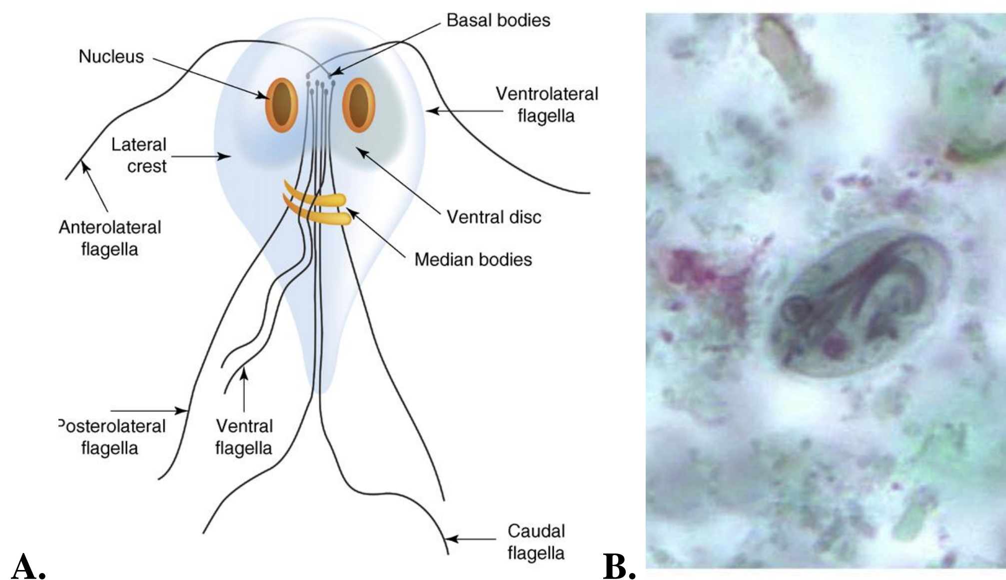

Figure (1): Morphology of *Giardia lamblia*. Quoted from Monis *et al*. (2009) & Cama and Mathison (2015).

A: trophozoite**, B:** cyst stained with trichrome.

2.1.6. Life cycle (Figure 2):

Giardia encompasses a comparatively easy life cycle that consists of: the trophozoite (vegetative form) which colonizes the host bowel and the dormant quadrinucleated cyst (infective form) that persists for months in cold fresh water. *Giardia* experiences two pathogenically critical sorts of differentiation: encystation is required for survival outside the host, and excystation is required for infection **(Gillin *et al*., 1996)**.

Giardia inhabits the human small intestine below the entrance of common bile duct where trophozoites adhere to enterocytes and mucus or swim in the intestinal liquid but do not hostile the epithelia, while *Giardia* cysts colonize the intestinal lumen **(Nohynkova *et al*., 2006)**.

Infection begins with the consumption of food defiled with cysts as ingestion of as few as 10 cysts can start disease, despite the fact that it is less common than waterborne transmission. Other potential mechanisms of transmission include: fecal-oral route, direct contact with infected person and zoonotic transmission from animals as livestock, cats, dogs and rodents. Following ingestion, the acidic environment of the stomach induces excystation, where each cyst generates two trophozoites. Then, the free trophozoites undergo mitotic divisions by longitudinal binary vision. As a result of growth, maturation and shedding down of intestinal cells and the excretion of intestinal contents, trophozoites must be motile to be able to stay in the bowel. Once trophozoites encounter changes within the environment of lower bowel during their migration, together with pH scale and levels of digestive fluid and bile they pass into encystation phase. The cysts are then discharged from the host with fecal material, to complete the transmission cycle upon infection of a new host **(Eckmann and Gillin, 2001)**.

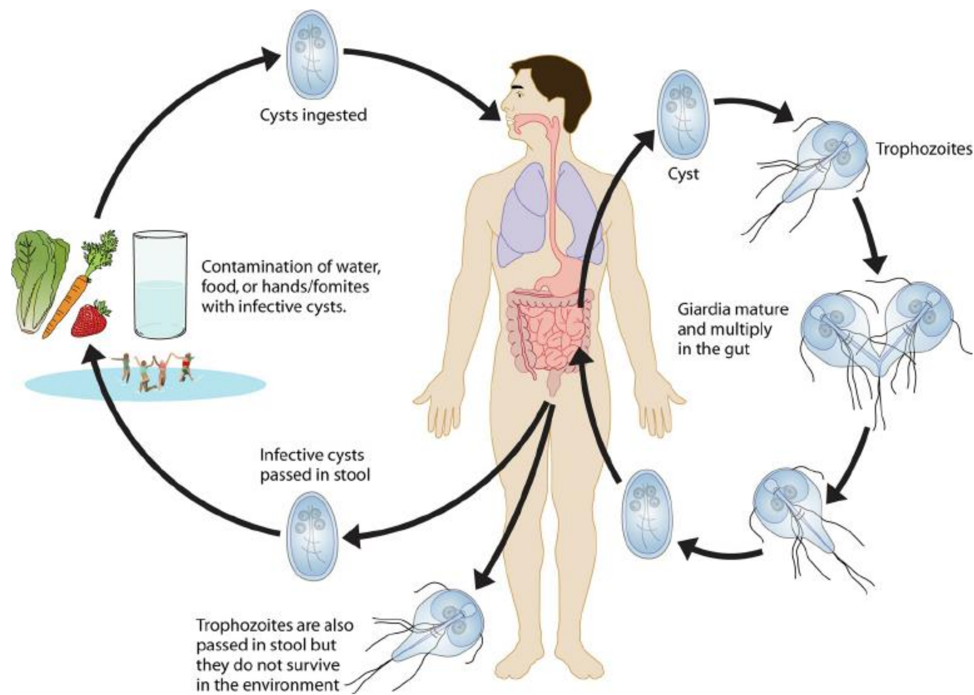

Figure (2): ***Giardia*** **life cycle. Quoted from Esch and Petersen (2013).**

2.1.7. Immunity to *Giardia* infection:

Host defense mechanisms to eradicate *Giardia* infection are mediated through a synergism between both innate and adaptive immunity **(Solaymani-Mohammadi and Singer, 2010).**

The first line of protection against *Giardia* multiplication is performed by innate immune response **(Lopez-Romero *et al.,* 2015),** through several mechanisms **(Figure 3):**

1. Mechanical barriers render *Giardia* attachment to bowel surface by mucus layer and peristalsis.
2. Paneth cells produce antimicrobial peptides (AMP) which destroy the trophozoites.
3. The microbiota of gut is able to compete with *Giardia*, causing direct toxicity, modifying the immune mechanism and finally preserving gut integrity.
4. Pro-inflammatory cytokines released by mast cells as interleukin-6 (IL-6) stimulates innate cellular responses, T-cell development into T helper-

17 (Th17) cells and finally fragmentation of mast cell that stimulate peristalsis.

5. Nitric oxide molecule produced by the epithelial and immune cells of intestine induces a cytostatic effect on *Giardia* trophozoite, preventing its excystation/encystation processes and promotes peristaltic movements.

6. Antigens within gut lumen are engulfed by microfold cell *via* endocytosis into peyer's patches in order to initiate immune responses.

7. Dendritic cells are considered as a 'linker' between innate and adaptive immune responses. As regard to their location in lamina propria and peyer's patches and their dendrites, they can easily identify, expand their dendrites and take up *Giardia* peptides.

8. Dendritic cells engulf, process, and present *Giardia* antigens to naive T cells by MHC class-II molecules. Then, activated T cells liberate an array of cytokines, which can modify the anti-*Giardia* immune response.

9. IL-6 released by mast cell, dendritic cells or T-cells is an important inducer of B-cell maturation and antibody class switching to produce IgA.

10. Plasma cells migrate to lamina propria to liberate IgA that surrounds *Giardia* trophozoite hindering its binding to bowel epithelium and also contributes to the maintenance of protective immunity against giardiasis.

11. Th17 and CD4+T cells activation during early adaptive immune response against *Giardia*, leads to production of pro-inflammatory cytokines such as IL-17, IL-21 and IL-22 responsible for recruitment of effector cells like neutrophils and macrophages.

12. Intra-epithelial lymphocytes mainly of CD8+T cells mediate toxic and pathological damage of intestine during giardiasis.

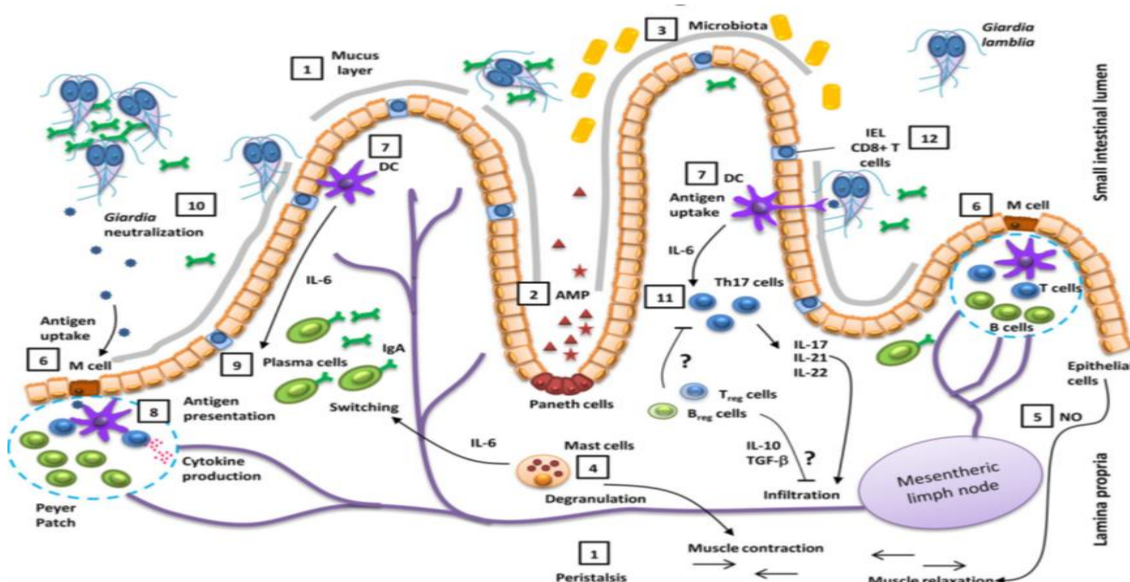

Figure (3): Host defense mechanisms against *Giardia lamblia*. Quoted from Lopez-Romero *et al.* (2015).

AMP: Antimicrobial peptides, **NO**: nitric oxide, **M cells**: microfold cells, **DC**: Dendritic cells, and **IEL**: Intra-epithelial lymphocytes.

In conclusion, if innate immune mechanisms cannot kill *Giardia* pathogen, it keeps developing, expressing, and discharging antigens, which activate specific adaptive cellular and humoral immune responses **(Jones, 2005)**. After taking up *Giardia* antigen, dendritic cells become activated through ligands of pattern-recognition receptors (PRRs), e.g. Toll-like receptors (TLRs), C-type lectins, and nucleotide-restricting oligomerization domain (NOD)-like receptors (NLRs), then process and present specific *Giardia* antigen to naive T lymphocytes, which initiate specific cellular resistance (**Ueno *et al*., 2007**).

A long-lasting cellular immunity against *G. lamblia* has been revealed in infected patients, and was mainly enhanced by CD4+T–cell activation **(Hanevik *et al*., 2011)**. In spite of involvement of CD4+T helper cells in immunity against *G. lamblia*, insufficient antigen presentation cannot effectively enhance protective cellular immunity, and chronic disease may develop **(Eckmann, 2003)**.

So, finally host defense against *Giardia* infection depends on both mucosal humoral and cellular immune responses, for example, an adjusted reaction of antigen-particular CD4+T cells, the release of cytokines, essentially IL-6, TNF-α, IFN-γ and IL-4, and the generation of particular IgA or IgG antibodies against parasite antigens **(Roxstrom-Lindquist *et al.,* 2006)**.

On the opposite side, *Giardia* pathogen has two mechanisms of host immune evasion to guarantee its survival inside and outside the host gut; antigenic variation and encystation, respectively. Antigenic variation is intervened by the consistent exchanging of particular antigens on the surface of parasite, and these antigens are vital for host immune evasion. Encystation, on the other hand, with the formation of a hard resistant cyst wall allows the parasite to maintain under unsuitable external environmental conditions and ensures the transmission of the infection to susceptible hosts **(Carranza and Lujan, 2010)**.

From the pathological point of view, **Kamda and Singer (2009)** declared that live parasite and parasite extracts activate dendritic cells for processing and presenting antigens with increasing the production of IL-10. On the contrary, they inhibit the secretion of IL-12, which is to some extent mediated by IL-10. Consequently, this hindrance potentially recommends that *Giardia* parasite effectively limits the advancement of extreme inflammation and this may be the cause of absent pathological profile often demonstrated during giardiasis.

Despite **Roxstrom-Lindquist *et al.* (2005)** speculations that *Giardia* trophozoites liberate chemokines able to attract dendritic cells to act as antigen-presenting cells, hence initiating T- and B-cell responses, **Grit *et al.* (2014a)** noted that neither dendritic cells nor B-cells are the main cells responsible for the enhancement of *Giardia* antigen presentation and thus the initiation of the cellular immunity. However, the

consumption of cells with high MHC-II particle essentially diminishes expansion of peripheral blood mononuclear cells (PBMCs) that are not also high MHC-II cells. The results demonstrated that antigen-presenting cells, other than dendritic cells and B-cells are required to initiate PBMCs multiplication. But the question is still which MHC-II cells are able to fully induce lymphocyte proliferation? Moreover, **Grit *et al.* (2014b)** reported that at the largest amount of proliferation of the antigen displaying PBMCs, the vast majority of cells were CD4+T-cells expressing mainly IL-17 and some FoxP3.

Other problems noted by **Lopez-Romero *et al.* (2015)** concerning the impressive defects in our knowledge about host-*Giardia* interaction as: What are the roles of B-regulatory and T-regulatory cells in regulating the inflammatory process during giardiasis? How can *Giardia* antigens effectively initiate both local and systemic immune responses in spite of the inability of *Giardia* trophozoites to penetrate the intestinal mucosa? How does *Giardia* infection disrupt mucosal tolerance? What type of *Giardia* antigens can enhance a protective immune response? Does *Giardia* modulate antigen pick up by antigen presenting cells (dendritic cells and macrophages)?

2.1.8. *Giardia* antigens:

Giardia antigens comprise variant specific surface proteins (VSPs), structurally conserved invariant proteins, cyst wall proteins (CWP) and heat shock proteins (HSP) **(Singer and Nash, 2000; Heyworth, 2014)**.

Although *Giardia* trophozoites attach to the mucosal surface, they produce a variety of immunogenic proteins that interface the host immune cells and elicit humoral immune response by secreting IgA in the mucosa as well as IgG in the serum, and activate the cellular immune response **(Velazquez *et al.*, 2005).** Although, the humoral antibodies are primarily

directed against the VSPs during *G. lamblia* infection, a humoral immune response is also generated against the invariant antigens **(Weiland *et al.*, 2003)**.

The VSPs are the most prominent *Giardia* trophozoite surface proteins and consist of a family of cysteine rich proteins that establish a dense coat on the parasite surface and flagella **(Prucca and Lujan, 2009)**. In addition, the non-reactive amino terminal part of VSPs is the most variable portion that interfaces the host immune system and thus permits the trophozoite survival in the small intestine **(Nash, 2002)**.

In spite that, each trophozoite contains at least 150 different VSP genes (representing 2.4% of the total parasite genome), the *Giardia* trophozoite expresses only one VSP at a time except during antigenic switching it expresses two distinct VSPs **(Nash *et al.*, 2001)**. Antigenic switching of VSPs occurs on the surface of proliferating trophozoite and the liberated ones from the non-proliferative cysts **(Bienz *et al.,* 2001)**.

Moreover, the greater quantity of VSP genes has a potential role in *Giardia* immune evasion mechanism through antigenic variation that is thought to play an important role in facilitating chronic as well as repeated infections, also the ability of *G. lamblia* parasite to infect a wide variety of hosts **(Singer *et al.*, 2001)**. Furthermore, the expression of a specific VSP could affect the capability of *G. lamblia* to infect a particular host **(Nash *et al.*, 2001)**. Indeed, the 5G8 *G. lamblia* protein is one of VSPs that settles on the surface and inner part of *G. lamblia* trophozoites and provokes effective humoral as well as cell-mediated immune responses and induces *in vitro* agglutination of *G. lamblia* trophozoites **(Quintero *et al.*, 2017)**.

It is worth noted that, the trophozoite invariant proteins include giardins and tubulin (cytoskeletal proteins recognized on trophozoite adhesive disc, surface membrane and flagella), fructose-1, 6 bisphosphate

aldolase as well as arginine deaminase and ornithine carbamoyl transferase; *G. lamblia* enzymes involved in arginine metabolism **(Palm *et al*., 2003)**.

Moreover, there are three abundant *Giardia* cyst wall proteins (CWP1, CWP2, and CWP3) synthesized and localized in encystation-specific secretory vesicles (ESVs) that are part of a Golgi-like apparatus **(Chatterjee *et al*., 2010)**. CWPs are developed during encystation process and throughout the lifetime of cyst stage **(Davids *et al*., 2006)**. The induction of specific cellular and humoral immunity against *Giardia* CWPs leads to significant reduction of cyst shedding **(Abdul-Wahid and Faubert, 2008)**.

Among the *Giardia* antigens, heat shock proteins (HSP) as Hsp70 and Hsp90 which are chaperone molecules are induced during stress conditions and encystation process, but their actual role in encystation process is not yet clear **(Kim *et al*., 2009)**.

2.1.9. Pathogenicity and clinical Picture:

Giardia trophozoites attach to epithelial cell surface but do not invade the surrounding tissues or the blood stream. This attachment contributes to different pathophysiological mechanisms which result in malabsorption of glucose, excretion of water and electrolyte, as well as reduction of disaccharidase activity **(Granados *et al*., 2012)**. These mechanisms are mediated through damage of intestinal absorptive surface, intercellular junctions, cytoskeleton rearrangement, and barrier dysfunction leading finally to diarrhea and malnutrition **(Certad *et al.*, 2017)**.

Clinical manifestations of *G. lamblia* infections contrast among people, starting from acute to chronic infection, whereas some hosts are symptomless. Patients with acute infection present with abdominal pain,

foul smelling explosive watery loose stool, greasy bulky fatty stool, innate reflex and nausea **(Cotton *et al.,* 2011)**.

Abdominal colic, loose watery stool, weight loss, malabsorption and impeded development are the characteristic features of chronic giardiasis **(Hanevik *et al.,* 2009)**. Post-infection disorder as irritable bowel syndrome is documented among individuals infected with *G. lamblia* more than in those with gut bacterial or viral infections **(Thabane and Marshall, 2009)**. Additionally, chronic extra-intestinal manifestations may be found affecting the joints, skin, eyes, and even the central nervous system of some patients in the absence of extra-intestinal spread of the parasite, in parallel with the increase of immunogenic basis of these abnormalities **(Halliez and Buret, 2013)**.

It has been reported that repeated symptoms as abdominal presentations and fatigue may be the result of re-infection, treatment failure or post-infection syndromes **(Spiller and Garsed, 2009)**.

Distinctive reports from around the world have correlated assemblage B infections with a lot of serious symptomatology, while others have linked more symptoms with assemblage A contaminations **(Robertson *et al*., 2010)**.

2.1.10. Diagnosis of giardiasis (Figure 4):

2.1.10.1. Microscopic examination:

Giardia cysts present in fecal specimens are detected principally by light microscopy. While, trophozoites are not always detected in stool because encystation occurs before *Giardia* passage in colon, but they can be detected in duodenal aspirates **(McHardy *et al*., 2014)**. *Giardia* stages; whether trophozoite or cyst, can be identified from other microorganisms by the usage of different stains as iodine, iron-haematoxylin, Giemsa or trichrome **(Smith and Paget, 2007)**.

Additionally, different *Giardia* species can be distinguished by electron microscopy on the basis of distinct features of their ventro-lateral flange, marginal groove, ventral disc and flagellum **(Adam, 2001)**.

2.1.10.2. Histopathological examination:

Upper endoscopic examination of chronic giardiasis patients usually shows normal luminal mucosa, while their duodenal biopsy specimens may show *Giardia* trophozoites reside between the villi with the characteristic pear shape **(Panarelli *et al*., 2017)**. *G. lamblia* trophozoites can also be demonstrated in biopsy specimens taken from the terminal ileum **(Oberhuber *et al*., 2016)**. In addition, a wide variety of histological changes following *G. lamblia* infection, ranging from mild microscopic duodenal inflammations to subtotal villous atrophy have been seen in severe giardiasis patients **(Troeger *et al.,* 2007)**.

2.1.10.3. Immunological techniques:

The detection of coproantigens; *Giardia* antigens in stool samples, is another procedure. Immuno-chromography tests were applicable with specificities ranging from 79%-100% and sensitivities from 26%-100% **(Elsafi *et al*., 2013)**. Various enzyme-linked immunoassays as ELISA were also used with specificities of 87%-100% and sensitivities of 63%-100% **(Schuurman *et al*., 2007)**. The sensitivity of coproantigen detection assays has been found lower than microscopic examination. This is attributed to low numbers of cysts in faeces or presence of trophozoites in the host intestine **(Johnston *et al*., 2003)**.

Among the immunological techniques, the direct fluorescence antibody (DFA) test has been reported to achieve high specificity (99.8%-100%) and sensitivity (93%-100%) for the identification of *Giardia* cysts in fecal and environmental samples **(Baig *et al*., 2012)**. Additionally, flow cytometry is a promising procedure for the detection and enumeration of

Giardia cysts **(Dixon *et al.*, 1997)**. The application of peptide nucleic acid probe to flow cytometry decreases false-positive results to zero percent **(Ferrari and Veal, 2003)**.

The variation in diagnostic performance of different assays may be related to cross-reactivity (affecting specificity) or discontinuous shedding of cysts and *Giardia* antigens, or the use of formalin as a fixative (reducing sensitivity) **(Strand *et al.*, 2008)**.

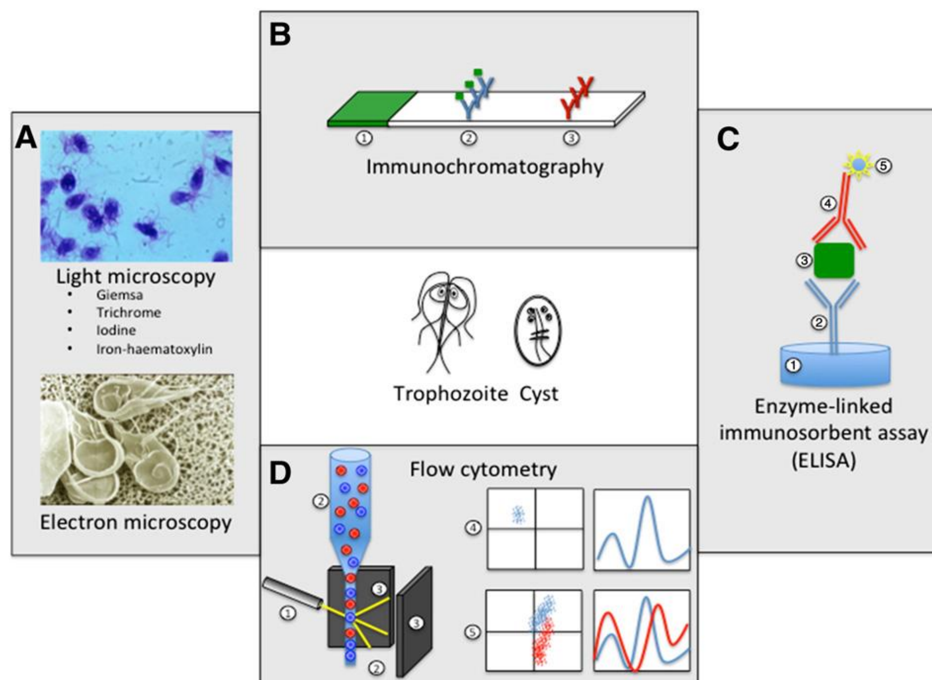

Figure (4): Some diagnostic approaches for the detection of *Giardia* trophozoite and cyst. Quoted from Koehler *et al*. (2014).

(A) Light and electron microscopic identification of *Giardia* stages; **(B)** Immuno-chromatography: **(1)** sample loading area, **(2)** positive-control antibody for the assay, **(3)** antibody for the specific antibody based detection of *Giardia* antigens; **(C)** Immunoassay: **(1)** ELISA well, **(2)** capture antibody, **(3)** *Giardia* antigen, **(4)** specific secondary antibody, **(5)** enzyme-labelled conjugate for colorimetric detection; **(D)** Flow cytometry: **(1)** laser emitter, **(2)** sample containing fluorescently labelled cysts, **(3)** light detectors, **(4)** display of results using one colour, **(5)** two-colour flow cytometry.

2.1.10.4. Nucleic acid techniques:

Different nucleic acid-coupled approaches could identify and analyze variant genes within different *Giardia* assemblages present in clinical samples. Most procedures depend on the specific magnification of one or more loci from small amounts of genomic DNA by PCR, and some of them can on the specific in situ hybridization of probes to specific genetic loci among *Giardia* genome **(Koehler *et al*., 2014)**.

2.1.11. Treatment:

More than six distinct classes of medications are effective against giardiasis e.g. 5-nitroimidazole derivatives (metronidazole, tinidazole and secnidazole; the most considerably specific medications), benzimidazole derivatives (albendazole and mebendazole), acridine derivative (quinacrine), nitrothiazolide (nitazoxanide), nitrofuran (furazolidone) and aminoglycoside (paromomycin). It has been reported that the single dosage of long acting 5-nitroimidazole derivatives is extremely successful than short course therapy with other anti-*Giardia* drugs **(Escobedo *et al.*, 2016)**.

These drugs exert their anti-*Giardia* effect through variable mechanisms of actions. For example, 5-nitroimidazole and nitrofuran derivatives interfere with both DNA and RNA synthesis, and benzimidazole derivatives attach to ß-tubulin, therefore prevent cytoskeleton polymerization. While, acridine derivative inhibits *Giardia* oxygen utilization with destruction of its plasma membrane, nitrothiazolide derivative suppresses nitro-reductase enzymes leading to changes in *Giardia* ventral disk and surface membrane and aminoglycoside compounds prevent *Giardia* protein synthesis **(Lalle, 2010)**.

In spite that metronidazole is the preference medication for many years, besides similar parasitological cure rates and symptom relief (>90% of patients) following metronidazole and tinidazole therapy **(Minetti *et al*., 2016)**, tinidazole is recommended as the first grade therapy in multiple states, owing to its high efficacy, less side effects, and prominent consistence **(Escobedo and Cimerman, 2007)**.

Owing to the fact that the other previous anti-*Giardia* drugs have serious symptoms as gastrointestinal discomfort, neuropathy, seizures and leucopenia in addition to potential toxicity, developing more compelling and less poisonous medications against this parasite is vital **(Loo *et al*., 2016)**. Among the anti-malarial drugs, dihydroartemisinin has revealed promising anti-*Giardia* effects, because it leads to *in vitro* alterations in morphology and cell cycle state of *G. lamblia* **(Tian *et al*. 2010)**.

2.2. Human leukocyte antigen

Human leukocyte antigen is a group of profoundly polymorphic qualities encoded by the MHC; a cluster of genes located on the short arm of chromosome 6 that is recognized as the most variable region in the human genome. These antigens have an important primary features for the maintenance of appropriate immune response. The human MHC is called HLA because of its first identification in humans by using alloantibodies against leukocytes (**Choo, 2007**).

2.2.1. Human leukocyte antigen structure and function (Figure 5, 6):

Human leukocyte antigen is divided into three regions; class-I, class-II, and class-III, where HLA class-I and class-II complexes convey the same function; display antigens to CD8+ and CD4+T cells, respectively **(Neefjes*, et al.*, 2011)**.

Human leukocyte antigen class-I molecules present on the surface of every single nucleated cells, however HLA class-II is expressed only by antigen-exhibiting cells such as B-cells, macrophages, monocytes and dendritic cells, which present peptides to $CD4^{+}$ T cells **(Kohaar *et al.*, 2009)**.

The antigens joined to class-I or class-II HLA molecules are naturally and originally diverse. For instance, HLA class-I focuses on T cells perceiving endogenous antigens synthesized within the objective cells (e.g. cellular or transformed). While, class-II specific T-cells recognize exogenous antigens. Moreover, there is an imperative connection; termed cross-presentation exists between these two pathways **(Cruse and Lewis, 2010)**.

It is far outstanding for the existence of a very high degree of polymorphism within class-I and class-II genes that is required for presenting pathogen-derived peptides and for accelerating the adaptive immune resistance **(Tsuji, *et al*., 2017)**.

Human leukocyte antigen class-I is marked by three classical polymorphic genes named MHC-A, MHC-B, and MHC-C, in addition to, the less polymorphic genes, MHC-E, MHC-F and MHC-G **(O'callaghan and Bell, 1998)**. While, MHC class-III region does not encode HLA molecules, but includes genes for complement components (C2, C4, factor B), 21-hydroxylase, tumor necrosis factors (TNFs) and lymphotoxin-α (LTA), where its region adjacent to class-I is called inflammatory region **(Beck and Trowsdale, 2000)**. On the opposite side, HLA class-II molecules are encoded by three different polymorphic genes (MHC-DR, -DQ, and -DP), each gene exists in multiple different allelic forms; containing one or more A and B genes that respond individually to various antigens **(Rammensee *et al*., 1997; Cruse and Lewis, 2010)**. HLA-DR sub-region is the most complex of the entire HLA-D region, and all HLA-DR haplotypes are carrying a single DRA gene, while the number of DRB genes is variable from one to nine **(Corell *et al*., 1991; Choo, 2007)**. The MHC class-II region also includes two catalysts that induce peptide exchange for specific peptides; tapasin (TAP) for MHC class-I and MHC-DM for HLA class-II molecule **(Brocke *et al*., 2002)**.

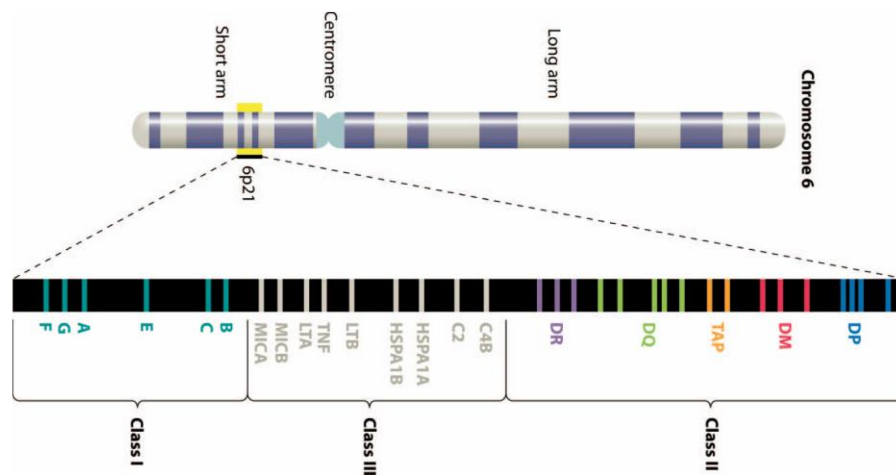

Figure (5): Human leukocyte antigen complex structure. Quoted from Blackwell *et al*. (2009).

C2: complement 2, **HSP:** heat shock protein, **TNFs:** tumor necrosis factors, **LTA:** lymphotoxin-α, **TAP:** tapasin.

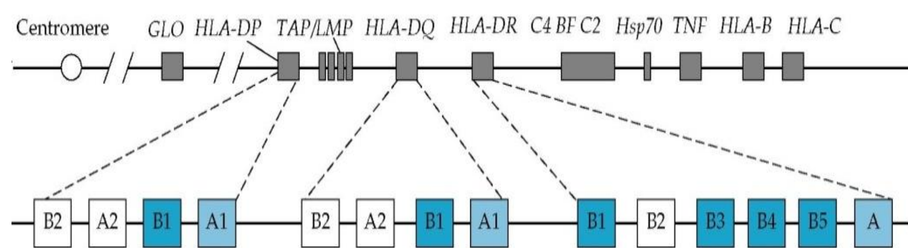

Figure (6): Loci of human leukocyte antigen (HLA-DR) region. Quoted from Milford and Carpenter (2004).

HLA: human leukocyte antigen, **TAP:** tapasin, **C4:** complement 4, **HSP:** heat shock protein, **TNFs:** tumor necrosis factors.

2.2.2. <u>Human leukocyte antigen and infection:</u>

Human leukocyte antigen molecule has been viewed as the most common polymorphic hereditary framework in human and this polymorphism is usually the cause of inter-individual variability in disease susceptibility or development of complication or disease regression (**Beskow *et al*., 2005**).

The HLA loci function as the main possibility for infectious illness susceptibility that emerges through the complex collaboration of environmental and host genetic elements. Hence, infectious diseases and parasites are thought to be the real drivers of HLA diversity **(Chong, 2009)**.

In this manner, a significant part of the current researches focus on the identification of functional variants at these loci and their influence on infection; where a significant positive genetic correlations were detected between the classical HLA loci and major infectious diseases as human immunodeficiency virus (HIV), hepatitis, leprosy, tuberculosis, malaria, leishmaniasis, and schistosomiasis **(Blackwell *et al*., 2009)**.

2.2.2.1. Human leukocyte antigen typing and giardiasis:

De Manueles *et al*. (1992) revealed a relationship between individuals with HLA-A1 and high susceptibility for acquiring *G. lamblia* infection. As well, other relationships were noticed between HLA variants and clinical symptoms of giardiasis, as HLA-A19 was associated with vomiting, HLA-A9 with abdominal pain and HLA-B7 with alterations in the gastrointestinal mucous. While, **El-Ganayni *et al*. (1994)** detected a strong correlation between HLA-A10, A11, B5, B7 as well as B17 phenotypes and the development of symptomatic giardiasis and between HLA-B7, B17, Bw14 as well as Bw40 and asymptomatic giardiasis development.

In addition, **Osipova *et al*. (1993)** revealed that HLA-B5, -B14, HLA-DR3, DR4, DR7 and haplotypes HLA-A9-B5 and HLA-A1-B5 were prevalent in patients with persistent giardiasis.

In experiment conducted by **Obendorf *et al*. (2013)** the expression of HLA-DR molecule decreased, when human dendritic cells incubated *in vitro* with assemblage A *Giardia* trophozoites. While, in other

experiment, using a mixture of *Giardia* assemblage A and E trophozoites; HLA class-II molecule expression increased on the surface of bovine dendritic cells (**Grit *et al.*, 2014b**).

2.3. Fecal calprotectin

Calprotectin is calcium and zinc binding protein found in both plasma and stool that is markedly elevated in infectious and inflammatory conditions (**Konikoff and Denson, 2006**). Calprotectin is abundant in neutrophils, accounts for 5% of total protein and 60% of the protein in the cytosol fraction, however lower concentrations are found in monocytes and reactive macrophages (**Bunn *et al.*, 2001**). This protein constitutes the fundamental product of neutrophil degranulation with an even dispersion all through the fecal matter (**Kopylov *et al.*, 2016**).

Neutrophils are the first line of defense against infectious diseases by phagocytosing and killing the invading microorganisms, and they also play a critical role in inflammation (**Witko-Sarsat *et al.*, 2000**). Accordingly, neutrophil stimulation during inflammation can lead to release of large amounts of toxic oxygen radicals and a variety of granule proteins and soluble proteins such as calprotectin (**Srinivasan, 2013**). Therefore, fecal calprotectin, a result of neutrophils' migration into the gastrointestinal tissue (**Damms and Bischoff, 2008**) can be used as a non-specific marker for neutrophils and mononuclear phagocytes activation as well as gastrointestinal inflammation (**Hestvik *et al.*, 2011**).

2.3.1. Calprotectin level in giardiasis:

Significant associations were noticed between persistent *Giardia* positivity especially for assemblage B infection, microscopic duodenal inflammation and a positive fecal calprotectin test (**Hanevik *et al.*, 2007**). Moreover, **Koot *et al.* (2009)** observed that neutrophilic and eosinophilic

granulocytes infiltrating the epithelial layer accounted for 16% and 9% of patient infected with giardiasis, respectively. While, **Chen *et al*. (2013)** suggested *in vivo* infiltration of neutrophils within mice infected with *G. lamblia* assemblage B occurred as an indirect sequel of microbiotic invasion into the intestinal gut mucosa.

Chapter III

Subjects, Materials and Methods

Subjects, Materials and Methods

3.1. Subjects:

3.1.1. Study location:

The study was conducted at departments of Medical Parasitology and Clinical Pathology (Immunology Unit), Faculty of Medicine, Mansoura University and Mansoura University Children Hospital, Mansoura, Egypt.

3.1.2. Study participant:

Pediatric cases attending Mansoura University Children Hospital were included.

3.1.2.1. Inclusion criteria:

The participants in the study were:

- **a.** Children.
- **b.** Both sexes.
- **c.** Complaining of gastrointestinal symptoms such as diarrhea, abdominal pain, or flatulence.
- **d.** Positive for *Giardia* by direct stool examination.

3.1.2.2. Exclusion criteria:

Children with:

- **a.** Other intestinal parasitic infections.
- **b.** Bacterial or viral infections that affect the gastrointestinal system.

3.1.2.3. Participant size:

Sample size (SS) was statistically calculated according to: $SS=Z^2*(p)*(1-p)/D^2$. Where: Z= 95% confidence level, p= prevalence by previous studies, d = 5% margin of error.

$SS= (95\%)^2*18\%*(1-18\%)/(5\%)^2=53$

Convenient sample size of 350 children was examined to obtain only 100 cases. The recruited children were divided into three groups:

- **Group I (symptomatic giardiasis):** forty children complaining of diarrhea or other gastrointestinal symptoms associated with detection of *G. lamblia* in stool in the absence of other diarrheal pathogens.
- **Group II (asymptomatic giardiasis):** forty children positive for *G. lamblia* infection with absence of diarrheal illness or other diarrheal pathogens.
- **Group III (control group):** twenty children free of gastrointestinal complaints and negative for *G. lamblia* infection and other intestinal pathogens.

3.1.3. A pre-planned sheet format: a detailed history was filled for each child including the following data:

- Personal history: name, age, sex and residence.
- Source of water supply.
- Social state: number of children/family, rooms/house, children/room and children/bed.
- Complaints.
- Ruuska score: numerical scoring system to assess the severity of diarrhea which accounts for duration of diarrhea, maximum number of diarrheal stools/day, vomiting duration, maximum number of vomiting/day, dehydration, temperature, and the level of clinical care required **(Lewis, 2011).**
- Other gastrointestinal symptoms: abdominal pain, constipation and diarrhea.
- Symptoms of vitamins deficiency: broken finger nail, night blindness and headache.

- Symptoms of anemia: pallor and headache.
- Growth assessment: weight and height.
- Previous investigations: hemoglobin concentration, total protein level and albumin level.

3.1.4. Clinical definitions:

- **Diarrhea** is considered when three or more unformed stool passed in a 24 hour period or passage of frequent stool than normal **(WHO, 2013)**.
- **Diarrheal episodes** are defined as diarrheal attacks separated by at least three diarrhea free days, and classified according to the duration of episodes into: acute (< 14 days), persistent (≥ 14 days but < 30 days) and chronic (≥ 30 days) **(Haque *et al*., 2003)**.
- **Intermittent diarrhea** means at least one diarrheal episode per month **(El-Basha *et al*., 2016)**.

3.1.5. Sample collection:

Stool and blood samples were collected from each participant enrolled in the study.

3.1.6. Study design:

3.1.6.1. Stool samples were examined by:

i. **Direct microscopic examination** for detection of *Giardia* parasite and exclusion of other intestinal parasitic infections through:

a. Wet mount examination by saline mount.

b. Concentration technique by formal ethyl acetate technique.

c. Staining by:

-Trichrome stain to exclude *Entamoeba histolytica*.

-Modified Trichrome stain to exclude *Microspora*.

-Modified Ziehl Neelsen stain to exclude *Cryptosporidium, Cystoisospora* and *Cyclospora.*

ii. **Culture of fecal specimens**: was conducted at department of Microbiology on microbiological medium to exclude intestinal bacterial infections through:

a. *Salmonella-Shigella*, MacConkey, as well as MacConkey-tellurite agars for exclusion of *Salmonellae* & *Shigella* species, and *Escherichia coli*, respectively **(Haque *et al*., 2003)**.

iii. **Immunological tests:**

a. *Giardia* antigen capture test was performed to confirm stool results in case of negative *Giardia* stool specimens.

b. ELISA for the detection of *Adenovirus* and *Rotavirus* antigens (Ridascreen, Germany) was reviewed by microbiologist according to manufacturer instructions.

iv. **Fecal calprotectin level** was measured using quantitative enzyme linked immunosorbent assay.

3.1.6.2. Blood samples were subjected to:

i. **Genomic DNA extraction** from 200µl of peripheral blood.

ii. **HLA class II-DRB1** was typed using PCR and sequence-specific oligonucleotide probes.

iii. **For genetic comparisons**: all siblings were excluded from analysis, as siblings share 50% of their alleles.

3.2. Materials:

3.2.1. Reagents:

Table (1): Reagents name, company and catalogue number

Reagent	Company	Catalogue number
For wet mount examination:		
Potassium iodide	El-Gomhoria for medical industries	L-26730
Powdered iodine crystal	Alpha chemicals	I 27561
For formal ethyl acetate concentration procedure:		
Formal ethyl acetate	POCH SA, Poland	405030115
Formalin	El-Gomhoria for medical industries	
Distilled water	El-Gomhoria for medical industries	
For Schaudinn's fixative:		
Mercuric chloride	Alpha chemicals	M 002671
Ethyl alcohol 70%	Natco. Laboratory Chemicals	ET-360-LR
Glacial acetic acid	EL-Nasr Pharmaceutical Chemicals	A0078111
For Trichrome stain (Modified Gomori):		
Chromotrope, 2 R	Alpha Chemical	C0408
Light green, SF	EL-Nasr Pharmaceutical Chemicals	
Fast green, FC	EL-Nasr Pharmaceutical Chemicals	F0138111
Phosphotungstic acid	Alpha Chemicals	P69371

Table (1) continued:

Reagent	Company	Catalogue number
Glacial acetic acid	EL-Nasr Pharmaceutical Chemicals	A0078111
Ethyl alcohol (95%, 90% and 70%)	Natco. Laboratory Chemicals	ET-360-LR
Iodine solution	International for medical industries	5822
Xylene	EL-Nasr Pharmaceutical Chemicals	X0018131
Canada balsam	Loba Chemie	LT0108032
For Modified Trichrome stain (Ryan-Blue):		
Chromotrope 2R	Alpha Chemicals	C0408
Aniline blue	Alpha Chemicals	A15904
Phosphotungstic acid	Alpha Chemicals	P69371
Glacial acetic acid	EL-Nasr Pharmaceutical Chemicals	A0078111
Ethyl alcohol 90%	Natco. Laboratory Chemicals	ET-360-LR
For Modified Ziehl Neelsen stain:		
Carbol fuchsin	Hexa-Biotech	
Methylene blue	Hexa-Biotech	
Hydrochloric acid	Salix	
Ethyl alcohol 70%	Natco. Laboratory Chemicals	ET-360-LR

3.2.2. Kits:

Table (2): Kits name, company and catalogue number

Kit	Company	Catalogue number
For *Giardia* antigen capture test:		
***Giardia*-Strip**	CorisBioconcept, Belgium	C-1013
For the detection of *Adenovirus* and *Rotavirus* antigens:		
RIDASCREEN®Adenovirus	R-Biopharm AG, Germany	C1001
RIDASCREEN®Rotavirus	R-Biopharm AG, Germany	C0901
For fecal calprotectin assessment:		
Calprest	Eurospital, Spain	9031
For blood DNA extraction:		
TIANamp Genomic DNA	TIANGEN, China	DP304-02
For DRB1 amplification:		
INNO-LIPA HLA-DRB1 Amp Plus	Innogenetics, Belgium	25831 V0
For DRB1 detection:		
INNO-LIPA HLA-DRB1 Plus	Fujirebio, Belgium	FRI66815

3.2.2.1. Kit contents:

i. For fecal calprotectin assessment:

Antibody coated plate 12x8 wells

Microtiter plate cover 1 piece

Enzyme conjugate antibody IgG 1x15 ml

Substrate 1x15 ml

Washing solution (20x)	50 ml
Diluent solution (10x)	20 ml
Extraction solution (x2.5)	50 ml
Standards	6x1 ml

At concentrations of 6.25, 12.5, 25, 50, 100 and 200 ng/ml

Control 1	low
Control 2	high

ii. For blood DNA extraction:

Buffer GA	15 ml
Buffer GB	15 ml
Buffer GD	15 ml
Buffer PW	15 ml
Buffer TE	15 ml
Proteinase K	1 ml
Spin Columns CB3	50 units
Collection Tubes 2ml	50 units

iii. For DRB1 amplification:

Amplification Buffer	0.3 ml
DRB1 Primer Solution	0.3 ml
DRB1*03,11,13,14 Primer Solution	0.07 ml
LIPA Taq	0.35 ml

iv. For DRB1 detection (Figure 7):

Conjugate (100x)	0.8 ml
Conjugate Diluent	80 ml
Denaturation Solution	1 ml
Hybridization Solution	80 ml
Rinse Solution (5x)	80 ml
Stringent Wash Solution	200 ml
Strips	20 units

Substrate (100x)	0.8 ml
Substrate Buffer	180 ml
Incubation tray	3 units
Troughs	24 units
Reading card	1 unit

Figure (7): The INNO-LIPA HLA-DRB1 Plus reagents and trough (https://www.fujirebio-europe.com/products-services/productbrowser/inno-lipa-hla-drb1-plus-0?destination=products-services/productbrowser).

3.2.3. Solutions:

Acid-alcohol (Ryan *et al.*, 1993):

Ethyl alcohol 90%: 995.5 ml

Glacial acetic acid: 4.5 ml

Formol-saline (10%):

Formalin: 100 ml

Saline: 1000 ml

Lugol's Iodine (Garcia, 2001):

Potassium iodide: 10 grams

Powdered iodine crystal: 5grams

Distilled water: 100 ml

3.2.4. Utensils:

Alcohol swabs

Automated micropipettes

Clean wide mouthed plastic containers with tight fitted lids

Cover slips: 22x 22 mm

Disposable breakable sterile inoculation loops

Disposable plastic gloves

Disposable polystyrene screw cap tubes

Eppendorf vials

Glass microscopic slides: 75x 25 mm

Potassium EDTA tubes

Surgical gauze

Syringes

Transport container

Wooden applicator sticks

3.2.5. Instruments:

Table (3): Instruments name, company and serial number

Instrument	Company	Serial number
Bench-tope centrifuge	Herolab, Wiesloch, Germany	D-69168
Electric light olympus microscope	Olympus Corporation, Tokyo, Japan	CX21FS1
Eppendorf tube centrifuge	Sigma Aldrich, Inc., Canada, US	155707
Incubator	Leader Engineering, Cheshire, England	99D031
Microwell Chromate reader	Awareness Technology Inc., USA	4300
Sensitive digital scale	Scaltec Instruments, Heiligenstadt, Germany	XX180011
Shaker incubator	Robbins Scientific, Sunnyvale, California, USA	A70624
Thermal cycler AG-9600	AcuGen Systems, Netherland	961107
Vortex	Scientific Industries Inc., Bohemia, USA	G-560E

3.3. Methods:

3.3.1. Methods for feacal samples examination:

3.3.1.1. Fecal samples collection (Garcia, 2007):

Three fecal specimens were collected from each participant over three successive days to overcome intermittent excretion of the parasites. Children or their parents or guardian were instructed to avoid contaminating the samples with tap water or urine. Stool samples were collected in clean, wide mouthed plastic containers with tight fitted lids, labeled with the patient's name, age, sex and date of collection, transferred to the laboratory of Medical Parasitology to be examined within one hour or stored at -20˚C until used.

3.3.1.2. Direct smears (Garcia, 2001):

a. One drop of normal saline and one drop of iodine solution were placed on two different separate slides.

b. Pin head part of the fecal material was then emulsified in saline and iodine.

c. Then, each specimen was covered with a separate cover slip to form two thin transparent films free of air bubbles, and examined under low and high powers of light microscope.

3.3.1.3. Formal ethyl acetate concentration procedure (Truant *et al.*, 1981):

a. Approximately 1gm of each stool specimen was emulsified in 10% formol-saline.

b. The suspension was strained through two layers surgical gauze into a conical 15ml centrifuge tube in order to obtain a pellet equal to 0.5 to 0.75 ml after centrifugation.

c. Then, 10 ml of saline was added to the suspension.

d. The mixture was efficiently mixed and centrifuged at 300 xg for 2 min.

e. The supernatant was discarded, while the sediment was repeatedly suspended in saline and centrifuged until the supernatant become clear.

f. This was followed by addition of distilled water to a volume of 10ml with 3 ml of ethyl acetate.

g. These contents were mixed and shaken vigorously for 30 sec.

h. Finally, four layers were formed after centrifugation at 300 xg for 2 min.

i. The top three layers were discarded, while the sediment was homogenized and suspended in few drops of saline.

3.3.1.4. <u>Schaudinn's fixative (Scholten and Yang, 1974):</u>

a. A total of 45 gm of mercuric chloride was dissolved into 625 ml distilled water in water bath to be completely dissolved, after cooling down crystals were formed.

b. To prepare 1000 ml of stock Schaudinn's solution, 600ml of saturated mercuric chloride was added to 300 ml ethyl alcohol 95%. Then each 95 ml of stock solution was added to 5 ml of glacial acetic acid immediately before use.

c. Fecal smears from the concentrated formol-ethyl-acetate deposits were spread over glass slides.

d. The glass slides were allowed to be air dried.

e. The smears were fixed with schaudinn's fixative for at least one hour and then placed in absolute methanol for 5 min.

3.3.1.5. Modified Gomori Trichrome stain (Wheatly, 1951):

a. The stain was prepared by adding 1 ml glacial acetic acid to the dry stains (0.6 gm Chromotrope 2 R, 0.15 gm Light green SF, 0.15 gm Fast green FC, and 0.7 gm Phosphotungstic acid) in a beaker and allowed to stand for 30 min to ripen.

b. A100 ml distilled water was added to the previous mixture and stored in a brown bottle.

c. Schaudinn's fixed fecal smears were immersed in 70 % ethanol containing five drops of iodine for 5 min.

d. Then the slides were placed twice in 70 % ethanol, firstly for 5 min then for 2 min.

e. The smears were stained with trichrome stain for 10 min.

f. The slides were put in acid alcohol for 3 sec, followed by absolute alcohol for 5 min, and then cleared by xylene solution for 2 min.

g. Finally, they were mounted with Canada balsam while wet and examined microscopically by x40 and x100 objectives.

3.3.1.6. Modified Trichrome stain (Ryan-Blue) (Ryan *et al.*, 1993):

a. Glacial acetic acid (3.0 ml) was added to the dry ingredients (6 gm Chromotrope 2R, 0.5 g Aniline blue, and 0.25 g Phosphotungstic acid). The mixture was maintained at room temperature for 30 min.

b. The solution was diluted by 100 ml of distilled water and the pH was adjusted to 2.5 with 1 ml HCl.

c. Schaudinn's fixed fecal smears were placed in trichrome stain for 90 min.

d. The slides were rinsed in acid-alcohol for 3 sec, and then they were dipped in 95% alcohol for several times for no more than 10 sec.

e. Smears were put twice in 95% alcohol, each for 5 min, then in absolute alcohol for 10 min, and lastly in xylene for 10 min.

f. Then, smears were mounted with coverslip using Canada balsam, and examined microscopically under x40 and x100 magnification.

3.3.1.7. Modified Ziehl Neelsen stain (Casemore *et al.*, 1985):

a. Acid-alcohol was prepared by mixing 3 ml of hydrochloric acid with 97ml of ethyl alcohol 70%.

b. Smears were stained with carbol fuchsin for 15-20 min, and then rinsed thoroughly in tap water.

c. Slides were decolorized in acid alcohol for 15-20 sec, and then rinsed in tap water.

d. Methylene blue was applied to the smears for 30-60 sec as counterstain.

e. The slides were rinsed in tap water and air dried.

f. Then, slides were mounted by Canada balsam, and examined by microscope using x40 and x100 objective lenses.

3.3.1.8. *Giardia* antigen capture test (Figure 8):

a. A total of 15 drops of the dilution buffer solution were added into each test tube.

b. Fecal material was collected with the inoculating loop, then the loop containing the fecal sample was inserted into the tube, agitated to homogenize the solution, and let stand for 1-2 min.

c. The inoculating loop was discarded, while the sensitized strip was immersed in the direction indicated by the arrow.

d. The strip was allowed to stand for 15 min.

e. Results must be read on wet strips after 15 min incubation.

Interpretation of result:

1 line (upper)	**Negative**
0 line	Invalid
2 lines	Positive

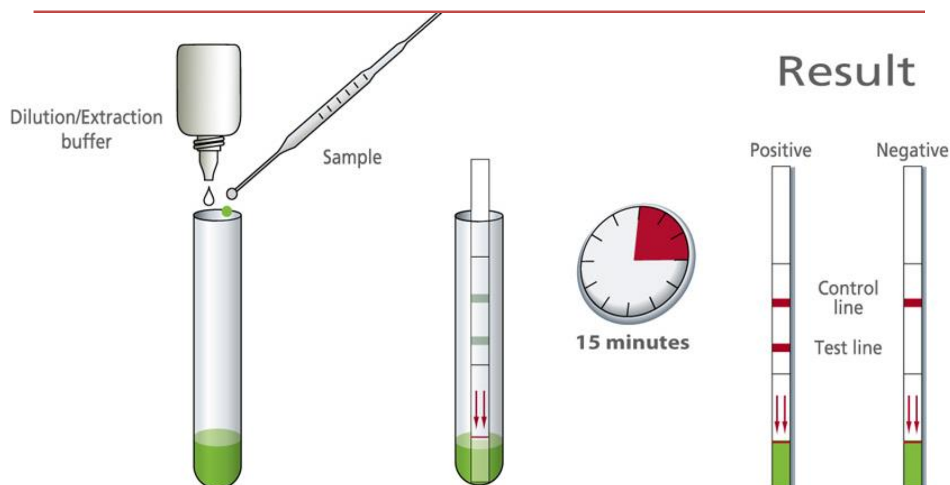

Figure (8): ***Giardia*** **antigen capture test technique (http://www.corisbio.com/Products/Human-Field/Giardia.php).**

3.3.1.9. <u>Fecal calprotectin measurement:</u>

3.3.1.9.1. <u>Fecal sample collection and preparation:</u>

a. The empty screw cap tube and the inoculation loop were weighed.

b. The tube containing the loop and faeces was weighed and calculated to obtain net fecal weight of 40 mg.

c. The diluted extraction buffer (50 ml extraction buffer +75 ml distilled water) was added to the pre-weighed fecal samples e.g. 2.0 ml diluted extraction solution to 40 mg faeces.

d. The tube was covered and shacked vigorously for 30 sec by vortex, then homogenized for 25± 5 minutes on a shaker with the loop inside the tube facilitating gentle stirring.

e. A 1ml of the homogenate was transferred to an Eppendorf and centrifuged at 10,000xg for 20 min. Then, 500 µl of the clear supernatant was transferred to a new Eppendorf to be examined

immediately or stored at -20˚C for maximum of 3 months for later measurement.

3.3.1.9.2. <u>ELISA procedure according to manufacturer procedure:</u>

a. All reagents and frozen samples were kept out of the refrigerator to reach room temperature of 20-25˚C.

b. The whole vial of washing solution was diluted in distilled water to obtain 1000 ml working washing solution. While, the whole vial of diluent solution (20 ml) was diluted in 180 ml of distilled water.

c. The fecal samples were diluted at concentration of 1:50 e.g. 20 µl sample to 980 µl dilution buffer).

d. All reagents and samples were tested in duplicates as shown in the following table:

A	Blank	STD4	CTRL2
B	Blank	STD4	CTRL2
C	STD1	STD5	Sample1
D	STD1	STD5	Sample1
E	STD2	STD6	Sample2
F	STD2	STD6	Sample2
G	STD3	CTRL1	Sample3
H	STD3	CTRL1	Sample3

e. Blank was prepared by adding 100 µl of dilution buffer to wells A1-B1.

f. Then, 100 µl of each standard was added in duplicate to wells; C1-D1, E1-F1, G1-H1, A2-B2, C2-D2, and E2-F2, followed by adding 100 µl of each control in duplicate to wells; G2-H2, and A3-B3.

g. Finally, diluted samples were mixed and 100 µl of each diluted sample was added in duplicate to wells.

h. The plate was covered and incubated at room temperature for 45min.

i. After incubation, the plate was washed 3 times by adding 300 µl of diluted washing solution to each well. At the end of washing steps, the plate was inverted and taped gently on absorbent tissue to completely remove any residuals of washing solution.

j. After that, 100 µl of conjugate was added to each well. Then, the plate was covered and incubated at room temperature for 45 min, and washed again as in step i.

k. Finally, 100 µl of substrate solution was added to each well. Then, the plate was incubated at room temperature for 30 min in dark place, and the optical density (OD) values were detected by ELISA reader at 405 nm.

Calculation of test results:

a. Mean OD was measured for all duplicates.

b. The mean blank OD was subtracted from all values.

c. Standard curve was plotted with actual calprotectin concentration of standards as ng/ml and corresponding OD values on an XY system **(Figure 9)**.

d. The samples concentration were detected from the standard curve and corrected for the dilution and converted to mg/kg by multiplying 2.5.

Interpretation of result:

< 50 mg/kg	**Negative**
50 - 100 mg/kg	Border line
> 100 mg/kg	Positive

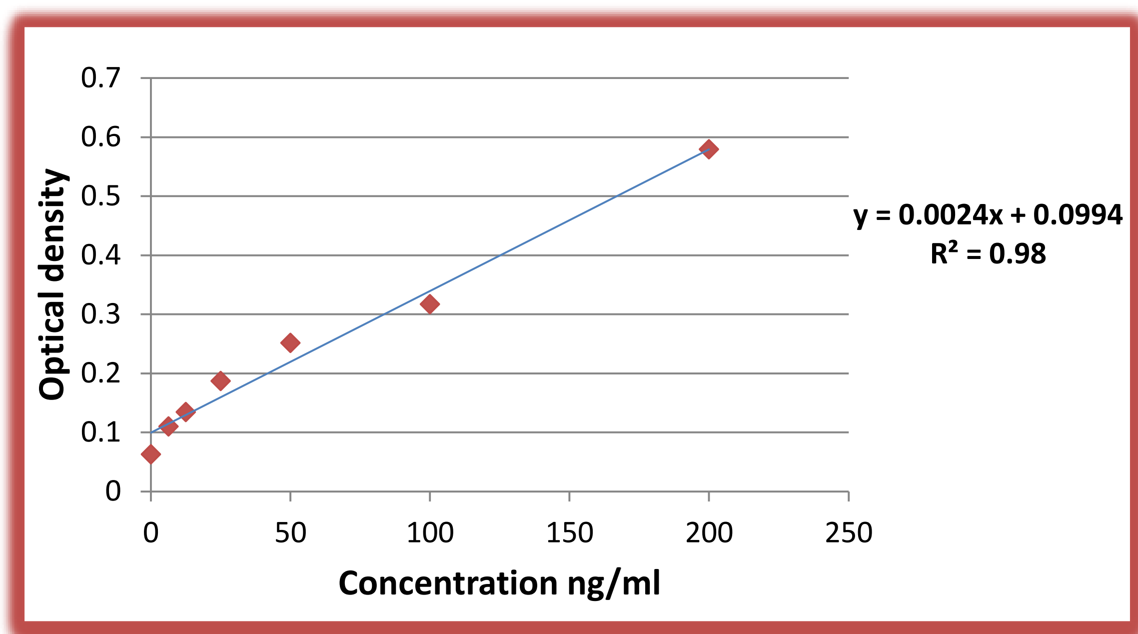

Figure (9): Standard curve on an XY system.

3.3.2. Methods for blood samples examination:

3.3.2.1. Blood samples collection:

Two ml venous blood was withdrawn from each child under aseptic condition, added to 50 µl potassium EDTA tubes and kept at -20° C until used later for DNA extraction and HLA typing.

3.3.2.2. Methods for DNA extraction:

- **a.** First, the buffer GD was diluted in 17 ml ethanol (96-100%), while the buffer PW was diluted by 60 ml of ethanol (96-100%).
- **b.** A 20 µl of proteinase K was added to 200 µl of anticoagulant blood samples and mixed thoroughly by vortex, followed by adding 200 µl of buffer GB to the samples, and mixing by vortex,
- **c.** A homogeneous solution was obtained by incubating the samples at 70°C for 10 min. Then, the samples were centrifuged to remove any drops from the inside of the lid.
- **d.** A 200 µl of ethanol (96-100%) was added to the samples and mixed by vortex for 15 sec.

e. The whole mixture was pipetted into the spin column CB3 in a 2 ml collection tube and centrifuged at 13,400 xg for 30 sec.

f. The flow-through in the collection tubes was discarded. The spin columns were placed again into the collection tubes.

g. A 500 µl of buffer GD was added to each spin column CB3 and centrifuged at 13,400 xg for 30 sec.

h. The flow-through in the collection tubes was discarded and the spin columns were placed into the collection tubes, then a 600 µl of buffer PW was added to each spin column CB3 and centrifuged at 13,400 xg for 30 sec. This step was repeated twice.

i. The spin columns were centrifuged at 13,400 xg for 2 min to ensure efficient dryness of the membrane.

j. The spin columns were placed into new clean micro-centrifuge tubes and about 200 µl of buffer TE was pipetted directly to the center of each membrane.

k. The spin columns were incubated at room temperature for 5 min and then centrifuged for 2 min at 13,400 xg.

l. DNA eluted in buffer TE was stored at -20°C.

3.3.2.3. <u>For HLA-DRB1 amplification:</u>

a. The vials were prepared and numbered as N, which indicated the numbers of DNA samples.

b. Master mix was prepared in an autoclaved 1.5 ml tube as follow:

(N x 12 µl) autoclaved distilled water

\+ (N x 5 µl) Amplification Buffer

\+ (N x 5 µl) Primer Solution DRB1

\+ (N x 0.5 µl) LIPA Taq

c. The whole mixture (N x 22.5 µl) was mixed thoroughly by vortex.

d. Approximately 22.5 µl of this master mix was added into each autoclaved amplification tubes.

e. Then, 5 µl of DNA samples was added into appropriate autoclaved amplification tubes.

f. These tubes were placed into the pre-heated and calibrated thermal cycler.

g. The DNA thermal apparatus was adjusted on the amplification program of the HLA-DRB1 amplification.

h. At the end of amplification process, the DNA products were used immediately with the INNO-LIPA HLA-DRB1 Plus test strip.

3.3.2.4. For HLA-DRB1 detection:

Firstly, conjugate working solution (20 µl was diluted in 2 ml of conjugate diluent for each trough), rinse working solution (10 ml was diluted in 40 ml of distilled water), and substrate working solution (20 µl was diluted in 2 ml of substrate buffer for each trough) were prepared.

Denaturation and hybridization

a. The hybridization solution and stringent wash solution were warmed in pre-heated shaking water bath at 56°C to dissolve all crystals.

b. The required troughs (one trough for each test sample) were placed into the tray.

c. Then, 10 µl of denaturation solution was pipetted into the upper corner of each trough with 10 µl of the appropriate sample and gently mixed by pipetting.

d. This denaturation process was incubated for 5 min at room temperature.

e. A 2 ml of pre-warmed hybridization solution was added and mixed with the denatured amplified sample into each trough.

f. The INNO-LIPA HLA-DRB1 Plus strip was taken by forceps; one strip per test sample, and then submerged completely into the mixture with the marked side of the membrane up in the trough.

g. The tray was incubated into the pre-heated shaking water bath at 56°C for 30 min.

Stringent wash:

a. At the end of hybridization process, the tray was removed from the shaking water bath and hold at a low angle to be able to suck the solution from the trough by a pipette.

b. A 2 ml of pre-warmed stringent wash solution was added into each trough and rinsed by gently rocking the tray for 20 sec.

c. The solution was aspirated from each trough.

d. This washing step was repeated twice.

Color development:

a. A total of 2 ml of the rinse working solution was added to wash each trough twice for about 1 min.

b. A 2 ml of the conjugate working solution was added to each strip, and then incubated on the shaker for 30 min**.**

c. About 2 ml of the rinse working solution was added to wash each trough twice for about 1 min. This was followed by washing once with 2 ml of substrate buffer.

d. A 2 ml of the diluted substrate working solution was placed into each trough and the tray was incubated on the shaker for 30 min**.**

e. Then, 2 ml of distilled water was used to wash the strips twice to stop the color development.

f. Finally, the strips were removed from the troughs by forceps, placed on absorbent paper to dry, and the dried strips were stored in the dark.

Reading:

When clear purple or brown bands were appeared at the end of the test procedure, these lines were considered positive as shown in **Figure (10)**.

Figure (10): The INNO-LIPA HLA-DRB1 Plus strip showing the marker line **(black),** conjugate control line **(conj. control),** HLA-DRB1 Plus control line **(HLA-DRB. control),** and the 37 sequence-specific DNA probes.

3.4. Statistical analysis:

Basic demographic information, as well as clinical and laboratory findings were collected, and then statistical analysis was done using statistical package for social science (SPSS) software (Version 20, USA). The qualitative data were presented in the form of number and percentage. Chi-square test was used for qualitative data. Yates correction was applied when indicated. Odds ratio and 95% confidence interval was calculated to estimate the risk. The quantitative data were presented in the form of mean and standard deviation (SD). Student t test was used as a test of significance. Significance was considered when P value less than 0.05.

3.5. Ethical clearance:

The research proposal was approved by the Review Board of the Department of Medical Parasitology and Mansoura University Children Hospital Authority. The study was conducted according to the ethical

guidelines of Mansoura Medical Research Ethics committee (Ph.D/34). Informed consent was obtained from parents or guardian of the recruited children after explaining the aim of the study. A preplanned sheet format was filled for each case and all data of the enrolled children were kept strictly confidential. Patients were referred to the physician for the treatment of diarrhea and *Giardia* infection.

All discarded blood samples were dealt with as a biohazard waste and all stored frozen blood samples were labeled as hazardous. All specimens and kits reagents were considered as potentially infectious.

Chapter IV

Results

Results

This case-control study was conducted on children attended out-patient clinics of Mansoura University Children Hospital, Mansoura, Egypt. Out of the 350 stool samples examined, 80 samples containing *G. lamblia* parasite only were included as cases and 20 parasitic free samples were used as controls. The study populations were divided on the basis of stool examination and clinical manifestations into three groups **(Figure 11 and 12**); **group (1):** children with gastrointestinal symptoms associated with presence of *G. lamblia* in stool samples (symptomatic giardiasis), **group (2):** children without any gastrointestinal symptoms but with *G. lamblia* detected in stool (asymptomatic giardiasis) and **group (3):** parasitic free children without any gastrointestinal symptoms (control group). Data were recorded and results were statistically analyzed and demonstrated in tables and figures.

A total of 350 children

Stool examination

Giardia positive

Multiple parasitic infections

No parasitic infection

Clinical manifestations

Excluded

Giardia antigen capture test

Present

Absent

Positive

Negative

Symptomatic giardiasis 40 cases

Asymptomatic giardiasis 40 cases

Excluded

Control group 20 cases

Total= 100 cases

Figure (11): Flow chart of the studied groups.

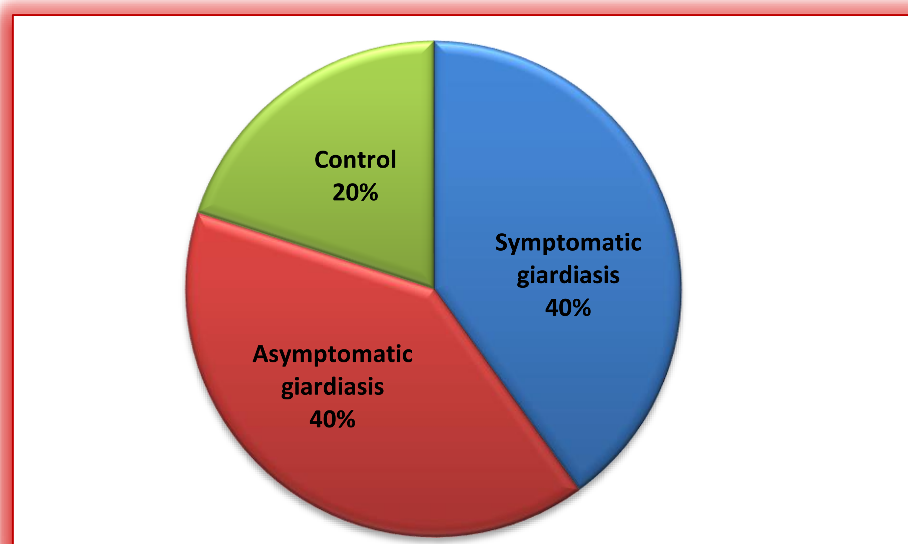

Figure (12): Distribution of the clinical groups.

Children participated in this study were mostly males among giardiasis cases; 22 cases (55%) and 24 cases (60%) in symptomatic and asymptomatic groups, respectively, while females were prominent (8 cases; 60%) in control group **(Figure 13)**. Their average age was 7.8±3.9 and 8.7±3.43 years for symptomatic and asymptomatic giardiasis, respectively, and 10.15±1.4 years for control group **(Figure 14)**.

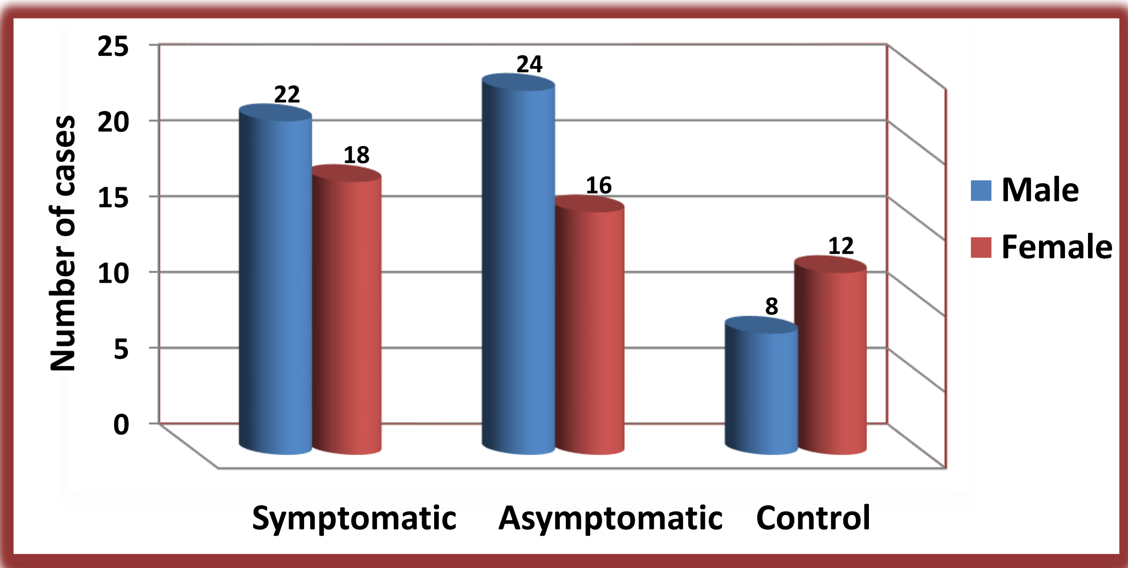

Figure (13): Gender of the studied groups.

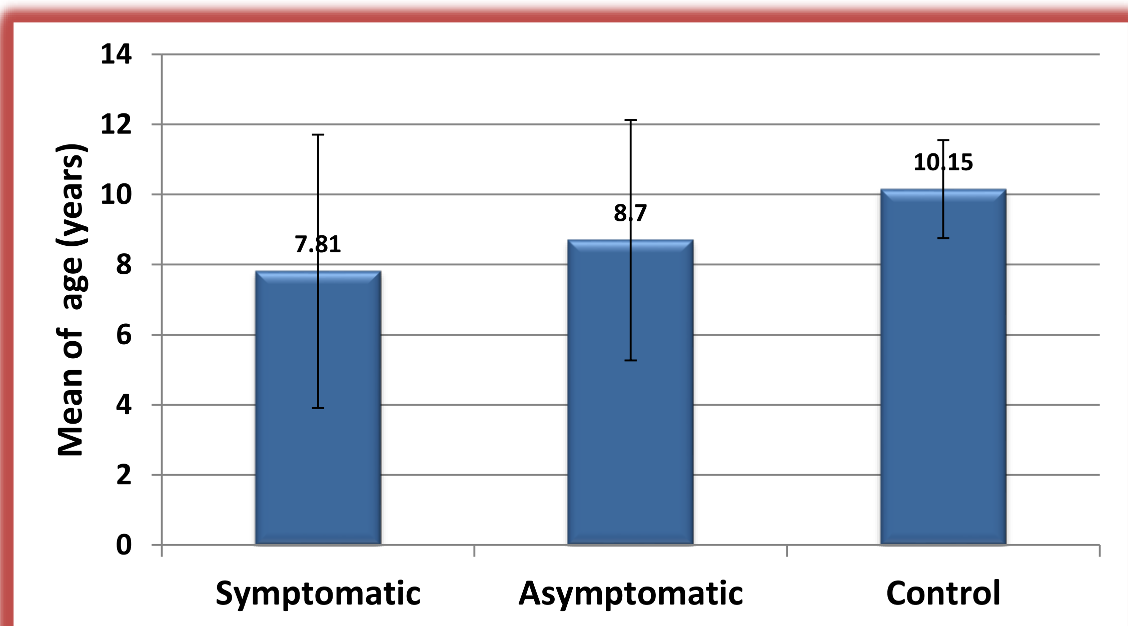

Figure (14): Age of the studied groups.

4.1. Demographic data of the study participants:

Baseline characteristics of the 100 children enrolled in this study are displayed in **Table (4)**, in order to understand the impact of these variables on *Giardia* infection in these study populations.

Analysis of the demographic data revealed that the majority of the recruited children were from villages; as 75% of symptomatic cases as well as 50% of asymptomatic cases and controls were rural residents, while the remaining were residing the cities; illustrating a significant correlation (***P*= 0.043**) between the residence in rural areas and the incidence of giardiasis.

Interestingly, two children per room were more common among *G. lamblia*-infected children and accounted for 60% and 62.5% of giardiasis cases in symptomatic and asymptomatic groups, respectively, although the difference was insignificant.

Moreover, no statistically significant correlation was detected between the variables of numbers of rooms per house, children per family or children per bed and *Giardia* infection.

Table (4): Demographic characteristics of the studied groups

Residence	Symptomatic		Asymptomatic		Control		Chi-Square test
	No.	(%)	No.	(%)	No.	(%)	
Urban	10	(25)	20	(50)	10	(50)	X^2=6.25
Rural	30	(75)	20	(50)	10	(50)	***P*=0.043**
No. of rooms/house							
1	2	(5)	2	(5)	1	(5)	
2	21	(52.5)	27	(67.5)	15	(75)	X^2=5.66
3	15	(37.5)	11	(27.5)	3	(15)	***P***=0.46
4	2	(5)	0	(0)	1	(5)	
No. of children/family							
1	2	(5)	2	(5)	2	(10)	
2	18	(45)	13	(32.5)	7	(35)	
3	11	(27.5)	15	(37.5)	10	(50)	X^2=7.58
4	8	(20)	8	(20)	1	(5)	***P***=0.67
5	1	(2.5)	1	(2.5)	0	(0)	
6	0	(0)	1	(2.5)	0	(0)	
No. of children/room							
1	7	(17.5)	2	(5)	6	(30)	
2	24	(60)	25	(62.5)	8	(40)	X^2=12.4
3	3	(7.5)	7	(17.5)	5	(25)	***P***=0.338
4	6	(15)	6	(15)	1	(5)	
No. of children/bed							
1	18	(45)	20	(50)	10	(50)	
2	21	(52.5)	19	(47.5)	9	(45)	X^2=0.64
3	1	(2.5)	1	(2.5)	1	(5)	***P***=0.94

No: number, values between parentheses refer to percentages.

4.2. Potential risk factors associated with *G. lamblia* infection among the study participants:

The incidence of *G. lamblia* infection was correlated with some risk factors in order to demonstrate their relevance for disease transmission **(Table 5)**. The results documented that the negligence of washing vegetables and fruits before eating and keeping domestic animals indoor were risky and significantly associated with *G. lamblia* transmission (*P* value=**0.014** and **0.012**, respectively).

Concerning other risk factors, 20% of giardiasis cases had other family member infected with the same parasite. Moreover, 66.25% and 2.5% of cases did not wash their hands before eating and after defecation, respectively. However, these factors were not significantly associated with *G. lamblia* infection.

It also appeared that the majority of the participants use tap water; 77.5% in both symptomatic and asymptomatic patients, as well as 80% in controls. Apart from them only 15 giardiasis children (18.75%) and two children (10%) from the control group were consuming boiled tap water.

Table (5): Analysis of potential risk factors associated with *G. lamblia* infection among the studied children

Variables	Symptomatic giardiasis No. (%)	Asymptomatic giardiasis No. (%)	Total giardiasis cases No. (%)	Control No (%)	Chi-Square test
Other family members infected with giardiasis					
Yes	9 (22.5)	7 (17.5)	16 (20)	4 (20)	***P***=0.23
No	31 (77.5)	33 (82.5)	64 (80)	16(80)	
Boiling water before consumption					
Yes	8 (20.0)	7 (17.5)	15 (18.75)	2 (10)	***P***=0.61
No	32 (80.0)	33 (82.5)	65 (81.25)	18(90)	
Washing hands before eating					
Yes	16 (40)	11 (27.5)	27 (33.75)	7 (35)	***P***=0.49
No	24 (60)	29 (72.5)	53 (66.25)	13(65)	
Washing hands after defecation					
Yes	39 (97.5)	39 (97.5)	78 (97.5)	17(85)	***P***=0.076
No	1 (2.5)	1 (2.5)	2 (2.5)	3 (15)	
Washing vegetables/fruits before consumption					
Yes	16 (40)	20 (50)	36 (45)	16(80)	***P*=0.014**
No	24 (60)	20 (50)	44 (55)	4 (20)	
Contact with domestic animals					
Yes	26 (65)	29 (72.5)	55 (68.75)	5(25)	***P*=0.012**
No	14 (35)	11 (27.5)	25 (31.25)	15(75)	
Source of drinking water					
Filter water	9 (22.5)	9 (22.5)	18 (22.5)	4(20)	***P***=0.97
Tap water	31 (77.5)	31 (77.5)	62 (77.5)	16(80)	

No: number, values between parentheses refer to percentages, data are presented as ***P* value**.

4.3. Clinical characters of the symptomatic cases:

Clinical symptoms and signs of the symptomatic giardiasis children revealed that the main gastrointestinal complaints were abdominal pain, diarrhea, flatulence, and vomiting in 95%, 47.5%, 40%, and 25%, respectively. In addition, there was a statistically significant correlation between the frequency of abdominal pain and diarrhea, flatulence as well as vomiting and giardiasis with *P* value of < **0.001**, =**0.001** and **0.011,** respectively **(Table 6)**.

Regarding the frequency of diarrhea, 17 cases (42.5%) had acute diarrheal attacks that last for less than 2 weeks and only two cases (5%) had diarrheal attacks continuing for less than 20 days (persistent diarrhea). Moreover, out of the 40 symptomatic cases; 23 cases (57.5%) suffered from intermittent diarrhea all over the year. Concerning the Ruuska score of disease severity, children with acute diarrheal attack were complaining of moderate diarrheal episodes (11 cases; 27.5%), while six cases (15%) were suffering from severe diarrheal attacks. It is worth noted that, among the 19 children experiencing diarrhea only three cases (7.5%) suffered from mild to moderate dehydration **(Table 6)**.

The average durations of diarrhea, abdominal pain and vomiting attacks were 5.47±2.789, 5.5±2.8, and 2.4±1.174 days, respectively. Moreover, the diarrheal attacks were repeated up to an average of 12.22±6.45 episodes every year **(Table 7)**.

Correlating the average number of diarrheal episodes with the age and sex of *Giardia*-infected children as shown in **Table (8)** demonstrated that 27 children (67.5%) out of symptomatic giardiasis cases had 11.44±6.07 episodes occurring at the age of 5-18 years, while children at the age of 2-5 years had 13.85±7.36 episodes annually. Furthermore,

female children were suffering from more annual diarrheal episodes (14.14±6.38) compared with males (10.64±6.37).

Table (6): Clinical features among symptomatic giardiasis group

Clinical manifestations	Symptomatic giardiasis No. (%)	Fisher exact
Abdominal pain	38 (95)	***F***=35.1 ***P<0*.001**
Diarrhea	19 (47.5)	***F***=35.1 ***P*<0.001**
Acute	17 (42.5)	
Ruuska score (mild)	0 (0)	
Ruuska score (moderate)	11 (27.5)	
Ruuska score (severe)	6 (15)	
Persistent	2 (5)	
Chronic	0 (0)	
Intermittent	23 (57.5)	
Flatulence	16 (40)	***F***=20.0 ***P*=0.001**
Vomiting	10 (25)	***F***=16.6 ***P*=0.011**
Mild to moderate dehydration	3 (7.5)	***F***=4.6 ***P***=0.098
Low grade fever	3 (7.5)	***F***=4.6 ***P***=0.098
Headache	21 (52.9)	***F***= 0.20 ***P***= 0.90
Pallor	11 (27.5)	***F***=0.43 ***P***=0.80

No: number, values between parentheses refer to percentages.

Table (7): Mean duration of some gastrointestinal symptoms among *Giardia*-infected children

Clinical symptoms	Mean±SD
Diarrhea duration (days)	5.47±2.789
Number of diarrheal episodes /year	12.22±6.45
Abdominal pain duration (days)	5.5±2.8
Vomiting duration (days)	2.40±1.174

SD: standard deviation.

Table (8): Correlation between age and gender of symptomatic giardiasis children with frequency of diarrheal episodes

Age group	No. of children		No. of diarrheal episodes/year	Student's t test
	No.	(%)	Mean±SD	
2-5 years	13	(32.5)	13.85±7.36	
5-18 years	27	(67.5)	11.44±6.07	*t*=1.18
All	40	(100)	12.22±6.45	*P*=0.38
Gender				
Male	22	(55)	10.64±6.37	
Female	18	(45)	14.14±6.38	*t*=1.72
All	40	(100)	12.22±6.45	*P*=0.091

No: number, values between parentheses refer to percentages, **SD**: standard deviation.

Comparing the average number and duration of diarrheal episodes among symptomatic children inhabiting urban and rural areas as shown in **Figure 15** and **16** revealed that these episodes were more common with longer duration in children residing in rural areas than those from urban localities. Obviously, 9-12 attacks annually lasting for a week were the most popular episodes recorded in rural inhabitants.

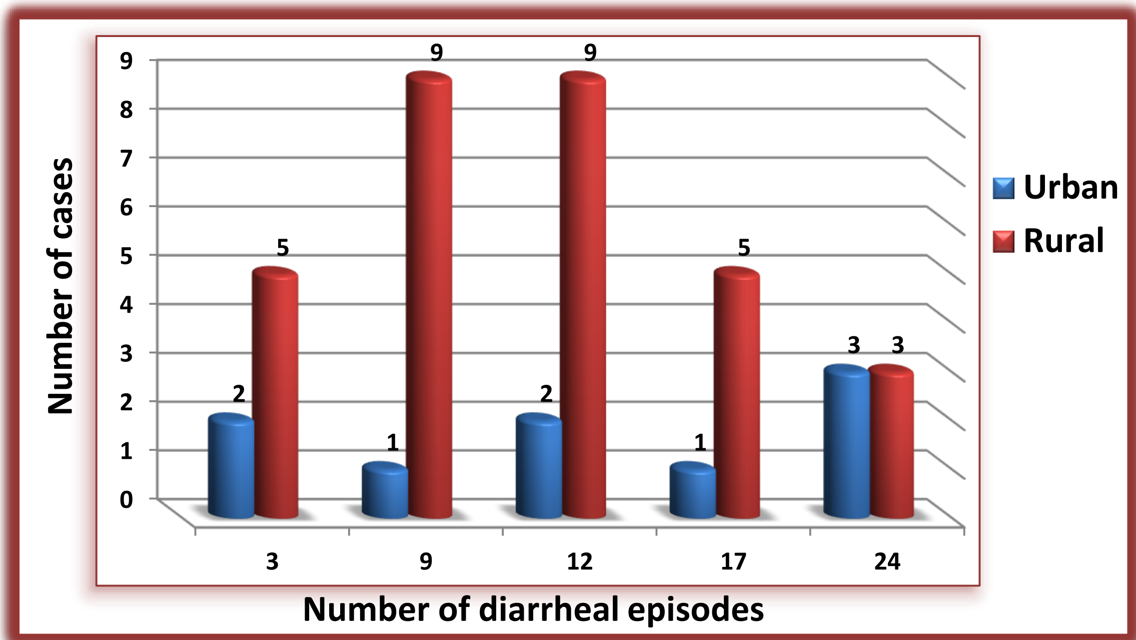

Figure (15): Diarrheal episodes distribution for symptomatic giardiasis children living in rural and urban areas.

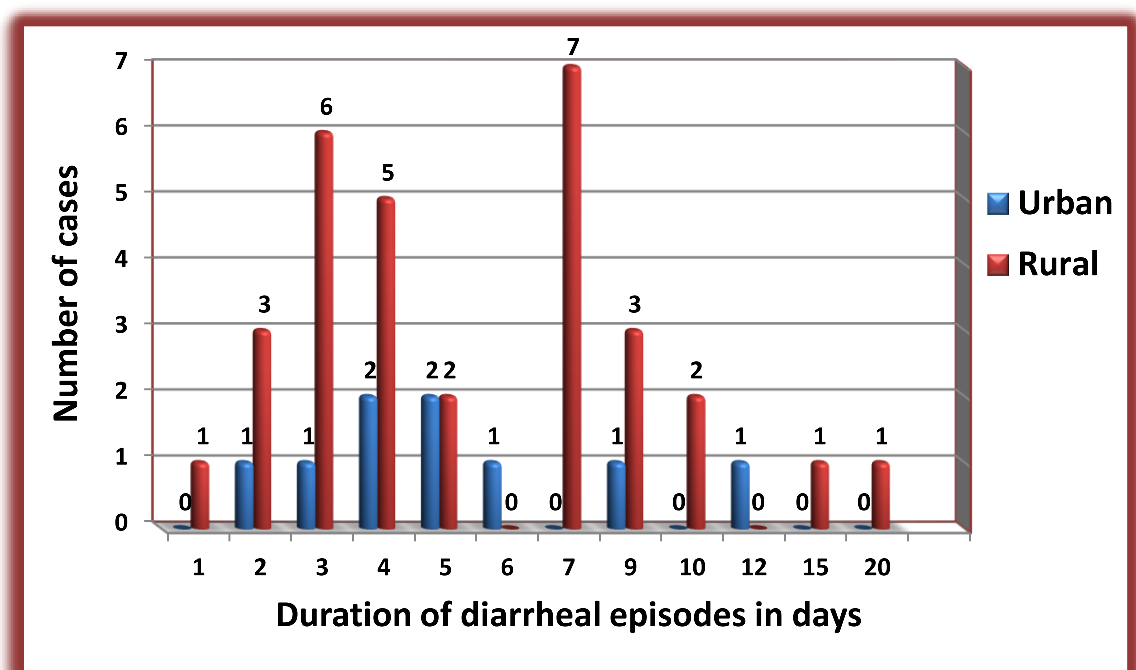

Figure (16): Duration of diarrheal episodes for symptomatic giardiasis children living in urban and rural areas.

In addition, growth assessment of the enrolled children was within the normal range of weight (weight for age, Z score) for the corresponding age and without any significant difference between groups. While, children infected with *G. lamblia* were shorter than *Giardia* free children revealing statistically significant correlation (***P*=0.031**) with *G. lamblia* infection **(Table 9)**. Interestingly, 13 symptomatic (32.5%) and 8 asymptomatic children presented with stunted growth (height for age, Z score), with no statistical significant difference was detected between both groups regarding the growth parameters **(Table 10)**.

Table (9): Growth assessment of the studied groups

Variables	Symptomatic Mean±SD	Asymptomatic Mean±SD	Control Mean±SD	One way ANOVA-F test
Age (years)	7.81±3.9	8.7±3.43	10.15±1.4	***F***=2.69 ***P***=0.73
Weight(Kg)	26.3±11.45	28.85±12.58	33.3±17.5	***F***= 1.85 ***P***= 0.162
Height(cm)	118.5±20.81	125.1±19.67	133±22.0	***F***= 3.67 ***P*= 0.031**

SD: standard deviation.

Table (10): Analysis of the growth measures for giardiasis children

Weight for age, Z score	**Symptomatic**		**Total No. (%)**	**Asymptomatic**		**Total No. (%)**	**Chi-square test**
	Male No.	**Female No.**		**Male No.**	**Female No.**		
Normal	18	17	35(87.5)	21	16	37(92.5)	X^2=0.59 P=0.74
Underweight	3	0	3 (7.5)	2	0	2 (5)	
Over-weight	1	1	2 (5)	1	0	1 (2.5)	
	X^2=2.66 P=0.26			X^2=2.16 P=0.33			
Height for age, Z score							
Stunted	10	3	13 (32.5)	6	2	8 (20)	X^2=1.61 P=0.201
Not stunted	12	15	27 (67.5)	18	14	32 (80)	
	X^2=3.74 P=0.53			X^2=0.94 P=0.33			
Total No.	22	18	40 (100)	24	16	40 (100)	

No: number, values between parentheses refer to percentages.

4.4. Human leukocyte antigen typing of the study participants:

The HLA-DRB1 alleles of the enrolled children were analyzed and reported **(Table 11)**, then the odds ratio (OR) with 95% confidence interval (CI) and *P* value were calculated for each detected allele to identify allele/s with relative risk or protective efficacy to *G. lamblia* infection **(Table 12)**. HLA-DRB1 loci had a large number of allelic variants, with frequencies ranging from 1.8% to 34.37%. In order to maximize the statistical analysis, alleles with frequencies ≤10% were excluded form tests of association analysis with *G. lamblia* infection.

HLA-DRB1*03 allele was the most common allele shared in both symptomatic and asymptomatic giardiasis representing 32.5% (26 alleles) and 36.25% (29 alleles), respectively. Children with *G. lamblia* infection (cases) were nearly 14 times as likely as the controls (2.5%), leading to a significant difference between cases and control children with odds ratio (OR, 40.7; 95% CI, 5.13-87.9; ***P*< 0.001**). The second most common allele detected in giardiasis cases was HLA-DRB1*13 allele accounting for 16.25% in both symptomatic and asymptomatic cases, but without any significant correlation with *G. lamblia* infection (OR, 4.33; 95% CI, 0.86 -4.32; ***P***= 0.084).

On the contrary, HLA-DRB1*14 allele showed higher rates within the control group than among cases with giardiasis, estimating for 15% in controls and 3.75% in cases, giving a significant protective association with *G. lamblia* infection (OR, 0.19; 95% CI, 0.09 -0.79; ***P*= 0.017**). In addition, HLA-DRB1*04 allele rate was elevated within the control group with a percentage of 20% compared to giardiasis cases (10%), but without any statistically different significance (OR, 0.38; 95% CI, 0.12 -

1.21; ***P***= 0.06). Interestingly, the other HAL-DRB1alleles did not reveal any significant relation with *G. lamblia* infection.

Table (11): Frequency of HLA-DRB1 alleles in the studied groups

HLA-DRB1 alleles	Symptomatic		Asymptomatic		Control	
	No.	(%)	No.	(%)	No.	(%)
01	**3.0**	**(3.75)**	**3.0**	**(3.75)**	**3.0**	**(7.5)**
01:01	3.0	(3.75)	0.0	(0.0)	1.0	(2.5)
01:02	0.0	(0.0)	3.0	(3.75)	2.0	(5.0)
03	**26.0**	**(32.5)**	**29.0**	**(36.25)**	**1.0**	**(2.5)**
03:01	18.0	(22.5)	26.0	(32.5)	1.0	(2.5)
03:02	8.0	(10.0)	0.0	(0.0)	0.0	(0.0)
03:05	0.0	(0.0)	3.0	(3.75)	0.0	(0.0)
04	**8.0**	**(10.0)**	**8.0**	**(10.0)**	**8.0**	**(20.0)**
04:01	3.0	(3.75)	0.0	(0.0)	0.0	(0.0)
04:02	3.0	(3.75)	0.0	(0.0)	4.0	(10.0)
04:03	0.0	(0.0)	5.0	(6.25)	3.0	(7.5)
04:05	0.0	(0.0)	0.0	(0.0)	1.0	(2.5)
04:15	2.0	(2.5)	0.0	(0.0)	0.0	(0.0)
04:22	0.0	(0.0)	3.0	(3.75)	0.0	(0.0)
07	**5.0**	**(6.25)**	**8.0**	**(10.0)**	**5.0**	**(12.5)**
07:01	5.0	(6.25)	8.0	(10.0)	5.0	(12.5)
08	**8.0**	**(10.0)**	**0.0**	**(0.0)**	**2.0**	**(5.0)**
08:02	8.0	(10.0)	0.0	(0.0)	1.0	(2.5)
08:09	0.0	(0.0)	0.0	(0.0)	1.0	(2.5)
09	**3.0**	**(3.75)**	**3.0**	**(3.75)**	**1.0**	**(2.5)**
09:01	3.0	(3.75)	3.0	(3.75)	1.0	(2.5)
10	**0.0**	**(0.0)**	**0.0**	**(0.0)**	**4.0**	**(10.0)**
10:01	0.0	(0.0)	0.0	(0.0)	4.0	(10.0)
11	**11.0**	**(13.75)**	**5.0**	**(6.25)**	**3.0**	**(7.5)**
11:01	5.0	(6.25)	0.0	(0.0)	1.0	(2.5)
11:02	3.0	(3.75)	0.0	(0.0)	0.0	(0.0)
11:4	0.0	(0.0)	0.0	(0.0)	1.0	(2.5)
11:6	0.0	(0.0)	0.0	(0.0	1.0	(2.5)
11:07	0.0	(0.0)	2.0	(2.5)	0.0	(0.0)
11:09	3.0	(3.75)	0.0	(0.0)	0.0	(0.0)
11:40	0.0	(0.0)	3.0	(3.75)	0.0	(0.0)
12	**0.0**	**(0.0)**	**3.0**	**(3.75)**	**0.0**	**(0.0)**
12:01	0.0	(0.0)	3.0	(3.75)	0.0	(0.0)
13	**13.0**	**(16.25)**	**13.0**	**(16.25)**	**2.0**	**(5.0)**
13:01	10.0	(12.5)	5.0	(6.25)	0.0	(0.0)
13:03	3.0	(3.75)	3.0	(3.75)	0.0	(0.0)
13:07	0.0	(0.0)	0.0	(0.0)	1.0	(2.5)
13:08	0.0	(0.0)	0.0	(0.0)	1.0	(2.5)
13:50	0.0	(0.0)	5.0	(6.25)	0.0	(0.0)
14	**3.0**	**(3.75)**	**3.0**	**(3.75)**	**6.0**	**(15.0)**
14:01	3.0	(3.75)	3.0	(3.75)	2.0	(5.0)
14:08	0.0	(0.0)	0.0	(0.0)	2.0	(5.0)

Table (11) continued:

14:13	0.0	(0.0)	0.0	(0.0)	1.0	(2.5)
14:35	0.0	(0.0)	0.0	(0.0)	1.0	(2.5)
15	**0.0**	**(0.0)**	**5.0**	**(6.25)**	**4.0**	**(10.0)**
15:01	0.0	(0.0)	3.0	(3.75)	4.0	(10.0)
15:05	0.0	(0.0)	2.0	(2.5)	0.0	(0.0)
16	**0.0**	**(0.0)**	**0.0**	**(0.0)**	**1.0**	**(2.5)**
16:01	0.0	(0.0)	0.0	(0.0)	1.0	(2.5)
Total	**80**	**(100)**	**80**	**(100)**	**40**	**(100)**

No: number, values between parentheses refer to percentages, data presented are numbers and percentages of HAL-DRB1alleles.

Table (12): Association between HAL-DRB1 alleles with giardiasis cases and controls

HLA-DRB1 alleles	Cases		Control		Odds Ratio (95% CI)	*P* value
	No.	(%)	No.	(%)		
01	6	(3.75)	3	(7.5)	0.46 (0.09-2.61)	0.29
03	55	(34.37)	1	(2.5)	40.7 (5.13-87.9)	**<0.001**
04	16	(10.0)	8	(20)	0.38 (0.12-1.21)	0.06
07	13	(8.12)	5	(12.5)	0.58 (0.16-2.21)	0.36
08	8	(5.0)	2	(5)	1 (0.17- 7.49)	0.99
09	6	(3.75)	1	(2.5)	1.54 (0.16-3.6)	0.69
10	0	(0.0)	4	(10)	0 (0.0-0.0)	0.45
11	16	(10.0)	3	(7.5)	1.42 (0.33-6.93)	0.61
12	3	(1.8)	0	(0)	0 (0.0-0.0)	0.37
13	26	(16.25)	2	(5)	4.33 (0.86-4.32)	0.084
14	6	(3.75)	6	(15)	0.19 (0.09-0.79)	**0.017**
15	5	(3.12)	4	(10)	0.27 (0.05-1.35)	0.075
16	0	(0.0)	1	(2.5)	0 (0.0-0.0)	0.69
Total	160	(100)	40	(100)		

No: number, values between parentheses refer to percentages, **CI:** confidence interval, test of significance: **Chi-Square test**.

Out of the 55 HLA-DRB1*03 alleles, HLA-DRB1*03:01 allele was the most frequent risky allele (44 alleles) among children infected with giardiasis, with significant difference between cases and controls (OR, 27.06; 95% CI, 3.51-58.0; ***P<0.001***). Another significant association (***P*=0.012**) was noticed between symptomatic and asymptomatic children **(Table 13)**. On the contrary, HLA-DRB1*14:01 variant was the most common protective allele against *G. lamblia* infection (OR, 0.19; 95% CI, 0.04-0.79; ***P*=0.005**) **(Table 14)**.

Table (13): Estimated risk of HLA-DRB1*03:01 allele among the studied groups

HLA-DRB1 allele	Symptomatic giardiasis No. (%)	Asymptomatic giardiasis No. (%)	Control No. (%)	Odds ratio (95% CI)	*P* value
03:01	18 (22.5)	26 (32. 5)	1 (2.5)	27.06 (3.51-58.0)	***P*<0.001**
Symptomatic versus control				15.55 (1.85-341.1)	***P*=0.001**
Symptomatic versus asymptomatic				0.31 (0.11-0.87)	***P*=0.012**
Asymptomatic versus control				50.09 (5.72-112.7)	***P*<0.001**

No: number, values between parentheses refer to percentages, **CI:** confidence interval, test of significance: **Chi-Square test**.

Table (14): Estimated risk of HLA-DRB1*14:01 allele among the studied groups

HLA-DRB1 allele	Symptomatic giardiasis No. (%)	Asymptomatic giardiasis No. (%)	Control No. (%)	Odds ratio (95% CI)	*P* value
14:01	3 (3.75)	3 (3.75)	6 (15)	0.19 (0.04-0.79)	***P*=0.005**
Symptomatic versus control				0.19 (0.03-1.03)	***P*=0.031**
Symptomatic versus asymptomatic				1 (0.15-6.76)	***P***=0.99
Asymptomatic versus control				0.19 (0.03-1.03)	***P*=0.031**

No: number, values between parentheses refer to percentages, **CI:** confidence interval, test of significance: **Chi-Square test**.

4.5. Laboratory investigations of the study participants:

Hemoglobin concentration, total protein, serum albumin and fecal calprotectin levels were assessed and results were statistically reported in **Table (15)**. The data denoted that hemoglobin concentration was 11.63±1.23 g/dl, albumin level was 4.39±0.56 g/dl and the total protein level was 6.9±0.51 g/dl in symptomatic children, with no significant association between any of these laboratory indices and *G. lamblia* infection.

Regarding the results of fecal calprotectin level, a significant correlation (***P*< 0.001**) was detected between *G. lamblia* infection and elevated fecal calprotectin level. As the average fecal calprotectin level was 141.4±67.7 mg/kg and 183±48.21 mg/kg within symptomatic and asymptomatic giardiasis, respectively, compared with its normal level in the control group (29.29±14.1 mg/kg). In addition, a significant correlation (***P*< 0.001**) was found regarding fecal calprotectin level between asymptomatic and symptomatic giardiasis **(Table 15)** with higher percentage of asymptomatic children (92.5%; 37 cases) had high calprotectin level compared with symptomatic patients (62.5%; 25 cases) **(Table 16)**. On the other hand, no significant correlation was noticed between the level of fecal calprotectin and the duration and severity of diarrhea or vomiting as well as abdominal pain and flatulence among the giardiasis children **(Table 17)**.

Table (15): Laboratory data of the studied groups

Laboratory investigations	Symptomatic Mean±SD	Asymptomatic Mean±SD	Control Mean±SD	Bonferronie test
Hemoglobin (g/dl)	11.63±1.23	11.59±1.25	12.13±1.5	***F***= 1.29 ***P***= 0.27
Protein (g/dl)	6.9±0.51	6.84±0.60	7.13±0.38	***F***= 1.98 ***P***= 0.143
Albumin (g/dl)	4.39±0.56	4.1±0.64	4.19±0.38	***F***= 2.75 ***P***= 0.69
Fecal calprotectin (mg/kg)	141.4±67.7 ***P***< 0.001*** ***P****= 0.002***	183±48.21 ***P***< 0.001***	29.29±14.1	***F***= 56.12 ***P< 0.001***

*: Significant difference from asymptomatic group at ***P*< 0.001**

: Significant difference from control group at ***P*= 0.002

***: Significant difference from control group at ***P*< 0.001**

Table (16): Fecal calprotectin level among the studied groups

Fecal calprotectin level	Symptomatic No. (%)	Asymptomatic No. (%)	Control No. (%)
Negative	0 (0)	0 (0)	15 (75)
Border line	15 (37.5)	3 (7.5)	5 (25)
Positive	25 (62.5)	37 (92.5)	0 (0)

No: number, values between parentheses refer to percentages.

Table (17): Correlation between the level of fecal calprotectin and some clinical data for giardiasis cases

Variables	Fecal calprotectin in symptomatic giardiasis
Maximum no. of stool/day	***R***= 0.054 ***P***= 0.74
Diarrhea duration	***R***= 0.047 ***P***= 0.77
Maximum no. of vomiting/day	***R***= 0.196 ***P***= 0.22
Vomiting duration	***R***=0.089 ***P***= 0.58
Abdominal pain	***R***=0.057 ***P***= 0.72
Flatulence	***R***=0.070 ***P***= 0.66

No: number, test of significance: **Pearson Correlation**.

4.6. Association of human leukocyte antigen alleles with some clinical and laboratory findings:

Among the most identified HLA alleles, the HLA-DRB1*03 alleles were correlated with the most common clinical outcomes in symptomatic giardiasis as abdominal pain, diarrhea, vomiting and flatulence. Most children (96.1%) who carried HLA-DRB1*03 alleles complained of abdominal pain that showed significant correlation with *G. lamblia* infection, with ***P*** value **<0.001**. Out of the 26 HLA-DRB1*03, only 5 alleles were associated with diarrhea, 2 alleles were correlated with vomiting and 11 alleles with flatulence in symptomatic giardiasis patients, but without any significant relation to *G. lamblia* infection **(Table 18)**.

Moreover, the different HLA-DRB1*03:01 alleles were detailed for homozygosity or heterozygosity of allotypes to identify which allotype was associated with the presence or absence of symptoms in giardiasis children. The results indicated that no statistically significant association was detected for children who were homozygous for HLA-DRB1*03:01 allotype **(Table 19)**. Interestingly, children with asymptomatic infection were not likely to carry HLA-DRB1*03:01/03:02 allotype, however a significant correlation **(*P*= 0.01)** to symptomatic giardiasis was detected; suggesting that this allotype may be associated with giardiasis symptoms. In addition, significant associations with other HLA-DRB1*03:01 allotypes in children who had both symptomatic and asymptomatic infections were not found **(Table 20)**. Furthermore, the most frequent HLA-DRB1*03 allotype (03:01/03:02) was associated with abdominal pain in all symptomatic children **(Table 21)**.

Table (18): Frequency of clinical symptoms associated with HLA-DRB1* 03 allele among symptomatic giardiasis children

HLA-DRB1* 03 allele	Abdominal pain No. (%)	Diarrhea No. (%)	Vomiting No. (%)	Flatulence No. (%)
No	1 (3.9)	21 (80.7)	24 (92.3)	15 (57.7)
Yes	25 (96.1)	5 (19.3)	2 (7.7)	11 (42.3)
Chi-Square test	X^2=20.91 ***P<0.001***	X^2= 0.70 ***P***=0.40	X^2=0.25 ***P***=0.61	X^2=0.09 ***P***=0.93

No: number, values between parentheses refer to percentages.

Table (19): Estimated homozygosity or heterozygosity of HLA-DRB1*03:01 allotypes for giardiasis cases

HLA-DRB1 allotypes	Symptomatic giardiasis No. (%)	Asymptomatic giardiasis No. (%)	Odds ratio (95% CI)	*P* value
HLA-DRB1*03:01 (heterozygous)	12 (30)	20 (50)	0.43 (0.15-1.18)	0.06
HLA-DRB1*03:01 (homozygous)	3 (7.5)	3 (7.5)	1 (0.15- 6.72)	0.99

No: number, values between parentheses refer to percentages, **CI:** confidence interval, test of significance: **Chi-Square test**.

Table (20): Frequency of HLA-DRB1*03:01 allotypes for giardiasis cases

HLA-DRB1 allotypes	Symptomatic No. (%)	Asymptomatic No. (%)	Chi-square test
03:01/03:01	3 (7.5)	3 (7.5)	X^2=3.6 ***P***= 0.99
03:01/03:02	6 (15)	0 (0)	X^2=6.49 **P= 0.01**
03:01/03:05	0 (0)	3 (7.5)	X^2=3.12 ***P***=0.077
03:01/13:03	3 (7.5)	0 (0)	X^2=3.12 ***P***=0.077
03:01/13:50	0 (0)	3 (7.5)	X^2=3.12 ***P***=0.077
03:01/14:01	3 (7.5)	3 (7.5)	X^2=3.6 ***P***=0.99

No: number, values between parentheses refer to percentages.

Table (21): Frequency of clinical symptoms associated with some HLA-DRB1*03 allotypes among symptomatic giardiasis children

HLA-DRB1* 03 allotypes	Abdominal pain No. (%)	Diarrhea No. (%)	Vomiting No. (%)	Flatulence No. (%)	Total No. (%)
03:01/03:02	6 (100)	0 (0)	0 (0)	3 (50)	6 (100)

No: number, values between parentheses refer to percentages.

It is worth noted that, no effect of the risky allele HLA- DRB1*03 was detected on fecal calprotectin, as the average level of fecal calprotectin (148.32± 66.75 mg/kg) was to some extent equal to its level (159.02± 69.69 mg/kg) detected with other HLA-DRB1 alleles. Also, there was no evidence of statistical correlation of HLA-DRB1*03 allele with fecal calprotectin level (***P***= 0.64) (**Table 22**).

Table (22): Comparison between the effect of HLA-DRB1*03 and other HLA-DRB1 alleles on fecal calprotectin level

	HLA-DRB1* 03 allele	Other HLA-DRB1alleles	Student's t test
	No.=55	No.=105	
Mean± SD	148.32 ± 66.75	159.02 ± 69.69	***t***= 0.46 ***P***= 0.64
Range	60.21-272.12	66.6-270.7	

No: total number**, SD:** standard deviation.

Chapter V

Discussion

Discussion

G. lamblia, a microscopic parasite that infects humans, causing the worldwide intestinal disease giardiasis **(Squire and Ryan, 2017)**. *Giardia* is scheduled as the second parasite detected in stool samples obtained from 1-2 year-old children, while it is the fourth parasite identified in children aged less than 1 year **(Platts-Mills *et al.,* 2015)**. The majority of affected cases are residing in developing countries where over 200 million symptomatic cases are detected every year **(Mbae *et al.*, 2016)**.

G. lamblia is widely transmitted from person to person, animal to person or from environment to person, that is supported by high temperatures and moist climate, poor personal hygiene, in addition to unsanitary habits of individuals **(Noor Azian *et al.*, 2007)**.

Giardia infection occurs through ingestion of cysts voided in human and animal defecations that can taint water and food. Following ingestion of cyst, it hatches liberating the actively replicating trophozoites which bind to the mucosa of small bowel, leading to various intestinal inflammatory changes responsible for clinical manifestations of the disease (**Minetti *et al.*, 2016**).

Giardiasis may be asymptomatic or symptomatic disease **(Escobedo *et al.,* 2010)**. Most *Giardia* infections in immunocompetent individuals are characterized by self-limited diarrhea within 2-4 weeks, but a high recurrence rates have been reported in endemic areas **(Thomas *et al.*, 2014)**. Moreover, as a consequence of long term giardiasis, post-infection irritable bowel syndrome has emerged **(Persson *et al.*, 2015)**. However, immunosuppressed individuals exhibit severe chronic infection as persistent diarrhea that leads to lactase, fat soluble vitamins (A, D, E

and K), vitamin B12 and folate deficiency, as well as disseminated giardiasis to distant organs (**Robertson *et al*., 2010**).

One of the great unsolved questions in giardiasis is why the majority of children infected with *G. lamblia* develop asymptomatic disease (**Ryan and Cacciò, 2013**). Moreover, what is the effect of different considerable factors as *Giardia* assemblages, host nutrition, gut microbiota and other enteropathogens as well as host immune status on the clinical manifestations of infection (**Certad *et al*., 2017**).

The effect of *G. lamblia* assemblages on the variability of clinical giardiasis is the subject of many studies, but they viewed contradictory results of assemblages A and B on the frequency of symptoms. However, others proved no role of these assemblages (**Faria *et al*., 2017**).

Regarding the role of nutritional status on clinical burden of infection, persistent giardiasis is manifested by chronic diarrhea that mostly develops in underweight children with pre-existing nutritional deficits (**DuPont, 2013**).

At the same time, gut microbiota plays a role in the development of different nutritional malabsorption during giardiasis (**Keselman *et al*., 2016**). Moreover, the persistent translocation of intestinal microbiota might contribute in the up-growth of post-infection irritable bowel syndrome and chronic fatigue (**Hanevik *et al*., 2014**). Additionally, experimental *Giardia* infection is associated with a rise in intestinal mucosal bacteria as *Lactobacillus*, *Streptococcus* and *Staphylococcus* species and the development of post-infection gastrointestinal disorders following the enhancement of mucosal influx of these bacteria (**Chen *et al*., 2013**).

Concerning the host immune status against *G. lamblia* infection, many defects still need an illustration, for example the role of B- and T-regulatory cells, the type of antigen presenting cells and *Giardia* antigen

that induce a protective immune response **(Lopez-Romero *et al.*, 2015)**. In addition, many researches have correlated HLA polymorphism with the susceptibility and clinical variability of multiple parasitic diseases **(Beskow *et al*., 2005)**.

This case-control study provided a unique opportunity to investigate the impact of human genes on the susceptibility to *G. lamblia* infection. Egyptian children involved in this study have been divided into two groups regarding the clinical symptoms of giardiasis; symptomatic and asymptomatic groups, besides a *Giardia*-free control group.

The enrolled children were 46 males and 34 females infected with *Giardia*, with average age of 8.3±3.7 years and *Giardia*-free 8 males and 12 females, aged 10.15±1.4 years; suggesting a higher incidence of *G. lamblia* infection in males than females. The high prevalence of giardiasis among male children could be explained by their outdoor active playing attitudes probably with animals and in the soil which bears *Giardia* cysts, as well as the diminished practice of some personal hygiene such as washing of hands with soap and water after playing in the soil or before eating. Additionally, the habit of purchasing food from street vendors who do not perform adequate personal cleanliness and may also be a good transporter of *Giardia* infective stage **(Nyarango *et al*., 2008; Ayeh-Kumi *et al*., 2009)**. Moreover, the increased frequency of *Giardia* infection among school-aged children may be attributed to the fact that children catch the infection in early age of life and acquire immunity subsequently resulting in some protection in later life **(Heresi and Cleary, 1997)**.

Similar result was reported by **Zylberberg *et al.* (2017)** who cleared that males were more susceptible to *G. lamblia* infection. In contrast, high susceptibility rates of giardiasis were recorded among females than males by **Gelani *et al*. (2009)**.

Additionally, the results of age match previous studies conducted in developing countries documented that the common incidence of giardiasis was in school-aged children mostly below 10 years (**Al-Mekhlafi *et al*., 2005; Ben Musa *et al*., 2007**).

The current study documented that the differences in socio-demographic status of people in different communities were associated with the epidemiology and transmission of *G. lamblia.* As the prevalence rates of *G. lamblia* infection were quite high in the inhabitants of rural areas; where 75% of symptomatic and 50% of asymptomatic *G. lamblia*-infected children were from villages and significantly (***P*= 0.043**) correlated with the incidence of giardiasis. This result is a direct consequence of daily activities of the rural inhabitants, especially children who are in close proximity with natural sources of soil and water as well as domestic animals **(Addy *et al*., 2004)**. Besides, soiled houses' floor, insufficient health services, deficient hygiene educations (e.g., use of soap), shortage of clean piped water and poor sanitary conditions are other important factors which rise the possibilities of *Giardia* cysts transmission in these regions **(Harhay *et al*., 2010)**.

The study result is in agreement with that of **Younas *et al.* (2008)** who contributed the highest rate of *Giardia* infection to residency in rural areas, but in contrast to **Dib *et al*. (2008)** who claimed that *Giardia* was more common in urban environments than in rural regions. While **Cancrini *et al*. (1988)** did not notice any significant differences between rural and urban environments in the prevalence of parasitic diseases as giardiasis.

Additionally, two children per room were more common among giardiasis cases; 60% and 62.5% of symptomatic and asymptomatic cases, respectively. However, similar to the number of children per room as well as children per family, children per bed and number of rooms,

none was significantly associated with *Giardia* infection. This result favors that overcrowding was not probably a predisposing factor for the increased incidence of giardiasis. Hence, *Giardia* transmission in rural regions depends mostly on bad hygienic practices more than overcrowding and consequently, person to person transmission. Contrastively, **Al-Mubrook *et al.* (2013)** significantly **(*P*= 0.011)** linked house overcrowding, more than three persons per room to higher incidence of giardiasis.

Previous studies suggested that higher incidence of *G. lamblia* infection is related to several risk factors, including contact with animals and manure **(Wegayehu *et al.*, 2013)**, ingestion of underground or tap water **(Helmy *et al.*, 2014)** or unwashed raw fruits and direct contact with another *Giardia*-infected family members **(Mengist *et al.*, 2015)**. Therefore, the work was conducted and various risk factors that might contribute to the transmission of *G. lamblia* infection were assessed. A notable finding is that the rate of giardiasis was significantly associated with the abstinence of washing vegetables or fruits before eating and handling domestic animals (***P*** value=**0.014** and **0.012**, respectively). While, other risk factors like washing hands before eating and after defecation, source of supplied water, as well as dealing with other family members infected with *G. lamblia* did not appear to play a definite role in disease transmission; suggesting that foodborne and zoonotic transmission may be responsible for the widespread of giardiasis.

Concerning foodborne giardiasis, consumption of raw fruits and vegetables that are contaminated by different methods, including their fertilization with inappropriately treated human sludge and animal fecal materials could increase the risk of *G. lamblia* transmission **(Pereira *et al.*, 2007)**.

Domestic animals are reservoirs for *G. lamblia*, therefore intimate close contact with animal facilitate its transmission **(Traub *et al.*, 2009)**. Besides, **Nkrumah and Nguah (2011)** described that farming and animal husbandry being the major occupation of the population in rural regions with domestic animals are usually kept in contact with children, favor the increased prevalence of giardiasis. Also, the authors suggested that lack of proper water supply on the farms hence the farmers and their children drink from streams and rivers which might be used by these animals too, could be another predisposing factor for increased infection.

The study data are well in line with the results of **Kiani *et al.* (2016)** who claimed that the presence of domestic animals in-door was significantly associated with *G. lamblia* infection (***P*=0.01**). Also, they are to some extent similar to those of **De Lucio *et al.* (2017)** who denoted that inadequate washing of raw vegetables and fruits was significant risk factor for giardiasis. While, the same authors found that zoonotic transmission of *G. lamblia* infection was an infrequent factor.

Furthermore, the present study attempted to estimate the clinical outcomes of giardiasis. The most common complaints of cases were abdominal pain (95%) and diarrhea (47.5%) followed by flatulence (40%) and then vomiting (25%), with statistically significant association (***P*** value < **0.001**, **=0.001** and **0.011**, respectively) with *G. lamblia* infection. **Omran and Mohammad (2015)** assumed that the most common symptom associated with giardiasis was abdominal pain (100%), while vomiting followed by flatulence and diarrhea were detected in 42.8% and 28.5%, respectively. In corroboration with the study results, **Ismail *et al.* (2016)** detected a positive significant association (***P*= 0.0001**) between *G. lamblia* infection and the development of abdominal pain, flatulence and diarrhea in Egyptian children.

Regarding the frequency of diarrhea reported in the present study, the result is contradictory to the previous observations of **Hollm-Delgado *et al.* (2008)** who emphasized that giardiasis was not associated with higher rates of diarrhea (6%), and referred that to the tolerance induced by multiple early life *G. lamblia* infections. Moreover, **Bodhidatta *et al.* (2010)** considered *G. lamblia* parasite as a protecting factor against acute diarrhea. Also, **Muhsen and Levine (2012)** confirmed the hypothesis of *Giardia* protective effect and did not correlate giardiasis with the occurrence of diarrhea in developing countries and hypothesized this was age dependent.

Concerning the diarrheal episodes, a total of 17 cases (42.5%) presented with acute diarrheal attacks, 23 cases (57.5%) had more than one diarrheal episode per month (intermittent diarrhea) and two cases (5%) suffered persistent diarrhea. While chronic diarrhea was not reported in this study; supposing that acute and intermittent diarrhea were more popular in Egyptian children infected with *G. lamblia* than chronic and persistent diarrhea. Additionally, the intensity of acute diarrheal attacks was moderate to severe and repeated up to 12.22±6.45 times every year. Moreover, these diarrheal episodes were more frequent in female patients mostly at the age of 2-5 years, as protection against the intensity and duration of diarrheal attacks is consistent with the development of host immune resistance **(Heyworth, 2014)**. Even more, the most common episodes (9 and 12 episodes) revealed higher incidence with longer duration (7 days) in the rural than the urban residents. The previous results support the concept that younger children infected with *Giardia* usually presented by severe acute diarrheal attacks (**Maikai *et al.*, 2012**). Hence, the age of children, in addition to increased outdoor activities that increased water consumption mostly untreated water

supplies and favored intimate contact with farm animals may be the reasons for the frequency and duration of these diarrheal attacks.

The fore mentioned study results are in harmony with those of **Yoder *et al.* (2007)** who reported that *G. lamblia* infection was mostly recognized by intermittent diarrheal episodes that might be preceded by acute episode. On the other side, the Global Enteric Multicenter Study (GEMS) claimed that giardiasis was not significantly associated with moderate to severe diarrhea in Africa **(Kotloff *et al.,* 2013)**. Besides, **Ismail *et al.* (2016)** revealed a statistically significant association between the detection of *Giardia* parasites and persistent diarrhea, while no association was revealed with intermittent diarrhea.

It is also clear that there was no significant association of headache, fever, pallor or dehydration with symptomatic giardiasis, besides absence of steatorrhea and symptoms of fat soluble vitamins deficiency as broken finger nails and night blindness among the participated children. These manifestations are believed to be more likely associated with chronic *Giardia* infection **(Robertson *et al*., 2010)**.

The consequence of *G. lamblia* infection on host nutritional condition is variable, but giardiasis is mostly associated with delayed growth **(Verhagen *et al*., 2013)** and delayed weight gain **(Ignatius *et al*., 2012)**. Therefore, the growth parameters were analyzed and found that 21 children (26.25%) infected with *G. lamblia* had stunted growth with statistically significant correlation (***P*=0.031**) with giardiasis. However, 72 cases (90%) had normal weight compared with their corresponding ages and this could be explained by the absence of chronic diarrhea that is usually associated with weight loss **(Hollm-Delgado *et al.,* 2008)**.

These results are more or less similar to those obtained by an epidemiologic study conducted in developing countries and correlated giardiasis with childhood growth retardation **(Prado *et al*., 2005)**.

Moreover, the findings are in agreement with the data of **Donowitz *et al.* (2016)** who announced that *Giardia* infection was responsible for only stunting of growth but not poor weight gain, and this was attributable to the motif that catching the infection before the age of 2 years had an effect on child height, as what was also proved by **Rogawski *et al.* (2017)**. On the other hand, this study did not confirm reports assuming that *G. lamblia* infection had a more prominent influence on the weight than the height of affected children and consequently, leads to a significantly more common wasting than stunting **(Carvalho-Costa *et al.,* 2007; Yones *et al.*, 2015)**.

According to my knowledge, no previous study assessed the correlation between HLA-DRB1 alleles and *G. lamblia* infection in humans. The biological role of HLA class-I and class-II is to present the processed peptide antigens to CD8+ and CD4+T-cells, respectively. Most HLA class-II processing occurs in the antigen-exhibiting cells as B cells, macrophages and dendritic cells **(Drozina *et al.*, 2005)**.

Because the peptide-restricting groove of HLA class-II molecule is open-ended and accommodates peptides longer than 15 residues, therefore polymorphisms occur mainly in the antigen-binding groove and affect HLA specificity as binding receptors **(Klein and Sato, 2000)**. So, a solitary HLA class-II allele is equipped for presenting distinct thousand peptides to a CD4+ T cell **(Duggal *et al.*, 2004)**. It is conceivable that the existence or absence of a specific HLA class-II allele and polymorphisms in HLA complex play a role in inter-individual variety in disease susceptibility **(Kohaar *et al.*, 2009)**. This is related to particular sequence-based typing of particular HLA variants mediated by distracts in specific amino acid that may modify antigen recognition and consequently, HLA-T cell receptor interaction **(Archbold *et al.*, 2009)**.

Furthermore, **Blackwell *et al.* (2009)** identified significant positive genetic correlations between HLA class-II variant and the major infectious diseases regarding the susceptibility or course of some parasitic diseases. For example, a research conducted by **Duggal *et al.* (2004)** demonstrated the protective association of the HLA class-II allele DQB1*06:01 and the heterozygous haplotype DQB1*06:01/DRB1*15:01 with *Entamoeba histolytica* infection. Also, **Watanabe *et al.* (2014)** proved the protective role of HLA DQB1*06:01 against the incidence of invasive amebiasis in patients with subclinical intestinal amebiasis. Moreover, HLA types could interfere with the disease severity as HLA-DR9, -DR11, -DR14, -DR17, -DQ2, -DQ7 and -DQ8 were high in patient with mild to moderate amoebic liver abscess, while HLA-DR4, -DR7, -DR13, -DR15, -DR16, and -DQ5 probably associated with severe manifestations (**Nazli *et al.*, 2016**).

Another study illustrated that children who carry DQB1*03:01/DRB1*11:01 haplotype were more susceptible to encounter both asymptomatic and symptomatic *Cryptosporidium* infection (**Kirkpatrick *et al.*, 2008**). On other hand, **Pantenburg *et al.* (2010)** evaluated the capability of HLA class-I alleles to resist *Cryptosporidium* infection, and revealed that both HLA-A and HLA-B presented *Cryptosporidium* antigen to CD8+T cells and presumably, eradicated the parasite burden.

Moreover, many studies linked different HAL-DRB1 alleles with malaria disease. For instance, **Hananantachai *et al.* (2005)** reported that DRB1*10:01 expression decreased in severe cerebral malaria patients, while **Osafo-Addo *et al.* (2008)** declared that DRB1*04 allele related to severe malaria. Another study documented that increased expression of the DRB1*13:01 allele, developed an anti-duffy binding protein-II IgG response against *Plasmodium* parasite (**Kano *et al.*, 2016**).

In respect to toxoplasmosis, **De Sorrentino *et al.* (2005)** examined HIV seropositive individuals and reported that patients expressing excess HLA-DRB1*08 and -DQB1*04:02 alleles were more likely to develop neurotoxoplasmosis than others. While, infants with high rates of HLA-DQA1*01:03, -DQA1*03:02 and -DQB1*05:04 alleles in amniotic fluid showed increased parasite burden with high susceptibility to develop congenital toxoplasmosis **(Shimokawa *et al.*, 2016)**.

Concerning leishmaniasis, **Ribas-Silva *et al.* (2013)** recorded that HLA-B and HLA-DRB1 alleles were risky and participated in exaggerated susceptibility to cutaneous leishmaniasis. While, **Samaranayake *et al.* (2016)** illustrated that several class-I and class-II HLA genes; other than HLA-B and HLA-DRB1 alleles had a pivotal role toward the susceptibility to localized cutaneous leishmaniasis through variations in their recognitions and interactions with different *Leishmania* antigens. In addition, neutrophils isolated from cutaneous leishmaniasis lesions and patients' circulation revealed increased expression of HLA-DR molecule to act as antigen presenting cell (**Davis *et al.*, 2017**).

Notably, individuals expressing HLA-DQA1*05:01 were capable of recognizing *Trypanosoma cruzi* B13 protein and this allele could clear the parasite and protect against the disease **(Abel *et al.*, 2005)**. Moreover, **Del Puerto *et al.* (2012)** revealed that HLA-DRB1*01 and HLA-B*14 variants had significantly lower frequencies in patients suffering from megacolon or those from electrocardiography changes, and this haplotype was therefore considered resistant against chronic Chagas' disease.

As regards the blood dwelling flukes, **Everts *et al.* (2010)** stated that chronic state of *Schistosoma haematobium* infection was mediated via decreased the expression of HLA-DR on myeloid dendritic cell leading to dysfunction of human dendritic cell *in vivo* and a state of T cell hyporesponsiveness. Moreover, **Huy *et al.* (2011)** linked antigen

presenting cells carrying DRB1*09:01 to the specific IgG4 elevation, Th2 cells activation and consequently, the incidence of post-schistosomal hepatic disorder (PSHD) as DRB1*09:01/DQB1*02:01, DRB1*09:01/DQB1*03:03 and HLA-A1/B8 haplotypes increased the susceptibility to PSHD development, whereas the haplotype DQA1*05:01/DQB1*03:01 resists the advancement of PSHD**.** However, **Fernandes *et al.* (2014)** illustrated that different phenotypes of monocytes (classical and intermediate) had a definite role on the development of periportal fibrosis in schistosomiasis through antigen presenting to T-cells and hence its activation, as they noticed excess expression of HLA-DR molecule on classical and intermediate monocytes in patients with periportal fibrosis.

Concerning the association of HLA loci and the susceptibility or resistance as well as clinical variability of *G. lamblia* infection, few studies were performed and examined primarily the HLA class-I alleles. For example, an association was noticed among individuals carrying HLA-A1 and their high susceptibility for developing *G. lamblia* infection **(De Manueles *et al.*, 1992)**. The authors also detected another correlation between the type of HLA antigens and the development of giardiasis clinical symptoms, as HLA-A19, HLA-A9, and HLA-B7 alleles were associated with vomiting, abdominal pain and alterations in the gastrointestinal mucous, respectively. Also, **El-Ganayni *et al.* (1994)** detected a strong correlation between HLA-A10, -A11, -B5, -B7 and -B17 phenotypes as well as HLA-B7, -B17, -Bw14 and -Bw40 with the development of symptomatic and asymptomatic giardiasis, respectively. In addition, **Osipova *et al.* (1993)** revealed that HLA-B5, -B14, -DR3, -DR4, and -DR7 as well as haplotypes HLA-A9-B5 and A1-B5 were the most common alleles in patients with persistent giardiasis.

The results presented in the current study highlighted that HLA class-II DRB1 alleles had an impact on human susceptibility to *G. lamblia* infection.

The most important finding is that children infected with *G. lamblia;* whether symptomatic or asymptomatic were more likely to carry the HLA class-II DRB1*03 allele (OR, 40.7; 95% CI, 5.13-87.9; ***P*< 0.001**). Among the distinct HLA-DRB1*03 alleles, HLA-DRB1*03:01 allele showed significant association with *G. lamblia* infection (OR, 27.06; 95% CI, 3.51-58.0; ***P*<0.001**); suggesting that this allele may be responsible for restricting antigen recognition and HLA-CD4+T-cell interaction and subsequently develops insufficient host immune resistance as well as greater host susceptibility to *G. lamblia* infection.

Additionally, significant correlation (***P*= 0.01**) was detected between children carrying the allotype HLA-DRB1*03:01/03:02 and the emergence of clinical symptoms, while asymptomatic children did not carry this allotype. Moreover, this allotype strongly present in symptomatic children suffering only of abdominal pain, but its presence was not associated with diarrhea or vomiting, supposing that HLA-DRB1*03:01/03:02 could play a role in susceptibility to clinical burden of giardiasis mainly to abdominal pain.

The results also hypothesized a protective association between the HLA class-II DRB1*14 allele and the development of giardiasis (OR, 0.19; 95% CI, 0.09 -0.79; ***P*= 0.017**), with HLA-DRB1*14:01 was the most mutual allele among controls (OR, 0.19; 95% CI, 0.04-0.79; ***P*=0.005**). Therefore, HLA-DRB1*14:01 variant could be an important component of the human immune response against *G. lamblia* infection through proper antigen recognition and presentation to the CD4+T-cell receptor.

It is worth noted that, other HAL-DRB1alleles showed no evidence of association with the incidence of giardiasis or accordingly the modification of immune response in the study population.

The results of the present study are more or less in accordance with that reported by **Grit *et al.* (2014b)** who experimentally utilized a mixture of *Giardia* assemblage A and E trophozoites, and noticed that the expression of HLA class-II molecule increased on the surface of bovine dendritic cells.

On the contrary, the present data do not support the conception that *G. lamblia* extracts could down regulate the induction of MHC-II molecules on the surface of dendritic cells **(Kamda and Singer, 2009)**, or the notion of diminished expression of HLA-DR molecules on the surface of activated human dendritic cells, when incubated with *Giardia* trophozoites *in vitro* and so, *G. lamblia* might interfere with human immune mechanisms **(Obendorf *et al.*, 2013)**.

It is worth noted that, this work enforces the concept that allelic frequencies of the different HLA class-II molecules have been associated with a positive influence on host protection or susceptibility to distinct parasitic infections **(Blackwell *et al.*, 2009)**, including *G. lamblia* infection.

In respect to laboratory investigations, it is well known that calprotectin is a major calcium and zinc binding protein found in the cytosol of inflammatory cells predominantly neutrophils and to a lesser extent, monocytes and macrophages **(Gisbert and McNicholl, 2009)**. Therefore, the detection of calprotectin in faeces is directly related to neutrophil migration towards the intestinal tract and to the extent of intestinal mucosal inflammation **(Vermeire *et al.*, 2006)**. Fecal calprotectin concentration increased in patients with various gastrointestinal infections e.g. viral and bacterial infections, but its level

is lower than in patients with inflammatory bowel disease **(Hanevik *et al*., 2007)**. As a consequence, the measurement of fecal calprotectin level is used as simple non-invasive screening test for various infectious and inflammatory bowel diseases **(Van Rheenen *et al*., 2010)**.

This work declared that hemoglobin concentration, total protein and serum albumin levels did not significantly correlate with *G. lamblia* infection. This might be due to minimal inflammation of intestinal mucosa in the giardiasis studied children, which was not severe enough to disturb the intestinal absorptive surface and its functional capability.

The laboratory work revealed that, fecal calprotectin level was significantly (***P*< 0.05**) elevated up to a mean of 141.4 and 183 mg/kg in symptomatic and asymptomatic *G. lamblia*-infected children, respectively. Even though, this elevation wasn't as high as fecal calprotectin level (797 mg/kg) in inflammatory bowel diseases **(Damms and Bischoff, 2008)**. Therefore, it was suggested that the increased fecal calprotectin levels in the recruited children may reflect a mild degree of inflammation mainly of neutrophil type occurred in the small intestine as a consequence of *G. lamblia* infection. Moreover, heightened fecal calprotectin level was more prominent among asymptomatic cases (37 cases; 92.5%) than symptomatic children (25 cases; 62.5%); reflecting that bowel inflammation occurred at higher frequencies in asymptomatic comparable with symptomatic *G. lamblia* infection. The lower fecal calprotectin level in symptomatic giardiasis cases could be related to the ability of *Giardia* to modulate host immune response through decreasing intestinal inflammatory markers **(Veenemans *et al*., 2011)**. As well, researchers suggested that low grade intestinal inflammation is probably a predisposing factor for the advancement of post-infection irritable bowel syndrome **(D'Anchino *et al*., 2002; Spiller and Campbell, 2006)** that is

characterized by persistent mucosal infiltration of immune cells as neutrophils **(Ibarra *et al.*, 2016)**.

Contrastively, fecal calprotectin elevation was not significantly associated with the frequency or duration of different clinical manifestations in *G. lamblia* infection. Even more, no significant effect of HLA-DRB1*03 was detected on the level of fecal calprotectin; thus, reducing the possibility of neutrophils involvement in giardiasis as antigen presenting cells.

Previous studies documented that fecal calprotectin level is elevated in cases of cystic fibrosis, rheumatoid arthritis, Crohn's disease, ulcerative colitis, neoplastic diseases and non-steroidal anti-inflammatory drugs induced enteropathy **(Hestvik *et al.*, 2011; Vrabie and Kane, 2014)**.

In correlation with giardiasis, human studies supporting the presence of heightened fecal calprotectin level and the development of mucosal inflammatory reaction were insufficient **(Cotton *et al.*, 2015)**. The present study data are in harmony with those of **Tibble *et al.* (2002)** who examined eight *Giardia*- infected children and observed excess fecal calprotectin level, and those of **Hanevik *et al.* (2007)** who demonstrated duodenal inflammations in patients infected with chronic *G. lamblia* with high fecal calprotectin levels. Similarly, **Hestvik *et al.* (2011)** observed that 11 out of 28 examined children with elevated fecal calprotectin level were infected with *G. lamblia*. Even more, **Garzon *et al.* (2017)** observed that most asymptomatic *Giardia* infection had the trend of heightened fecal inflammatory markers and supposed local inflammatory reaction with a definite role of neutrophils.

Chapter VI

Summary and Conclusions

Summary and Conclusions

G. lamblia is a globally disseminated enteric protozoan parasite causing the disease giardiasis that is more prevalent in low-income countries, owing to the poor sanitary supplementation.

One of the most characteristic features of giardiasis is the myriad of clinical manifestations, ranging from absence of clinical symptoms to various gastrointestinal symptoms.

Even more, the frequency of different gastrointestinal symptoms is debated. As a result, a recent intriguing question is why and how *G. lamblia* develops asymptomatic infection in some individuals, while causing severe disease in others.

Additionally, histological examination of small intestinal biopsy specimens following *G. lamblia* infection revealed significant vigorous intestinal inflammatory responses and even villous atrophy, while others are deprived of any indications of bowel inflammation.

As *Giardia*-induced intestinal inflammatory reaction shows conflicting responses, additionally some studies have correlated HLA polymorphism with common infectious diseases, this study was conducted aiming to evaluate the influence of HLA-DRB1 variant on the susceptibility and clinical variability of *G. lamblia* infection. Also, the study tried to assess the fecal level of calprotectin in case of giardiasis, and finally to detect any correlation between fecal calprotectin level and the development of clinical manifestations in giardiasis.

The study was conducted on 100 children aged from 2-16 years, divided to 3 groups; symptomatic giardiasis, asymptomatic giardiasis and controls.

The present study documented that the prevalence of *G. lamblia* infection was quite high among rural inhabitants. Moreover, *G. lamblia*

was widely distributed by the habit of eating unwashed vegetables and fruits, besides contact with domestic animals, which supports the potential of foodborne and zoonotic transmission of giardiasis.

G. lamblia infection was mostly associated with abdominal pain (95%) followed by diarrhea (47.5%), flatulence (40%) and then vomiting (25%). The diarrheal attacks were acute or intermittent with moderate to severe degree than being persistent or chronic. The frequency and duration of diarrheal attacks were higher in children residing in rural areas than those from urban areas. Besides, these diarrheal episodes were more prominent in females (14.14±6.38 episodes/year), mostly at the age 2-5 years (13.85±7.36 episodes/year).

Moreover, giardiasis was associated with stunted growth (26.25%), with statistically significant correlation of ***P*=0.031**, but the body weight of the affected children was normal compared with their corresponding ages.

The most important finding of the present study was the significant association of the HLA class-II DRB1*03:01 allele with the advancement of *G. lamblia* infection. In addition, the significant association of the heterozygous allotype HLA-DRB1*03:01/03:02 with the development of clinical manifestations of giardiasis mainly abdominal pain was revealed.

On the other hand, the HLA class-II DRB1*14:01 allele played a protective role against the development of *G. lamblia* infection and probably involved in *Giardia* antigen presentation to CD4+T cells, hence the establishment of host immune resistance against the disease.

Nevertheless, other HLA-DRB1 alleles did not correlate with the incidence of giardiasis.

Furthermore, fecal calprotectin level was high in *G. lamblia* infected children, irrespective of *G. lamblia* association with clinical symptoms or not. Obviously, elevated fecal calprotectin level was more

common amongst asymptomatic *G. lamblia*-infected children (92.5%). Presumably, *G. lamblia* infection induced a degree of enteric inflammation mainly of neutrophil type, and its elevation in giardiasis cases may be a predisposing factor for the incidence of post-infection irritable bowel syndrome.

Conclusively, the present study demonstrated that host genetics underlie human susceptibility or resistance as well as clinical variability to *G. lamblia* infection. Besides, fecal calprotectin can be used as a marker of *G. lamblia*-induced intestinal inflammation.

Recommendations

i. Further studies should be conducted on larger sample sizes to confirm the results.

ii. Edify the community and the children on the best personal hygienic measures to minify giardiasis prevalence.

iii. Future analysis of other HLA class-II genes (HLA-DQ and -DP) is needed to enlighten the components of immune system that influence the susceptibility and resistance to *G. lamblia* infection.

iv. Promote examination of HLA class-I alleles to test for linkage disequilibrium across the class-I and class-II alleles to better determine the degree of genetic protection against giardiasis.

v. *Giardia* genotyping studies should be conducted hand in hand with HLA typing to assess for possible correlation with the emergence of symptomatic or asymptomatic infections.

vi. Histopathological studies of duodenal biopsy specimens are needed to verify the extent of duodenal inflammation with fecal calprotectin level.

vii. Follow-up of asymptomatic giardiasis cases is a must to link the level of fecal calprotectin with the advancement of post-infection irritable bowel syndrome.

Chapter VIII

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Appendix

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Title: Clinical and laboratory findings in children infected with *Giardia lamblia*: A case control study.

Authors: Ayat A. El-Belehy *et al*. (2017).

Protocol

Introduction

Giardia duodenalis (*G. duodenalis*) is an intestinal flagellated protozoan parasite of the upper small intestine which was recently included in the World Health Organization's Neglected Disease Initiative **(Samad, 2011).**

In developing countries, the prevalence rate of human giardiasis commonly ranges from 20% to 30%, with reports of 100% prevalence in some populations, while in developed countries, prevalence ranges from 3% to 7% **(Halliez and Buret, 2013).**

The clinical manifestations of giardiasis are highly variable among individuals and can range from acute to chronic infections, while some hosts may remain asymptomatic. *Giardia* infections tend to be self-limiting in individuals with competent immune systems. Immunocompromised people are at increased risk of developing chronic giardiasis and yet it is also common in immunocompetent individuals (**Cotton *et al.*, 2011**).

Human major histocompatibility complex (MHC) is called human leukocyte antigen (HLA) and is expressed on antigen-presenting cells such as B cells and macrophages, which present antigen fragments to $CD4^+$ T cells (**Kohaar *et al.*, 2009**).

Human MHC maps to the short arm of chromosome 6 and is divided into three regions; class I, class II, and class III, where class II consists of a series of sub-regions (DR, DQ, and DP), each contains one or more A, and B gene, respectively (**Choo, 2007**).

Solaymani-Mohammadi and Singer (2010) stated that immune response to microbial pathogens, including *Giardia* sp., depends on both innate and adaptive components. Although, the actual host defense mechanisms responsible for controlling *Giardia* infections are poorly

understood, many studies have demonstrated the development of adaptive immune responses as well as innate mechanisms in humans and animals.

G. duodenalis is a non-invasive parasite which stays in the intestinal lumen where it adheres to epithelial cells (**Singer and Nash, 2000**). Since CD4+ T helper cells are included in protection against *G. duodenalis*, chronic infection may result from inadequate antigen presentation that does not lead to the development of protective cellular immunity **(Eckmann, 2003**).

Dendritic cells (DCs) might be precisely involved in the induction of anti-*Giardia* immune responses as they are able to reach with their cellular protrusions into the gut lumen and sample antigen (**Obendorf *et al*., 2013**). Following antigen-uptake, DCs may process and present specific peptides to naïve T lymphocytes, and activated through ligands of pattern-recognition receptors (PRRs), e.g., Toll-like receptors (TLRs), C-type lectins, and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), are essential for the induction of specific cellular immunity (**Ueno *et al*., 2007**).

Grit *et al*. (2014) noted that DCs are not the leading cells in the induction of the cellular response. Nor do B-cells play a role in *Giardia* antigen presentation. However, the depletion of cells with high MHC II molecule significantly reduced proliferation of peripheral blood mononuclear cells (PBMC). Since the proliferating PBMC themselves are not MHC II high cells, the results indicate that *Giardia* trophozoites have little or no direct effect on blood lymphocytes and that antigen-presenting cells, other than DC and B-cells are required to initiate proliferation. Which MHC II cells are able to induce lymphocyte proliferation remains unclear.

Koot *et al.* (2009) observed that neutrophilic and eosinophilic granulocytes infiltrate the epithelial layer (16% and 9%, respectively) in

giardiasis. Neutrophils are the first line of defense against infectious disease by phagocytosing and killing invading microorganisms, and play a critical role in inflammation **(Witko-Sarsat, *et al*., 2000).** They contain primary (azurophil) and secondary (specific) granules, which are formed at different stages of neutrophil maturation **(Carlson *et al*., 2002).**

Primary granules contain several proteolytic enzymes and bactericidal proteins including serine proteases, cathepsin-G (cat-G), elastase, and myeloperoxidase (MPO), while the secondary or specific granules contain a wide variety of different components, including lactoferrin, lysozyme, collagenase, and lipocalins **(Mollinedo, 2003).** Release of granule contents may modify the inflammatory process as secondary granules promote phagocytic capacity, while primary and secondary granules each contribute the major anti-microbial arsenal **(Scott, and Krauss, 2012).**

During inflammation, neutrophil stimulation can lead to its release of large amounts of toxic oxygen radicals and a variety of granule proteins and soluble proteins such as calprotectin **(Srinivasan, 2013).**

Calprotectin is abundant in neutrophils, accounts for 5% of total protein and 60% of the protein in the cytosol fraction, however lower concentrations are found in monocytes and reactive macrophages **(Bunn *et al*., 2001).** It is calcium and zinc binding protein that found in both plasma and stool and is markedly elevated in infectious and inflammatory conditions **(Konikoff and Denson, 2006).**

Faecal calprotectin is used as a non-specific marker for neutrophils and mononuclear phagocytes activation, and gastrointestinal inflammation **(Hestvik *et al.,* 2011).**

Aim of work:

1- The correlation between host genetics, including the human leukocyte antigen (HLA-DRB1) and the development of susceptibility (symptomatic) or resistance (asymptomatic) to *Giardia* infection needs to be measured.

2- Assessment of faecal level of calprotectin (neutrophil activation marker) in children with giardiasis.

3- Report the assosiation between faecal calprotectin level and the development of symptomatic or asymptomatic giardiasis.

Patients

Study design:

A comparative study will be performed within one year.

Sample size:

Convenient sample of about 100 children:

1- 80 children as patient cases.
2- 20 children as control cases.

Study location:

Study will be conducted at Parasitology and Clinical Pathology Departments, Faculty of Medicine, Mansoura University and Mansoura University Children Hospital.

Study population:

Pediatric cases attending Mansoura University Children Hospital.

Cases' selection:

Inclusion criteria:

The participants in the study will be

1- Children.
2- Both sexes.
3- Complaining of gastrointestinal symptoms such as abdominal pain, diarrhea or flatulence.

4- Positive for *Giardia* by direct stool examination.

Exclusion criteria:

1- Those who have other parasitic infections.

2- Those who have bacterial infection.

Clinical groups:

Are divided into three groups:

Group I: those who have diarrhea associated with detection of *G. duodenalis* in stool in the absence of other diarrheal pathogens.

Group II: those who are positive for *G. duodenalis* infection only with absence of diarrheal illness or other diarrheal pathogens.

Group III (control): are children without symptomatic or asymptomatic *Giardia* infection during the study period.

Methods:

-Stool and blood samples will be collected from each participant enrolled in the study.

-The severity of diarrhea will be defined by a numerical scoring system known as the Ruuska score, which accounts for duration of diarrhea, maximum number of diarrheal stools/day, vomiting, fever, dehydration, and the level of clinical care required (**Lewis, 2011**).

I. Stool specimens will undergo:

1- Direct microscopic examination for detection of giardiasis and exclusion of other parasitic infections through:

A- Wet mount examination by saline mount.

B- Concentration procedure by formal ethyl acetate technique.

2- *Giardia* antigen capture test will be performed to confirm stool results in the case of negative *Giardia* stool specimens.

3- Culture onto *Salmonella-Shigella*, MacConkey, and MacConkey-tellurite agars for the identification of *Salmonellae* and *Shigella* species, and *Escherichia coli*.

4- Measurement of faecal calprotectin using quantitative enzyme linked immunosorbent assay (Epitope Diagnostics, USA).

II. Blood samples will be subjected to:

1- Genetic analysis**:** Genomic DNA will be extracted from 200µL of peripheral blood, using the Qiagen DNA extraction kit (Qiagen, USA). Human leukocyte antigen (HLA) class II-DRB1 typing will be performed using polymerase chain reaction (PCR) and sequence-specific oligonucleotides. For genetic comparisons, all siblings will be excluded from analysis, since siblings share 50% of their alleles.

Statistical analysis: Basic demographic information, and clinical and laboratory findings will be collected and the statistical package SPSS software (V. 21, USA) will be used for data analysis with the application of appropriate statistic significant tests.

Ethical clearance:

The study will be conducted according to the ethical guidelines of Mansoura Medical Research Ethics committee. Informed consent will be obtained from parents or guardian of the children in the study. Patients will be referred to the physician for the treatment of diarrhea.

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الملخص العربى

يعتبر طفيل الجيارديا لامبليا بأنه الطفيل المعوي المنتشر على الصعيد العالمي ومسببا لمرض الجيارديا، المرض الأكثر انتشارا في البلدان المنخفضة الدخل وذلك بسبب سوء المكملات الصحية.

من أكثر السمات المميزه لمرض الجيارديا، التنوع فى المظاهر السريرية للمرض بدءا من غياب تلك الأعراض السريرية إلى حدوث معظم أعراض الجهاز الهضمي.

وأكثر من ذلك، فإن مختلف أعراض الجهاز الهضمي مازالت موضع جدال. ونتيجة لذلك، ظهرت مؤخرا أسئله مثير للاهتمام مثال علي ذلك لماذا وكيف لاتسبب عدوى الجيارديا لامبليا أى أعراض في بعض الأفراد، في حين تسبب مرضا شديدا في الآخرين.

بالإضافة إلى ذلك، فإن الفحص النسيجي لعينات مأخوذه من الأمعاء الصغيرة ومصابه بعدوى الجيارديا اللامبليا قد كشف وجود التهابات معوية شديده وقد تصل لحد ضمور الزغابات وفي الوقت نفسه فإن عينات أخرى لم تظهر أي مؤشرات لإلتهاب الأمعاء.

وإذا كانت التهابات الأمعاء التي تسببها الجيارديا أظهرت ردود متضاربة، بالإضافة إلى ذلك بعض الدراسات قد ربطت تنوع مستضد الكريات البيضاء البشريه مع الأمراض المعدية الشائعة، لذلك تم إجراء هذه الدراسة التي تهدف إلى تقييم تأثير بديل مستضد الكريات البيضاء البشريه (DRB1) على عدوى الجيارديا لامبليا من حيث قابليتها للحدوث وتنوع ظواهرها السريرية.

كما حاولنا أيضا تقييم مستوى الكالبروتكتين فى البراز لمرض الجيارديا، وأخيرا للكشف عن أي علاقة بين مستوى الكالبروتكتين فى البراز وتطور المظاهر السريرية في الجيارديا.

فقد أجريت الدراسة على مائه طفل تتراوح أعمارهم بين ٢-١٦ سنة، ومقسمين إلى ثلاث مجموعات: مجموعه تظهر أعراض مرض الجيارديا، ومجموعه بدون أعراض لمرض الجيارديا، بالإضافه إلى مجموعه ضابطه.

وقد وثقت الدراسه الحاليه أن انتشار عدوى الجيارديا لامبليا وجد مرتفعا جدا في المناطق الريفية. وعلاوة على ذلك، فإن انتشار الجيارديا لامبليا على نطاق واسع قد تم من خلال عادة تناول الخضار والفواكه الغير مغسولة، وإلى جانب الاتصال مع الحيوانات الأليفة، والذي دعم إمكانيه إنتقال الجيارديا المنقولة بالأغذية والناشئه من الأمراض الحيوانية.

وقد ارتبطت العدوى في الغالب بألم في البطن (٩٥٪) يليه الإسهال (٤٧,٥٪)، وانتفاخ البطن (٤٠٪)، ثم القيء (٢٥٪)، وقد كانت نوبات الإسهال حادة أو متقطعة وبدرجة معتدلة إلى

شديدة أكثرمن كونها مستمرة أو مزمنة. وكان معدل وفتره نوبات الإسهال أعلى لدى الأطفال المقيمين في المناطق الريفية من أولئك الذين يعيشون في المناطق الحضرية. وبالإضافة إلى ذلك، كانت هذه النوبات الإسهالية أكثر بروزا في الإناث (١٤,١٤±٦,٣٨)، ومعظمهم في سن ٢-٥ سنوات (١٣,٨٥± ٧,٣٦).

وعلاوة على ذلك، فقد ارتبط مرض الجيارديا مع التقزم (٢٦,٢٥٪)، مع وجود علاقة ذات دلالة إحصائية (٠,٠٣١)، بينما وزن الجسم من الأطفال المتضررين ظهر طبيعيا مقارنة مع الأعمار المقابلة لها.

فأهم النتائج التي توصلت إليها الدراسة الحالية هي الارتباط الكبير بين بديل مستضد الكريات البيضاء البشريه من الدرجة الثانية (DRB1* 03: 01) مع تقدم عدوى الجيارديا لامبليا، وبالإضافة إلى ذلك، تم الكشف عن وجود ارتباط كبير بين النمط المتخالف لمستضد الكريات البيضاء البشريه (DRB1*03:01/03:02) مع تطور المظاهر السريرية لمرض الجيارديا أساسا مع آلام البطن.

من ناحية أخرى، لعب بديل مستضد الكريات البيضاء البشريه من الدرجة الثانية (DRB1*14:01) دورا وقائيا ضد تطور عدوى الجيارديا لامبليا، وربما يشارك في عرض مستضد الجيارديا إلى خلايا CD4+ T ، وبالتالي إنشاء المقاومة المناعية للمضيف ضد المرض. ومع ذلك، فإن أليلات مستضد الكريات البيضاء البشريه الأخرى ليس لها أى تأثير على حدوث مرض الجيارديا.

وعلاوة على ذلك، فإن مستوى الكالبروتكتين البرازي كان عاليا في الأطفال المصابين بالجيارديا لامبليا، بغض النظر عن إرتباطها بالأعراض السريرية من عدمها. ومن الواضح أن ارتفاع مستوى الكالبروتكتين البرازي كان أكثر شيوعا بين الأطفال المصابين بعدوى الجيارديا لامبليا (٩٢,٥٪). وعلى نحو محتمل، فإن عدوى الجيارديا لامبليا تسببت في حدوث درجة من الألتهاب المعوي أساسا من نوع العدلات، وارتفاعاته في حالات الجيارديا قد يكون مسببا لحدوث متلازمة لما بعد العدوى القولون العصبي.

وبشكل قاطع، فإن نتائج الدراسه الحاليه أوضحت أن علم الوراثة المضيف يكمن وراء قابلية الإنسان أو مقاومته لعدوى الجيارديا لامبليا وكذلك مظاهر التقلب السريري. وإلى جانب ذلك، فإن الكالبروتكتين البرازي يمكن استخدامه كعلامة لإالتهاب الأمعاء الناجم من عدوى الجيارديا لامبليا.

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أليات مستضد الطبقة الثانية لخلايا الدم البيضاء البشرية في الأطفال المصابين بمرض الجيارديا

رسالة مقدمه للحصول على درجة الدكتوراة فى العلوم الطبية الاساسية (الطفيليات الطبية)

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