بسم الله الرحمن الرحيم

Sudan University of Science and Technology

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Molecular Characterization and Serological Cut-off (Copro-Antigen ELISA) of *Entamoeba Histolytica* and *Giardia Lamblia* - Khartoum State

التوصيف الجزيئي والفرق المناعي (كوبرو أنتجين إليزا) للأميبا المحللة للنسيج والقارضيا لامبليا- ولاية الخرطوم

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الآيـــــة

بسم الله الرحمن الرحيم

قال تعالي:

﴿ إِنَّ فِي خَلْقِ السَّمَاوَاتِ وَالْأَرْضِ وَاخْتِلَافِ اللَّيْلِ وَالنَّهَارِ وَالْفُلْكِ الَّتِي تَجْرِي فِي الْبَحْرِ بِمَا يَنفَعُ النَّاسَ وَمَا أَنزَلَ اللَّهُ مِنَ السَّمَاءِ مِن مَّاءٍ فَأَحْيَا بِهِ الْأَرْضَ بَعْدَ مَوْتِهَا وَبَثَّ فِيهَا مِن كُلِّ دَابَّةٍ وَتَصْرِيفِ الرِّيَاحِ وَالسَّحَابِ الْمُسَخَّرِ بَيْنَ السَّمَاءِ وَالْأَرْضِ لَآيَاتٍ لِّقَوْمٍ يَعْقِلُونَ **(164)** ﴾

صدق الله العظيم

سورة البقرة

الاية (164)

Dedication

To the soul of my father

To my lovely mother

To my brothers

To my sisters

I dedicate this work.

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Firstly, I would like to express my thanks and gratitude to my supervisors, Prof. Mohamed Baha Aldeen Ahmed Saad and Dr. Tayseer Elamin Mohamed for their advice, guidance and support.

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Abstract

This study aimed to determine the molecular characterization and serological cut-off (Copro-Antigen ELISA) of *E. histolytica* and *G. lamblia* in Khartoum State. A cross-sectional study was conducted in period between August, 2014 to August, 2017. Epidemiological and parasitological data were obtained and recorded. 300 stool samples were collected from all participants, (100 infected with *E. histolytica*, 100 infected with *G. lamblia* and 100 healthy individuals as control group). All samples were diagnosed by formal ether technique as gold standard method. The results showed that, the point prevalence of *E. histolytica* and *G. lamblia* during data collection were (5.8, 7.2) respectively. The sensitivity and specificity of Copro antigen ELISA and PCR techniques against microscopy were (88%, 49%) for *E. histolytica* and (90, 42%) for giardiasis respectively. The clinical cut off Copro ELISA among Sudanese population of *E. histolytica* and *G. lamblia* of case study and control group were (0.98±0.20), (0.49±0.25) and (0.13±0.01), (0.07±0.08) respectively. The higher prevalence rate 45% occurred at eastern part of Khartoum State (*P* value= 0.04) among males group 55% at the age of 21-30 years (43.8%) *(P* value < 0.01). The study showed that, 80% of the infection occured as acute phase of the disease at $P < 0.01$ level. The study indicated that, the bioinformatics analysis results in 80-100% similarity together with variant alteration of 18s gene of *E. histolytica* EH strains and GDH gene for *G. lamblia on* NCBI with the following sequences ID: KT253454.1, KT253453.1, KJ870202.1, and: JF918446.1, JF918441.1 JF968194.1 respectively. 41 (84%) out of 49 positive PCR samples were single infection of *E. histolytica*; while 8 (16 %) out of 49 samples were co infection with *E. dispar*. When RFLP genotyping was performed, the most prevalent strain for *G. lamblia were* group AI (81%), group AII (14%) and group B(5%) respectively at (*P* value =0.01). The association of symptoms related to *G. lamblia* assemblage was found to be (81%) with Group AI sub genotype as virulence strain with acute phase of the disease, while (5%) of Group BIII sub genotype were strongly related to chronic diarrhea with very low prevalence rate at *P* value < 0.01.

الخلاصة

هدفت هذه الدراسة لتحديد التوصيف الجزيئي و التعرف على الفرق المناعي للاميبا المحللة للنسيج والقارضيا لامبليا في ولاية الخرطوم . الدراسة المستعرضة نفذت في الفترة من اغسطس 2014 حتي اغسطس 2017. البيانات الوبائية والطفيلية تم اخذها وتسجيلها. تم فحص 300 عينة تمثل مجتمع البحث ، منها 100 عينة إيجابية للاميبا المحللة للنسيج و 100 عينة إيجابية للقارضيا و 100 عينة كمجموعة كنترول مقارن وفقا للفحص المركز الذي يعتبر الطريقة الرسمية و القياسية المتبعةلاجراء التشخيص. اظهرت النتائج أن معدل انتشار الاميبا المحللة للنسيج و القارضيا خلال فترة جمع البيانات تساوي (5.8، 7.2) على التوالي. وكانت مستضدات كوبرو إليزا هي افضل طريقة للتشخيص مقارنة مع تقنيات الاحياء الجزيئية كمايلي: (88٪،٪ 49) للاميبا المحللة للنسيج و (% 90،٪ 42) للقارضيا على التوالي. متوسط الفرق المناعي السوداني لتشخيص الاميبا المحللة للنسيج يساوي (0.20±0.98)، اما في القارضيا كان الفرق المناعي يساوي (0.13 ± 0.01) على التوالي. أعلى معدل انتشار و تردد للمرضي يوجد في المنطقة الشرقية من ولاية الخرطوم بنسبة 45٪ (p <0.04) والذي ينحصر بصورة اعلي في مجموعة الذكور بنسبة 55٪ في الفئة العمرية 21-30 سنة (43.8٪) (p <0.0). اظهرت الدراسة ان 80٪ من الاصابات كانت من الحالات الحادة من المرض (P <0.01). واظهرت نتائج التحليل علي المركز الوطني لمعلومات التقانة الحيوية NCBI وجود تشابه بنسبة 80-100٪ مع وجود بعض التغيرات علي الجين 18S للاميبا المحللة للنسيج مع الجين HE بالتسلسلات التالية: تسلسل رقم: KT253454.1، KJ870202.1 KT253453.1. و للقارضيا مع الجين NADP بالتسلسلات رقم: JF918446.1، JF968194.1 JF918441.1 علي التوالي. كذلك عند استخدام التنميط الجينيRFLP، وجد ان اكثر انواع القارضيا انتشاراً من نوع A1 بنسبة (81٪) ،وهو الطراز العرقي الاكثر امراضاً بينما نوع A2 يمثل انتشار بنسبة (14٪) واقل الانواع انتشاراً هو BIII بنسبة 5٪ على التوالي في قيمة معنوية = 0.01 وهذا النوع يرتبط عادة بالإسهال المزمن مع معدل انتشار منخفض.

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CHAPTER ONE

Introduction

Chapter One

Introduction

Gastrointestinal infections are major causes of morbidity and mortality throughout the world and particularly in developing countries. While diarrhea does not typically cause serious complications for most patients, it can be a fatal ailment for young children and elderly, especially those who are malnourished or have compromised immune systems (WHO, 2014). Diarrhea is the passage of watery stool for more than three times in twenty-four hours caused by consumption of contaminated food or drinks by a variety of pathogens including viruses, bacteria, and parasites, and in this context the most important members of the latter group are *E. histolytica* and *G. lamblia* (Buzigi, 2015). Diarrhea can also be caused by intolerance to certain types of food or drugs and sometimes stress (Vargas *et al*., 2004). More than 90% of the deaths from infectious diseases worldwide are caused by only a handful of diseases. The World Health Organization ranks diarrheal disease as the second disease after acute respiratory infections, most common cause of morbidity and mortality in children in the developing world (WHO, 2013). Intestinal parasites are associated with serious clinical diseases, severe dehydration may lead to mortality and also are known to cause malnutrition and impairment of physical development in children and affect their growth and learning. The primary complication of diarrhea is dehydration caused by the loss of large amounts of water, salt and nutrients. Dehydration can lead to other serious conditions such as low blood pressure, seizures, kidney failure or even death (Clark, 2000). *G. lamblia* and *E. histolytica* are considered as two of the most important parasites of the gastrointestinal tract causing gastroenteritis in human population. They are eukaryotic protozoa cells distributed worldwide in nature and are receiving increasing attention as reservoirs and potential vectors for the transmission of pathogenic bacteria in the environment. Only limited information is available on these organisms in developing countries and so far no information on their presence is available from Sudan. They have a low infectious dose, are spread through feces contaminated food and water, have similar clinical presentations and are commonly found in areas that lack sanitation and clean water. They have comparable, simple life cycles that comprise a resistant, infectious cyst form and a fragile, disease-causing trophozoite, which are also the diagnostic stages. Another common feature called cryptic genetic variation, meaning that it is not possible to distinguish species or genotypes by morphological criteria (Clark, 2000).

Rationale:

Diarrheal disease constitutes a major cause of morbidity and mortality in children in the developing world. WHO ranks diarrheal disease as the second after acute respiratory tract infections. Also the most common illnesses in all age groups worldwide with high incidence rate about 1.7 to 5 billion cases of diarrhea occur per year. One of the etiological agents of the acute diarrhea is protozoa and in this context the most important members of the latter group are *E. histolytica* and *G. lamblia* (WHO, 2013). No research done or data base reported in Sudan about the molecular characterization and genotyping in relation to the severity of diarrhae. Also no data related to the real clinical cut-off level for those parasites among Sudanese population. Diagnosis of intestinal protozoa by traditional microscopy is the most widely method used throughout the world which is fast and cheap, but it's less sensitive (Verweiji *et al.*, 2004). Direct fluorescent antibody tests and enzyme-linked immunosorbent assays are still commonly confirmatory techniques used for their diagnosis (Weitzel *et al.*, 2006). The sensitivity of these methods may be inadequate for detecting low levels of infections (Verweiji *et al.*, 2003). Serologic antibody detection is insensitive early in disease and unable to distinguish active infection from previous exposure (Pillai DR, 1999). For the above mentioned reasons we are trying to use other immunodetection tests for identifying parasite antigens in clinical samples, highly specific Enzyme linked Immunosorbent Assay (ELISA) reagents using copro antigens can detect parasites antigen in stool samples with high sensitivity and specificity. Real-time PCR is the most sensitive and accurate method used for diagnosis. DNA isolation from stool can be processed in a solid phase and can be simultaneously amplified. The molecular diagnostic approach was merged with an alternative diagnostic strategy where clusters of patients with shared characteristics are routinely screened for a selected number of parasites species (Caballero, 1994). The purpose of this study is to design the Sudanese serological cut-off copro antigen ELISA and molecular characterization for *E.histolytica* and *G. lamblia* in Khartoum state which is considered as a new study in Sudan.

Objectives of the study:

General objective:

-To designate Sudanese Serological Cut-off (Copro-antigen ELISA) and molecular Characterization of *E. histolytica* and *G. lamblia* in Khartoum State.

Specific objectives:

1- To genotype *E. histolytica* and *G. lamblia* isolates from Sudanese population.

2- To investigate the correlation between *Entamoeba histolytica* and *Giardia lamblia* assemblages and clinical symptoms of the disease.

3- To compare the sensitivity and specificity of serological and molecular techniques against conventional methods.

4- To determine the intensity of infection with *Entamoeba histolytica* and *Giardia lamblia* according to age groups and gender.

CHAPTER TWO

Literature Review

Chapter Two

Literature review

2.1 Entamoeba histolytica:

2.1.1Transmission:

The *E. histolytica* parasite lives in the intestine of infected humans and can be released in a bowel movement of the intestine from an infected human and transmitted through oral route. *E. histolytica* is found in soil, food, water, or surfaces that have been contaminated with the faeces from infected humans (Kato *et al.,* 2013). Man can become infected after accidentally swallowing the infective mature cyst with four nuclei. Insects play a role in mechanical transmission. *E. histolytica* can be spread through contaminated food or recreational water. A limited outbreak of symptomatic intestinal and extraintestinal amoebiasis within a family complex was occured. The infection was almost certainly transmitted by a Philippino housemaid, who was an asymptomatic carrier of *E. histolytica* infection acquired in her native country. Starch-gel electrophoresis showed isoenzyme patterns characteristic of pathogenic zymodeme XIX in all the *Amoebic* isolates. (Mortimer *et al.*, 2014).

2.1.2 Life cycle and morphology of *Entamoeba*:

The life cycle of *E. histolytica* is comparatively simple, the resistant infective cysts, formed in the lumen of the large intestine, pass out in the faeces, and after an extracorporeal existence, are ingested by new host, infection occurs when cysts are transmitted by the fecal-oral route or through person to person contact. Few cysts are voided in acute dysentery, but they predominate in chronic infection and carrier's (Rivera *et al.,* 2010). The infectious dose is considered to be low, around 10 cysts, even though the only experimental transmission of *Entamoeba* that has been done used cysts of *Entamoeba coli* (Kato *et al.,* 2013). Excystation occurs in the small intestine, and the trophozoites that are released migrate to the large intestine, where they reproduce by binary fission. Encystation takes place in the colon, thus completing the life cycle by excretion of cysts in the stool. In cases involving diarrhea, trophozoites can be excreted as well, but they can survive for only a short time outside the body of the host. Infections that remain luminal are usually asymptomatic, and clinical amoebiasis occurs only when the trophozoites disrupt the mucosal barrier and penetrate the colon wall, which causes ulcers that lead to amoebic dysentry. Much less frequently, trophozoites are spread through the portal vein to the liver, and, very rarely, they even disseminate to other organs such as lungs and brain. Amoebic liver abscess (ALA) is more common in

adult men than in adult women, and the typical patient of 20–40 year old male with a 1–2 week history of fever and diffuse abdominal pain in the right upper quadrant (Carrero, 2007). Humans and occasionally non-human primates are the only natural hosts of *E. histolytica,* thus the zoonotic importance of this parasite is limited (Rivera *et al.,* 2010). Man is the principle host; other mammals are a negliable source of infection. Spontaneous natural infections with *Amoebic* indistinguishable from *E.histolytica* have been reported in monkeys, dogs and laboratory rodents (Ackers and Mirelman, 2006).

2.1.3 Epidemiology:

The actual incidence of ameobiasis throughout the world, especially in the temperate zone, remains unknown (Woodhall *et al.*, 2014). Surveys indicate that the incidence of infection varies from 0.2-50% and is directly correlated with sanitary conditions (Betancourt *et al.*, 2014). In travellers, *E. histolytica* and *G. lamblia* are the most frequent causes of intestinal protozoan infection (Al-Mekhlafi *et al.,* 2013). *E. histolytica* is estimated to infect about 50 million people worldwide. Previously, it was thought that 10% of the world population was infected, but these figures predate the recognition that at least 90% of these infections were due to a second species *E. dispar* (Woodhall *et al.,* 2014). Mammals become infected transiently, but are not thought to contribute significantly to transmission (Bakre *et al.,* 2009). The recognition of *E.dispar* as a separate non-pathogenic species means that the results of all previous prevalence studies based on microscopy were not reliable. It was realized that *E. dispar* gave rise to about 90% of 500 million new *Amoeba* infections occur each year (Woodhall *et al.*, 2014). It also became evident that, only one in four real *E. histolytica* infections progresses to disease (Ali *et al.*, 2008). All the same, amoebiasis does have a marked impact on global public health and has been estimated to cause around 40.000–100.000 deaths annually, making it one of the leading causes of mortality from a parasitic disease (Betancourt *et al.*, 2014). Many prevalence studies have been performed since the introduction of PCR methods and antigen tests that can distinguish between *E. histolytica* and *E. dispar*. The most wide spread occurrence of *E. histolytica* has been reported from certain countries in Latin America, Asia, and Africa, although local prevalence is highly variable (Ali *et al.*, 2008). However, even in countries such as South Africa and Mexico with a high prevalence of *E. histolytica*, *E. dispar* is responsible for a considerable portion of the infections (Betancourt *et al.,* 2014).

2.1.4 Pathophysiology of amoebiasis:

The major limitation one faces in studying pathogenesis is the lack of a satisfactory animal model which can duplicate the spectrum of human disease. Nonetheless several species have been used as animal models to study various aspects of pathogenesis (Husain *et al.*, 2010). For example, hamsters and gerbils are most commonly used as models for liver disease. Trophozoites produce lesions when injected directly into the liver of these animals. In vitro models are also available for studying various steps involved in pathogenesis (Ralston *et al.*, 2014). For example, adherences can be scored by using Chinese hamster ovary (CHO) cells, erythrocytes or bacteria. Lysis can be scored as percent cell culture monolayers disrupted. The number of erythrocyte digested per trophozoite can be used as a measure of phagocytosis or more experimental approaches have been taken to study the killing of target cells by *E. histolytica* trophozoites. The processes interactions which are thought to influence or implicated in pathogenesis (Ackers and Mirelman, 2006).In the gut, the trophozoites are constantly interacting with the intestinal flora. Studies have shown that trophozoites undergo changes on interacting with bacteria. Axenic *E. histolytica* which have lost virulence can regain it if associated with bacteria like *Escherichia coli, Salmonella typhosa* or *S. paratyphi.* Bacterial strains which do not attach to, and get ingested by trophozoites do not affect virulence (Haque *et al.*, 2003).Virulence of trophozoites of strain 200: NIH varied depending on culture associates. When cultured with NRS bacteria or rabbit intestinal flora, these trophozoites caused acute disease in animals but very little disease when cultured with *Trypanosoma cruzi.* Reassociation with rabbit flora returned their infectivity. Samie *et al*., (2006) showed that direct association of *E. histolytica* with viable bacteria was required for virulence. Heat killed or glutaraldehyde-fixed bacteria do not increase virulence.Soluble bacterial factors were not implicated (Haque *et al.*, 2003). *E. histolytica* exposed to live bacteria that are known to adhere *Amoeba* for 30 min, showed that increased in virulence invivo measurement; however, it appears that association with bacteria is not an absolute requirement for invasion by *E. histolytica.* Association of specific bacteria with *E. histolytica* could change the architecture of the cell surface leading to altered properties of the cell. Adherence to establish direct contact between trophozoite and target cell. Adherence of trophozoites to target cells is a necessary prerequisite for cytotoxicity. Evidence for this is provided by the following observations (Petri *et al.*, 1989). Cinemicrography of *Amoeba* interacting with CHO cells on a glass coverslip showed

that the CHO cells in direct contact with *Amoeba* displayed membrane blebbing and release from cover slip, while those not in direct contact, remained viable (Ralston *et al.*,2014). When CHO cells and trophozoites were mixed and incubated in the presence of high molecular weight dextran (10%), lysis did not occur as dextran prevented adherence of trophozoites to target cells (Ravdin and Guerrant, 1981). In another experiment, erythrocytes and trophozoites were mixed so as to allow adherence. Cells were centrifuged through a ficoll gradient. Trophozoites that banded on top of the gradient had not adhered to erythrocytes. These were found to be much less virulent in a hamster liver model. Adherence to CHO cells at 37°C is inhibited by cytochalasins B and D, implicating the need for intact *amoebic* microfilament function in the process (Stanley, 2003). Adherence is also inhibited by the Ca_{2+} channel blocker, Bepridil possibly by preventing intracellular Ca2+ flux which is thought to be necessary for microfilament function (Haque *et al.*, 2003). Two surface molecules responsible for adherence have been identified, one inhibitable by galactose or N-acetyl-D-galactosamine (GalNAc) (Reither *et al.*, 2007), and the other inhibitable by N- acetyl-D- glucosamine (GlcNAc) polymers (Kobiler and Mirelman, 1981). Pretreatment of *amoeba* with galactose or GalNAc inhibits adherence whereas pretreatment with neuraminic acid, maltose, mannose and GlcNAc has no effect (Betancourt *et al.*, 2014). The Gal/GalNAc inhibitable lectin of *E. histolytica* has been characterized in considerable detail. The following data suggest that this molecule plays an essential role in *amoebic* adherence to target cells like binding of trophozoites to CHO cells was inhibited 90-95% by 50 Mm galactose and GalNAc while other sugars had no effect (Johnston *et al.*, 2003). Also mutant of CHO cell defective in production of N- and O-linked galactose-terminal oligosaccharides was almost completely resistant to adherence; complex branched polysaccharides containing galactose groups at their termining were 1.000 fold more effective by weight than galactose in inhibiting adherence to CHO cells (Samie *et al.*, 2006). The lectin has a molecular weight of 260 kDa and dissociates into heavy (170 kDa) and light (35-31 kDa) subunits in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Kato *et al.*, 2013).Three genes (hgl1-3) encoding the 170 kDa subunit have been identified and characterized. Analyses of deduced amino acid sequences of the three genes indicate that this subunit of the lectin is a transmembrane protein. Northern blot analyses show that all the three genes are expressed in *E. histolytica* and the mRNAs were of the same size (4·0 kb) (Ryan and Ray, 2004). Two light subunit genes have also been identified

and characterized (McCoy *et al.,* 1993, Tannich *et al.,* 1992). These genes have hydrophobic amino- and carboxy-terminal signal sequences. The 31 kDa isoform of the light subunit has a putative glycosyl-phosphatidyl-inositol (GPI) anchor cleavage addition site while the 35 kDa isoform seems to lack it. Lectin heterodimers.The purified lectin showed at least two major heterodimers, one containing the170 KDa subunit with 35 kDa isoform and another 170 and 31 kDa isoform. Minor heterodimers with 160 and 150 kDa heavy subunit isoforms were also present (McCoy *et al.,* 1993). The native lectin probably exists as oligomers of 400 kDa and 660 kDa. Apart from its function in adherence, the lectin appears to mediate *amoebic* resistance to complement lysis. The lysis of target cells by release of toxins and introduction of membrane channels prior to mucosal invasion by *E. histolytica* cause depletion of mucous and disruption of epithelial barrier. Cytolysis of the target cell is thought to require *amoebic* microfilament function, Ca2+ flux and phospho-lipase A, among others. Microfilament function seems to be necessary because lysis is inhibited at 25°C, a temperature at which act in gelation ceases (Pollard, 1976), the optimal temperature being 37°C. Studies with the Ca2+ binding fluorescent dye FURA-2 showed 20-fold increase in intracellular Ca2+in target cells within seconds of direct contact. Actual cell death occurred 5-15 min after the lethal hit. Possible roles of Ca2+ are in contact dependent release of cytotoxic enzymes and toxins, cytoskeletal changes and activation of $Ca2^{+}$ dependent enzymes, for example, phospholipases. Bos (1979) proposed that *E. histolytica* has two ways of killing host cells, one is a rapid process occurring at close contact; other is slow, operating through soluble substances. Contact-dependent cytolethal effect of *E. histolytica* is not inhibited by serum but contact-independent effect is inhibited. Lushbaugh *et al*. (1978) showed that cell free extracts from axenically grown trophozoites caused cytopathic effect on cell cultures, in the absence of serum independently purified a "cytotoxic" substance from trophozoite extracts which caused cell rounding and release from monolayer (Ravdin *et al.,* 1985). The activity was associated with a protein (34-40 kDa) activated by thiols (Kato *et al*., 2013). It is believed that these thiol-proteases may be one of the molecules involved in pathogenesis (McKerrow *et al*., 1993). This is based on the fact that there seems to be a correlation between clinical severities with the level of thiol-protease in clinical isolates (Reed *et al.,* 1989). HM- 1: IMSS (more virulent of the two strains) has greater thiol protease activity than HK-9 strain (Lushbaugh *et al.,* 1989). Patients with invasive disease produce antibodies against this enzyme; those with non-invasive disease do not

(Reed *et al.,* 1989). The enzyme has broad substrate specificity. It can utilize case in, gelatin, insulin type I collagen, fibronectin and laminin as substrates (Kato *et al.*, 2013).It is a cathepsin B-like enzyme. Similar enzymes are found in extra cellular milieu of invasive tumour cells (Rosales *et al*., 2005). The protease may assist trophozoite to gain access to target cells by degrading the extra cellular matrix. A candidate for the toxin responsible for cytolysis may be a pore-forming peptide.Various *amoebic* pore-forming proteins (30, 14 and 5 kDa proteins) have been described by Loftus *et al*. (2005). A 30 kDa *Amoebic* protein was purified and shown to lyse erythrocytes and insert into and create pores in lipid bilayers. A 14 kDa pore forming protein was described as an ion-channel forming protein. Of these, the 5 kDa protein (*amoeba* pore) has been the best characterized (Rosales *et al*., 2005). The primary structure of the 5 kDa *Amoeba* pore from pathogenic *E. histolytica* was determined by sequencing the purified peptide and the corresponding cDNA. It is composed of 77 amino acids, including 6 cysteine residues. Like other membrane penetrating polypeptides, it too has an all α helical conformation. The cellular immune response of the host may contribute to destruction of the local host tissue. In hamster liver model recruitment of neutrophils is the initial host response to *E. histolytica* infection (Kato *et al*., 2013). Neutrophils are lysed when they come in contact with *E. histolytica* trophozoites releasing toxic products which lyse distant hepatocytes (Magdalena *et al*., 2015). Leukocytes have the potential to lyse *E. histolytica* trophozoites and vice versa. *E. histolytica* is cytolytic to human leukocytes on contact. Only virulent *amoeba* can lyse polymorphonuclear leukocytes (PMNs) and lysis is blocked by GalNAc. At a ratio of 1000 PMNs per *amoeba*, trophozoites of the highly virulent strain HM-1: IMSS were not killed but those of the less virulent strain 303 were killed (Kato *et al*., 2013). At a ratio of 100 PMNs per-*amoeba*, HM- 1: IMSS trophozoites killed a high percentage of PMNs while killing was less with 303 trophozoites. *E. histolytica* could kill macrophages and T lymphocytes invitro. Conversely, macrophages activated with concanavalin A could kill *Amoeba*. T lymphocytes from immune individuals, following incubation with *amoebic* antigen, were capable of killing *E. histolytica* trophozoites (Salata and Ravdin, 1986). Phagocytosis trophozoites from stools of many invasive patients contain ingested erythrocytes and have much higher rate of erythrophagocytosis than healthy human carrier. Phagocytosis of mammalian tissue culture grown cells was observed by transmission electron microscopy. Cells with intact plasma membrane were phagocytosed, showing that prior cell lysis was not

required for endocytosis (Magdalena *et al*., 2015). A phagocytosis deficient mutant of *E. histolytica* has been isolated by (Orozco *et al.* (2003). This mutant part from being poor in phagocytosis was also found to be low in virulence, when tested in the hamster liver model. Thus there seems to be a correlation between phagocytosis and virulence (Magdalena *et al*., 2015).

2.1.5 Diagnosis:

The outcome of any diagnostic procedure depends largely on the methods used for sample collection, preservation, and different approaches are necessary for the two diagnostic stages of *Entamoeba* species, the cyst and the trophozoite. A formed stool is likely to contain cysts, whereas a watery or dysenteric stool will usually harbor trophozoites (Mahmood *et al*., 2014). A fresh stool specimen obtained directly from the patient is seldom available but is essential if the aim is to detect mobile trophozoites. Fecal samples are often forwarded to the diagnostic laboratory, thus it is necessary to use fixatives to preserve the trophozoites (Parameshwarappa *et al*., 2012).

2.1.5.1 Microscopy:

For over 100 years, microscopy remained the only method for diagnosing intestinal *Entamoeba* infection, and even though it cannot differentiate between *E. histolytica* and *E. dispar*, it is still the technique of choice in many parasitology laboratories worldwide. In light of our present knowledge, microscopy must be considered as a screening method for the *E. histolytica*/*E. dispar* complex and not as a technique to confirm the diagnosis of *E. histolytica* (AL-Kubaisy *et al*., 2014).

2.1.5 .2 Direct wet smears:

Direct microscopy of a wet smear requires a minimum of equipment and it is probably the most widely used method for diagnosing intestinal protozoa throughout the world. It is fast and cheap, but its outcome depends entirely on the skills of the microscopist (Mahmood et *al*., 2014). A crude fecal sample contains numerous structures such as undigested food particles and human cells, which might be mis-interpreted as protozoan cysts or trophozoites. Nonetheless, if interpreted correctly, microscopy is the simplest technique for diagnosing an invasive intestinal *amoeba* infection, because findings of *Entamoeba* trophozoites with ingested erythrocytes strongly indicate *E. histolytica*. This is, however, rarely seen in a setting such as Sweden, where most of the *Entamoeba* patients harbor either only cysts or trophozoites without erythrocytes (Parameshwarappa *et al*., 2012).

2.1.5 .3 Concentration techniques:

In all parasitology laboratories, the standard method for detecting fecal parasites is more or less modified form of the formal ether concentration technique that was developed by Ritchie in 1948. The method has been changed very little since that time, except that ether has been replaced by ethyl acetate (Young *et al.*, 1979), and sodium acetate-acetic acid-formalin (SAF) may be used instead of formalin as the fixative (Arora, 2010). With this technique, *Amoeba* cysts are concentrated, whereas trophozoites are found only occasionally (AL-Kubaisy *et al.*, 2014).

2.1.5.4 Permanent staining techniques:

The most commonly used permanent staining techniques for cyst and trophozoite identification are hematoxylin, trichrome staining of fixed feces (Van Gool *et al.*, 2003). American literature recommends that a permanent staining should be included in all parasite and ova examinations, whereas traditions in Europe have been more directed towards cyst identification after concentration techniques. However, the rediscovery of *Dientamoeba fragilis,* a diarrhea-related protozoan that lacks a cyst form, has prompted the use of permanent staining methods in Europe (Schuster and Jackson, 2009).

2.1.6 Extra-intestinal amoebiasis:

Few patients with extraintestinal amoebiasis have *E. histolytica* in their stool samples (Fotedar *et al.*, 2007). Thus other methods must be used for diagnosis in such cases. Antibody detection is the preferred laboratory technique in that context, and many different serology methods have been developed (Verweij *et al.*, 2001). The most widely used is enzyme linked immunosorbent assay (ELISA), for which a recent study in Bangladesh indicated a sensitivity of 96% for ALA patients but only 46% for *amoebic* colitis patients (Haque *et al.*, 2010). When using aspirated abscess material to diagnose *E. histolytica* infection, both conventional and real-time PCR have proven to be valuable methods (Zaman *et al.*, 2000; Othman *et al.*, 2010). However, sample from invasive patient should be avoided due to the risk of bacterial super infection or spillage of abscess contents, and they should be used only if absolutely necessary to confirm a diagnosis (Pritt and Clark, 2008). Haque and colleagues have observed that the combined results of real-time PCR performed on the non-invasive fluids, urine and saliva reached a sensitivity of 97% for ALA and 89% for *Amoebic* colitis. This is a promising new approach for diagnosis of ALA, especially in endemic areas where

serology is of limited value due to the difficulty of distinguishing between past and present infections (Haque *et al*., 2010).

2.1.7 Differentiation of *Entamoeba* species:

The zymodeme technique was the first procedure used to characterize different *Entamoeba* isolates and was long regarded as the gold standard for this purpose. However, it requires the use of cultured *amoeba* trophozoites, and it is tedious and time consuming to perform (González *et al*., 1994). Furthermore, the sensitivity is low, because many samples that are positive by microscopy are culture negative. Although the zymodeme method is not currently used for routine species identification, it did play a major role in the early differentiation of *E. histolytica* and *E. dispar* (Sargeaunt *et al*., 1978). Specific detection of *E.histolytica* can be achieved by employing commercially available antigen detection kits, such as *Entamoeba* CELISA PATH and Tech Lab *E. histolytica* II, both of which use monoclonal antibodies against the same target: the Gal/GalNAc specific lectin (adhesin molecule) of *E. histolytica*. Several studies carried out in Bangladesh, an area with high *E. histolytica* transmission, have shown that antigen detection has a high sensitivity, equal to PCR (Haque *et al*., 1998), whereas investigations in non-endemic areas have demonstrated a poor sensitivity for antigen detection compared to PCR (Gonin and Trudel, 2003; Stark *et al*., 2008). This difference in test results between endemic and non-endemic areas is not well understood, although it might reflect the fact that PCR is 100–1000 times more sensitive in detection of trophozoites than antigen tests (Stark *et al*., 2008). The conclusion so far is that the antigen testing which is both rapid and technically simple, is appropriate in areas with high prevalence of *E. histolytica* but is not useful in settings where there are few cases of *E. histolytica* infection (Gonin and Trudel, 2003; Visser *et al*., 2006; Stark *et al*., 2008). The first *Entamoeba* PCR assays were performed on cultured trophozoites, thus DNA isolation was a minor challenge compared to the situation today, when DNA usually is extracted directly from stool samples. It is known that feces contain several PCR inhibitors (Abu Al-Soud and Radstrom, 2000), most of which should be removed by an optimal extraction procedure. Commercial spin columns (QIAampTM DNA mini kit or QIAampTM DNA stool mini kit) are among the most widely used devices for extraction of *Entamoeba* DNA directly from stool samples (Verweij *et al*., 2000; Gonin and Trudel, 2003). The extraction procedures often include initial cyst disrupting steps such as freezing and thawing or boiling. Nonetheless, manual extraction is time consuming and inconvenient when analyzing a

large number of samples. Since molecular biology methods are becoming part of routine diagnostic techniques for detecting intestinal parasites, use of automatic extraction procedures is increasing (Bruijnesteijn *et al*., 2009; Calderaro *et al*., 2010), although there is a lack of studies comparing manual and automatic extraction of stool parasites. The earliest methods for differentiating *E. histolytica* and *E. dispar* included two single PCRs that target either the small subunit ribosomal RNA (*ssrRNA*) gene (Clark and Diamond, 1991) or the gene encoding peroxiredoxin (a 30-kDa protein) (Tachibana and Ihara, 1991). According to a very informative review published by Fotedar *et al,* (2007), these techniques have been the most commonly used worldwide. Several more recent protocols for detection and differentiation of *E. histolytica* and *E. dispar* are now available, which include duplex PCR, multiplex PCR, nested PCR, and real-time PCR. In addition, a number of procedures for detecting *E. moskhovskii* have been described (Ali *et al*., 2003; Hamzah *et al*., 2006b; Mojarad *et al*., 2010). *Entamoeba* PCR assays target different loci, but the *ssrRNA* gene has been used most often for all *Entamoeba* species and, due to its multi-copy nature, the analytical sensitivity is usually high. Sargeaunt (1987) observed very few mixed infections while performing isoenzyme electrophoresis on cultured trophozoites from thousands of *Entamoeba* isolates from many different settings. A plausible reason for this is that, in culture, one species may easily outgrow the other, as has been demonstrated in a model system using invitro co-cultures and PCR detection (Pysova *et al*., 2009; Verweij *et al*., 2003); in which *E. dispar* trophozoites outgrow *E. histolytica* within 48 hours in all assays. It remains to be determined whether the same is true for natural co-infections. PCR performed on DNA extracted directly from stool samples has indicated that mixed infections seem to occur mainly in endemic areas (Ramos *et al*., 2000; Nunez *et al*. 2001) and are rarely reported in European settings, although most *E. histolytica* or *E. dispar* infections that are diagnosed in Europe are acquired in endemic areas. Investigations of genetic variation in coding and non-coding regions of different *E. histolytica* isolates might explain why some infections remain asymptomatic whereas others do not (Ali *et al*., 2008). In a study performed in Bangladesh, different genotypes were found in isolates from various patient groups (asymptomatic or suffering from colitis or ALA). Moreover, genotyping used in the investigation of a sexually transmitted outbreak of severe amoebiasis in Canada suggested that the patients involved were infected with a highly virulent genotype that differed from simultaneously examined strains that caused less severe infections (Salit *et al*., 2009). It

has also been observed that paired isolates from stool samples and liver abscesses from the same patients differed genetically which implies that the patients in that study were either infected with mixed genotypes, or that some kind of DNA reorganization had taken place in the intestine or liver (Ali *et al.*, 2008).

2.1.8 Treatment:

The WHO recommends that *E. histolytica/E. dispar* should be differentiated when-ever possible and that patients should not be treated based on microscopy findings alone. Yet, regardless of symptoms, all cases identified as *E. histolytica* should be treated due to the risk of invasive disease, whereas cases found to involve only *E. dispar* should not be treated. If a patient with *E. dispar* (or *E. moshkowskii*) has intestinal symptoms, a search should be made for other causes of disease. Asymptomatic *E. histolytica* infection should be treated with a luminal amoebicide (diloxanide furoate or paromomycin), and invasive intestinal or extra-intestinal amoebiasis should be handled by administering a tissue amoebicide (metronidazole) followed by luminal treatment (WHO, 2014).

2.2 Giardiasis:

2.2.1 Transmission:

Giardiasis is the disease of all ages; however, its prevalence is higher in children.*Giardia* infection can occur through ingestion of dormant microbial cysts in contaminated water, food, or by the faecal-oral route (through poor hygiene practices). The cyst can survive for weeks to months in cold water,so can be present in contaminated wells and water systems, especially stagnant water sources, such as naturally occurring ponds, storm water storage systems, and even clean-looking mountain streams (Ghosh *et al.,* 2001*)*. They may also occur in city reservoirs and persist after water treatment, as the cysts are resistant to conventional water treatment methods, such as chlorination and ozonolysis. Zoonotic transmission is also possible, so *Giardia* infection is a concern for people camping in the wilderness or swimming in contaminated streams or lakes, especially the artificial lakes formed by beaver dams (popular name for giardiasis, "beaver fever"). In addition to water-borne sources, fecal-oral transmission can also occur, for example in day-care centers, where children may have poor hygiene practices (Huang and White, 2006).

2.2.2 Life cycle:

Giardia has a simple life cycle that comprises a resistant infective cyst stage and a mobile disease-causing trophozoite. The cysts are transmitted via water, food, or person-to-person contact, and it has been estimated that 10–25 cysts are sufficient to achieve infection (Paniker, 2002). A cyst is oval in shape, measures 8–12 µm x 7–10 µm, and has four nuclei. The nuclei are hardly visible in unstained preparations but are distinct after trichrome or DAPI (4'-6-diamidino-2-phenylindole) staining. Excystation is triggered by exposure to the gastric acid in the host's stomach and continues in the small intestine. This process starts with a shortlived excyzoite, which divides twice and gives rise to four trophozoites that measure 5–9 µm x 12–15 µm and have two nuclei, eight flagella, and a median body with unknown function. The living trophozoite moves in a very characteristic way, like a falling leaf, and this feature is used as a diagnostic criterion to discriminate *Giardia* trophozoites from other protozoan flagellates in fresh stool samples. The trophozoites are very photogenic in Giemsa-stained preparations, appearing as a face in which the two nuclei form the eyes and the median body constitutes the smile. The trophozoites are either free or become attached to the intestinal epithelium via an adhesive disc. Encystation into new cysts takes place in the lower part of the small intestine, and the excreted cysts, which are immediately infectious, are also resistant to environmental factors and can survive for a long time under favorable conditions (Ankarklev *et al*., 2010).

2.2.3 Pathophysiology of giardiasis:

Once excystation occurs, *Giardia* trophozoites use their flagella to swim to the microvillus covered surface of the duodenum and jejunum, where they attach to enterocytes using a special disk located on their ventral surface (Faubert, 2000). In addition, lectinson the surface of *Giardia* bind to sugars on the surface of enterocytes (Ghosh *et al*., 2001). The attachment process damages microvilli, which interferes with nutrient absorption (Buret *et al*., 1992). Rapid multiplication of trophozoites eventually creates a physical barrier between the enterocytes and the intestinal lumen (Dagci *et al.,* 2002). Further interfering with nutrient absorption (Faubert, 2000). This process leads to enterocyte damage, villus atrophy, crypt hyperplasia and intestinal hyperpermeability (Chin *et al*. 2002). The brush border damage that causes a reduction in disaccharidase enzyme secretion (Nain *et al*., 2002). Recent research also demonstrates the presence of cytopathic substances, such as glycoproteins, proteinases and lectins that may cause direct damage to the intestinal mucosa (Kaur *et al*., 2001). Trophozoites do not usually

penetrate the epithelium, invade surrounding tissues, or enter the bloodstream (Jimenez *et al.,* 2000). Thus, infection is generally contained within the intestinal lumen (Faubert, 2000). Interestingly, the mechanism leading to *Giardia* induced diarrhea has not been fully characterized, although one or a combination of the following factors is believed to be involved a glycoprotein located on the surface of *G. lamblia* trophozoites has been demonstrated to induce fluid accumulation in ligated ileal loops in rabbits giardiasis results in decreased jejunal electrolyte, water and 3-O-methyl-Dglucose absorption, thus leading to electrolyte, solute and fluid malabsorption (Buret *et al*,. 1992). Damage to the intestinal brush border and the corresponding decrease in disaccharidase activity may lead to increased quantities of disaccharides in the intestinal lumen, which can result in osmotic diarrhea (Nain *et al*., 2002). *Giardia* infection in gerbils accelerates intestinal transit time and increases smooth muscle contractility, both of which may play a role in *giardial* diarrhea (Adam, 2001).

2.2.4 Epidemiology:

G*iardia* is a parasite found in all parts of the world and in a large number of mammals, including humans, livestock, pets, wildlife, and aquatic animals (Thompson, 2000; Nesselquist *et al*., 2010). Several recent reports have also described *G. lamblia* in various birds and even fish, although true infections remain to be confirmed in these animals (Yang *et al*., 2010). The prevalence of *Giardia* in humans varies in and between countries, and it is higher in areas where environmental hygiene is low. According to estimates from the WHO about 200 million people have symptomatic giardiasis, and around 500,000 new cases occur each year (WHO, 2013). Different studies in European countries have indicated that prevalence of giardiasis about 1–17%, and up to 100% of the population can be infected in certain highly endemic areas (Plutzer *et al*., 2010). High prevalence rate of giardiasis about 200 million people in Asia, Africa and Latin America with 280 million infections per year (Gatti *et al*., 2002, Roy *et al*., 2005). Epidemiological studies have shown that most cases of parasitic diarrhoea in children are due to *G. lamblia* infection, especially in areas with poor sanitation (Visser *et al*., 2006).

2.2.5 Diagnosis:

2.2.5.1 Microscopy:

Microscopic detection of *Giardia* cysts in a stool specimen, either directly in a wet smear or after formol-ethyl acetate concentration, is the most frequently used method for diagnosis of giardiasis worldwide. Less often, diagnosis is based on detection of

trophozoites in fresh stool samples or SAF-fixed material. Compared to identification of *Entamoeba* spp., microscopy of *Giardia* cysts and trophozoites is more straightforward, and there is little risk of confusion with other parasites. Moreover, only ghost cysts with an empty appearance are sometimes not recognized as *Giardia* parasites. However, the sensitivity of microscopy is quite low due to the intermittent excretion of *Giardia* cysts, and thus it is recommended that at least three samples be examined in order to rule out giardiasis (Jaco *et al*., 2011).

2.2.5.2 Antigen detection methods:

Quite a few commercial kits are available for detection of *Giardia* antigen. Two techniques that are often used are enzyme-linked immune-sorbent assay (ELISA) that assesses soluble antigens and a direct fluorescent antibody (DFA) test that detects intact organisms. Several studies have shown that these two methods offer greater sensitivity compared to light microscopy (Garcia and Shimizu, 1997), but they are not available in all parasitology laboratories due to the high cost and substantial workload they entail, and also limited access to the required equipment. An alternative technique involves a solid-phase immune chromatographic test card system (Immuno Card STAT *Cryptosporidium/ Giardia* rapid assay), which allows concurrent detection of *Cryptosporidium* and is also fast, easy to use, and does not require extra equipment (Johnston *et al*., 2003). Unfortunately, the sensitivity of such a system is lower than microscopy, and thus this test is not recommended for follow-up of patients with *Giardia* treatment failure (Strand *et al*., 2008).

2.2.5.3 PCR:

Conventional single or nested PCR analyses are not often performed for diagnosing giardiasis, except at specialized centers. In contrast, there is increasing use of real-time PCR as a diagnostic tool that can detect *Giardia*, often in combination with other enteric protozoa, such as *Cryptosporidium* spp.*, E. histolytica* and *D. fragilis*. This approach has proven to provide higher sensitivity compared to conventional methods, and it also entails a lower workload However, in the parts of the world where giardiasis is highly endemic, microscopy will probably remain the routine procedure for detecting stool parasites, including *Giardia*, for a long time (Calderaro *et al*., 2010).

2.2.6 Treatment:

The most common antibiotics used for the treatment of giardiasis are the 5-Nitroimidazoles (5-NIs); these include metronidazole, tinidazole, secnidazole and ornidazole, of which metronidazole is the most common (Nash *et al*., 2001).

Alternative agents which are less commonly used in giardiasis treatment are quinacrine, furazolidone, benzimidazoles (albendazole and mebendazole), paromomycin, bacitracin zinc, chloroquine and nitazoxanide (Lopez *et al*., 2010). Depending on local epidemiology, availability, and cost, these drugs have been widely available for the curative treatment of cases; however, several cases of treatment failure have been reported (Munoz *et al*., 2013). With the advent of newer agents which might have similar efficacies as 5-NIs,and also offer an added advantage of more simplified regimens, fewer adverse effects or less drug resistance, it is of considerable interest to determine whether 5-NIs are still the best available option in the treatment of giardiasis (Lopez *et al*., 2010). Different factors may shape how effective a drug regimen will be, including medical history, nutritional status, and condition of the immune system (Munoz *et al*., 2013, Lofmark *et al.,* 2010). This wide spectrum usage of 5-NIs could have led to an increased occurrence of *G. lamblia* resistance; in fact, *Giardia* resistance towards common antigiardials has been demonstrated or induced in vitro and also cross-resistance between metronidazole and tinidazole has been demonstrated (Arguello *et al*., 2009).

2.2.7 Prevention and control:

The prevention of amoebiasis and giardiasis hinges upon the proper sanitation of water sources and the avoidance of fecal-oral exposures. Effective preventive measures include the adequate treatment of water for consumption and appropriate sanitary practices such as hand washing, the proper disposal and handling of human and animal waste, and not allowing children with diarrhea to participate in recreational water activities. Hand washing for the prevention from any fecal-oral pathogen is a universal precaution and should be performed regularly after handling soil, diapers, animal feces, or garbage; treating a wound; or going to the bathroom (Coles *et al*., 2009). Surface water for drinking should be boiled at a rolling boil for 1 min or filtered with an approved water filtration device with a National Safety Foundation Standard 53 or Standard 58 rating for cyst reduction. Fresh fruits and vegetables should be adequately washed prior to consumption. Travelers in areas where water treatment capabilities are unknown should avoid consuming water or ice in drinks and drink only bottled beverages. In dogs, prevention depends upon the prompt removal of fecal material, preventing dogs from consuming contaminated surface water or feces, and the disinfection and cleaning of kennels. The disinfection of kennels can be accomplished with 1% sodium hypochlorite (20% commercial bleach), 2% glutaraldehyde, or

quaternary ammonium compounds. Cysts are relatively resistant to chlorination; levels of chlorine in drinking water are inadequate to inactivate cysts. (Coles *et al*., 2009).

2.3 Theory of techniques:

2.3.1 Stool examination:

Stool examination is carried out in laboratories for various diagnostic purposes. Mostly fresh faecal specimen in a clean container which does not contain any detergent or disinfectant is sufficient for all types of stool examinations including stool culture. In physical examination of stool, the consistency of normal stools iswell formed (AL-Kubaisy *et al*., 2014). In diarrhea and dysentery, the stools are semi solid or watery in nature. In malabsorption states also the stool will be semi solid or watery depending on the severity of the disease. In cases of malabsorption of fats the stools are pale bulky and semi solid. Normal stools are light to dark brown in colour due to the presence of stercobilinogen which is a product of bilirubin metabolism. In cases with bleeding into the intestinal tract the stools become dark tarry in nature due to the formation of acid hematin if the bleeding within the small intestines. If bleeding in the large intestine or rectum, the blood may be bright red. In cholera, the stools have a rice water appearance as there is no fecal matter and there is presence of flakes of epithelial cells in it. In biliary tract obstruction, the stools may be clay coloured due to absence of sterobilinogen. Pateints diet may also lead to alteration in the colour of the stools (Arora, 2010). For instance if the patient had spinach earlier, the stools may be green in colour. In those who had a barium examination the stools may be white in colour. The fecal odour of stools may become offensive in conditions like intestinal amoebiasis. In cases of bacillary dysentery and cholera the stools are not foul smelling due to the absence of fecal matter.Blood should be noted in stools if present as it is indicative of ulceration or presence of any other pathology like malignancy (AL-Kubaisy *et al*., 2014). It should also be noted if the blood is bright red or is altered in colour as it may be a clue to the site of pathology in the intestinal tract.Mucus is present in certain conditions like Giardiasis; only mucus known as steatorrhea or in amoebic and bacillary dysentery.Stools may contain adult helminthes. Nematodes like *Ascaris* are easily visible as their size is large. *Hook* worms and proglotids of cetodes may also be present. These may be visible to the naked eye. The Microscopic laboratory diagnosis of most parasitic infections is by the demonstration of ova of the parasite in the stools of the infected person (Arora, 2010).

2.3.1.1 Saline wet mount examination:

The stool is emulsified in normal saline to allow study the parasite shape and motility. This is then examined under a light microscope. It is preferable to keep the condenser down and the intensity of the light low for proper visualization of the ova and cysts. The thickness of the film should be such that one is able to see the printed letters of the newspaper through it (AL-Kubaisy *et al.*, 2014). Iodine preparation leads to better visualization of morphological details of ova and cysts as it stains the glycogen in them. It however has the disadvantage that the live trophozoites of *Entamoeba histolytica* cannot be seen as the iodine kills it. One gram of iodine and two gram of potassium iodide is mixed in 100 ml of distilled water. Potassium iodide is mixed in water and then the iodine crystals are added and it is shaken vigorously. The solution is then filtered into a dark glass bottle and kept away from light (Arora, 2010).

2.3.1.2 Faecal concentration techniques:

The need for faecal concentration techniques has been discussed at the beginning of this subunit. Thefollowing techniques are commonly used to concentrate faecal parasites in district laboratories: Sedimentation techniques in which parasites are sedimented by gravity or centrifugal force, e.g. formol ether concentration method which is the most frequently used technique because it concentrates a wide range of parasites with minimum damage to their morphology. The other technique is the floatation methods in which parasites are concentrated by being floated in solutions of high specific gravity (Arora, 2010).

2.3.1.3 Formal ether concentration technique:

This is recommended for use in district laboratories because it is rapid and can be used to concentrate a wide range of faecal parasites from fresh or preserved faeces. Eggs that do not concentrate well by this technique are those of *Fasciola* species and *Vampirolepis nana* but concentration of these parasites is not usually required. Risk of laboratory acquired infection from faecal pathogens is minimized because organisms are killed by the formalin solution (AL-Kubaisy *et al.*, 2014). The technique requires the use of highly flammable ether or less flammable ethyl acetate. When concentrating the oocysts of *coccidia*, an additional centrifugation stage is required. In the widley modified method, faeces are emulsified in formol water, the suspension is strained to remove large faecal particles, ether is added, and the mixed suspension is centrifuged (Coles *et al.*, 2009). Cysts, oocysts, eggs, and larvae are fixed and sedimented while the faecal debris is separated in a layer between the ether and the formal water. Faecal fat is

dissolved in the ether. Prepare by mixing 50 ml of strong formaldehyde solution with 450 ml of distilled water. Diethyl ether is a highly flammable and volatile chemical and therefore its replacement by a less flammable chemical such as ethyl acetate or acetone is recommended by some workers. When using ethyl acetate, greater care needs to be taken when discarding the faecal debris layer to prevent remixing with the sediment. Insoluble particles of ethyl acetate may form under the cover glass. In some countries ethyl acetate is difficult to obtain. It has an unpleasant odour. Strainer, sieve with small holes, preferably 400–450 nm in size (Arora, 2010).

2.3.2 Enzyme linked immunosorbent assay (ELISA):

It's a commonly used technique for the determination of known analytes for food allergens, pesticides, herbicides, PCBs and veterinary drug residues. ELISAS are routinely used in scientific research, veterinary medicine, environmental and agricultural applications, and in healthcare (Aikyo and Oh, 2014). The fundamental principle of the ELISA is that the target analyte (the antigen) is recognised with high specificity by antibodies, which are proteins produced by the immune system. The immune system of animals produces antibodies in response to the presence of antigens. These antibodies can recognise and bind to the antigens, the labelling of the bound antibody forms the basis of the detection.The history of the ELISA was developed in the 1960s independently at the same time by two research groups; Peter Perlmann and Eva Engvall at Stockholm University, and the Dutch research group of Anton Schuurs and Bauke van Weemen. The assay was based on the underlying principles of conventional radio-immunoassay, with the key difference that the antibodies are labelled with an enzyme, rather than radio-isotopes. The sandwich ELISA measures the amount of antigen between two layers of antibodies (i.e. capture and detection antibody) (Aikyo and Oh, 2014). The antigen to be measured must contain at least two antigenic sites capable of binding to antibody, since at least two antibodies act in the sandwich. Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in Sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible. The advantage of Sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive (up to 2 to 5 times more sensitive than direct or indirect. This ensures the antibodies are detecting different epitopes on the target protein so they do not interfere with the other antibody binding.

Therefore, we are unable to guarantee our antibodies in sandwich Elisa unless they have been specifically tested for sandwich ELISA (Aikyo and Oh, 2014).

2.3.2.1 Coating with capture antibody:

Coat the wells of a PVC microtiter plate with the capture antibody at a concentration of 1-10 μg/ml in carbonatebicarbonate buffer (PH9.6). If an unpurified antibody is used (eg ascites fluid or antiserum), you may need to compensate for the lower amount of specific antibody by increasing the concentration of the sample protein (try 10ug/ml). Cover the plate with an adhesive plastic and incubate overnight at 4°C. Remove the coating solution and wash the plate twice by filling the wells with 200 μl PBS. The solutions are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel (Aikyo and Oh, 2014).

2.3.2.2 Blocking and adding samples:

Block the remaining proteinbinding sites in the coated wells by adding 200 μl blocking buffer, 5% non fat dry milk/PBS, per well. Cover the plate with an adhesive plastic and incubate for at least 1-2 h at room temperature or, if more convenient, overnight at 4°C. Add 100 μl of appropriately diluted samples to each well. For accurate quantitative results, always compare signal of unknown samples against those of a standard curve. Standards (duplicates or triplicates) and blank must be run with each plate to ensure accuracy. Incubate for 90 min at 37°C. For quantification, the concentration of the standard used should span the most dynamic detection range of antibody binding. You may need to optimize the concentration range to ensure you obtain a suitable standard curve. For accurate quantitation, always run samples and standard in duplicate or triplicate. Remove the samples and wash the plate twice by filling the wells with 200 μl PBS (Alberts *et al.*, 2008).

2.3.2.3 Incubation with detection antibody and then secondary antibody:

Add 100 μl of diluted detection antibody to each well.Ensure the secondary detection antibody recognizes a different epitope on the target protein than the coating antibody. This prevents interference with the antibody binding and ensures the epitope for the second antibody is available for binding. Cover the plate with an adhesive plastic; incubate for 2 h at room temperature. Wash the plate four times with PBS. Add 100 μl of secondary antibody conjugated, diluted at the optimal concentration in blocking buffer immediately before use. Cover the plate with an adhesive plastic; incubate for 1-2 h at room temperature. Wash the plate four times with PBS (Alberts *et al.*, 2008).

2.3. 2.4 Detection:

Although many different types of enzymes have been used for detection, horse radish peroxidase (HRP) and alkaline phosphatase (ALP) are the two most widely used enzymes employed in ELISA assay. It is important to consider the fact that some biological materials have high levels of endogenous enzyme activity (such as high ALP in alveolar cells, high peroxidase in red blood cells), and this may result in a nonspecific signal. If necessary, perform an additional blocking treatment with Levamisol (for ALP) or with 0.3% solution of H_2O_2 in methanol for peroxidase (Alberts *et al.*, 2008).

2.3.2.5 ALP substrate:

For most applications pNPP (p-Nitrophenyl-phosphate) is the most widely used substrate.The yellow colour of nitrophenol can be measured at 405 nm after 15-30 min incubation at room temperature. This reaction can be stopped by adding equal volume of 0.75 M NaOH (Aikyo and Oh, 2014).

2.3.2.6 HRP chromogenes:

The substrate for HRP is hydrogen peroxide. Cleavage of hydrogen peroxide is coupled to oxidation of a hydrogen donor which changes colour during reaction. TMB (3,3',5,5'-tetra-methyl-benzidine) solution is added to each well, incubate for 15-30 min, equal volume of stopping solution (2 M H_2SO4) is added and the optical density is read at 450 nm. OPD (o-phenylene-diamine dihydro-chloride) the end product is measured at 492 nm. As the substrate is light sensitive so it is kept and stored in the dark. ABTS (2, 2-azino-di- [3-ethyl-benzo- thiazoline-6 sulfonic acid] diammonium salt). The end product is green and the optical density can be measured at 416 nm. Some enzyme substrates are considered hazardous (potential carcinogens), therefore is always handled with care and wearing gloves. 100 µl (or 50 µl) of the substrate solution were dispensed per well with a multichannel pipet (Alberts *et al.*, 2008).

2.3.3 Thermal cyclers:

The first PCRs were performed using multiple water baths or heat blocks set at the required temperatures for each of the steps. The tubes were moved from one temperature to another by hand. In addition, before the discovery of thermostable enzymes, new enzyme had to be added after each denaturation step, further slowing the procedure and increasing the chance of error and contamination. Automation of this tedious process was greatly facilitated by the availability of the heat stable enzymes (Obeid *et al.,* 2003).To accomplish the PCR, then, an instrument must only manage

temperature according to a scheduled amplification program. Thermocyclers were thus designed to rapidly and automatically ramp or change through the required incubation temperatures, holding at each one for designated periods (Klaschik *et al*., 2002). Early versions of thermal cyclers were designed as heater/coolers with programmable memory to accept the appropriate reaction conditions. Compared with modern models, the available memory for recording the reaction conditions was limited, and sample capacity was small. Wax or oil (vapor barriers) had to be added to the reactions to prevent condensation of the sample on the tops of the tubes during the temperature changes. (Waltenbury *et al*., 2005). The layer of wax or oil made subsequent sample handling more difficult. Later, thermal cycler models were designed with heated lids that eliminated the requirement for vapor barriers. There are numerous manufacturers of thermal cyclers. These instruments differ in heating and/or refrigeration systems as well as the programmable software within the units. Samples may be held in open chambers for air heating and cooling or in sample blocks designed to accommodate 0.2mL tubes, usually in a 96 well format (Hui *et al*., 2004). Some models have interchangeable blocks to accommodate amplification in different sizes and numbers of tubes or slides (Casabianca *et al*., 2003). A cycler may run more than one block independently at the same time, so that different PCR programs can be performed simultaneously (Menard *et al*., 2005). Rapid PCR systems are designed to work with very small sample volumes in chambers that can be heated and cooled quickly by changing the air temperature surrounding the samples. Realtime PCR systems are equipped with fluorescent detectors to measure PCR product as the reaction proceeds. PCR can also be performed in a microchip device in which 1–2 L samples are forced through tiny channels etched in a glass chip, passing through temperature zones as the chip rests on a specially adapted heat block. For routine PCR in the laboratory, an appropriate amount of DNA that has been isolated from a test specimen is mixed with the other PCR components, either separately or as part of a master mix in 0.2–0.5mL tubes. Most thermal cyclers take thin-walled tubes, 0.2Ml tube strips or 96 well plates (Aliyu *et al*., 2004). Preparation of the specimen for PCR is often referred to as pre-PCR work. To avoid contamination, it is recommended that the pre-PCR work be done in a designated area that is clean and free of amplified products. The sample tubes are then loaded into the thermal cycler. The computer is programmed with the temperatures and times for each step of the PCR cycle, the number of cycles to complete (usually 30–50), the conditions for ramping from step to step, and the temperature at which to

hold the tubes once all of the cycles are complete. The technologist starts the run and walks away until it is complete. After the PCR, a variety of methods are used to analyze the PCR product. Most commonly, the PCR product is analyzed by gel or capillary electrophoresis. Depending on the application, the size, presence, or intensity of PCR products is observed on the gel (Caliendo *et al.*, 2003).

2.3.3.1 Components of PCR:

2.3.3.2 Primers:

Primers determine the specificity of the PCR. They are chemically synthesized on an oligonucleotide synthesizer. Primers sequences should be homologous to sequences flanking the target sequence. Primers are single-stranded DNA fragments, usually 20–30 bases in length. The forward primer must bind to the target DNA sequence just 5′ to the sequences intended to be amplified. The reverse primer must bind just 5′ to the sequence to be amplified on the opposite strand of the DNA. Thus, the design of primers requires some knowledge of the target sequence. The placement of the primers will also dictate the size of the amplified product. Binding of primers is subject to the same physical limitations as probe binding (Han *et al.*, 2013). The primer sequence (GC %) and length affect the optimal conditions in which the primer will bind to its target. The approximate melting temperature, or Tm, of the primers can be calculated usingthe equation for short DNA fragments [Tm = 2* (AT) +4*(GC)]. The primer Tm can serve as a starting point for optimizing annealing temperature, this can be done by changing the length and the GC content of the primers (Aikyo and Oh, 2014).

2.3.3.3 Primer design guidelines:

Primer length is generally accepted that the optimal length of PCR primers is 18-22 bp. This length is long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature. Primer melting temperature (Tm) is the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability. Primers with melting temperatures in the range of 52-58 ℃ generally produce the best results. Primers with melting temperatures above 65C° have a tendency for secondary annealing. The GC content of the sequence gives a fair indication of the primer Tm. The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature. Too high Ta will produce insufficient primer-template hybridization resulting in low PCR product yield (Han *et al.*, 2013). Too low Ta may possibly lead to non-specific products caused by a high number of base pair mismatches. Mismatch

tolerance is found to have the strongest influence on PCR specificity. GC content The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer should be 40-60%. GC clamp: the presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer. Presence of the primer secondary structures produced by intermolecular or intramolecular interactions can lead to poor or no yield of the product. They adversely affect primer template annealing and thus the amplification. They greatly reduce the availability of primers to the reaction. Hairpins: it is formed by intramolecular interaction within the primer and should be avoided (Aikyo and Oh Ishi, 2014). Optimally a 3' end hairpin with a ΔG of-2 kcal/mol and an internal hairpin with a ΔG of-3 kcal/mol are tolerated generally. A primer self-dimer is formed by intermolecular interactions between the two (same sense) primers, where the primer is homologous to itself. Generally a large amount of primers are used in PCR compared to the amount of target gene. When primers form intermolecular dimers much more readily than hybridizing to target DNA, they reduce the product yield. Optimally a 3' end self dimer with a ΔG of -5 kcal/mol and an internal self dimer with a ΔG of -6 kcal/mol is tolerated generally. Primer cross dimers are formed by intermolecular interaction between sense and antisense primers, where they are homologous. Optimally a 3' end cross dimer with a ΔG of-5 kcal/mol and an internal cross dimer with a ΔG of-6 kcal/mol is tolerated generally. Repeats: A repeat is a di-nucleotide occurring many times consecutively and should be avoided because they can misprime. For example: ATATATAT. A maximum number of di-nucleotide repeats acceptable in an oligo are 4 di-nucleotides. Runs: primers with long runs of a single base should generally be avoided as they can misprime. For example, AGCGGGGGATGGGG has runs of base 'G' of value 5 and 4. A maximum number of runs accepted are 4 bp. End stabilityit is the maximum ΔG value of the five bases from the 3' end. An unstable 3' end (less negative ΔG) will result in less false priming. Avoid template secondary structure: a single stranded Nucleic acid sequences is highly unstable and fold into conformations (secondary structures). The stability of these template secondary structures depends largely on their free energy and melting temperatures(Tm). Consideration of template secondary structures is important in designing primers, especially in qPCR. If primers are designed on a secondary structure which is stable even above the annealing temperatures, the primers are unable to bind to

the template and the yield of PCR product is significantly affected. Hence, it is important to design primers in the regions of the templates that do not form stable secondary structures during the PCR reaction. To improve specificity of the primers it is necessary to avoid regions of homology. Primers designed for a sequence must not amplify other genes in the mixture. Commonly, designed primers are blasted to test to avoid regions of cross homology (Han *et al.*, 2013).

2.3.3.4 DNA Template:

The template may be single-stranded or double-stranded DNA.In a clinical sample, depending on the application, the template may be derived from the patient's genomic ormitochondrial DNA or from viruses, bacteria, fungi, orparasites that might be infecting the patient. Genomic DNA will have only one or two copies per cell equivalentof single-copy genes to serve as amplification targets.With robust PCR reagents and conditions, nanogram amounts of genomic DNA are sufficient for consistent results. For routine clinical analysis, 100 ng to 1µg of DNA is usually used. Lesser amounts are required formore defined template preparations such as cloned target DNA or product from a previous amplification. The best templates are in good condition, free of contaminating proteins, and without nicks or break that canstop DNA synthesis or cause mis-incorporation of nucleotide bases. Templates with high GC content and secondary structure may prove more difficult to optimize for amplification. The DNA region affected in Fragile Xsyndrome, 5′ to the FMR-1 gene, is an example of such aGC-rich target (Casabianca *et al.*, 2003).

2.3.3.5 Deoxyribonucleotide bases:

Nucleotide triphosphates are the building blocks ofDNA. An equimolar mixture of the four deoxy-nucleotide-triphosphates (dNTPs), adenine, thymine, guanine, and cytosine, is added to the synthesis reaction in concentrations sufficient to support the exponential increase of copies of the template. Standard procedures require 0.1–0.5 mM concentrations of each nucleotide (Aikyo and Oh, 2014). Substituted or labeled nucleotides, such as deaza GTP, may beincluded in the reaction for special applications. These nucleotides will require empirical optimization for bestresults.Reagent systems that are designed to amplify targets optimally with high GC content are available. These systems incorporate an analog of dGTP, deaza GTP, to de-stabilize secondary structure. Deaza-GTP interferes with EtBr staining in gels and is best used in

procedures with other types of detection, such as auto-radiography (Han *et al*., 2013).

2.3.3.6 DNA polymerase:

The DNA polymerase Taq polymerase isolated from the thermophilic bacterium Thermu saquaticus greatly facilitate the automation of the PCR procedure. Using a thermo stable polymerase enzyme meant that the DNA polymerase could be added once at the beginning of the procedure and it would maintain its activity throughout the heating and cooling cycles. Other thermostable enzymes, such as Tth polymerase from Thermus thermophilus, were subsequently exploited for laboratory use. Tth polymerase also has reverse transcriptase activity so that it can be used in reverse transcriptase PCR where the starting material is an RNA template. The addition of proof reading enzymes, e.g., Vent polymerase allows Taq or Tth polymerase to generate large products over 30,000 bases in length (Casabianca *et al*., 2003). Cloning of the genes coding for these polymerases has led to modified versions of the polymerase enzymes, such as the Stoffel fragment, which lacks the 5' to 3' exonuclease activity that normally hydrolyses blocking DNA strands during primer extension. The halflife of the Stoffel fragment at high temperatures is about twice that of Taq polymerase, and it has a broader range of optimal $MgCl_2$ concentrations (2–10 mM) than Taq. This enzyme is recommended for allele-specific PCR and for amplification of regions with high GC content (Aikyo and Oh, 2014). The Klenow fragment, derived from the original DNA polymerase I from *E. coli*, was the first enzyme used in PCR. Because of its lack of stability at high temperature, it needs be replenished during each cycle, and therefore is not commonly used in PCR. The bacteriophage T4 DNA polymerase was also initially used in PCR (Aikyo and Oh, 2014). It has a higher fidelity of replication than the Klenow fragment, but is also destroyed by heat. The DNA polymerase from Thermus aquaticus (or Taq), was the first thermostable polymerase used in PCR, and is still the one most commonly used. The enzyme can be isolated from its native source, or from its cloned gene expressed in *E. coli*. The Stoffel fragment is made from a truncated gene for Taq polymerase and expressed in *E. coli*. It is lacking 5'-3' exonuclease activity, and may be able to amplify longer targets than the native enzyme. Hot-start PCR polymerase is a variant of Taq polymerase that requires strong heat activation, thereby avoiding non-specific amplification due to polymerase activity at low temperature. Pfu DNA polymerase, isolated from the Archean *Pyrococcus furious*, has proofreading activity, and a 5-fold decrease in the error rate of replication compared to Taq. Since errors increase as PCR progresses, Pfu is the preferred polymerase when products are to be

individually cloned for sequencing or expression. Vent polymerase is an extremely the rmost able DNA polymerase isolated from *Thermococcus litoralis* Tth polymerase is a thermostable polymerase from *Thermous thermophilus*. It has reverse transcriptase activity in the presence of Mn^{2+} ions, allowing PCR amplification from RNA (Han *et al*., 2013).

2.3.3.7 PCR buffer:

PCR buffers provide the optimal conditions for enzyme activity. Monovalent cations such as potassium chloride (20–100 mM) and ammonium sulfate (15–30 mM), or other salts are important buffer components. Thesesalts affect the denaturing and annealing temperatures of the DNA and the enzyme activity. An increase in salt concentration makes longer DNA products denature moreslowly than shorter DNA products during the amplification process, so shorter molecules will be amplified preferentially (Casabianca *et al*., 2003).The influence of buffer/salt conditions varies with different primers and templates. Magnesium chloride also affects primer annealing and is very important for enzyme activity (Han *et al*., 2013). Magnesium requirements will vary with each reaction, because each NTP will take up one magnesium atom. Furthermore, the presence of ethylene-diamine-tetraacetic acid (EDTA) or other chelators will lower the amount of magnesium available for the enzyme. Too few Mg_2 ions lower enzyme efficiency, resulting in a low yield of PCR product.Overly high Mg_2 concentrations promote misincorporation and thus increase the yield of nonspecific products. Lower Mg_2 concentrations are desirable when fidelity of the PCR is critical. The recommended range of $MgCl_2$ concentration is 1–4 mM, in standard reaction conditions. If the DNA samples contain EDTA or other chelators, the $MgCl_2$ concentration in the reaction mixture should be adjusted accordingly. As with other PCR components, the optimal conditions are established empirically. Tris buffer and accessory buffer componentsare also important for optimal enzyme activity and accurate amplification of the intended product; 10 mM Tris-HCl maintains the proper pH of the buffer, usuallybetween pH 8 and pH 9.5. Accessory components sometimes used to optimize reactions include: bovineserum albumin (B.S.A) de-acetylated (10–100 μg/mL) binds inhibitors and stabilizes the enzyme. Di-thiothreitol (DTT) (0.01 mM) provides reducing conditions that mayenhance enzyme activity. Formamide (1%–10%) added to the reaction mixture will lower the denaturing temperature of DNA with high secondary structure, thereby increasing the availability for primer binding.

Chaotropic agents such as triton X-100, glycerol, and dimethyl sulfoxide (D.M.S.O) added at concentrations of 1%–10% may also reduce secondary structure to allow polymerase extension through difficult areas. These agents contribute tothe stability of the enzyme as well. Enzymes are usually supplied with buffers optimized by the manufacturer. Commercial PCR buffer enhancers of proprietary composition may also be purchased to optimize difficult reactions. Often, the buffer and its ingredients are mixed with the nucleotide bases andstored as aliquots of a master mix. The enzyme, targetand primers are then added when necessary. Dedicated master mixes will also include the primers, so that onlythe target sequences must be added (Aikyo and Oh, 2014).

2.3.3.8 Controls for PCR:

With every PCR run, the appropriate controls must be included. Positive controls ensurethat the enzyme is active, the buffer is optimal, the primers are priming the right sequences, and the thermal cycler is cycling appropriately (Aikyo and Oh, 2014).A negative control without DNA (also called a contamination control or reagent blank) ensures that the reaction mix is not contaminated with template DNA or amplified products from a previous run. A negative control with DNA that lacks the target sequence (negative template control) ensures that the primers are not annealing to unintended sequences of DNA. In some applications of PCR, an internal control is included. In this type of control (amplification control), a second set of primers and an unrelated target are added to the reaction mix to demonstrate that the reaction is working even if the test sample is not amplified. Amplification controls are performed, preferably in the same tube with the test reaction; although it is acceptableto perform the amplification control on a duplicate sample. This type of control is most important when PCR results are reported as positive or negative, by which"negative" means that the target sequences are not present.The amplification control is critical to distinguish between a true negative for the sample and an amplification failure (false-negative) (Casabianca *et al*., 2003).

2.3.3.9 PCR lab setup and control of contamination:

Although genomic DNA is a source of spurious PCR targets, the major cause of contamination is PCR products from previous amplifications. Unlike the relatively large and scarce genomic DNA, the small, highly concentrated PCR product DNA can aerosol when tubes are uncapped and when the DNA is pipetted. This PCR product is a perfect template for primer binding and amplification in a subsequent PCR using the

same primers. Contamination control procedures, therefore, are mainly directed toward eliminating PCR product from the setup reaction. Contamination is controlled both physically and chemically. Physically, the best way to avoid PCR carry over is to separate the pre-PCR areas from the post PCR analysis areas. Positive airflow, air locks, and more extensive measures are taken by high throughput laboratories that process large numbers of samples and test for a limited number of amplification targets. Most laboratories can separate these areas by assigning separate rooms or using isolation cabinets. Equipment, including laboratory gowns and gloves, and reagents should be dedicated to either pre- or post PCR. Items can have unidirectional flow from the pre- to the post PCR area but not in the opposite direction without decontamination (Aikyo and Oh, 2014). Ultra violet light (UV) has been used to decontaminate and maintain pre-PCR areas. UV light catalyzes single and double-strand breaks in the DNA that will then interfere with replication. Isolation cabinets are equipped with UV light sources that are turned on for about 20 minutes after the box has been used. The effectiveness of UV light may be increased by the addition of psoralens to amplification products after analysis. Psoralens intercalate between the bases of double-stranded DNA, and in thepresence of long-wave UV light they covalently attach to the thymidines, uracils, and cytidines in the DNA chain.The bulky adducts of the psoralens prevent denaturation and amplification of the treated DNA. The efficiency of UV light treatment for decontamination depends on the wave length, energy, and distance of the light source. Care must be taken to avoid skin or eye exposure to UV light. UV light will also damage some plastics, so that laboratory equipment may be affected by extended exposure. Although convenient, the efficiencyof UV treatment may not be the most effective decontaminant for every procedure. A widely used method for decontamination and preparation of the work space is 10% bleach (7 mM sodium hypochlorite). Frequently wiping bench tops (hoods preferably 5% to avoid corrosion); or any surface that comes in contact with specimen material with dilute bleach or alcohol removes most DNA contamination (Casabianca *et al.*, 2003). As a common practice in forensic work, before handling evidence or items that come in contactwith evidence, gloves are wiped with bleach and allowedto air-dry. Another widely used chemical method of contamination control is the dUTP-UNG system. This requires substitution of dTTP with dUTP in the PCR reagent mastermix, which will result in incorporation of dUTP instead of dTTP into the PCR product. Although some polymerase enzymes may be more or less efficient in incorporation of the nucleotide,

the dUTP does not affect the PCR product for most applications. At the beginning ofeach PCR, the enzyme uracil-N-glycosylase (UNG) isadded to the reaction mix. This enzyme will degrade anynucleic acid containing uracil, such as contaminatingPCR product from previous reactions. A short incubation period is added to the beginning of the PCR amplification program, usually at 50°C for 2–10 minutes to allow theUNG enzyme to function. The initial denaturation step in the PCR cycle will degrade the UNG before synthesis ofthe new products. Note that this system will not work with some types of PCR, such as nested PCR, because a second round of amplification requires the presence of the first round product, also this method is not appropriate for single step PCR amplification of RNA. The dUTP-UNG system is used routinely in real-time PCR procedures. This method is not ideal for decontamination for the following reasons. Only portion of the contaminating PCR product from previous PCR will be degraded. Inactivation of uracil-N-glycosylase might be incomplete. It might affect the efficiency of PCR (Han *et al.*, 2013).

2.3.3.10 Prevention of mispriming:

PCR products are analyzed for size and purity by electrophoresis. The amplicon size should agree with the size determined by the primer placement. For instance, if two 20 bp primers were designed to hybridize to sequences flanking a 100 bp target, the amplicon should be 140 bp in size (Hui *et al.*, 2004). Much larger or smaller amplicons are due to mispriming or primer dimers or other artifacts of the reaction. For some procedures, these artifacts do not affect interpretation of results and, as long as they do not compromise the efficiency of the reaction, can be ignored. For other purposes, however, extraneous PCR products must be avoided or removed.Misprimes is initially averted by good primer designand optimal amplification conditions. Even with the best conditions, however, misprimes can occur during preparation of the reaction mix. This is because Taq polymerase has some activity at room temperature. While mixes are prepared and transported to the thermal cycler, the primers and template are in contact at 22–25ºC, a conditionof very low stringency these conditions; the primers can bind sequences other than their exact complements in the target (Waltenbury *et al.*, 2005). This mispriming can be avoided by the use of hot start PCR in which the enzyme is inactive until it is activated by heat in the first denaturation step of the PCR program, preventing any primer extension during reagent mix preparation (Waltenbury *et al.*, 2005).

2.3.3.11 PCR product cleanup:

A direct way of obtaining clean PCR product for subsequent analytical procedures that demand pure product, such as sequencing or some mutation analyses is to resolve the amplification products bygel electrophoresis and then cut the desired bands fromthe gel and elute the PCR product. The gel slice cans be digested with enzymes such as β-agarase or iodine. The agarase enzyme digests the agarose polymer and releases the DNA into solution for further purification (Aliyu *et al.*, 2004).Residual components of the reaction mix, such as leftover primers and unused nucleotides, also interfere with some post-PCR applications. Moreover, the buffers usedfor the PCR may not be compatible with post-PCR procedures. Amplicons free of PCR components are most frequently and conveniently prepared using spin columnsor silica beads. Addition of shrimp alkaline phosphatase (SAP) in combination with exonuclease I (ExoI) is an enzymatic method for removing nucleotides and primers from PCRproducts prior to sequencing or mutational analyses. During 15minute incubation at 37 ° C, SAP dephosphorylates nucleotides, and ExoI degrades primers. Theenzymes must then be removed by extraction or inactivated by heating at 80 ° C for 15 minutes. This method is convenient as it is performed in the same tube as the PCR (Caliendo *et al.,* 2003).

2.3.3.12 Nucleic acid extraction:

The purpose of extraction is to release the nucleic acid from the cell for use in subsequent procedures. Ideally, the target nucleic acid should be free of contamination with protein, carbohydrate, lipids or other nucleic acid, i.e., DNA free of RNA or RNA free of DNA (Dani *et al*., 2003). The initial release of the cellular material is achieved by breaking the cell and nuclear membranes (cell lysis) (Aplenc *et al*.; 2002). Lysis must take place in conditions that will not damage the nucleic acid. Following lysis, the target material is purified, and then the concentration and purity of the sample can be determined. Miescher first isolated DNA from human cells in 1869 (McOrist *et al*., 2002).The initial routine laboratory procedures for DNA isolation were developed from density gradient centrifugation strategies. Meselson and Stahl (1958) used such a method to demonstrate semi-conservative replication of DNA. Later procedures made use of the differences in solubility of large chromosomal DNA, plasmids and proteins in alkaline buffers. Large (50 kbp) chromosomal DNA and proteins cannot renature properly when neutralized in acetate at low pH after alkaline treatment, forming large aggregates instead. As a result, they precipitate out of solution (Medeiros *et al*.,

2003).The relatively small plasmids return to their supercoiled state and stay in solution. Alkaline lysis procedures were used extensively for extraction of 1–50kb plasmid DNA from bacteria during the early days of recombinant DNA technology (Byers *et al*., 2004).

2.3.3.13 DNA isolation methods:

In organic method, after release of DNA from the cell, further purification requires removal of contaminating proteins, lipids, carbohydrates and cell debris. This is accomplished using a combination of high salt, low pH and organic mix- DNA associated proteins (Medeiros *et al*., 2003). Isolation of small amounts of DNA from challenging samples such as fungi can be facilitated by pretreatment with acetyl-trimethyl-ammonium bromide, a cationic detergent that efficiently separates DNA from polysaccharide contamination (Byers *et al*., 2004). To avoid RNA contamination, RNAse, an enzyme that degrades RNA, can be added at this point. Alternatively, RNAse may also be added to the resuspended DNA at the end of the procedure (Dani *et al*., 2003). When phenol and chloroform are added to the hydrophilic cleared cell lysate, a biphasic emulsion forms. Centrifugation will settle the hydrophobic layer on the bottom, with the hydrophilic layer on top. Lipids and other hydrophobic components will dissolve in the lower hydrophobic phase. DNA will dissolve in the upper aqueous phase. Amphiphilic components, which have both hydrophobic and hydrophilic properties as well as cell debris, will collect as a white precipitate at the interface between the two layers (Byers *et al*., 2004). The upper phase containing the DNA is collected, and the DNA is then precipitated using ethanol or isopropanol in a high concentration of salt (ammonium, sodium acetate or lithium or sodium chloride). The ethyl or isopropyl alcohol is added to the upper phase solution at 2:1 or 1:1 ratios, respectively, and the DNA forms a solid precipitate (Medeiros *et al*., 2003). The DNA precipitate is collected by centrifugation. Excess salt is removed by rinsing the pellet in70% ethanol, centrifuging and discarding the ethanol supernatant, and then dissolving DNA pellet in rehydration buffer, usually 10 mM Tris, 1 mM EDTA (TE), or water (McOrist *et al*., 2002). Inorganic Isolation method was developed to avoid using corrosive organic solvents. First step is cell lyses and enzyme treatment (SDS–proteinase K,). DNA isolation is achieved through the addition of high salt concentration (Sodium Chloride, Sodium Acetate). After centrifugation, hydrophilic DNA is left in solution while proteins are precipitated due to their relative hydrophobicity. The supernatant containing the DNA is transferred to a new tube and

precipitated with absolute ethanol or isopropanol. Purity of DNA isolated by this method may be of less quality than organic solvents method. Proteinase K needs long incubation times and is relatively expensive, which lead to developing alternative methods for deproteinization of DNA without using the enzyme (Dani *et al*., 2003). Solid-phase purification is based on passing the cell lysate through a solid support (mostly positioned in a spin column). DNA will be selectively adsorbed to the solid support, while proteins and other cell components will pass through it. This filtration process is facilitated by centrifugation or through application of vacuum. Common examples on solid supports used in solid-phase extraction method include Silica matrices, glass particles and anion-exchange carriers (Byers *et al*., 2004).

2.3.3.14 Measurement of nucleic acid quality and quantity:

Laboratory analysis of nucleic acids produces variable results, depending on the quality and quantity of input material. This is an important consideration in the clinical laboratory, as test results must be accurately interpreted with respect to disease pathology. Consistent results require that run-to-run variation be minimized. Fortunately, measurement of the quality and quantity of DNA and RNA is straight forward (Caliendo *et al*., 2003).The use of a Thermo Scientific NanoDrop spectrophotometer to QC nucleic acid samples can result in significant savings in time and money. The micro volume capability of NanoDrop™ spectrophotometers allow the researcher to quickly and easily run quality control checks of nucleic acid and protein samples. In addition, the instrument's short measurement cycle and general ease of use greatly increases the rate at which samples can be processed, making it possible to implement multiple quality control checks throughout a procedure or process (William *et al.,* 1997).

2.3.3.15 Electrophoresis:

DNA and RNA can be analyzed for quality by resolving an aliquot of the isolated sample on an agarose gel fluorescent dyes such as ethidium bromide or SybrGreen I bind specifically to DNA and are used to visualize the sample preparation. Ethidium bromide or SybrGreen II can be used to detect RNA. Less frequently, silver stain has been used to detect small amounts of DNA by visual inspection (Matsuno *et al*., 2013). The appearance of DNA on agarose gels depends on the type of DNA isolated. A good preparation of plasmid DNA will yield a bright, moderate-mobility single band of supercoiled plasmid DNA with minor or no other bands that represent nicked or broken plasmid (Caliendo *et al*., 2003). High molecular weight genomic DNA should collect as

a bright band with low mobility. A highquality preparation of RNA will yield two distinct bands of rRNA. The integrity of these bands is an indication of the integrity of the other RNA species present in the same sample. If these bands are degraded (smeared) or absent, the quality of the RNA in the sample is deemed unacceptable for use in molecular assays (Matsuno *et al.*, 2011).When fluorescent dyes are used, DNA and less accurately, RNA can be quantitated by comparison of the fluorescence intensity of the sample aliquot run on the gel with that of a known amount of control DNA or RNA loaded on the same gel. Densitometry of the band intensities gives the most accurate measurement of quantity. For some procedures, estimation of DNA quantity can be made by visual inspection (McOrist *et al.*, 2002).

2.3.3.16 Spectrophotometry:

Nucleic acids absorb light at 260 nm through the adenine residues using the Beer-Lambert Law, concentration can be determined from the absorptivity constants (50 for DNA, 40 for RNA)(Han *et al.*, 2012).The relationship of concentration toabsorbance is expressed as where A the absorbance, molar absorptivity (L/molcm), b the path length (cm), and c the concentration (mg/L) (Han *et al.*, 2013).The absorbance at this wavelength is thus directly proportional to the concentration of the nucleic acid in the sample. Using the absorptivity as a conversion factor from optical density to concentration, one optical density unit (or absorbance unit) at 260 nm is equivalent to 50 mg/L (or 50 _g/mL) of DNA and 40 _g/mL of RNA (Phillips and Wellner *et al.,* 2013). To determine concentration, multiply the spectrophotometer reading in absorbance units by the appropriate conversion factor. Phenol absorbs ultraviolet light at 270–275 nm, close to the wavelength of maximum absorption by nucleic acids. This means that residual phenol from organic isolation procedures can increase 260 readings, so phenol contamination must be avoided when measuring concentration at 260 nm (Medeiros *et al.*, 2003). Most DNA and RNA preparations are of sufficient concentration to require dilution before spectrophotometry in order for the reading to fall within the linear reading range (0.05–0.800 absorbance units, depending on the instrument) (Shia *et al.*, 2002). If the sample has been diluted before reading, the dilution factor must be included in the calculation of quantity. Multiply the absorbance reading by the conversion factor and the dilution factor to find the concentration of nucleic acid. Spectrophotometric measurements also indicate the quality of nucleic acid (McOrist *et al.*, 2002). Protein absorbs light at 280 nm through the tryptophane

residues. The absorbance of the nucleic acid at 260 nm should be 1.6–2.00 times more than the absorbance at 280 nm. If the 260 nm280 nm ratio is less than 1.6, the nucleic acid preparation may be contaminated with unacceptable amounts of protein and not of sufficient purity for use. Such a sample can be improved by re-precipitating the nucleic acid or repeating the protein removal step of the isolation procedure. It should be noted that low pH can affect the 260/280 nm ratio. Some what alkaline buffers pH 7.5 is recommended for accurate determination of purity (Shimura and Kasani, 2014). RNA affords a some what higher 260 /280 nm ratio, 2.0–2.3. DNA preparation with a ratio higher than 2.0 may be contaminated with RNA. Some procedures for DNA analysis are not affected by contaminating RNA, in which case the DNA is still suitable for use. If, however, RNA may interfere or react with DNA detection components, RNase should be used to remove the contaminating RNA. Because it is difficult to detect contaminating DNA in RNA preparations, RNA should be treated with RNase free DNase where DNA contamination may interfere (Byers *et al*., 2004).

2.3.3.17 Agarose gels:

Agarose is a polysaccharide polymer extracted from seaweed. It is a component of agar used in bacterial culture dishes. Agarose is a linear polymer of agarobiose, which consists of 1, 3 linked-D-galactopyranose and 1, 4 linked 3, 6-anhydro-L-galactopyranose (Aikyoand and Oh Oshi, 2014).Hydrated agarose gels in various concentrations, buffers, and sizes can be purchased ready for use. Alternatively, agarose can be purchased and stored in the laboratory in powdered form. For use, powdered agarose is suspended in buffer, heated, and poured into a mold. The concentration of the agarose dictates the size of the spaces in the gel and will, therefore, be determined by the size of DNA to be resolved. Small pieces of DNA (50–500 bp) are resolved on more concentrated agarose gels, e.g., 2%–3%. Larger fragments of DNA (2000–50,000) are best resolved in lower agarose concentrations, e.g., 0.5%–1%. Agarose concentrations above5% and below 0.5% are not practical. High-concentration agarose will impede migration, whereas very low concentrations produce a weak gel with limited integrity (Morais *et al*., 2010).

2.3.3.18 Polyacryl amide gels:

Very small DNA fragments and single-stranded DNA are best resolved on polyacrylamidegels in polyacrylamide gel electrophoresis. Acrylamide, in combination with the cross-linker methylene bisacrylamide, polymerizes into a gel that has consistent resolution characteristics (McOrist *et al*., 2002). Polyacrylamide was

originally used mostly for protein separation, but it is now routinely applied to nucleic acid analysis. Polyacrylamide gels are used for sequencing nucleic acids, mutation analyses, nuclease protection assays, and other applications requiring the resolution of nucleic acids down to the single-base level. Acrylamide is supplied to the laboratory in several forms. The powdered form is a dangerous neurotoxin and must be handled with care (Shia *et al*., 2002). Solutions of mixtures of acrylamide and bis-acrylamide are less hazardous and more convenient to use. Preformed gels are the most convenient, as the procedure for preparation of acrylamide gels is more involved than that for agarose gels (Aplenc *et al*., 2002). The composition of polyacrylamide gels is represented as the total percentage concentration (w/v) of monomer (acrylamide with cross-linker) T and the percentage of monomer that is cross-linker C. For example, a 6% 19:1 acrylamide: bis gel has a T value of 6% and a C value of 5%. Unlike agarose gels that polymerize upon cooling, polymeration of polyacrylamide gels requires the use of a catalyst. The catalyst may be the nucleation agents, ammonium persulfate (APS) plus N, N, N, N-tetra-methyl-ethylene-diamine (TEMED), or light activation. APS produces free oxygen radicals in the presence of TEMED to drive the free-radical polymerization mechanism. Free radicals can also be generated by a photochemical process using riboflavin plus TEMED. Excess oxygen inhibits the polymerization process. Therefore, deaeration, or the removal of air, of the gel solution is often done before the addition of the nucleation agents. Polyacrylamide gels for nucleic acid separation are very thin, e.g., 50 _m, making gel preparation difficult. Systems have been designed to facilitate the preparation of single and multiple gels. Increasing numbers of laboratories are using preformed polyacrylamide gels to avoid the hazards of working with acrylamide and the labor time involved in gel preparation. Use of preformed gels must be scheduled; keeping in mind the limited shelf life of the product (Shimura and Kasai, 2014).The main advantage of polyacrylamide over agarose is the higher resolution capability for small fragments that can be accomplished with polyacrylamide. A variation of1 base pair in a 1-kb molecule (0.1% difference) can be detected in a polyacrylamide gel. Another advantage of polyacrylamide is that, unlike agarose, the components of polyacrylamide gels are synthetic; thus, there is not as much difference in batches obtained from different sources. Further, altering T and C in a polyacrylamide gel can change the pore size and, therefore, the sieving properties in a predictable and reproducible manner. Increasing T decreases the pore size proportionally. The minimum pore size (highest resolution for small molecules) occurs at a C value of 5%.

Variation of C above or below 5% will increase pore size. Usually, C is set at3.3% (29:1) for native and 5% (19:1) for standard DNA and RNA gels (Matsuno *et al*., 2013).

2.3.3.19 Buffer systems:

The purpose of a buffer system is to carry the current and protect the samples during electrophoresis. This is accomplished through the electrochemical characteristics of the buffer components. A buffer is a solution of a weak acid and its conjugate base. The pH of a buffered solution remains constant as the buffer molecules take up or release protons given off or absorbed by other solutes (Aikyo and Oh, 2014).The equilibrium between acid and base in a buffer is expressed as the dissociation constant, Ka: If the acidic and the basic forms of the buffer in solution are of equal concentration, pH pKa. If the acidic form predominates, the pH will be less than the pKa; if the basic form predominates, the pH will be greater than the pKa. The Henderson-Hasselbach equation predicts that, in order to change the pH of a buffered solution by one point, either the acidic or basic form of the buffer must be brought to a concentration of 1/10 that of the other form. Therefore, addition of acid or base will barely affect the pH of a buffered solution as long as the acidic or basic forms of the buffer are not depleted. Control of the pH of a gel by the buffer also protects the sample molecules from damage. Furthermore, the current through the gel is carried by buffer ions, preventing severe fluctuations in the pH of the gel. A buffer concentration must be high enough to provide sufficient acidic and basic forms to buffer its solution. Raising the buffer concentration, however, also increases the conductivity of the electrophoresis system, generating more heat at a given voltage. This can cause problems with gel stability and can increase sample denaturation. High buffer concentrations must therefore be offset by low voltage. In order for nucleic acids to migrate properly, the gel system must be immersed in a buffer that conducts the electric current efficiently in relation to the buffering capacity (Shimura and Kasai, 2014). Ions with high charge differences move through the gel more quickly; that is, they increase conductivity without increasing buffering capacity. This results in too much current passing through the gel as well as faster depletion of the buffer. Therefore, buffer components such as Tris base or borate is preferred because they remain partly uncharged at the desired pH and thus maintain constant pH without high conductivity. In addition to pKa, charge, and size, other buffer characteristics that can be taken into account when choosing a buffer include toxicity, interaction with other components, solubility, and ultraviolet absorption. The

Tris buffers Tris borate EDTA (TBE; 0.089 M Trisbase, 0.089 M boric acid, 0.0020 M EDTA), Tris phosphate EDTA (TPE; 0.089 M Tris-base, 1.3% phosphoric acid, 0.0020 M EDTA) and Tris acetate EDTA (TAE; 0.04 M Tris-base, 0.005 M sodium acetate, 0.002 M EDTA) are most commonly used for electrophoresis of DNA(Aikyo and Oh Hishi, 2014).There are some advantages and disadvantages of both TBE and TAE that must be considered before one of these buffers is used for a particular application. TBE has a greater buffering capacity than TAE. Although the ion species in TAE are more easily exhausted during extended or high-voltage electrophoresis, DNA will migrate twice as fast in TAE than in TBE in a constant current. TBE is not recommended for some post-electrophoretic isolation procedures. When using any buffer, especially TBE and TPE, care must be taken that the gel does not overheat when run at high voltage in a closed container. Finally, stock solutions of TBE are prone to precipitation. This can result in differences in concentration between the buffer in the gel and the running buffer. Such a gradient will cause localized distortions in nucleic acid migration patterns, often causing a salt wave that is visible as a sharp horizontal band through the gel (Shimura and Kasai, 2014).

2.3.3.20 Electrophoresis equipment:

Gel electrophoresis can be done in one of two conformations, horizontal or vertical. In general, agarose gels are run horizontally, and polyacrylamide gels are run vertically. Horizontal gels are run in acrylic containers called gel boxes or baths that are divided into two parts with a platform in the middle on which the gel rests. The gel in the box is submerged with electrophoresis buffer filling both compartments and making a continuous system through which the current flows. The thickness of the gel and the volume of the buffer affect migration, so these parameters should be kept constant for consistent results (Singer *et al*., 1999).As the gel is submerged through the loading and electrophoresis process, horizontal gels are sometimes referred to as submarine gels. The power supply will deliver voltage, setting up a current that will run through the gel buffer and the gel, carrying the charged sample through the matrix of the gel at a speed corresponding to the charge mass ratio of the sample molecules. Horizontal agarose gels are cast as square or rectangular slabs of varying size. Purchased gel boxes come with casting trays that mold the gel to the appropriate size for the gel box. The volume of the gel solution will determine the thickness of the gel. Agarose, supplied as a dry powder, is mixed at a certain percentage with electrophoresis buffer and heated on a heat block or by microwave to dissolve and melt the agarose. The molten agarose is

cooled to 55–65 C, and a certain volume is poured into the casting tray as dictated by the gel box manufacturer or application. A comb is then inserted into the top of the gel to create holes, or wells, in the gel into which the sample will be loaded. The size of the teeth in the comb will determine the volume of loaded sample and the number of teeth will determine the number of wells that are available in the gel to receive samples. The gel is then allowed to cool, during which time it will solidify. After the gel has polymerized, the comb is carefully removed and the gel is placed into the gel box and submerged in electrophoresis buffer (Singer *et al*., 1999)

2.3.3.21 Gel loading:

Prior to loading the sample containing isolated nucleic acid onto the gel, tracking dyeand a density agent are added to the sample. The density agent (ficoll, sucrose, or glycerol) increases the density of the solution as compared with the electrophoresis buffer. When the sample solution is dispensed into the wells of the gel below the surface of the buffer, it sinks into the well instead of floating away in the buffer. The tracking dyes are used to monitor the progress of the electrophoresis run. The dyes migrate at specific speeds in a given gel concentration and usually run ahead of the smallest fragments of DNA. They are not associated with the sample DNA, and thus they do not affect the separation of the sample DNA. The movement of the tracking dye is monitored, and when the tracking dye approaches the end of the well electrophoresis is terminated. Bromo-phenol blue is a tracking dye that is used for many applications. Xylene cyanol green is another example of chromophores that are used as tracking dyes for both agarose and polyacrylamide gels (Han *et al*., 2013).

2.3.3.22 Detection systems:

Following the status of samples during and after electrophoresis is accomplished using dyes that specifically associate with nucleic acid. The agents used most frequently for this application are fluorescent dyes and silver stain (Matsuno *et al*., 2011).

2.3.3.23 Nucleicacid specific dyes:

Intercalating agents intercalate, or stack, between the nitrogen bases in double-stranded nucleic acid. Ethidium bromide, 3,8 diamino 5-ethyl-6phenyl-phenanthridinium bromide (EtBr), is one of these agents. Under excitation with ultraviolet light at 300 nm, EtBr in DNA emits visible light at 590 nm. Therefore, DNA separated in agarose or acrylamide and exposed to EtBR will emit orange light when illuminated by ultraviolet light at 300 nm (Miller *et al*., 1999).EtBr was the most widely used dye in

early DNA and RNA analyses. Care must be taken in handling EtBr because it is carcinogenic. After electrophoresis, the agarose or acrylamide gel can be soaked in a solution of0.1–1–mg/ml EtBr in running buffer (TAE, TBE, or TPE) or TE. Alternatively, dye can be added directly to the gel before polymerization or to the running buffer. The latter two measures save time and allow visualization of the DNA during the run. Dye added to the gel, however, may form a bright front across the gel that could mask informative bands. Dye added to the running buffer produces more consistent staining, although more hazardous waste is generated by this method. Some enclosed gel systems contain EtBr inside a plastic enclosed gel cassette, limiting exposure and waste. After soaking or running in EtBr, the DNA illuminated with ultraviolet light will appear as orange bands in the gel. The image can be captured with a camera or by digital transfer to analytical software. SyBr green is one of a set of stains introduced in 1995 as another type of nucleic acid specific dye system. It differs from EtBr in that it does not intercalate between bases; it sits in the minor groove of the double helix. SyBr green in association with DNA or RNA also emits light in the orange range (Aliyu *et al*., 2004). SyBr green staining is 25–100 times more sensitive than EtBr (detection level: 60 pg of double stranded DNA vs. 5 ng for EtBr). This is due, in part, to background fluorescence from EtBr in agarose. A1 dilution of the manufacturer's 10.000X stock solution of SyBr green in TAE, TBE, or TE can be used in methods described for EtBr. A 1/100 dilution of Sybr green can also be added directly to the DNA sample before electrophoresis. DNA prestaining decreases the amount of dye required for DNA visualization but lowers the sensitivity of detection, at higher DNA concentrations, interfere with DNA migration through the gel. Because SyBr green is not an intercalating agent, it is not as mutagenic. Although SyBr green has some advantages over EtBr, many laboratories continue to use the latter dye due to the requirement for special optical filters for detection of SyBr green. Scanning and photographic equipment optimized for EtBr would have to be modified for optimal detection of the SyBr green stains. New instrumentation with flexible detection systems allows utilization of the SyBr green stains. SyBr green is the preferred dye for real-time PCR (Singer *et al*., 1999).

2.3.3.24 DNA sequencing:

In the clinical laboratory, DNA sequence information, the order of nucleotides in the DNA molecule is used routinely for a variety of purposes, including detecting mutations, typing microorganisms, identifying human haplotypes, and designating polymorphisms. Ultimately, targeted therapies will be directed at abnormal DNA sequences detected by these techniques (Menard *et al.,* 2005).

2.3.3.24.1 Direct sequencing:

The importance of knowing the order, or sequence, of nucleotides on the DNA chain was appreciated in the earliest days of molecular analysis. Elegant genetic experiments with microorganisms detected molecular changes indirectly at the nucleotide level. Indirect methods of investigating nucleotide sequence differences are still in use. Molecular techniques, from Southern blot to the mutation detection methods are aimed at identifying nucleotide changes. Without knowing the nucleotide sequence of the targeted areas, results from many of these methods would be difficult to interpret; in fact, some methods would not be useful at all. Direct determination of the nucleotide sequence, or DNA sequencing, is the most definitive molecular method to identify genetic lesions (Hui *et al*., 2004).

2.3.3.24.2 Manual sequencing:

Direct determination of the order, or sequence, of nucleotides in a DNA polymer is the most specific and direct method for identifying genetic lesions, mutations or polymorphisms, especially when looking for changes affecting only one or two nucleotides. Chemical, Maxam-Gilbert sequencing method was developed in the late 1970s by Allan M. Maxam and Walter Gilbert. Maxam-Gilbert sequencing requires a double- or single-stranded version of the DNA region to be sequenced, with one end radioactively labeled. For sequencing, the labeled fragment, or template, is aliquoted into four tubes. Each aliquot is treated with a different chemical with or without high salt.Upon addition of a strong reducing agent, such as 10% piperidine, the single-stranded DNA will break at specific nucleotides.After the reactions, the piperidine is evaporated and the contents of each tube are dried and resuspended in formamide for gel loading. The fragments are then separated by size on a denaturing polyacrylamide gel (Klaschik *et al.*, 2002).

2.3.3.24.3 Parasites sequencing:

Parasites are typically detected and identified by morphology directly in clinical specimens. This method of diagnosis is subject to false negatives because of low

organism concentrations and depends greatly on appropriately trained personnel. Molecular-based testing has been limited for the parasites mainly because parasites are not a major cause of disease in developed countries. Recognition that travelers from parasite-endemic countries bring the parasite to developed countries and can serve as a reservoir for transmitting the parasite and that expertise in identifying parasites by morphology is declining have made the development of molecularbased assays for parasite detection and identification more of a need than a luxury. PCR assays have been developed for the following parasites: *Trypanosoma cruzi* in patients with chronic Chagas' disease, *Trypanosoma brucei* subspecies *gambiense* and *rhodesiense, Plasmodium* in blood and speciation, *Leishmania* and differentiation to the species level, *Toxoplasma gondii* in suspected congenital and central nervous system infections, *Entamoeba histolytica, Cryptosporidium* in water and microsporidial detection in stool and small intestine biopsies. The development of multiplex PCR assays to detect multiple parasites in stool samples would be extremely useful. First, multiple parasites can cause diarrhea, and morphology is the only way to differentiate between causative agents. Second, patients can have multiple intestinal parasites at the same time and laboratory detection of the presence of all parasites is important. Finally, multiple parasites are transmitted in the same water supply; thus, detection of all parasites and appropriate water treatment will reduce largescale outbreaks of water borne parasites (Petri, 2004).

2.3.3.25 Polymerase chain reaction:

Kary Mullis conceived the idea of amplifying DNA in vitro in 1983 while driving one night on a California highway. In the process of working through a mutation detection method, Mullis came upon a way to double his test target, a short region of double-stranded DNA, giving him 21 or 2, copies. The PCR is a method of in vitro DNA synthesis. Therefore, to perform PCR, all of the components necessary for the replication of DNA in vivo are combined in optimal concentrations for replication of DNA to occur in vitro. This includes the template to be copied, primers to prime synthesis of the template, nucleotides, polymerase enzyme, and buffer components including monovalent and divalent cations to provide optimal conditions for accurate and efficient replication (Menard *et al*., 2005).

2.3.3.25.1 Basic PCR procedure:

When the cell replicates its DNA it requires the existing doublestranded DNA that serves as the template to give the order of the nucleotide bases, the deoxyribonucleo-

tide bases themselves: adenine, thymine, cytosine, and guanine; DNA polymerase to catalyze the addition of nucleotides to the growing strand, and a primer to which DNA polymerase adds subsequent bases (Han *et al*., 2013). PCR essentially duplicates the in vivo replication of DNA in vitro, using the same components to replicate DNA as the cell does in vivo, with the same end result, one copy of double-stranded DNA becoming two copies. Within one to two hours PCR can produce millions of copies called amplicons of DNA. In contrast it would probably take days for a cell to produce the same number of copies in vivo. The real advantage of the PCR is the ability to amplify specific targets. Just as the Southern blot first allowed analysis of specific regions in a complex background, PCR presents the opportunity to amplify and essentially clone the target sequences. The amplified target, then, can be subjected to innumerable analytical procedures. The components of the PCR, DNR template, primers, nucleotides, polymerase, and buffers, are subjected to an amplification program (Menard *et al*., 2005).The amplification program consists of a specified number of cycles that are divided into steps during which the samples are held at particular temperatures for designated times. The temperature will then determine the reaction that occurs and changing the temperature changes the reaction. Denaturation: the double-stranded DNA is denatured into two single strands in order to be replicated. This is accomplished by heating the sample at 94–96°C for several seconds to several minutes, depending on the template. The initial denaturation step is lengthened for genomic or other large DNA template fragments. Subsequent denaturations can be shorter. Annealing is the next and most critical step for the specificity of the PCR. In this step of the PCR cycle, the two oligonucleotides that will prime the synthesis of DNA anneal (hybridize) to complementary sequences on the template. The primers dictate the part of the template that will be amplified; in other words, the primers determine the specificity of the amplification. It is important that the annealing temperature be optimized with the primers and reaction conditions. Annealing temperatures will range 50–70 °C and are usually established empirically. A starting point can be determined using the Tm of the primer sequences for a discussion of stringency and hybridization. Reaction conditions, salt concentration, mismatches, template condition, and secondary structure will all affect the real Tm of the primers in the reaction. Extension is the third and last step of the PCR cycle. This is essentially when DNA synthesis occurs. In this step, the polymerase synthesizes a copy of the template DNA by adding nucleotides to the hybridized primers. DNA polymerase

catalyzes the formation of the phosphodiester bond between an incoming dNTP determined by hydrogen bonding to the template (A: T or G: C) and the base at the 3′end of the primer. In this way, DNA polymerase replicates the template DNA by simultaneously extending the primers on both strands of the template. This step occurs at the optimal temperature of the enzyme, 68–72 °C. In some cases, the annealing temperature is close enough to the extension temperature that the reaction can precede with only two temperature changes. This is two-step PCR, as opposed to threesteps PCR that requires a different temperature for all three steps. At the end of the three steps, or one cycle (denaturation, primer annealing, and primer extension), one copy of double stranded DNA has been replicated into two copies. Increasing the temperature back up to the denaturing temperature starts another cycle, with the end result being a doubling in the number of double stranded DNA molecules again). At the end of the PCR program, millions of copies of the original region defined by the primer sequences will have been generated (Casabianca *et al*., 2003).

2.3.4 *Amoeba* and *Giardia* in Sudan:

Intestinal parasitic infections are public health problem worldwide, particularly in developing countries (Ullah *et al*., 2009). The prevalence of intestinal parasites in Sudan is more in displaced camp due to poor sanitation, primitive standard of living and personal habits of cleanliness (Suliman, 2011). Most studies in Sudan deal with children as they bearing the brunt of infections. A cross sectional study was carried out in Elengaz area, Khartoum, the capital of Sudan.The overall prevalence was 64.4% which is a high percentage and may be attributed to the poor environmental condition of the area in addition to poor quality of life and behavior. The findings of this study indicated that the common intestinal parasites in primary school children were higher *in Giardia lamblia* (33.4%), *and low for Entamoebia histolytica* (3.6%) (Abdelsafi *et al*., 2014). The epidemiology of *Entamoeba histolytica* infection in Sudan is poorly understood. This is due to the inability to differentiate *E. histolytica* from the non pathogenic, *Entamoeba dispar*. (Ali *et al*., 2003). Old methods used such as direct microscopy and culturing are insensitive compared to polymerase chain reaction. By microscopy, 196 stool samples were reported as positive for *E. histolytica*. PCR detected infections caused by *E. histolytica* in 54% (106 of 196), and *Entamoeba dispar* in 51% (100 of 196) of stool samples. By PCR also mixed infections were detected with both *E. histolytica* and *E. dispar* in 5% (10 of196) of stool samples. All 50 negative stool samples examined by microscopy were negative by PCR. The inability to

distinguish *E. histolytica* from the morphologically similar *E. dispar* in stool samples is the main limitation of microscopic methods used mainly in all laboratories in Sudan. Out of 196 positive samples for *E. histolytica* by microscopy were tested by PCR, shown that only 54 % (106 of 196) were positive for *E. histolytica*. The other 45% (90 of 196) were positive for E. *dispar*, which were misdiagnosed as *E. histolytica* infections and mistreated with anti-amoebic drugs. Thus, PCR is recommended for detection and accurate identification of *Entamoeba* species in stool samples (Stanley, 2003). Few studies have shown that the correct incidence and prevalence of *E. histolytica* and *E. dispar* in South Africa, Egypt and Sudan (Stauffer *et al*., 2006). Although in order to report a complete picture of the epidemiology of *E. histolytica* infection and illuminate the disease in Sudan, further epidemiological studies from other part of Sudan need to be done (Saeed *et al*., 2011).

CHAPTER THREE

Materials and Methods

Chapter Three

Materials and Methods

3.1Study design:

It is a cross sectional hospital based study.

3.2 Study area:

The study was carried out in Khartoum State in various hospitals localities.

3.3 Study population:

The study was carried out on outpatients visiting hospitals.The populations were comprised various age groups and gender.

3.4 Sample size:

The sample size was obtained according to the following equation:

$SS= Z^{2*} (P)* (1-P)/ C^2$

Where: SS= Sample size

Z= Z value (1.96 for 95% confidence level).

C= confidence interval (0.05).

P= prevelance annual rate (10%)

1.96* 1.96* 0.1* 0.9/ 0.05* 0.05= 138.

According to the research committee approval this study was conducted on 300 subjects. 100 sample truely positive *Giardia lamblia*, 100 samples truely positive *Entamoeba histolytica* and 100 truly negative samples as a control group. Traditional microscopy and formal ether techniqes are the gold standard methods that were used in the study.

3.5 Data collection:

A questionnaire (appendix 4) was designated to collect data from all participants.

3.6 Techniques:

3.6.1Wet preparation:

This is a routine stool examination procedure for ideal recovery and identification of protozoan parasites and helminths egg (Arora, 2010). The motility of protozoan trophozoite was observed in normal saline preparation, whereas iodine preparation may demonstrate the nuclear details of protozoan cysts. For the use of small amount of sample for preparation, light parasitic infections may not be detected (Lehmann, 1998). The appearance of faecal specimens report the color of the specimens, consistency, whether formed, semiformed or unformed and presence of blood and mucus (Magdalena *et al.*, 2015).The quality of the normal saline was detected under the

microscope using 40X objective lense before used. The methods of wet preparation were performed with high quality (Arora, 2010).

3.6.2 Stool concentration techniques:

Fecal concentration techniques are a routine examination of the stool specimen to assess gastro-intestinal diseases, allowing the detection of small numbers of organisms that may be missed by direct wet smears i.e. concentrate parasites that are low in their secretion. These techniques are also used in surveys and epidemiological studies or in assessing the efficiency of treatment (Garcia, 1997 and Lehmann, 1998). Concentration techniques are divided into formal ether concentration method which is the most frequently used technique because it concentrates a wide range of parasites with minimum damage to their morphology and flotation techniques, Sodium chloride and zinc sulphate in which parasites were concentrated by being floated in solutions of high specific gravity (Magdalena *et al.*, 2015).

3.6.2.1 Formal-ether sedimentation technique:

Parasites were sedimented by using centrifuge, 1-2 cc of faeces equivalent to 1-2 g were collected by cut head syringe, and mixed well with 4 ml of prepared formalin. The suspension was sieved by strainer of mesh size 400-500 um and transferred to a labeled conical centrifuge tube. Further 3-4 ml of diethyl ether was added and shacked well; the tube was centrifuged at 3000 rpm for 1min. Then the tube was inverted to discard the ether, faecal debris and formal saline, the remaining sediment with a little part of fluid was resuspended and mixed well. One drop from the sediment was transferred to a clean slide and covered with a cover glass and examined under microscope using 10 X and 40 X objective lenses. A small drop of Lugol's iodine solution was run under the cover glass to assist the identification of cysts. The numbers of parasites were counted per gram of feces (AL-Kubaisy *et al.*, 2014).

3.6.3 ELISA (copro-antigen detection test):

Stool Ag ELISA kits from Demeditec Diagnostics GmbH of serine-rich 30 KD membrane proteins (SREHP) of *Entamoeba histolytica* and *Giardia lamblia* specific Ag were used (Mirelman *et al.,* 1997). Stool specimens as well as positive and negative controls were diluted and react in the first incubation step of 60 minutes at room temperature with the solid phase bound antibodies. Unbound components were removed from the wells after washing step. Then, horseradish-peroxidase (HRP) labelled antibodies were reacted with solid phase bound antibody-antigen-complexes for 30 minutes at room temperature. Non-bound material was separated from the solid

phase immune complexes by a subsequent washing step (Nar *et al.,* 2003). HRP converts the subsequently added colorless substrate solution of 3, 3, 5, 5'-tetra-methyl-benzidine (TMB) into a blue product. The enzyme reaction was terminated by sulphuric acid that was dispensed into the wells for 10 min incubation at room temperature; the solution was changed from blue to yellow. The optical density (OD) of the solution was read at 450/620 nm which directly proportional to the specifically bound amount of *Entamoeba histolytica* antigen (Uyar and Ozkan, 2009). Stool samples were stored at 2 °C - 8 °C immediately after collection. All dilutions of unpreserved stools were made with the dilution buffer provided. All reagents were warmed to room temperature (RT) before used and mixed gently without causing foam (Gonin and Trudel, 2003). 100 µL of positive control, 100 µL of negative control and 100 µL diluted samples were added in the wells plate. Then the plate was covered with parafilm and incubated for 60 min at RT. Each well was washed 5 times with 300 µL washing solution and tapped to dry onto absorbent paper. 3 drops or 100 µL Conj HRP were dispensed per well. Plate was covered and incubated for 30 min at RT, and then each well was washed 5 times with 300 µL washing solution and inverted to dry onto absorbent paper. 100 µL substrate TMB were dispensed per well and incubated for 10 min at RT. 100 µL stop solution were dispensed and mixed gently. OD was read at 450 nm / ≥ 620 nm with a microplate reader within 30 min after reaction stops. Samples with absorbances equal to or higher than the cut-off value were considered positive; samples with absorbances below the cut-off value were considered negative for *E.histolytica* antigen. The test run was valid if the mean OD of the negative control is > 0.20 for manual performance and > 0.30 for automatic performance (Nar *et al.,* 2003). The mean OD of the positive control was 0.80. Absorbance reading of 0.08 OD units and above indicates the sample contains *Giardia* antigen. Absorbance reading less than 0.08 OD units indicates the sample does not contain detectable levels of *Giardia* antigen (Delialigo *et al.*, 2008).

3.6.4 PCR amplification and detection:

3.6.4.1 Specimen preparation:

DNA was extracted from all fecal specimens without fixatives after storage at −20°C. Briefly, the stool sample (200 mg) was washed twice with 1 ml of sterile phosphate-buffer saline (pH 7.2), centrifuged for 5 min at 14,000 × g and DNA was extracted according to the manufacturer's instructions with the G-spin™ Total DNA Extraction Kit, iNtRON Biotechnology, Korea.

3.6.4.2 Extraction of nucleic acids:

DNA from faecal specimens was obtained according to the protocol previously described by Tovar *et al*., (2003) with mild modification at the incubation time in water path in order to release all DNA from cystic stage. Approximately one g of unpreserved fresh stool or stored at -20°C was homogenized in PBS and passed through gauze to discard larger detritus. The homogenates was centrifuged at 2500 rpm for 3 min. The sediment was resuspended in 3 ml of distilled water. The fecal suspension was stored at - 20°C (Gustavo *et al*., 2015). For extraction, aliquots of 300 µl from fecal suspension were placed in a 1.5 ml eppendorff tube.20 µl of Proteinase K and 5 µl of RNase solutions were added into sample tube and gently mixed. Then 200 µl of Buffer BL was added into upper sample tube and mixed thoroughly. The lysate was incubated at 56°C for 40 min. The 1.5 ml tube was briefly centrifuged to remove drops from the inside of the rid. 200 µl of absolute ethanol was added into the lysate, and mixed well by gently inverting 5 - 6 times or by pipetting. Carefully mixture from step 6 was appllied to the spin column (in a 2 ml collection tube) without wetting the rim, cap was closed, and centrifuged at 13000 rpm for one min. The filtrate was discarded and the spin column was placed in a 2 ml collection tube (reuse). 700 µl of Buffer WA was added to the spin column without wetting the rim, and centrifuge for one min at 13000 rpm. The flow-through was discardedand reused the collection tube. 700 µl of Buffer WB was added to the spin column without wetting the rim, and thencentrifuged for one min at 13000 rpm. Discarded the flow-through and the column wasplacedinto a 2.0 ml collection tube (reuse), then again centrifuged for additionally one min to dry the membrane. The flow-through was discarded and collection tube altogether. The spin column was placed into a new 1.5 ml tube (not supplied), and 30 - 100 µl of Buffer CE was added directly onto the membrane. Incubated for one min at room temperature and then centrifuged for 1 min at 13000 rpm to elute. Elution with 30 µl (instead of 50 µl) increases the final DNA concentration, but reduces overall DNA yield conventionally. A new 1.5 ml tube was used for the second elution step to prevent dilution of the first eluate. Alternatively, the tube was reused for the second elution step to combine the eluates (Aikyo and Oh Ishi, 2014).The concentration and purity of the extracted DNA was assessed by NanoDrop 2000 USA. Concentrations of all DNA samples were ranged from 20 pg and 35 pg, and DNA purity between 1.6-1.9. Amplification of the 432 bp *sequence from GDH* genes for *G. Lamblia* was accomplished as the single PCR run (Read *et al*.; 2004). Both positive and negative controls were included in each PCR run to validate results.

3.6.4.3 Polymerase chain reaction for *Entamoebae:*

Single-round PCR amplification as well as multiplex PCR were used in the study. The sequence of a forward primer used (EntaF, 5’- ATGCACGAGAGCGAAAGCAT-3’) was conserved in all *Entamoeba* spp., whereas the specific reverse primers, EhR (5’-GATCTAGAAACAATGCTTCTCT-3’. Accession number X64142), EdR (5’-CACCACTTACTATCCCT-ACC-3’.Accession number Z49256), and EmR (5’-TGACCGGAGCCAGAGACAT-3’ AF149906), were specific for *E. histolytca, E. dispar and E. moshkovskii* respectively (Hamza *et al.,* 2006 b). PCR was performed using Amplicon (i- master mix PCR, iNtRON) as a ready-made solution. The reaction mixture contained 5 µl of distilled water, 7.5 µl of amplicon, 20 pmol of forward and reverse primers, and about 5-10 ng of extracted DNA template in a final volume of 25 µl. Amplification started with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for one min, 55°C for one min, and 72°C for one min, with a final extension at 72°C for 7 min. Amplified products were visualized after electrophoresis on 1.5% agarose gels by ETBr staining (Fallah *et al.;* 2014). Extraction of fecal DNA when using G spin extraction kits, protocol A for body fluid with mild modification in the incubation time at water path from 10 min to 20 min, result in very good DNA purity and concentration. Concentration of DNA samples were found to be in range from 20 pg and 35 pg, and DNA purity ranging between 1.6 - 1.9. The specific band size resulted by multiplex PCR for *E.histolytca* 166 bp, *E. dispar* 752 bp and for *E. moshkovskii* 580 bp, as published by Zebardast *et al.*, (2014).

3.6.4.4 Polymerase chain reaction for *Giardia lamblia:*

PCR amplification of 432 bp fragment of GDH gene was conducted using PCR protocol of forward primer 5'-CAG TACAACTCYGCTCTC GG-3 'and reveres primers 5'-GTT RTCCTTGCA CAT CTC C-3 (Read and Monis*,* 2004). The mixture of PCR reaction contained Intron master mix, 10 µL of DNA template, 50 pmol of each primer in a final volume of 25 µl. DNA were reproduced using Corbbet Germany under the following conditions: 1 cycle in 94 °C for 8 min (denaturation), 35 cycles in 94 °C for 1 minute, 60 °C for 90 sec, 72 °C for 2 min and a final cycle of 72 °C for 7 minutes . Known *E. histolytica* DNA and DH2O were used as positive and negative control in each run. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide. PCR-RFLP at the gdh locus was performed using restriction enzyme of BspLI (NIaIV, Fermentase) to identify *G. lamblia* genotype A1, A2 and B. RsaI (Fermentase) respectively was used to separate assemblage B genotypes into BIII, BIV.

15 μl of PCR product with 0.5 U of each enzyme along with 1.5 of 1X buffer enzyme, 1μl distilled water in final volume of 18 μl was kept in 37 °C for 2 hours and then the fragments resulted from enzyme restriction were isolated on 3% high resolution agarose gels stained by ethidium bromide after electrophoresis, a ladder of 50 bp was used as a size marker (Read and Monis, 2004).

3.7 Data analysis:

Statistical analysis was performed using SPSS version16 (Statistical Package for Social Sciences). P value ≤ 0.05 was considered statistically significant. The obtained mean of the case study and control group represent the positive and negative clinical cut off level of *E.histolytica* and *G. lamblia* in Sudanese population. Kappa test was done to measure the agreement between the methods. It explains the strength between the two tests, when the value equal 0.5 it represent mirror agreement, above 0.7 represent good agreement and above 0.8 represent very good agreement. Since microscopy after formol ether test was reported as a reference standard test. The sensitivity, specificity, PVP, NPV and accuracy of ELISA copro antigen were calculated with the following formula to analyze data: sensitivity: A/(A+C) × 100]; Specificity: D/(D+B) × 100]; PVP: A/(A+B) × 100]; NPV: D/(D+C) × 100], and accuracy:(TN + TP)/(TN+TP+FN+FP)], where a = true positive samples, b = false positive samples, c = false negative samples and d = true negative samples.

3.8 Ethical consideration:

The study was approved by the ethics in Research Committee of College of Medical Laboratory Science at Sudan University of Science and Technology. Objective of the study was explained at the beginning to all individuals under the study and written consent was obtained from each participant in the study.

CHAPTER FOUR

Results

Chapter Four

Results

Within the100 positive cases diagnosed by formal ether, as gold standard method, *E. histolytica* was detected in 85 (85%) using wet preparation, while it was detected in 88(88%) when ELISA technique was applied, whereas 100 samples in control group was free of *E. histolytica* by both wet preparation and formal ether technique. Only 5 (5%) from case control were detected as false positives by ELISA (Table 4.1). Accuracy of ELISA test and simple microscopy were measured against formol ether as gold standard for diagnosis of *E. histolytica* in fecal sample (Table 4.2). Out of 200 samples of total study population, 93 (46.5%) were detected as positive cases of *E. histolytica* and 107 (53.5%) were negative by copro ELISA (Table 4.3).The clinical cut off ELISA among Sudanese population infected with *E. histolytica* against control group were (0.98±0.20, and 0.49±0.25) respectively (Table 4.4).The point prevalence of *E. histolytica* among study population during data collection equal 5.8%.

Table (4.1): Results of wet preparation and Copro ELISA tests among *Entamoeba SPP.* study group using formal ether as a reference standard:

Techniques	Case group		Control group	
	positive	negative	positive	negative
Formal ether(gold standard)	100	0	100	0
Wet prep	85	15	100	0
ELISA	88	12	95	5

Table (4.2): Accuracy of wet preparation and Copro ELISA: *Entamoeba SPP.*:

Statistics	Wet prep.	Copro ELISA
Sensitivity	85%	88%
Specificity	100%	95%
PPV	100%	94.6%
NPV	86.9%	88.7%
Accuracy	92.5%	91.5%

Table (4.3): Number of positive and negative cases in study population by ELISA:

			Entamoeba cases		
			Positive	Negative	
		Count	88	12	100
		expected count	46.5	53.5	100.0
	negative	count	5	95	100
		expected count	46.5	53.5	100.0
Total		count	93	107	200

Table (4.4): Determination of clinical copro ELISA cut off among case study:

Group	No	Mean	Std. deviation
E.histolytica case group	100	0.98	0.21
E.histolytica control group	100	0.49	0.25

Out of 100 cases of amoebiasis, 55(55%) were males and 45(45%) were females (Table 3. 5). Age of patients ranged from 2-60 years and mean age was 26.8± 12.9 years. Among studied variables, 80% of cases were acute phase of disease. Highest cases of amoebiasis diagnosed by ELISA test were detected in age group 21-30 years (43.8%), followed by 31-40 years age groups (21.3%) and least number of cases (2.2%) in age group above 60 years (Table 4.6). Microscopical appearance of stool samples among case study group of *Entamoeba* complex, cysts were detected in 26 % (n = 100). 30% of *E. histolytica* positive cases had pus in their stool, while RBCs were detected in 20% from all positive cases. None of the *E. histolytica* cases show co-pathogen with *E. coli* trophozoites or cysts. Kappa test showed strong agreement between copro ELISA and microscopy among case and control group (p value ≤ 0.00) (Table 4.7).

Table (4.5): Sex distribution in *E.histolytica* case group:

		Frequency	Percent	Valid percent	Cumulative percent
Valid	males	55	55.0	55.0	55.0
	females	45	45.0	45.0	100.0
	Total	100	100.0	100.0	

Table (4.6): Distribution of *E.histolytica* disease according to sex and age:

E.histolytica			Case group:sex		Total
			males	females	
possitive	Age	> 10	6	4	10
		10 - 20	6	3	9
		21 - 30	20	19	39
		31 - 40	12	7	19
		41 - 50	4	3	7
		51 - 60	1	1	3
		61+	1	1	2
	Total		50	38	88

Table (4.7): Measurement of Kappa between results obtained by Copro ELISA and formol ether in case and control group:

		Value	Asymp. std. error[a]	Approx. T[b]	Approx. sig.
Measure of agreement	Kappa	0.830	0.039	11.767	0.00
N of Valid Cases		200			

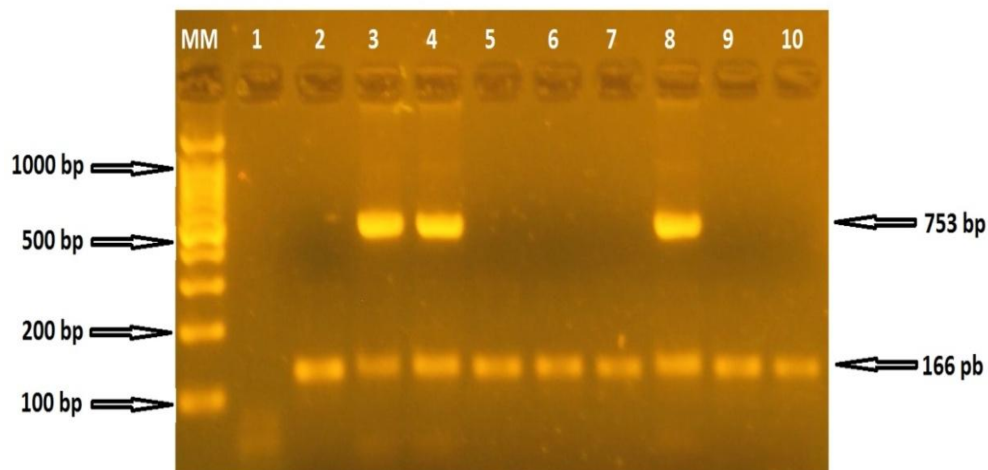

Figure (4.1): PCR results for *Entamoeba* SPP. on agarose:

MM: 100 bp molecular markers, Line 1 is negative control, Line 2, 5,6,7,9 and 10 shows only *E. histolytica* infection, Line 3, 4 and 8 shows co-infection with *E. dispar*.

Only 49 out of 100 parasitized samples (49%) were positive for *E. histolytica* and/or *E. dispar*; 41/49 samples corresponded to individuals infected with *E. histolytica* (84 %);

while 8/49 samples present as coinfection with *E. dispar* (16 %) (Fig 4.1). All the 100 samples of the negative control were negative by PCR. No epidemiology of *E. moshkovskii* occurs in Sudan. Out of 100 positive cases detected by microscopy after formal ether, as gold standard method, Copro Ag ELISA for *G. lamblia* result in 90% sensitivity and 94% specificity (Table 4.8), while 100 samples in control group was free of giardiaisis by wet preparation and concentration technique, 96 (96%) were negative by ELISA, while only 6 (6%) from control group were detected as false positive by ELISA (Table 4.9). Out of 200 participants, 96 (48%) were detected as positive cases of giardiasis and 104 (53.5%) were negative by copro ELISA (Table 4.8).

Table (4.8): Accuracy for giardiasis by Copro Ag ELISA among study population using microscopy as a reference standard:

Parameters	Accuracy
Sensitivity	90%
Specificity	94%
PPV	90%
NPV	93.75%
Accuracy	92%

Table (4.9): Analysis of Copro Ag ELISA results in case and control group:

<table>
<tr><td colspan="3" rowspan="2"></td><td colspan="2">Giardia Copro ELISA</td><td rowspan="2">Total</td></tr>
<tr><td>possitive</td><td>negative</td></tr>
<tr><td rowspan="6">Status</td><td rowspan="3">Case</td><td>Count</td><td>90</td><td>10</td><td>100</td></tr>
<tr><td>% within</td><td>90.0%</td><td>10.0%</td><td>100.0%</td></tr>
<tr><td>% of Total</td><td>45.0%</td><td>5.0%</td><td>50.0%</td></tr>
<tr><td rowspan="3">control</td><td>Count</td><td>6</td><td>94</td><td>100</td></tr>
<tr><td>% within</td><td>6.0%</td><td>94.0%</td><td>100.0%</td></tr>
<tr><td>% of Total</td><td>3.0%</td><td>47.0%</td><td>50.0%</td></tr>
<tr><td colspan="2" rowspan="3">Total</td><td>Count</td><td>96</td><td>104</td><td>200</td></tr>
<tr><td>% within</td><td>48.0%</td><td>52.0%</td><td>100.0%</td></tr>
<tr><td>% of Total</td><td>48.0%</td><td>52.0%</td><td>100.0%</td></tr>
</table>

The clinical cut off Copro antigen ELISA among Sudanese population of giardia case and control group equal (0.13 ± 0.01) and (0.07 ± 0.08)] respectively (Table 4.10). Out of 100 cases of giardiasis, 65 (72.2%) were males and 25 (27.8%) were females (Table 4.11).

Table (4.10): Mean cut off Copro Ag ELISA among case and control groups:

Group	No	Minimum	Maximum	Mean	Std.
Giardia case group	100	0.04	0.65	0.13	0.01
Giardia control group	100	0.02	0.13	0.07	0.08

Table (4.11): Gender distribution among study population:

			Males	Females	Total
Giardiasis	possitive	Count	65	25	90
		% within	72.2%	27.8%	100.0%
		% of Total	65.0%	25.0%	90.0%
	negative	Count	7	3	10
		% within	70.0%	30.0%	100.0%
		% of Total	7.0%	3.0%	10.0%
Total		Count	72	28	100
		% within	72.0%	28.0%	100.0%
		% of Total	72.0%	28.0%	100.0%

The mean age of patients was 26.84 ± 12.9 y. Among studied variables, 80% of cases were acute phase of disease. Highest cases of giardiasis diagnosed by ELISA test were detected in age group of 21-30 years, 43% (Table 4.12). The point prevalence of *G. lamblia* infection during data collection equal 7.4%. Microscopical appearance of stool samples of *G. lamblia* case study group, 88 (88%) with characteristics of acute phase of the disease. Cysts were detected in 15 (15%) (n = 100). 40% of *G. lamblia* positive cases contained pus in stool, while no RBCs were detected in all cases. Accuracy of ELISA was obtained by Kappa test (p value= 0.001) (Table 4.13). The validation and sensitivity of ELISA was measured by ROC curve [fig 2], an area under the curve equal to 0.97, which represent excellent test with high sensitivity and specificity (Table 4.14). Only 42 out of 100 parasitized samples (42%) were positive for *G. lamblia* by PCR (Fig4.3).

Table (4.12): Distribution of *Giardia* infection according to age group:

Age groups:	Gender		Total
	Males	Females	
> 10 years	9	4	13
10 – 20 years	7	2	9
21 – 30 years	30	13	43
31 - 40 years	10	3	13
41 - 50 years	3	2	5
51 - 60 years	3	2	5
More than 61years	1	1	2
Total	63	26	90

Table (4.13): Kappa agreement between Copro Ag ELISA and microscopy results in case and control groups of giardiasis:

		Value	Asymp. std. error[a]	Approx. T[b]	Approx. sig.
Measure of agreem.	Kappa	0.830	0.039	11.767	0.001
N of valid cases		200			

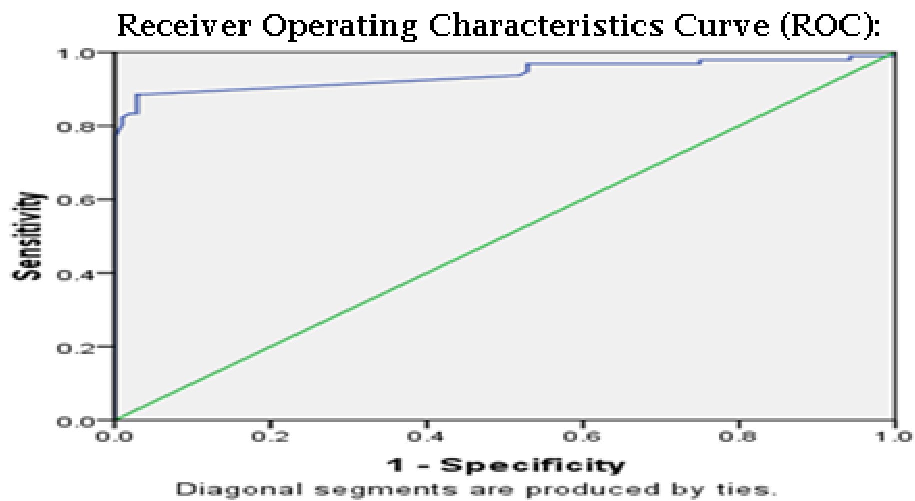

Figure (4.2): ROC curve for diagnosis of giardiasis by Copro Ag ELISA:

Table (4.14): Accuracy an area under the curve (AUC):

Area	Std. error[a]	Asymptotic sig.[b]	Asymptotic 95% confidence interval	
			Lower	Upper
0.939	0.020	.000	0.900	0.978

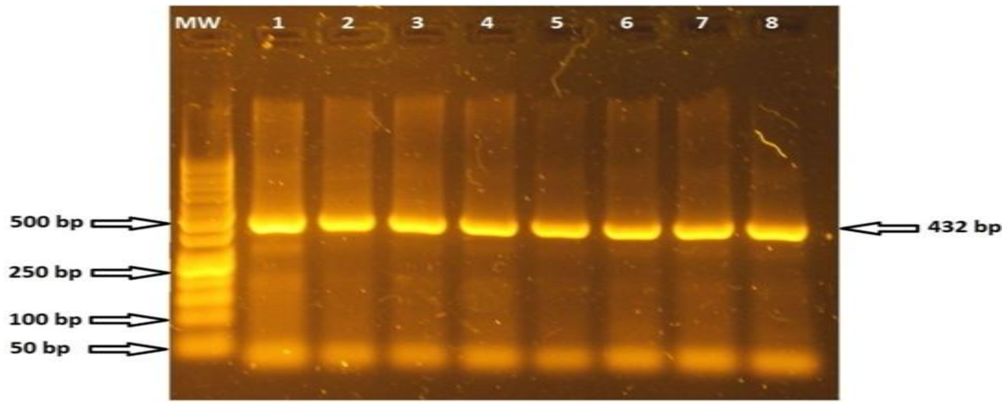

Fig (4.3): Gdh gene on 2%agarose gel with 50bp size marker:

Lane 1 MM: 50 bp molecular markers, sample 1 is positive control, lane 2, 3,4,5,6, 7 and 8 shows positive 432 bp bands for giardiasis.

According to the RFLP results obtained by NIaVI fermentase enzyme at 37°C., from 42 tested samples, 34 (81%) were with AI type assemblage, 6 (14%) with All type assemblage and 2 (5%) with B type assemblage, While the RsaI (Fermentase) was identified the two samples of assemblage B into BIII genotypes.it is noteworthy that BIV type assemblage was not detected in the isolated genes.

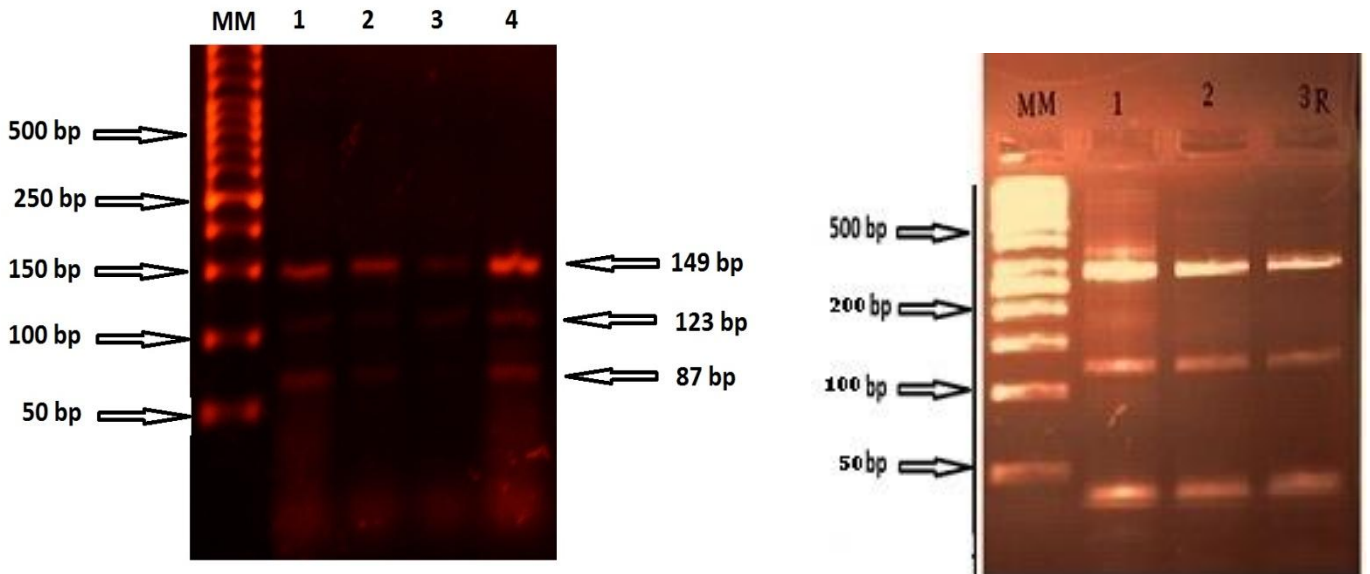

Fig (4.4): PCR-RFLP: fragments size for GDH gene of *Giardia lamblia*:

The effect of BspLI enzyme on PCR products of *Giardia lamblia* on high resolation 3% agar Gel. Lane MM: 50 bp markes, Lane 1 -4 AI genotype.	The effect of RsaI enzyme on PCR products of *Giardia lamblia* on high resolation 3% agar gel. Lane M size marker 50bp and Lane 1, 2 and 3 Blll genotype.

Action of restriction enzymes on GDH gene: fragments size result:

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAGTACAACTCTGCTC TCGG	20	57.73	55.00	4.00	1.00
Reverse primer	GTTGTCCTTGCACATCT CC	19	56.22	52.63	4.00	2.00

Products on target templates

>KT728539.1 *Giardia* intestinalis isolate D06RGDH glutamate dehydrogenase (gdh) gene, partial cds: NIaVI fermentase enzyme

product length = 432
Forward primer 1 CAGTACAACTCTGCTCTCGG 20
Template 4 23
Reverse primer 1 GTTGTCCTTGCACATCTCC 19
Template 435 417

CAGTACAACTCTGCTCTCGGCCCC 24

TACAAGGGTGGCCTCCGCTTCCACCCCTCTGTCAATCTTTCGATTCTCAAGTTCCTC GGTTTCGAGCAGATCCTGAAGAACTCCCTCACCACGCTCCCGATGGGCGGCGGCAA GGGCGGCTCC 123

GACTTTGACCCAAAGGGCAAGTCCGACAACGAGGTCATGCGCTTCTGCCAGTCCTT CATGACCGAGCTCCAGAGGCACGTCGGCGG 87

GACACTGACGTTCCTGCCGGCGACATCGGCGTCGGCGCC 39

CGCGAGATCGGGTACC16

TGTACGGACAGTACAAGCGCCTGAGGAACGAGTTCACAGGCGTCCTCACAGGCAA GAACGTCAAGTGGGGCGGGTCC 77

TTCATCAGGCCGGAGGCTACGGGCTATGGCGCTGTCTACTTCCTGGAGGAGATGTG CAAGGACAACA 67

Table (4.15): Action of RFLP enzymes upon GDH gene:

Diagnostic genotyping profile	Predicted fragment sizes	Enzyme	Assemblage
90, 120, 150	16, 18, 39, 87, 123, 149	NlaIV	AI
40,70, 80, 90, 120	18, 16, 39, 72, 77, 87, 123	NlaIV	AII
40,120, 290	39,18, 123 291	*Nla*IV	B
40,130, 300	2,39, 133, 297	*Rsa*I	BIII
430	2, 430	*Rsa*I	BIV

Bioinformatics Analysis:

The nucleotide sequences of the amplified portion of 18S gene of *E. histolytica* (166 bp) and GDH gene for *Giardia lamblia* (432 bp) were achieved https://dna.macrogen.com/eng/support/contact/contact_us.jsp and viewed for sequences chromatograms by FinchTV program (FinchTV 1.4.0 Windows) (Fig 3.5) and searched 0n (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for sequence similarity using nucleotide BLAST. Highly variant in similarity sequences were retrieved from NCBI and subjected to multiple sequence alignment using the BioEdit software (A biological sequence alignment editor, In GeneMarkS BioEdit v7.0.9). Matching of 18s gene of *E. histolytica* and GDH gene for *Giardia lamblia* with the following sequences ID: KT253454.1 (Iraq), KT253453.1 (Iraq), KJ870202.1 (Cameroon), and ID: JF918446.1 (India), JF918441.1 (India) , JF968194.1 (Iran) respectively. A number of repetitive DNA-containing loci showing PCR fragment size polymorphism are present in *E. histolytica* and *G. lamblia* gene, and significant levels of variation can be detected even among samples from geographically restricted regions.

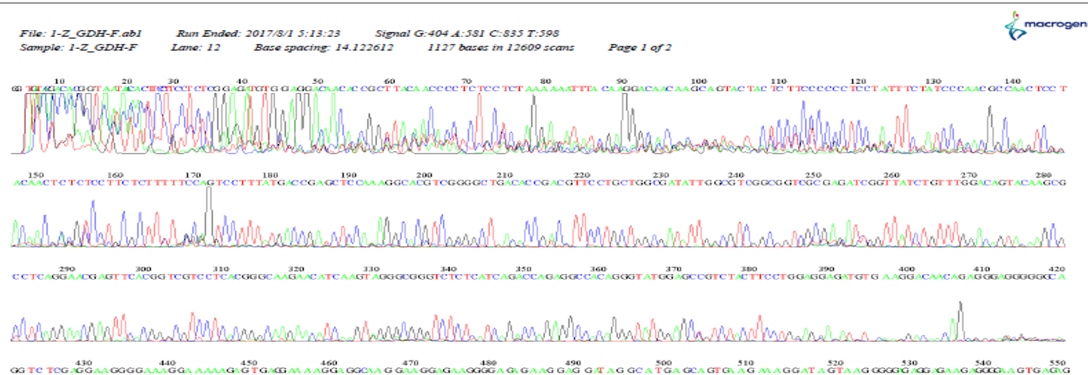

Fig (4.5): Sequencing result from macrogen.

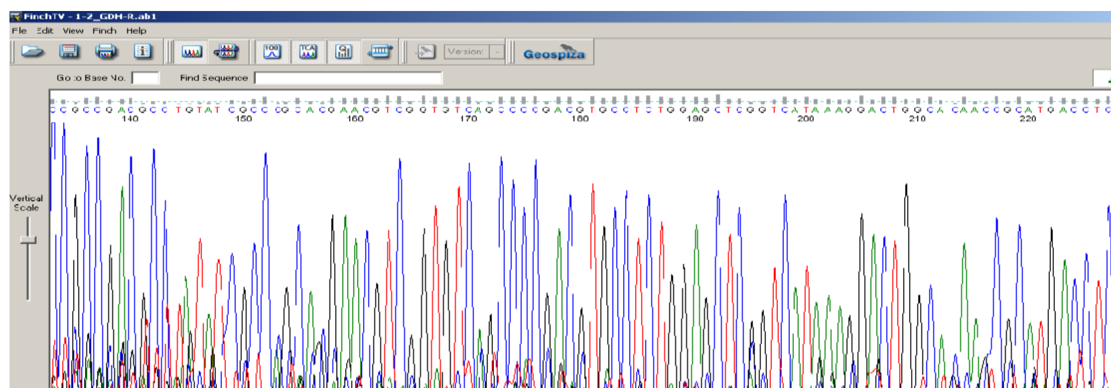

Fig (4.6): GDH gene chromatogram on Finch TV after correction: peaks less than 20% not acceptable. Selected bases range 140-220. (Q 25-42).

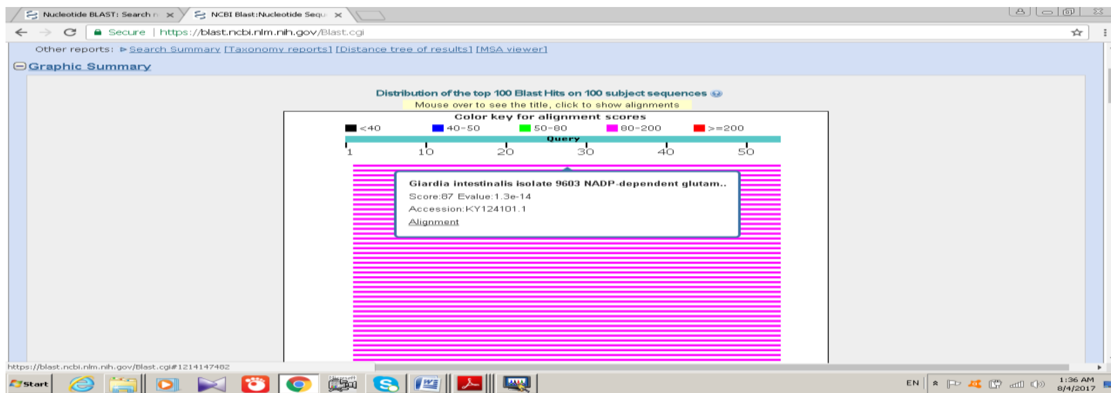

Fig (4.7): **Blast result: GDH gene:** alignment, 98 % homology.

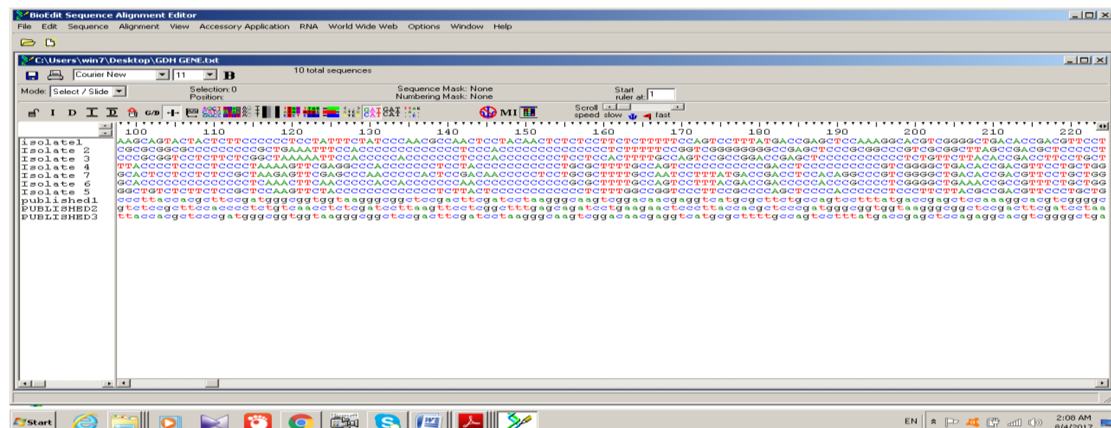

Fig (4.8): BioEdit: Multiple sequence alignment for GDH gene: Showed polymorphism between isolated and published genes**.**

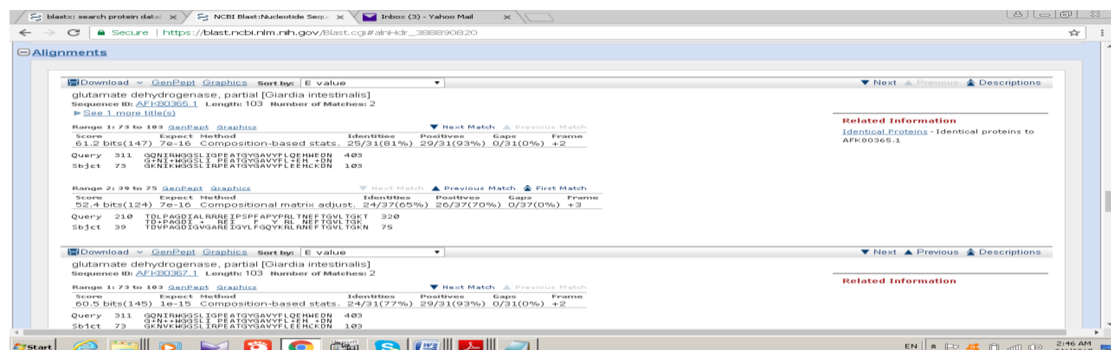

Fig (4.9): NCBI: FASTA: Similarity sequencing for GDH gene.

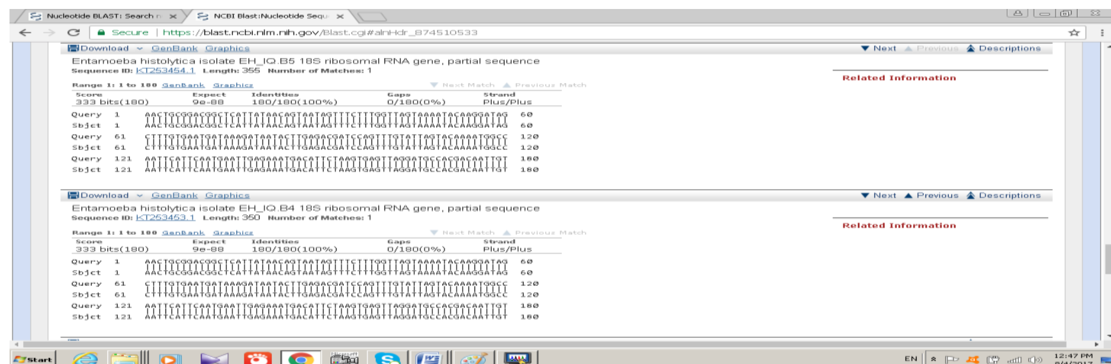

Fig (4.10): NCBI: FASTA: Similarity sequencing for *E. histolytica* 18s gene.

CHAPTER FIVE

Discussion, Conclusions and Recommendations

Chapter Five

Discussion, Conclusions and Recomendations

5.1 Discussion

E. histolytica is the causative agent of amebic dysentry. The prevalence of amoebiasis differs among countries, a fact that was attributed to environmental, socioeconomic, demographic, host hygiene-related behavioral factors (Norhayati *et al.*, 2003; Al-Harthi and Jamjoom, 2007). Our test results showed that out of 100 samples diagnosed by microscopy as positive *E. histolytica*, only (88%) (n=100) were truely positive by copro ELISA and a result of (5%) from control group diagnosed as false negative. While PCR results in (49%) out of 100 truly positive samples diagnosed by microscopy were also positive by PCR, co-infection with *E. dispar* occurs in (16%) of positive PCR samples. This result was agreed with Yvonne (Yvonne *et al.*, (2005), who found that, the limits of detection and efficiency of real-time PCR assay were better than conventional PCR approach for differential diagnosis of two species of *Entamoebas*, *E. histolytica*, and *E. dispar,* which share identical morphology as both cysts and trophozoites. The WHO recommends that *E. histolytica/E. dispar* should be differentiated when ever possible and those patients should not be treated based on microscopy findings alone. Yet, regardless of symptoms, all cases identified as *E. histolytica* should be treated due to the risk of invasive disease, whereas cases found to involve only *E. dispar* should not be treated. The benefit of copro ELISA is valuable in epidemiological study and in diagnosis, especially for those who had low parasites secretion in their stool samples ie negative result by microscopy with continuous sign and symptoms. Our successful PCR result agrees with Zaki and Clark (2001), Haghighi *et al*., (2002). The fact that some of the samples did not give amplification products at any of the loci suggests that the DNA might have been lost or degraded during the extraction procedure. Another explanation for low PCR results done by Zaki *et al*. (2003) who found that a significant proportion of the *E. histolytica* (25%) and a few of the *E. dispar* (5%) samples gave amplification products at some, but not all, of the loci. There is evidence that this may be due to sequence diversity among isolates in the primer binding regions. Only 8 of 47 *E. histolytica* samples tested with the original primer pair 9-11 amplified successfully, but, using modified primer 11A, we were able to amplify 37 samples. Most outcomes of researchers suggest that the diagnosis of amebiasis based only on microscopy is inadequate (WHO, 2013; Norhayati *et al*. 2003). Thus differentiating it from non-pathogenic species is important (Ruiz *et al*., 1994; Jasim and Alradi, 2015). Our finding

was agreed with the result performed by (Roy *et al.* 2005; Visser *et al.* 2006), they found that, ELISA II for *E. histolytica* compared to real-time PCR as a reference test demonstrsted good level of sensitivity and pecificity 79% and 96% respectively. Also (*Gatti et al.* 2002), demonstrated that very low sensitivity (14.3%) and specificity (98.4%), were obtained when were compared to culture. In another comparative study done by *Hamzah et al*. (2006a) showed that, out of 27 stools sampled positive for *Entamoeba* sp. by microscopy, only 7 (25%) were successfully identified at species level by PCR, which included only one sample positive for *E. histolytica* and 6 samples positive for *E dispar*. The WHO recommends that *E. histolytica/E. dispar* should be differentiated when-ever possible and that patients should not be treated based on microscopy findings alone. Yet, regardless of symptoms, all cases identified as *E. histolytica* should be treated due to the risk of invasive disease, whereas cases found to involve only *E. dispar* should not be treated. Our finding indicates that 86% (43/49) of amoebiasis were acute phase of disease and very low prevalence of chronic symptoms equal 14% (7/49). Similar result was btained by Saeed *et al* (2011), (54%) of positive *E. histolytica* in stool samples were detected by PCR, and *Entamoeba dispar* in (51%) of stool samples. These results allow us to improve the gold standard method that must differentiate between pathogenic *E. histolytica* and non pathogenic *E. dispar* to be available in routine diagnostics. Acceptable mean cut off results between case study group and case control when using copro ELISA. This result is considered as a reference data which helps in diagnosis of *E. histol*ytica by copro antigen ELISA in Sudan. The diagnosis of giardiasis is based primarily on microscopic examination of stool samples through the identification of motile trophozoites or cystic stage (Hamzah *et al*., 2006a). Microscopic examination requires examining three consecutive stool samples in order to obtain higher sensitivity (over 90 %). Lower sensitivity (approximately 50 %) of a single sample examination may be attributed to low parasite density, sporadic excretion of cysts or the possibility of the parasite being masked with bile pigments (Al-Harthi and Jamjoom, 2007). The need for a more robust diagnostic techniques lead to the development of rapid, sensitive and specific diagnostic methods (Hamzah *et al*., 2006a). ELISA is a rapid, sensitive and cost effective method for detection of specific antigens in stools and confirmation of certain infection. Copro antigens of a parasite could be traced and diagnosed even if the live parasite is absent in the fecal samples (Saeed *et al*., 2011). The present study showed that the percentage of positive rates of *G. lamblia* that were detected by using direct wet mount was (45%),

while it was increased to reach more than (50%) when using formal either concentration technique (FECT).These results were similar to results obtained by (Eltayeb *et al.* 2012) and disagreed with the result of (Gabbad and Elawad, 2014) in Khartoum State. The current study revealed that the prevalence of *G. lamblia* infection among males was (70 %) higher than in females (30%); these results were in agreement with Yakoob *et al.,* (2005) who found that the prevalence of *G. lamblia* was 38.9% higher in males than in females in Pakistan. The present study showed that the prevalence rate of *G. lamblia* was higher (43%) in the age group 21-30 years old; it was found that drinking tap water and contaminated foods from out side were associated with increased risk of infection. These results were not in line with Iraqian study which was done by Raza and Sami (2009) who showed that the highest rate of infection (17%) was among the age group (6-10) years old. Our study demonstrated that the sensitivity and specificity of the copro ELISA test for detection of *G. lamblia* versus microscopy were 90 % and 94%. These results were similar to an Iraqian study conducted by Mahmood *et al* (2014), 76.4% and 100 %. *Giardia* cysts or trophozoites are difficult to recover from infected patients. Quality of the diagnosis can be confirmed by examining at least three stool samples over several days. Copro antigen ELISA test is more sensitive and specific. Several immunological tests can detect *Giardia* antigens in stool specimen but until now does not replace the simple microscopy. We recommend using ELISA in epidemiological surveys in Sudan, same as a result obtained by Al-Saeed and Issa, (2010), and to confirm the diagnosis in patients with typical clinical symptoms of giardiasis but with negative results by direct microscopy. PCR for *Giardia* GDH gene results in 42% sensitivity, RFLP showed that, 81% (34/42) group AI, 14% (6/42) group AII and 5% (2/42) group B. 86% positive giardiasis with acute phase of the disase were significanyly associated with the virulence strain Group AI at p value = 0.01, while group B related to chronic diarrhea. High proportion of infection refers to the high virulence of strain A1 genotype among Sudanese population. This result disagrees with Rashidul *et al*., (2005); who showed that, assemblage B was the more prevalent genotype in the region,247 infections were with assemblage B, and 36 were with assemblage A. our finding that assemblage A infection was the most prevalent genotype in Khartoum State and significantly associated with increased OR of acute diarrhea. In contrast, assemblage B infection was statistically associated with asymptomatic giardiasis, which was found to occur at a significant rate of (14.0%) among Sudanese population.

5.2 Conclusions

The study concluded that, the point prevalence of diarrhea caused by *E. histolytica* and *G. lamblia* in Khartoum State was 13.2 %. The clinical cut-off ELISA that were obtained from our data were higher than the diagnostic cut off. The genetic characterization indicated that the present severity of infections by *E. histolytica* due to virulent HE strain, and A1 sub genotype of *G. lamblia and* were considered as the most prevalent strains within the Sudanese population. Low prevalence rate (16%) of *E. dispar* occurred as co-infection with *E. histolytica* .The diagnosis of *E. histolytica* and *G. lamblia* by copro antigen ELISA has high sensitivity and specificity, incompared to PCR which required highly QC during procedure and avoid inhibitors from fecal samples. Phylogenetic analysis revealed that the isolated HE genes of the *E.histolytica* were related to the genes isolated from Iraq and Cameroon. While *G. lamblia* isolated genes were not genetically differentiated among shared isolates of India and Iran.

5.3 Recommendations:

1- Large scale study is needed to measure the prevalence of the disease and the factors that play a role in continuous infection including hospitals data, contamination of raw vegetables and water resources.

2-Improving the hygienic standards among the population in Khartoum state to help in controlling the disease.

3- Recommend to use Copro Ag ELISA as a routine test in diagnosis of giardiasis and amoebiasis and in epidemiological surveys in Sudan.

4- Using of isoenzyme analysis to differentiate between pathogenic and non pathogenic strain of *E. histolytica* especially for patient admitted with severe dysentery.

5- Using our cut off results as a reference data in diagnosis of *E. histolytica* and *G. lamblia when using copro* antigen ELISA among Sudanese population.

6- Introduction of PCR technique for specific identification of *Entamoeba* spp. in clinical specimens which is an important confirmatory diagnostic step in the management of patients who may be infected with *E. histolytica.*

7- Continue the study upon the genes that were isolated to detect the presence of mutation and rates of effectiveness or resistant to drugs, mainly metronidazole.

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APPENDICIES

Appendicies

Appendix (1): Raw data:

Table (1): *E. histolytica* case group:

no	Area	disease	stage	formal	age	color	stool	blood	mucus	Pus cell	RBCs
1	خ شرق	acute	trophozoite	4/hpf	22	brown	soft	1	2	1…3	0-1
2	خ شرق	acute	cyst	5/hpf	25	brown	soft	nil	1	2…4	0-2
3	خ شرق	acute	trophozoite	2/hpf	5	yellow	watery	nil	2	2…4	0-3
4	خ شرق	acute	trophozoite	3/hpf	7	reddish	soft	2	1	1…3	0-3
5	خ شرق	acute	trophozoite	3/hpf	8	yellow	soft	nil	3	5…7	0-1
6	خ شرق	acute	cyst+troph	5/hpf	28	brown	fluid	1	4	2…4	2..4
7	خ شرق	acute	trophozoite	3/hpf	28	brown	solid	1	2	2…4	0-4
8	خ شرق	acute	cyst	2/hpf	22	yellow	soft	nil	2	2…3	1 3
9	خ شرق	acute	cyst	9/hpf	44	brown	fluid	nil	1	1…3	0-1
10	خ شرق	acute	cyst+troph	4/hpf	49	brown	fluid	4	4	4…6	uncount
11	جنوب	acute	trophozoite	2hpf	19	yellow	soft	3	2	6…8	6..8
12	خ شمال	acute	trophozoite	4/hpf	4	yellow	soft	nil	1	3…5	0-2
13	خ شمال	acute	trophozoite	6/hpf	55	yellow	soft	1	2	2…4	0-3
14	خ شرق	acute	trophozoite	9/hpf	31	yellow	soft	nil	1	2…4	0-3
15	جنوب	acute	trophozoite	1/hpf	33	d.brown	fluid	nil	1	1…3	2..4
16	خ شمال	acute	trophozoite	1/hpf	35	green	fluid	nil	2	1…3	0-2
17	خ شمال	chronic	trophozoite	5/hpf	23	yellow	soft	2	4	uncount	0-4
18	خ شمال	acute	cyst+troph	6/hpf	30	yellow	solid	nil	2	0…3	1 3
19	خ غرب	acute	trophozoite	3/hpf	30	brown	soft	1	1	2…4	2..4
20	جنوب	chronic	trophozoite	4/hpf	33	brown	solid	1	2	uncoun	0-1
21	جنوب	chronic	trophozoite	5hlpf	28	brown	fluid	nil	2	2..4	0-2
22	خ غرب	acute	cyst+troph	2/hpf	13	yellow	fluid	2	1	4…6	3..5
23	غرب	acute	trophozoite	10/hpf	26	reddish	fluid	nil	1	2…4	0-2
24	جنوب	acute	trophozoite	3/hpf	24	yellow	fluid	nil	2	1…3	0-3

25	خ شمال	acute	cyst	1/hpf	10	brown	fluid	nil	4	2…4	0-3
26	خ غرب	acute	troph+cyst	3/hpf	25	brown	watery	3	2	uncoun	4..6
27	خ غرب	acute	troph+cyst	2/hpf	27	yellow	soft	nil	1	1…3	0-2
28	جنوب	acute	troph+cyst	6/hpf	29	yellow	soft	2	2	2…4	2..5
29	خ شرق	acute	troph+cyst	8/hpf	28	green	fluid	nil	2	2…4	1 3
30	خ شرق	acute	troph+cyst	2hpf	29	brown	solid	nil	1	1…3	0-1
31	خ شرق	chronic	troph+cyst	4/hpf	27	yellow	soft	nil	3	5…7	0-2
32	خ شرق	acute	trophozoite	5/hpf	30	yellow	fluid	3	2	uncoun	uncount
33	خ غرب	acute	trophozoite	7/hpf	28	reddish	fluid	2	1	uncoun	6..8
34	جنوب	acute	trophozoite	3/hpf	34	yellow	fluid	nil	1	2…3	0-3
35	خ شمال	chronic	trophozoite	4/hpf	11	brown	soft	nil	1	1…3	0-1
36	خ شرق	chronic	cyst	1/hpf	5	brown	solid	4	2	uncoun	uncount
37	خ شرق	acute	trophozoite	5/hpf	4	yellow	soft	nil	2	2..4	0-4
38	خ شمال	chronic	trophozoite	3/hpf	9	brown	solid	2	1	3…5	4..6
39	خ شرق	acute	trophozoite	4/hpf	42	brown	fluid	1	2	4..6	3..5
40	خ غرب	acute	cyst+troph	4/hpf	40	yellow	fluid	nil	2	5…7	0-1
41	خ شرق	acute	trophozoite	2/hpf	33	yellow	soft	nil	1	2…4	0-2
42	جنوب	acute	cyst	1/hpf	26	yellow	watery	nil	2	2…4	0-1
43	جنوب	acute	cyst	3/hpf	41	yellow	soft	nil	1	2…3	0-2
44	جنوب	acute	cyst+troph	1/hpf	30	brown	soft	nil	1	1…3	0-3
45	خ غرب	acute	cyst	3/hpf	57	green	fluid	2	2	4…6	5..7
46	خ شرق	acute	cyst	2/hpf	25	yellow	solid	3	4	6…8	uncount
47	خ غرب	acute	cyst	1/hpf	44	yellow	soft	nil	2	3…5	0-2
48	خ شمال	chronic	cyst	2/hpf	32	brown	fluid	nil	1	2…4	0-4
49	خ شمال	acute	cyst	2hpf	29	brown	fluid	nil	2	2…4	1 3
50	خ شمال	chronic	trophozoite	4/hpf	60	brown	soft	nil	2	1…3	0-1
51	خ شمال	acute	cyst+troph	1/hpf	30	yellow	soft	nil	1	1…3	0-2

52	جنوب	acute	trophozoite	1/hpf	34	reddish	soft	2	1	uncount	4..6
53	جنوب	acute	cyst	5/hpf	40	yellow	soft	2	2	uncoun	3..5
54	جنوب	chronic	cyst	2/hpf	40	brown	fluid	nil	2	4..6	0-3
55	جنوب	acute	cyst+troph	3/hpf	54	brown	fluid	nil	1	3…5	0-3
56	خ شرق	acute	trohozoite+cyst	5/hpf	34	yellow	soft	nil	3	2…4	0-1
57	خ شرق	acute	trophozoite	3/hpf	20	yellow	solid	3	2	5…7	uncount
58	خ شرق	acute	trophozoite	4/hpf	21	green	soft	1	1	2…4	0-4
59	خ شرق	acute	trophozoite	7/hpf	22	brown	solid	nil	1	2…4	1 3
60	خ شرق	acute	trophozoite	2/hpf	35	yellow	fluid	nil	1	2…3	0-1
61	خ شرق	acute	trophozoite	6//hpf	18	green	fluid	nil	2	1…3	0-2
62	خ شمال	acute	trophozoite	3/hpf	12	yellow	fluid	1	2	4…6	0-3
63	خ شمال	acute	trophozoite	1/hpf	6	yellow	fluid	nil	1	0..2	0-3
64	جنوب	acute	trophozoite	3/hpf	11	brown	fluid	2	2	3…5	4..6
65	جنوب	chronic	cyst	2/hpf	30	brown	watery	nil	2	2…4	0-2
66	جنوب	acute	trophozoite	8/hpf	32	brown	soft	1	2	2…4	0-4
67	جنوب	acute	trophozoite	2/hpf	55	yellow	soft	nil	1	1…3	1 3
68	خ شرق	chronic	cyst	4/hpf	25	reddish	fluid	nil	2	1…3	0-1
69	خ شرق	chronic	cyst	4/hpf	42	yellow	solid	2	3	uncoun	3..5
70	خ شرق	acute	trophozoite	4/hpf	24	brown	soft	1	3	5…7	2..4
71	خ شرق	acute	cyst+troph	1/hpf	6	brown	fluid	4	4	uncoun	uncount
72	خ شرق	acute	trophozoite	1/hpf	36	yellow	fluid	nil	2	2…5	0-3
73	خ شرق	acute	cyst+troph	6/hpf	39	brown	fluid	nil	2	1…3	0-3
74	خ شرق	acute	trophozoite	2h/pf	64	brown	soft	2	3	uncount	3..5
75	خ شرق	acute	cyst	1/hpf	30	yellow	solid	3	4	uncoun	uncount
76	خ شرق	acute	cyst	3/hpf	29	reddish	soft	3	2	uncoun	6..8
77	خ شرق	acute	cyst+troph	4/hpf	30	yellow	fluid	1	1	6…8	1 3
78	خ شرق	chronic	trophozoite	4/hpf	24	brown	solid	nil	2	4…6	0-1

79	خ شرق	acute	trophozoite	2/hpf	22	brown	soft	nil	1	2…4	0-1
80	خ شرق	chronic	trophozoite	1/hpf	29	yellow	fluid	nil	3	3…5	0-2
81	خ شرق	acute	trophozoite	4/hpf	27	yellow	solid	3	4	uncount	uncount
82	جنوب	acute	trophozoite	1/hpf	25	green	soft	nil	2	2…4	0-2
83	خ شمال	acute	cyst	3/hpf	22	brown	fluid	nil	2	1…3	0-3
84	خ شمال	chronic	trophozoite	8/hpf	24	yellow	fluid	2	1	uncoun	3..5
85	خ شرق	acute	trophozoite	1/hpf	28	green	soft	1	2	4..6	2..4
86	خ شرق	acute	cyst	2/hpf	23	yellow	soft	nil	2	1…3	0-2
87	خ شرق	acute	cyst	2hpf	29	yellow	soft	3	1	uncoun	5..7
88	خ شرق	acute	trophozoite	4/hpf	26	brown	soft	nil	2	1…3	1 3
89	خ شرق	acute	cyst+troph	1/hpf	29	brown	fluid	nil	1	2…4	0-1
90	خ شرق	acute	trophozoite	1/hpf	28	brown	fluid	nil	1	2…4	0-2
91	خ شمال	acute	cyst+troph	2hpf	18	yellow	soft	nil	2	1…3	0-1
92	خ شمال	acute	trophozoite	4/hpf	14	reddish	fluid	4	4	uncount	uncount
93	جنوب	acute	cyst	6/hpf	10	brown	soft	nil	2	2…4	0-3
94	جنوب	acute	cyst	9/hpf	22	yellow	solid	1	1	2…4	0-3
95	خ شرق	chronic	cyst+troph	1/hpf	25	brown	soft	nil	2	2…3	0-1
96	خ شرق	acute	trophozoite	1/hpf	5	brown	fluid	2	2	3..5	4..6
97	جنوب	acute	trophozoite	5/hpf	7	yellow	solid	nil	1	4…6	0-4
98	شمال	chronic	cyst	6/hpf	8	yellow	soft	2	3	6…8	3..5
99	شمال	chronic	cyst	3/hpf	28	yellow	fluid	1	2	4..6	1..3
100	شمال	Acute	trophozoite	4/hpf	14	reddish	fluid	4	4	uncount	uncount

Table (2): *Giardia lamblia* case group:

no	العمر	السكن	disease	wet preparation	conc	color	stool	mucous	blood	pus cells	RBCs
101	12	خ شمال	acute	trophozoite	2/hpf	yellow	fluid	1	nil	2…4	0-2
102	5	خ شمال	chronic	trophozoite+cyst	2hpf	brown	soft	2	nil	6…8	0-1
103	3	خ شمال	acute	trophozoite+cyst	4/hpf	brown	soft	1	nil	1…3	0-1
104	9	خ شمال	acute	trophozoite	1/hpf	yellow	soft	nil	nil	0…2	0-1
105	26	خ جنوب	acute	trophozoite	1/hpf	yellow	soft	4	nil	uncoun	0-2
106	27	خ جنوب	acute	trophozoite	1/hpf	green	fluid	2	nil	1…3	0-1
107	28	خ جنوب	acute	trophozoite	1/hpf	brown	fluid	1	nil	1…2	0-2
108	29	خ جنوب	acute	trophozoite	1/hpf	yellow	soft	1	nil	1…3	0-3
109	30	خ شرق	acute	trophozoite	1/hpf	yellow	solid	1	nil	2…4	0-3
110	24	خ شرق	acute	trohozoite+cyst	3/hpf	brown	soft	3	nil	5…7	0-1
111	22	خ شرق	acute	trophozoite	4/hpf	bloody	solid	4	nil	uncoun	0-2
112	29	خ شرق	acute	trophozoite	1hpf	brown	fluid	2	nil	2…5	0-4
113	27	خ شرق	acute	trophozoite	2/hpf	yellow	fluid	2	nil	1…3	1 3
114	25	خ شرق	acute	trophozoite	1/hpf	yellow	fluid	1	nil	0…3	0-1
115	22	خ شرق	chronic	trophozoite	3/hpf	yellow	fluid	2	nil	2….3	0-2
116	24	خ شرق	acute	trophozoite	1/hpf	brown	fluid	1	nil	0…2	0-1
117	28	خ شرق	acute	trophozoite	3/hpf	brown	soft	4	nil	6…8	0-2
118	23	خ شرق	acute	trophozoite	2/hpf	yellow	soft	3	nil	4…6	0-3
119	29	خ شرق	acute	cyst	1/hpf	yellow	watery	2	nil	2…4	0-3
120	26	خ شرق	acute	trophozoite	2/hpf	yellow	soft	4	nil	uncoun	0-1
121	29	خ شرق	acute	trophozoite	2hpf	yellow	soft	3	nil	2…4	0-2
122	28	خ شرق	acute	trophozoite	4/hpf	brown	fluid	2	nil	uncoun	0-4
123	18	خ جنوب	acute	trophozoite	1/hpf	green	solid	1	nil	1…3	1 3
124	14	خ شمال	acute	trophozoite	1/hpf	yellow	soft	2	nil	2…4	0-1
125	10	خ شمال	acute	trophozoite	1/hpf	yellow	fluid	1	nil	2…4	0-1
126	22	خ شرق	acute	trophozoite	1/hpf	brown	fluid	nil	nil	1…3	0-2
127	25	خ جنوب	acute	trophozoite	1/hpf	brown	fluid	4	nil	5…7	0-1
128	5	خ شمال	acute	trophozoite	1/hpf	brown	soft	2	nil	2…4	0-2
129	7	خ شمال	acute	trophozoite	3/hpf	yellow	solid	1	nil	2…4	0-3
130	8	خ شمال	acute	trophozoite	4/hpf	reddish	soft	1	nil	2…3	0-3
131	28	خ غرب	acute	trophozoite	1hpf	yellow	solid	1	nil	1…3	0-1
132	28	خ جنوب	acute	trophozoite	2/hpf	brown	fluid	3	nil	4…6	0-2
133	22	خ جنوب	acute	trophozoite	1/hpf	brown	fluid	4	nil	6…8	0-4

134	44	خ غرب	acute	trophozoite	3/hpf	yellow	fluid	2	nil	3…5	1 3
135	49	خ غرب	acute	trophozoite	1/hpf	brown	fluid	2	nil	2…4	0-1
136	19	خ جنوب	acute	trophozoite	3/hpf	brown	fluid	1	nil	2…4	0-2
137	4	خ شمال	acute	trophozoite	2/hpf	yellow	soft	2	nil	1…3	0-1
138	55	خ غرب	acute	trophozoite	1/hpf	yellow	soft	1	nil	1…3	0-2
139	31	خ غرب	acute	trophozoite	2/hpf	yellow	watery	4	nil	0…2	0-3
140	33	خ جنوب	acute	trophozoite	2hpf	yellow	soft	3	nil	uncoun	0-3
141	35	خ شرق	acute	trophozoite	4/hpf	brown	soft	2	nil	1…3	0-1
142	23	خ شرق	acute	trophozoite	1/hpf	green	fluid	4	nil	4…6	0-2
143	30	خ شرق	acute	cyst	1/hpf	yellow	solid	3	nil	1…3	0-4
144	30	خ شرق	acute	trophozoite	1/hpf	yellow	soft	2	nil	2…4	1 3
145	33	خ غرب	acute	trophozoite	1/hpf	brown	fluid	nil	nil	5…7	0-1
146	28	خ جنوب	acute	trophozoite	1/hpf	brown	fluid	1	nil	2…4	0-2
147	13	خ شمال	chronic	cyst+troph	1/hpf	brown	soft	2	nil	2…5	0-3
148	26	خ شرق	acute	trophozoite	3/hpf	yellow	soft	4	nil	3…5	0-3
149	24	خ شرق	acute	cyst	4/hpf	reddish	soft	nil	nil	0…3	0-1
150	10	خ شمال	chronic	cyst	1hpf	yellow	soft	nil	nil	2…4	0-2
151	25	خ شرق	chronic	cyst+troph	2/hpf	brown	fluid	4	nil	uncoun	0-4
152	27	خ غرب	acute	trophozoite	1/hpf	brown	fluid	2	nil	6…8	1 3
153	29	خ شرق	acute	trophozoite	3/hpf	yellow	soft	1	nil	4…6	0-1
154	28	خ جنوب	acute	trophozoite	1/hpf	yellow	solid	1	nil	2…4	0-2
155	29	خ جنوب	acute	trophozoite	3/hpf	green	soft	1	nil	1…3	0-1
156	27	خ جنوب	acute	trophozoite	2/hpf	brown	solid	3	nil	2…4	0-2
157	30	خ غرب	acute	trophozoite	1/hpf	yellow	fluid	4	nil	uncoun	0-3
158	28	خ شرق	acute	trophozoite	2/hpf	yellow	fluid	2	nil	1…3	0-3
159	34	خ غرب	acute	cyst+troph	2hpf	brown	fluid	2	nil	2…4	0-1
160	11	خ شمال	acute	trophozoite	4/hpf	bloody	fluid	1	nil	2…4	0-2
161	5	خ شمال	chronic	trophozoite	1/hpf	brown	fluid	2	nil	1…3	0-4
162	4	خ شمال	acute	cyst	1/hpf	yellow	soft	1	nil	5…7	1 3
163	9	خ شمال	acute	troph+cyst	1/hpf	yellow	soft	4	nil	uncoun	0-1
164	42	خ جنوب	acute	troph+cyst	1/hpf	yellow	watery	3	nil	uncoun	0-1
165	40	خ جنوب	chronic	troph+cyst	1/hpf	brown	soft	1	nil	2…3	0-2
166	33	خ جنوب	chronic	troph+cyst	1/hpf	brown	soft	nil	nil	1…3	0-1
167	26	خ جنوب	acute	troph+cyst	3/hpf	yellow	fluid	4	nil	uncoun	0-2
168	41	خ شرق	chronic	troph+cyst	4/hpf	yellow	solid	2	nil	6…8	0-3

169	30	خ شرق	acute	trophozoite	1hpf	yellow	soft	1	nil	3…5	0-3
170	57	خ شرق	acute	trophozoite	2/hpf	yellow	fluid	1	nil	2…4	0-1
171	25	خ شرق	acute	trophozoite	1/hpf	brown	fluid	1	nil	2…4	0-2
172	44	خ شرق	acute	trophozoite	3/hpf	green	fluid	3	nil	5…7	0-4
173	32	خ شرق	acute	trophozoite	1/hpf	yellow	soft	4	nil	uncoun	1 3
174	29	خ شرق	acute	trophozoite	3/hpf	yellow	solid	2	nil	5…7	0-1
175	60	خ شرق	acute	trophozoite	2/hpf	brown	soft	2	nil	uncoun	0-2
176	30	خ شرق	acute	trophozoite	1/hpf	brown	solid	1	nil	2…5	0-1
177	34	خ شرق	acute	trophozoite	2/hpf	brown	fluid	2	nil	1…3	0-2
178	40	خ شرق	chronic	trophozoite	2hpf	yellow	fluid	1	nil	0…3	0-3
179	40	خ شرق	acute	trophozoite	4/hpf	reddish	fluid	4	nil	uncoun	0-3
180	54	خ شرق	chronic	trophozoite	1/hpf	yellow	fluid	3	nil	uncoun	0-1
181	34	خ شرق	acute	trophozoite	1/hpf	brown	fluid	2	nil	6…8	0-2
182	20	خ جنوب	acute	trophozoite	1/hpf	brown	soft	4	nil	4…6	0-4
183	21	خ شمال	acute	trophozoite	1/hpf	yellow	soft	3	nil	2…4	1 3
184	22	خ شمال	chronic	cyst+troph	1/hpf	brown	watery	2	nil	3…5	0-1
185	35	خ شرق	acute	cyst	1/hpf	brown	soft	nil	nil	2…4	0-2
186	18	خ جنوب	acute	trophozoite	3/hpf	yellow	soft	1	nil	2…4	0-3
187	12	خ شمال	acute	trophozoite	4/hpf	yellow	fluid	2	nil	1…3	0-3
188	6	خ شمال	acute	trophozoite	1hpf	yellow	solid	4	nil	uncoun	0-1
189	11	خ شمال	acute	trophozoite	2/hpf	yellow	soft	nil	nil	2…4	0-2
190	30	خ غرب	acute	trophozoite	1/hpf	brown	fluid	nil	nil	1…3	0-4
191	32	خ جنوب	acute	trophozoite	3/hpf	green	soft	4	nil	uncoun	1 3
192	55	خ جنوب	acute	trophozoite	1/hpf	yellow	soft	2	nil	2…4	0-1
193	25	خ غرب	acute	trophozoite	3/hpf	yellow	fluid	1	nil	2…4	0-2
194	42	خ غرب	acute	trophozoite	2/hpf	brown	fluid	1	nil	2…3	0-1
195	24	خ جنوب	acute	trophozoite	1/hpf	brown	fluid	1	nil	1…3	0-2
196	6	خ شمال	acute	trophozoite	2/hpf	brown	fluid	3	nil	4…6	0-3
197	36	خ غرب	acute	trophozoite	2hpf	yellow	fluid	4	nil	6…8	0-3
198	39	خ غرب	acute	trophozoite	4/hpf	reddish	soft	2	nil	1…3	0-1
199	64	خ غرب	acute	trophozoite	1/hpf	yellow	fluid	2	nil	2…4	0-2
200	30	خ غرب	acute	trophozoite	1/hpf	brown	fluid	1	nil	1…3	0-4

Table (3): Control group data:

	العمر	السكن	disease	wet preparation	conc technique	color	consistency	mucous	blood	RBCs
201.	51	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
202.	6	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
203.	10	خ شمال	healthy	negative	negative	yellow	soft	1..2	nil	0-1
204.	3	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
205.	57	خ غرب	healthy	negative	negative	brown	soft	0..1	nil	0-2
206.	42	خ شرق	healthy	negative	negative	brown	soft	0..3	nil	0-1
207.	11	خ جنوب	healthy	negative	negative	brown	solid	0..1	nil	0-1
208.	44	خ جنوب	healthy	negative	negative	brown	soft	1..3	nil	0-1
209.	21	خ جنوب	healthy	negative	negative	yellow	soft	0..2	nil	0-3
210.	14	خ غرب	healthy	negative	negative	brown	soft	0..1	nil	0-1
211.	35	خ شرق	healthy	negative	negative	brown	fluid	0..1	nil	0-1
212.	18	خ غرب	healthy	negative	negative	yellow	semi solid	0..1	nil	0-1
213.	8	خ شمال	healthy	negative	negative	yellow	soft	0..2	nil	0-1
214.	6	خ شمال	healthy	negative	negative	brown	soft	0..1	nil	0-1
215.	5	خ شمال	healthy	negative	negative	brown	soft	0..1	nil	0-2
216.	11	خ شمال	healthy	negative	negative	brown	soft	0..1	nil	0-1
217.	56	خ جنوب	healthy	negative	negative	brown	soft	0..1	nil	0-1
218.	65	خ جنوب	healthy	negative	negative	brown	soft	0..1	nil	0-1
219.	44	خ جنوب	healthy	negative	negative	yellow	soft	0..1	nil	0-1
220.	22	خ جنوب	healthy	negative	negative	brown	semi solid	2..4	nil	0-1
221.	36	خ شرق	healthy	negative	negative	brown	soft	0..3	nil	0-1
222.	21	خ شرق	healthy	negative	negative	yellow	soft	1..3	nil	0-1

223.	35	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
224.	22	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
225.	12	خ شرق	healthy	negative	negative	yellow	soft	0..2	nil	0-1
226.	70	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
227.	50	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-2
228.	25	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
229.	36	خ شرق	healthy	negative	negative	dark brown	soft	0..2	nil	0-1
230.	88	خ شرق	healthy	negative	negative	green	solid	0..1	nil	0-1
231.	22	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-3
232.	39	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
233.	23	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
234.	45	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
235.	30	خ جنوب	healthy	negative	negative	brown	soft	0..1	nil	0-1
236.	9	خ شمال	healthy	negative	negative	yellow	soft	1..3	nil	0-1
237.	6	خ شمال	healthy	negative	negative	yellow	soft	0..1	nil	0-2
238.	36	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
239.	40	خ جنوب	healthy	negative	negative	brown	soft	0..2	nil	0-1
240.	3	خ شمال	healthy	negative	negative	brown	soft	0..1	nil	0-1
241.	4	خ شمال	healthy	negative	negative	yellow	soft	0..1	nil	0-1
242.	2	خ شمال	healthy	negative	negative	yellow	soft	0..1	nil	0-1
243.	39	خ غرب	healthy	negative	negative	green	solid	0..2	nil	0-1
244.	32	خ جنوب	healthy	negative	negative	brown	soft	0..1	nil	0-1
245.	22	خ جنوب	healthy	negative	negative	yellow	soft	0..1	nil	0-1
246.	66	خ غرب	healthy	negative	negative	yellow	soft	0..1	nil	0-2
247.	18	خ غرب	healthy	negative	negative	brown	solid	0..1	nil	0-1
248.	21	خ جنوب	healthy	negative	negative	bloody	soft	0..1	nil	0-1
249.	13	خ شمال	healthy	negative	negative	brown	soft	0..1	nil	0-1

250.	22	خ غرب	healthy	negative	negative	yellow	soft	0..1	nil	0-1
251.	26	خ غرب	healthy	negative	negative	yellow	fluid	0..1	nil	0-1
252.	25	خ جنوب	healthy	negative	negative	yellow	soft	0..2	nil	0-2
253.	18	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
254.	63	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
255.	54	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
256.	36	خ شرق	healthy	negative	negative	brown	soft	0..2	nil	0-1
257.	65	خ غرب	healthy	negative	negative	brown	soft	0..1	nil	0-1
258.	25	خ جنوب	healthy	negative	negative	brown	soft	0..1	nil	0-1
259.	8	خ شمال	healthy	negative	negative	brown	solid	0..1	nil	0-1
260.	2	خ شمال	healthy	negative	negative	brown	soft	0..1	nil	0-1
261.	6	خ شمال	healthy	negative	negative	brown	soft	0..1	nil	0-1
262.	9	خ شمال	healthy	negative	negative	yellow	soft	0..1	nil	0-1
263.	51	خ غرب	healthy	negative	negative	yellow	soft	1..3	nil	0-1
264.	5	خ شمال	healthy	negative	negative	brown	soft	0..1	nil	0-2
265.	24	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
266.	2	خ شمال	healthy	negative	negative	brown	soft	0..2	nil	0-1
267.	9	خ شمال	healthy	negative	negative	yellow	soft	0..1	nil	0-1
268.	29	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
269.	45	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
270.	4	خ شمال	healthy	negative	negative	brown	soft	0..2	nil	0-2
271.	12	خ شمال	healthy	negative	negative	brown	soft	0..1	nil	0-1
272.	38	خ جنوب	healthy	negative	negative	yellow	solid	0..1	nil	0-1
273.	14	خ غرب	healthy	negative	negative	brown	soft	0..1	nil	0-1
274.	49	خ جنوب	healthy	negative	negative	brown	soft	0..1	nil	0-3
275.	67	خ غرب	healthy	negative	negative	brown	soft	0..1	nil	0-1
276.	21	خ شرق	healthy	negative	negative	brown	solid	0..1	nil	0-1

277.	28	خ شرق	healthy	negative	negative	brown	soft	1..3	nil	0-1
278.	52	خ جنوب	healthy	negative	negative	brown	soft	0..1	nil	0-1
279.	10	خ غرب	healthy	negative	negative	brown	soft	0..1	nil	0-1
280.	70	خ شرق	healthy	negative	negative	brown	soft	0..2	nil	0-2
281.	11	خ شرق	healthy	negative	negative	yellow	fluid	0..1	nil	0-1
282.	56	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
283.	61	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
284.	16	خ جنوب	healthy	negative	negative	brown	soft	0..2	nil	0-1
285.	66	خ غرب	healthy	negative	negative	brown	soft	0..1	nil	0-1
286.	53	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
287.	13	خ شمال	healthy	negative	negative	yellow	soft	0..1	nil	0-1
288.	19	خ شرق	healthy	negative	negative	yellow	soft	0..1	nil	0-1
289.	12	خ شمال	healthy	negative	negative	brown	solid	0..1	nil	0-2
290.	14	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
291.	22	خ شرق	healthy	negative	negative	yellow	solid	1..3	nil	0-1
292.	35	خ جنوب	healthy	negative	negative	yellow	soft	0..1	nil	0-1
293.	17	خ شرق	healthy	negative	negative	green	soft	0..1	nil	0-1
294.	51	خ جنوب	healthy	negative	negative	brown	soft	0..1	nil	0-1
295.	6	خ شرق	healthy	negative	negative	yellow	solid	0..1	nil	0-2
296.	38	خ شرق	healthy	negative	negative	yellow	soft	1..3	nil	0-1
297.	33	خ شرق	healthy	negative	negative	brown	soft	0..1	nil	0-1
298.	10	خ شمال	healthy	negative	negative	bloody	soft	0..1	nil	0-1
299.	7	خ شرق	healthy	negative	negative	brown	soft	0..3	nil	0-1
300.	35	خ جنوب	healthy	negative	negative	yellow	soft	0..1	nil	0-1

Table (4): Results of Copro ELISA for *E. histolytica* and *G. lamblia*:

no	OD E Control	Cut off E control	OD E Case	Cut off E case	OD G Control	Cut off G control	OD G case	Cut off G case
1.	1.564	positive	0.79	negative	0.07	negative	0.20	positive
2.	0.432	negative	0.80	negative	0.07	positive	0.108	positive
3.	0.356	negative	0.80	positive	0.07	negative	0.089	positive
4.	0.384	negative	0.89	positive	0.063	negative	0.096	positive
5.	0.556	negative	0.90	positive	0.06	negative	0.139	positive
6.	1.108	positive	0.96	positive	0.084	positive	0.277	positive
7.	1.068	positive	0.83	positive	0.06	negative	0.267	positive
8.	0.576	negative	1.0	positive	0.084	positive	0.65	positive
9.	0.76	negative	0.95	positive	0.05	negative	0.19	positive
10.	0.38	negative	1.28	positive	0.077	negative	0.095	positive
11.	0.336	negative	1.22	positive	0.07	negative	0.084	positive
12.	0.32	negative	1.0	positive	0.07	positive	0.08	negative
13.	0.408	negative	1.34	positive	0.06	negative	0.102	positive
14.	0.55	negative	1.16	positive	0.04	negative	0.276	positive
15.	1.712	positive	1.22	positive	0.07	negative	0.428	positive
16.	0.348	negative	1.48	positive	0.098	positive	0.087	positive
17.	0.744	negative	1.06	positive	0.126	positive	0.186	positive
18.	0.324	negative	1.18	positive	0.07	negative	0.081	positive
19.	0.38	negative	0.88	positive	0.07	negative	0.095	positive
20.	0.348	negative	1.06	positive	0.07	negative	0.087	positive
21.	0.412	negative	1.22	positive	0.063	negative	0.103	positive
22.	0.444	negative	1.06	positive	0.06	negative	0.111	positive
23.	0.776	negative	1.08	positive	0.07	negative	0.194	positive
24.	0.304	negative	1.3	positive	0.07	negative	0.076	positive
25.	0.588	negative	0.84	positive	0.07	negative	0.147	positive
26.	0.304	negative	1.06	positive	0.063	negative	0.076	positive
27.	0.404	negative	1.1	positive	0.06	negative	0.101	positive
28.	0.292	negative	0.96	positive	0.07	negative	0.073	positive
29.	0.48	negative	0.90	positive	0.07	negative	0.12	positive
30.	0.712	negative	1.12	positive	0.07	negative	0.178	positive

31.	0.6	negative	1.16	positive	0.063	negative	0.291	positive
32.	0.408	negative	0.86	positive	0.06	negative	0.102	positive
33.	0.604	negative	0.96	positive	0.07	negative	0.151	positive
34.	0.42	negative	1.16	positive	0.07	negative	0.105	positive
35.	0.4	negative	0.86	positive	0.07	negative	0.10	positive
36.	0.376	negative	1.43	positive	0.063	negative	0.094	positive
37.	0.80	negative	0.98	positive	0.06	negative	0.193	positive
38.	0.80	negative	1.18	positive	0.07	negative	0.196	positive
39.	0.524	negative	1.1	positive	0.07	negative	0.131	positive
40.	0.288	negative	0.92	positive	0.07	negative	0.072	negative
41.	0.34	negative	1.02	positive	0.063	negative	0.085	positive
42.	0.44	negative	1.0	positive	0.06	negative	0.11	positive
43.	0.52	negative	0.82	positive	0.07	negative	0.13	positive
44.	0.368	negative	1.0	positive	0.07	negative	0.092	positive
45.	0.8	negative	1.18	positive	0.07	negative	0.161	positive
46.	0.48	negative	0.92	positive	0.063	negative	0.12	positive
47.	0.864	positive	0.9	positive	0.06	negative	0.216	positive
48.	0.272	negative	0.88	positive	0.084	negative	0.068	negative
49.	0.80	negative	0.94	positive	0.07	negative	0.222	positive
50.	0.372	negative	1.16	positive	0.07	negative	0.093	positive
51.	0.532	negative	1.3	positive	0.07	negative	0.133	positive
52.	0.452	negative	0.88	positive	0.063	negative	0.113	positive
53.	0.456	negative	1.04	positive	0.06	negative	0.114	positive
54.	0.456	negative	0.18	negative	0.07	negative	0.114	positive
55.	0.208	negative	0.77	negative	0.07	negative	0.052	negative
56.	0.248	negative	0.79	negative	0.07	negative	0.062	negative
57.	0.80	negative	0.9	positive	0.063	negative	0.247	positive
58.	0.80	negative	1.28	positive	0.06	negative	0.207	positive
59.	0.436	negative	0.88	positive	0.07	negative	0.109	positive
60.	0.392	negative	1.0	positive	0.07	negative	0.098	positive
61.	0.536	negative	1.1	positive	0.07	negative	0.134	positive
62.	0.468	negative	0.88	positive	0.063	negative	0.117	positive

63.	0.272	negative	0.81	positive	0.06	negative	0.068	positive
64.	0.268	negative	0.8	negative	0.07	negative	0.067	positive
65.	0.536	negative	0.24	negative	0.07	negative	0.134	positive
66.	0.28	negative	0.3	negative	0.07	negative	0.07	positive
67.	0.484	negative	0.7	negative	0.063	negative	0.121	positive
68.	0.388	negative	0.85	positive	0.06	negative	0.097	positive
69.	0.512	negative	0.65	negative	0.07	negative	0.128	positive
70.	0.48	negative	1.44	positive	0.07	negative	0.12	positive
71.	0.252	negative	0.96	positive	0.07	negative	0.063	negative
72.	0.264	negative	1.22	positive	0.063	negative	0.066	negative
73.	0.40	negative	1.22	positive	0.06	negative	0.10	positive
74.	0.06	negative	0.79	positive	0.07	negative	0.015	positive
75.	0.30	negative	0.9	positive	0.07	negative	0.075	positive
76.	0.268	negative	0.92	positive	0.07	negative	0.067	positive
77.	0.504	negative	0.94	positive	0.063	negative	0.126	positive
78.	0.628	negative	1.1	positive	0.06	negative	0.157	positive
79.	0.204	negative	1.1	positive	0.07	negative	0.051	positive
80.	0.244	negative	1.0	positive	0.07	negative	0.061	positive
81.	0.328	negative	0.98	positive	0.07	negative	0.082	positive
82.	0.436	negative	0.92	positive	0.063	negative	0.109	positive
83.	0.436	negative	1.4	positive	0.06	negative	0.109	positive
84.	0.48	negative	0.84	positive	0.07	negative	0.12	positive
85.	0.58	negative	0.81	positive	0.07	negative	0.145	positive
86.	0.436	negative	1.16	positive	0.07	negative	0.109	positive
87.	0.256	negative	1.04	positive	0.063	negative	0.064	negative
88.	0.304	negative	1.04	positive	0.06	negative	0.076	positive
89.	0.56	negative	1.1	positive	0.04	negative	0.14	positive
90.	0.396	negative	0.92	positive	0.07	negative	0.099	positive
91.	0.452	negative	0.96	positive	0.07	negative	0.113	positive
92.	0.476	negative	0.98	positive	0.063	negative	0.119	positive
93.	0.428	negative	1.03	positive	0.06	negative	0.107	positive
94.	0.536	negative	0.86	positive	0.05	negative	0.35	positive

95.	0.76	negative	0.96	positive	0.07	negative	0.07	positive
96.	0.75	negative	0.85	positive	0.07	negative	0.07	positive
97.	0.45	negative	0.8	negative	0.063	negative	0.052	negative
98.	0.35	negative	0.9	positive	0.05	negative	0.062	negative
99.	0.43	negative	1.0	positive	0.063	negative	0.08	positive
100.	0.57	negative	0.96	positive	0.05	negative	0.09	positive

Table (5): SPSS analysis: *Giardia lamblia* frequency table:

		Frequency	Percent	Valid percent	Cumulative %
Valid	case	100	50.0	50.0	50.0
	control	100	50.0	50.0	50.0
	Total	200	100.0	100.0	100.0

Table (6): ELISA data: *Giardia lamblia Descriptive statistic:*

		Frequency	Percent	Valid percent	Cumulative %
Valid	positive	95	47.5	47.5	47.5
	negative	105	52.5	52.5	100.0
	Total	200	100.0	100.0	

Table (7): ELISA result: *Giardia lamblia Descriptive statistic:*

		Frequency	Percent	Valid percent	Cumulative percent
Valid	positive	96	48.0	48.0	48.0
	negative	104	52.0	52.0	100.0
	Total	200	100.0	100.0	

Table (8): Formal ether result: *Giardia lamblia*

Frequency		Percent	Valid Percent	Cumulative percent
Valid	positive	100	50.0	50.0
	negative	100	50.0	50.0
	Total	200	100.0	100.0

Table (9): Sex group in *E. histolytica*

	Frequency	Percent	Valid percent	Cumulative percent
male	115	57.5	57.5	57.5
female	85	42.5	42.5	100.0
Total	200	100.0	100.0	

Table (10): Sex group in *Giardia lamblia*:

	Sex	Frequency	Percent	Valid percent	Cumulative %
Valid	male	132	66.0	66.0	66.0
	female	68	34.0	34.0	100.0
	Total	200	100.0	100.0	

Table (11): Distribution of giardiasis disease in Khartoum region:

	Area	Frequency	Percent	Valid percent	Cumulative %
Valid	north	45	22.5	22.5	22.5
	south	40	20.0	20.0	42.5
	east	85	42.5	42.5	85.0
	west	30	15.0	15.0	100.0
	Total	200	100.0	100.0	

Table (12): Distribution of amoebiasis disease in Khartoum region:

	Area	Frequency	Percent	Valid Percent	Cumulative %
Valid	north	43	21.5	21.5	21.5
	south	43	21.5	21.5	43.0
	east	91	45.5	45.5	88.5
	west	23	11.5	11.5	100.0
	Total	200	100.0	100.0	

Table (13): Crosstabulation microscopy:

			microscopy		Total
			positive	negative	
Status	Case	Count	100	0	100
		% within Status	100.0%	0.0%	100.0%
		% of Total	50.0%	0.0%	50.0%
	control	Count	0	100	100
		% within Status	0.0%	100.0%	100.0%
		% of Total	0.0%	50.0%	50.0%
Total		Count	100	100	200

Table (14): Chi-Square Tests: sig. microscopy:

	Value	df	sig.	Exact sig.	Exact sig.
Pearson chi-square	200.000[a]	1	.000		
continuity correction[b]	196.020	1	.000		
Likelihood ratio	277.259	1	.000		
Fisher's exact test				.000	.000
Linear-by-linear association	199.000	1	.000		
N of valid cases	200				

Table (15): Crosstabtabulation *E. histolytica* ELISA:

			ELISA		Total
			positive	negative	
Status	Case	Count	90	10	100
		% within Status	90.0%	10.0%	100.0%
		% of Total	45.0%	5.0%	50.0%
	control	Count	5	95	100
		% within Status	5.0%	95.0%	100.0%
		% of Total	2.5%	47.5%	50.0%
Total		Count	95	105	200
		% within Status	47.5%	52.5%	100.0%
		% of Total	47.5%	52.5%	100.0%

Table (16): Chi-Square Tests: *E. histolytica* ELISA:

	Value	df	Asymp. Sig.	Exact Sig.	Exact sig.
Pearson chi-square	144.862[a]	1	.000		
continuity correction[b]	141.474	1	.000		
Likelihood ratio	172.039	1	.000		
Fisher's exact test				.000	.000
Linear-by-linear association	144.138	1	.000		
N of valid cases	200				

Table (17): Crosstabulation ELISA giardiasis:

			ELISA giardiasis		Total
			positive	negative	
Status	Case	Count	90	10	100
		% within Status	90.0%	10.0%	100.0%
		% of Total	45.0%	5.0%	50.0%
	control	Count	6	94	100
		% within Status	6.0%	94.0%	100.0%
		% of Total	3.0%	47.0%	50.0%
Total		Count	96	104	200
		% within Status	48.0%	52.0%	100.0%
		% of Total	48.0%	52.0%	100.0%

Table (18): Chi-Square Tests

	Value	df	Asymp. Sig.	Exact sig.	Exact sig.
Pearson chi-square	141.346[a]	1	.000		
continuity correction[b]	138.001	1	.000		
Likelihood ratio	166.529	1	.000		
Fisher's exact test				.000	.000
Linear-by-linear association	140.639	1	.000		
N of valid cases	200				

Table (19): Crosstabulation Giardiasis microscopy:

			microscopy		Total
			positive	negative	
Status	Case	Count	100	0	100
		% within Status	100.0%	0.0%	100.0%
		% of Total	50.0%	0.0%	50.0%
	control	Count	0	100	100
		% within Status	0.0%	100.0%	100.0%
		% of Total	0.0%	50.0%	50.0%
Total		Count	100	100	200
		% within Status	50.0%	50.0%	100.0%
		% of Total	50.0%	50.0%	100.0%

Table (20): Chi-Square Tests: sig. giardiasis microscopy:

	Value	df	Asymp. sig.	Exact sig.	Exact sig.
Pearson chi-square	200.000[a]	1	.000		
continuity correction[b]	196.020	1	.000		
Likelihood ratio	277.259	1	.000		
Fisher's exact test				.000	.000
Linear-by-linear assoc	199.000	1	.000		
N of valid cases	200				

Table (21): Crosstabulation: amoebiasis:

			ELISA giardiasis		Total
			positive	negative	
Status	Case	Count	90	10	100
		% within Status	90.0%	10.0%	100.0%
		% of Total	45.0%	5.0%	50.0%
	control	Count	6	94	100
		% within Status	6.0%	94.0%	100.0%
		% of Total	3.0%	47.0%	50.0%
Total		Count	96	104	200
		% within Status	48.0%	52.0%	100.0%
		% of Total	48.0%	52.0%	100.0%

Table (23): Crosstabulation: amoebiasis

			microscopy		Total
			positive	negative	
Status	Case	Count	100	0	100
		% within Status	100.0%	0.0%	100.0%
		% of Total	50.0%	0.0%	50.0%
	control	Count	0	100	100
		% within Status	0.0%	100.0%	100.0%
		% of Total	0.0%	50.0%	50.0%
Total		Count	100	100	200
		% within Status	50.0%	50.0%	100.0%
		% of Total	50.0%	50.0%	100.0%

Table (22): Chi-Square Tests

	Value	df	Asymp. Sig.	Exact Sig.	Exact Sig.
Pearson chi-square	141.346[a]	1	.000		
continuity correction[b]	138.001	1	.000		
likelihood ratio	166.529	1	.000		
Fisher's exact test				.000	.000
Linear-by-linear association	140.639	1	.000		
N of valid cases	200				

Table (24): Chi-Square Tests: amoebiasis

	Value	df	Asymp. sig.	Exact Sig.	Exact sig.
Pearson chi-square	200.000[a]	1	.000		
continuity correction[b]	196.020	1	.000		
Likelihood ratio	277.259	1	.000		
fisher's exact Test				.000	.000
Linear-by-Linear association	199.000	1	.000		
N of valid cases	200				

Table (25): Distribution of giardiasis among age group:

		Frequency	Percent	Valid percent	Cumulative %
Valid	0 - 9	32	16.0	16.0	16.0
	10 - 19	32	16.0	16.0	32.0
	20 - 29	59	29.5	29.5	61.5
	30 - 39	35	17.5	17.5	79.0
	40 - 49	16	8.0	8.0	87.0
	50 - 59	14	7.0	7.0	94.0
	60+	12	6.0	6.0	100.0
	Total	200	100.0	100.0	

Table (26): Crosstabulation amoebiasis according to sex:

			sex		Total
			male	female	
Amoebiasis	positive	Count	50	39	89
		% within remarkteste	56.2%	43.8%	100.0%
		% of Total	50.0%	39.0%	89.0%
	negative	Count	5	6	11
		% within remarkteste	45.5%	54.5%	100.0%
		% of Total	5.0%	6.0%	11.0%
Total		Count	55	45	100
		% within remarkteste	55.0%	45.0%	100.0%
		% of Total	55.0%	45.0%	100.0%

Table (33): Chi-Square Tests

	Value	df	Asymp. sig. (2-
Pearson chi-square	3.959[a]	3	0.266
Likelihood ratio	5.552	3	0.136
Linear-by-linear association	.010	1	0.921
N of valid cases	100		

Table (27): Chi-Square Tests:

	Value	df	Asymp. sig.	Exact Sig.	Exact sig.
Pearson Chi-Square	.455[a]	1	.500		
Continuity Correction[b]	.125	1	.724		
Likelihood Ratio	.452	1	.501		
Fisher's Exact Test				0.536	0.360
Linear-by-Linear Association	.450	1	0.502		
N of Valid Cases	100				

Table (28): Chi-Square Tests

	Value	df	Asymp. Sig.
Pearson Chi-Square	8.201[a]	3	0.042
Likelihood Ratio	7.872	3	0.049
Linear-by-Linear Association	.574	1	0.449
N of Valid Cases	100		

Table (29): Chi-Square Tests

	Value	df	Asymp. sig.
Pearson Chi-Square	4.795[a]	6	0.570
Likelihood Ratio	4.834	6	0.565
Linear-by-Linear Association	.007	1	0.932
N of Valid Cases	100		

Table (32): Chi-Square Tests

	Value	df	Asymp. sig.
Pearson chi-square	3.889[a]	6	0.692
Likelihood ratio	5.588	6	0.471
Linear-by-linear association	.636	1	0.425
N of valid cases	100		

Table (30): Crosstabulation: giardiasis in sex group:

			sex		Total
			Male	Female	
Giardiasis	positive	Count	65	25	90
		% within remarktestg	72.2%	27.8%	100.0%
		% of Total	65.0%	25.0%	90.0%
	negative	Count	7	3	10
		% within remarktestg	70.0%	30.0%	100.0%
		% of Total	7.0%	3.0%	10.0%
Total		Count	72	28	100
		% within remarktestg	72.0%	28.0%	100.0%
		% of Total	72.0%	28.0%	100.0%

Table (31): Chi-Square Tests

	Value	df	Asymp. sig.	Exact Sig.	Exact sig.
Pearson Chi-Square	.022[a]	1	0.882		
Continuity Correction[b]	0.000	1	1.000		
Likelihood Ratio	.022	1	0.883		
Fisher's Exact Test				1.000	0.570
Linear-by-Linear Association	.022	1	0.883		
N of Valid Cases	100				
		% within remarktestg	14.0%	10.0%	48.0%
		% of Total	14.0%	10.0%	48.0%

Table (34): distribution of amoebiasis according to area:

			locality				Total
			north	south	east	west	
Amoebiasis	positive	Count	19	16	45	9	89
		% within remarkteste	21.3%	18.0%	50.6%	10.1%	100.0%
		% of Total	19.0%	16.0%	45.0%	9.0%	89.0%
	negative	Count	1	6	4	0	11
		% within remarkteste	9.1%	54.5%	36.4%	0.0%	100.0%
		% of Total	1.0%	6.0%	4.0%	0.0%	11.0%
Total		Count	20	22	49	9	100
		% within remarkteste	20.0%	22.0%	49.0%	9.0%	100.0%
		% of Total	20.0%	22.0%	49.0%	9.0%	100.0%

Table (35): Crosstabulation: amoebiasis age group:

Amoebiasis		Age							Total
		> 10	10 - 20	21 - 30	31 - 40	41 - 50	51 - 60	60+	
positive	Count	10	9	39	19	7	3	2	89
	% within remarkteste	11.2%	10.1%	43.8%	21.3%	7.9%	3.4%	2.2%	100.0%
	% of Total	10.0%	9.0%	39.0%	19.0%	7.0%	3.0%	2.0%	89.0%
negative	Count	2	2	2	2	2	1	0	11
	% within remarkteste	18.2%	18.2%	18.2%	18.2%	18.2%	9.1%	0.0%	100.0%
	% of Total	2.0%	2.0%	2.0%	2.0%	2.0%	1.0%	0.0%	11.0%
Total	Count	12	11	41	21	9	4	2	100
	% within remarkteste	12.0%	11.0%	41.0%	21.0%	9.0%	4.0%	2.0%	100.0%
	% of Total	12.0%	11.0%	41.0%	21.0%	9.0%	4.0%	2.0%	100.0%

Table (36): Crosstabulation: giardiasis age group:

Giardiasis		Age							Total
		> 10	10 - 20	21 - 30	31 - 40	41 - 50	51 - 60	60+	
Positive	Count	14	9	43	13	5	5	1	90
	% within remarktestg	15.6%	10.0%	47.8%	14.4%	5.6%	5.6%	1.1%	100.0%
	% of Total	14.0%	9.0%	43.0%	13.0%	5.0%	5.0%	1.0%	90.0%
negative	Count	0	1	5	3	1	0	0	10
	% within remarktestg	0.0%	10.0%	50.0%	30.0%	10.0%	0.0%	0.0%	100.0%
	% of Total	0.0%	1.0%	5.0%	3.0%	1.0%	0.0%	0.0%	10.0%
Total	Count	14	10	48	16	6	5	1	100
	% within remarktestg	14.0%	10.0%	48.0%	16.0%	6.0%	5.0%	1.0%	100.0%
	% of Total	14.0%	10.0%	48.0%	16.0%	6.0%	5.0%	1.0%	100.0%

Table (37): Crosstabulation: distribution of giardiasis according to area

			Locality				Total
			north	south	east	west	
Giardiasis	positive	Count	21	16	37	16	90
		% within remarktestg	23.3%	17.8%	41.1%	17.8%	100.0%
		% of Total	21.0%	16.0%	37.0%	16.0%	90.0%
	negative	Count	1	3	6	0	10
		% within remarktestg	10.0%	30.0%	60.0%	0.0%	100.0%
		% of Total	1.0%	3.0%	6.0%	0.0%	10.0%
Total		Count	22	19	43	16	100
		% within remarktestg	22.0%	19.0%	43.0%	16.0%	100.0%
		% of Total	22.0%	19.0%	43.0%	16.0%	100.0%

Appendix (2)

Reagents:

- PBS phosphate buffer saline
- 10 X buffer.
- G-spin™ Total DNA Extraction Kit
- Maxime PCR PreMix Kit (i-Taq) (Korea)
- Ethidium promide
- Rsa fermentase (*New England Bioloabs*)
- *NIaVI* (*New England Bioloabs*)
- *Giardia lamblia* ELISA DE610001 Demeditec Diagnostics GmbH
- *Entamoeba histolytica* Ag ELISA DE4454 Demeditec Diagnostics GmbH
- TBE Tris EDTA buffer
- Agarose powder bio-rad
- Absolute alcohol and 70% alcohol
- Sodium hypochlorite (bleach) solution (10 %; v/v)
- Normal saline
- Distled H_2o

Appendix (3)

Materials and Equipments:

- Thermocycler (SensoQuest brand-Germany)
- Gel electrophoresis system (Biometra GmbH- Germany)
- Ultraviolet trans-illuminator (Biometra GmbH- Germany).
- Nanodrop spectrophotometer (ND 1.000 - USA).
- Cryo tube
- Eppendorf tube
- Biometra product line gradient PCR Biometra GmbH- Germany
- PCR racks
- Yellow and white tips.

-Refrigerator -20 °C

-Microscope- Olympus Cx21GERMANY

-Sensitive balance (Boa Phie-2006) Electronic scale

-Slides- 26x76x1mm (CITOGLAS)

-Cover glass -22x22(OMEGALAB)

-Wood sticks 53/4-1/12(FISHERBRAND-USA)

-Stool containers 60ML (MDM)

-Sieve (strainer)

-Centrifuge EBA 20 (Hettich)

-Test tubes different size

-Electronic pipette (Accumax Pro)

- Pasteur pipette, Graduate pipette

-Plastic and metal rack

-Cotton, gauze and Syringe (RAMY)

-Papers, pens and marker

- Questionnaire for data collection
- SPSS 16.0 - for data analysis

Appendix (4)

Sudan University of Science and Technology

College of Graduate Studies

Department of Parasitology and Medical Entomology

'Questionnaire'

Sample Patient Information

Name: .... Gender: ....

Nationality: .... Age: ....

Place of residence: ....

Occupation: ....

Sample no.	***Remarks***	**Blood**	**Mucus**	**Wet prep**	**FECT**
Asymptomatic					
Acute diarrhea					
Chronic diarrhea					
Entamoeba histolytica					
Giardia lamblia					
Other parasitic					
Bacteria					
Yeast					

ELISA OD	**DNA purity**	**DNA Conc.**	**PCR result**	**RFLP1**	**RFLP2**	**Sequencing**

Researcher signed: .... Date: ....

Appendix (5)

Sudan University of Science and Technology

College of Graduate Studies

Department of Parasitology and Medical Entomology

<u>Informed Consent Form</u>

Thank you for kindly participating in this questionnaire. This information is provided to help you in understand the aim of the research that I recommend it for you. Before I begin collection, I want to be certain that I have provided you with enough information in a way you can understand, so that you're well informed and confident that you wish to proceed. I will also have a discussion with you.

I have received information about the research and have been given an opportunity to ask questions and have them fully answered. I understand the nature of the specimen, there are no risks.

Participant signed: ………………………………………… Date: ……………………………………..

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Discrepancy in Diagnosis of *Giardia Lamblia* Infection among Sudanese Population in Khartoum State-A Comparison between Direct microscopy, Formal Ether and Copro Antigen Elisa

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Abstract: *The objective of this case control study was to detect discrepancy in the diagnosis of Giardia lamblia among Sudanese patients by comparing the sensitivity and specificity of copro antigen ELISA aginst microscopy and FECT. Method: 100 patients diagnosed with Giardiasis, and100 healthy controls were included in this study. Fresh stool specimens were collected from each study participants and were examined microscopically for cysts and trophozoites by wet preparation, formal-ether technique and Copro antigen ELISA. Results: No differences between wet preparation and formal ether techniques. Out of 100 true positive samples after microscopy, only 90(90%) were positive by ELISA, while 96(96%) were true negative from 100 samples of control group. Copro-antigen ELISA for detection of Giardia lamblia results a sensitivity of 90% and a specificity of 94%. With 93.8% and 90.3% PPV and NPV. The obtained clinical cut off copro ELISA among Sudanese population infected with Giardiasis and non-infected group were [(0.98±.21) and (0.16±.18)] respectively. Higher cases of Giardiasis were detected in age group of 21-30 years 43% (30+13). Strong agreement between copro antigen ELISA and microscopy was obtained by Kappa test (P value 0.00). The level of accuracy was obtained by area under the curve 0.939, which represent excellent test also highly sensitive and specific. Difference between these techniques was found to be statistically significant (p=0.001). We recommend using ELISA in epidemiological studies also to confirm the diagnosis in patients with continuous symptoms of Giardiasis with no results by direct microscopy. Such technique would be helpful at early infection, when the level of parasite is quite low.*

Keywords: *Giardiasis, microscopy, Formol ether, Copro antigen Elisa.*

I. Introduction

Gastrointestinal infections consider the most causes of morbidity and mortality over the world and mainly in countries under developing. While diarrhea does not typically cause serious complications for most patients, it can be a fatal ailment for young children and elderly, especially those who are malnourished or have compromised immune systems [1].There were a variety of pathogens causing diarrhea as viruses, bacteria, and parasites. The most groups of parasitic diarrhea are *Entamoeba histolytica* and *Giardia lamblia* [2]. Some literature reported that 90% of the parasitic diarrhea is due to *Giardia* infections. G*iardia* is a parasite found in all parts of the world and in a large number of mammals, including humans, livestock, pets, wildlife, and aquatic animals [3, 4]. Several recent reports have also described *G. lamblia*in various birds and even fish, although true infections remain to be confirmed in these animals [5]. Different percentage of Giardiasis occurs between countries, and it is higher in areas with low sanitary condition. According to estimates from the WHO about 200 million people have symptomatic giardiasis, and around 500,000 new cases occur each year [6]. Studies in different European countries have indicated prevalence of 1–17%, and up to 100% of the population can be infected in certain highly endemic areas [7]. Microscopic detection of *Giardia* cysts in a stool specimen, either directly in a wet smear or after formol-ethyl acetate concentration, is the most frequently used method for diagnosis of giardiasis worldwide. Usually, most used is the basic technique for cysts and trophozoites in fecal specimens. Compared to identification of *Entamoeba* spp., microscopical diagnosis of *Giardiasis is* simple and cheap, but still with quite low the sensitivity due to the intermittent excretion of *Giardia* cysts, and thus it is recommended that at least three samples be examined in order to rule out giardiasis [8]. Quite number of commercial kits is available for detection of *Giardia* antigen. Two techniques that are often used are enzyme-linked immune-sorbent assay (ELISA) that assesses soluble antigens and a direct fluorescent antibody (DFA) test that detects intact organisms. Several studies have shown that these two methods offer greater sensitivity compared to light microscopy [9], but they are not available in all parasitology laboratories due to the high cost and substantial workload they entail, and also limited access to the required equipment. An alternative technique involves a solid-phase immune chromatographic test card system (Immuno Card STAT *Cryptosporidium /Giardia* rapid assay), which allows concurrent detection of *Cryptosporidium* and is also fast, easy to use, and does not require extra equipment but low sensitivity may occur [10, 11].

II. Materials And Methods:

2.1. Study design: Case control study was conducted during period from August 2014 to June 2016 in Khartoum state, Sudan.

2.2. Subjects: A total sample size of 200 patients were divided into two groups, 100 patients with Giardiasis as study group and 100 healthy individuals as control group, confirmed by microscopy after formal ether as gold standard method for diagnosis .Permission of this study were obtained from the Research Committee, College of Medical Laboratory Science at Sudan University. The aim of the study was explained to all participants in this study. Informed consent was obtained from each participant. Also, a questionnaire was designed to collect data from the patients.

2.3. Sample: Stool samples from all individuals were collected in clean, leak proof container and were divided into two parts, one part in preservation media of 10% formalin was used for standard microscopic for O&P and confirmed by formal ether concentration technique, a second part was stored in frozen condition at - 20º C until used for Elisa technique.

2.4. Measurements of *Giardia* copro antigen: All faecal samples were analyzed for qualitative determination of *Giardia* specific antigens according to the manufacturer instructions. ELISA kits from Demeditec Diagnostics GmbH. It is an enzymometric two step immune assay based on polyclonal peptide antibodies. OD was measured at 450 nm / ≥ 620 nm using semi automated Elisa. 0.08 OD and above indicates the samples contains *Giardia* antigen. All samples were analyzed in the laboratory of Alrayan centre.

2.5. Statistic evaluation: Statistical analysis was performed using SPSS 16 (Statistical Package for Social Sciences).The obtained mean of the case and control group represent the clinical cut off level of Giardiasis in Sudanese population. Since microscopy test was reported as a reference standard test. Sensitivity, specificity, PVP, NPV and accuracy were calculated with the following formula to analyze data: sensitivity: sensitivity: $A/(A+C) \times 100$]; Specificity: $D/(D+B) \times 100$]; PVP: $A/(A+B) \times 100$]; NPV: $D/(D+C) \times 100$], and accuracy:$(TN + TP)/(TN+TP+FN+FP)$], where a = true positive samples, b = false positive samples, c = false negative samples and d = true negative samples. Kappa test was done to measure the agreement between the methods. P value ≤ 0.05 was statistically significant. while the Kappa value explains the strength between the two tests, when the value equal 0.5 it represent mirror agreement, above 0.7 represent good agreement and above 0.8 represent very good agreement. Receiver Operating Characteristic (ROC) curve was obtained to determine the excellent, good, and worthless tests plotted on the same graph (validation of ELISA). Accuracy is measured by the area under the ROC curve. An area of .90-1 represents an excellent test; .80-.90 = good .70-.80 = fair .60-.70 = poor an area of .5 represents a worthless (fail) test. p-value ≤ 0.05 was considered as statistically significant.

III. Results

Within the100 positive cases detected by microscopy after formol ether, as gold standard method, Giardia was detected in 90(90%) in ELISA technique, whereas 100 samples in control group was free of Giardia by both wet preparation and concentration technique, 96(96%) were negative by ELISA, while only 6(6%) from control were detected as false positive by ELISA [Table1]. Out of 200 participants, 96 (48%) were detected positive cases of *Giardia* and 104 (53.5%) were negative by copro ELISA [Table1]. The clinical cut off copro ELISA among Sudanese population of Giardiasis infected and non-infected group [(0.98±.21) and (0.16±.18)] respectively [Table3]. Out of 100 cases of Giardiasis 65(72.2%) were males and 25(27.8%) were females [Table4]. Age of patients ranged from 2-80 y and mean age was 26.84± 12.9y. Among studied variables 80% of cases were acute phase of disease. Highest cases of Giardiasis diagnosed by ELISA test were detected in age group of 21-30 years, 43% (30+13) [Table5]. Microscopical appearance of stool samples among case study group of Giardiasis, 88 (88%) with acute phase of the disease. cysts were detected in15 (15%) (n = 100). 40% of Giardia positive cases had pus in their stool, while no RBCs were detected in all cases. Accuracy of Elisa was obtained by Kappa test (P value 0.001) [Table 6]. The validation and sensitivity of Elisa was measured by ROC curve, area under the curve 0.939 represent excellent test with high sensitivity and specificity [fig 1].

Table (1): Analysis of Copro Ag ELISA results in case and control group:

			Giardia copro ELISA		Total
			possitive	negative	
Status	Case	Count	90	10	100
		% within Status	90.0%	10.0%	100.0%
		% of Total	45.0%	5.0%	50.0%
	control	Count	6	94	100
		% within Status	6.0%	94.0%	100.0%
		% of Total	3.0%	47.0%	50.0%
Total		Count	96	104	200
		% within Status	48.0%	52.0%	100.0%
		% of Total	48.0%	52.0%	100.0%

Table (2): Accuracy for Copro Ag Elisa among study population using microscopy as a reference standard:

Parameters	Accuracy
Sensitivity	90%
Specificity	94%
PPV	90%
NPV	93.75%
Accuracy	92%

Table (3): Mean cut off Copro Ag ELISA among case and control groups:

Group	N	Minimum	Maximum	Mean	Std. Deviation
Giardia case group	100	0.04	0.65	0.13	0.009
Giardia control group	100	0.02	0.13	0.07	0.08
Disease prevalence (purposive)	47%				

Table (4): Sex distribution among study population:

			SAE		Total
			MALE	FEMALE	
remarktestg	possitive	Count	65	25	90
		% within remarktestg	72.2%	27.8%	100.0%
		% of Total	65.0%	25.0%	90.0%
	negative	Count	7	3	10
		% within remarktestg	70.0%	30.0%	100.0%
		% of Total	7.0%	3.0%	10.0%
Total		Count	72	28	100
		% within remarktestg	72.0%	28.0%	100.0%
		% of Total	72.0%	28.0%	100.0%

Table (5): Distribution of *Giardia* infection according to age group:

Age groups:	sex		Total
	MALE	FEMALE	
> 10 years	9	4	13
10 – 20 years	7	2	9
21 – 30 years	30	13	43
31 - 40 years	10	3	13
41 - 50 years	3	2	5
51 - 60 years	3	2	5
More than 61+	1	1	2
Total	63	26	90

Table (6): Kappa agreement between Copro Ag Elisa and microscopy results in case and control group:

		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement	Kappa	0.830	0.039	11.767	0.001
N of Valid Cases		200			
a. Not assuming the null hypothesis					
b. Using the asymptotic standard error assuming the null hypothesis.					

Figure (1): ROC curve for diagnosis of Giardiasis by copro Ag ELISA:

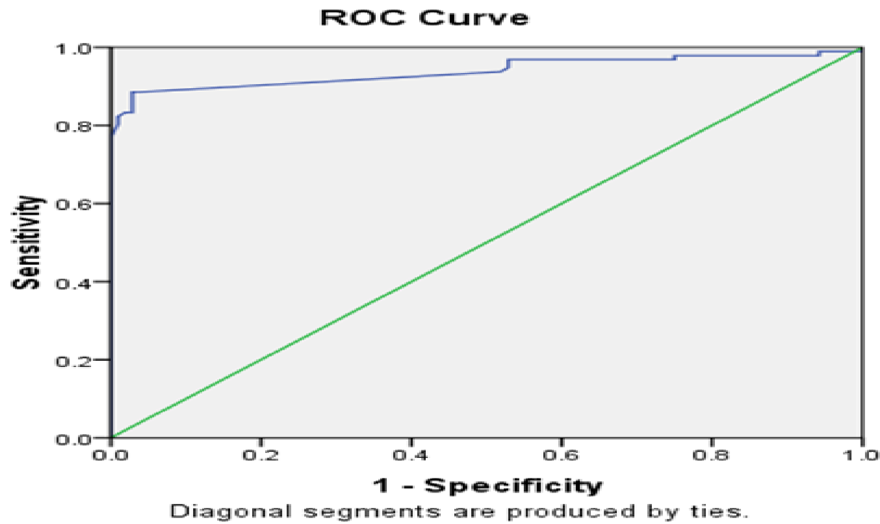

Table (7): Accuracy Area under the Curve (AUC):

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.939	0.020	.000	0.900	0.978
a. Under the nonparametric assumption				
b. Null hypothesis: true area = 0.5				

IV. Discussion

G. lamblia is one of the most common intestinal protozoan parasites which affect about 200 million people in Asia, Africa and Latin America with 280 million infections per year [12, 13]. Epidemiological studies have shown that most cases of parasitic diarrhoea in children are due to *G. lamblia* infection, especially in areas with poor sanitation. [14].The diagnosis of giardiasis is based primarily on microscopic examination of stool samples through the identification of motile trophozoites or the cyst phase. [15]. Microscopic examination requires examining three consecutive stool samples in order to obtain higher sensitivity (over 90 %). Lower sensitivity (approximately 50 %) of a single sample examination may be attributed to low parasite density, sporadic excretion of cysts or the possibility of the parasite being masked with bile pigments. [17]. The need for a more robust diagnostic techniques lead to the development of rapid, sensitive and specific diagnostic methods [16]. ELISA is a rapid, sensitive and cost effective method for detection of specific antigens in stools and confirmation of certain infection. Copro antigens of a parasite could be traced and diagnosed even if the live parasite is absent in the fecal samples [18]. The present study showed that the percentage of positive rates of G. lamblia that were detected by using direct wet mount was (45%), while it was increased to reach more than (50%) when using formal either concentration technique (FECT).These results were similar to results obtained by (Eltayeb et al. 2012) [19] and disagreed with the result of Gabbad and Elawad (2014) [20] in Khartoum State. The current study revealed that the prevalence of *G. lamblia* infection among males was higher (30 %) than in females (13%), these results were in agreement with *Yakoob et al*. (2005) [21] who found that the prevalence of G. lamblia was 38.9% higher in males than in females in Pakistan. The present study showed that the prevalence rate of G. lamblia was higher (43%) in the age group 21-30 years old; these results were not in line with Iraqian study which was done by Raza and Sami (2009) [22] who showed that the highest rate of infection (17%) was among the age group (6-10) years old. Our study demonstrated that the sensitivity and specificity of the copro ELISA test for detection of *G. lamblia* versus microscopy were 90% and 94%. These results were similar to an Iraqi study conducted by Souhaila, [23], 76.4% and 100%. *Giardia* cysts or trophozoites are difficult to recover from infected patients. Quality of the diagnosis can be confirmed by examining at least three stool samples over several days. Copro antigen ELISA test is more sensitive and specific. Several immunological tests can detect *Giardia* antigens in stool specimen but until now does not replace the simple microscopy. We recommend using ELISA in epidemiological surveys in Sudan, same as a result obtained by Al-Saeed, [24] and to confirm the diagnosis in patients with typical clinical symptoms of giardiasis but with negative results by direct microscopy

V. Conclusion

The study concluded that diagnosis of *G. lamblia*by copro ELISA has high sensitivity and specificity, compare to microscopy that required highly qualified microscopist. No apparent differences between the three techniques in diagnosis of giardiasis. The benefit of copro ELISA is valuable in epidemiological study and in diagnosis, especially for those who had low parasitessecretionin their stool samples and was negative by microscopy with continuous sign and symptoms. The clinical cut off obtained is considered as a reference range for diagnosis of Giardiasis by copro ELISA in Sudan.

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1 INTRODUCTION

Entamoeba histolytica is the causative agent of amoebiasis (amoebic dysentery, amoebic liver abscess). Only trophozoites of virulent strains invade the intestinal wall and cause ulcers releasing blood and mucous into the faeces. Invasive Amoebae reach the liver via the enteral vascular system and cause extensive abscesses (1). The infectious cysts are spread via faecal-oral transmission. The vegetative trophozoites are released by excystation and multiply by bisection.

Meanwhile molecular biological (PCR) and biochemical (isoenzyme analysis) methods confirm the classification of the pathogenic, invasive strains as separate species *Entamoeba histolytica*. The non-invasive strains are classified as *Entamoeba dispar*. The two species share an identical morphology and therefore a microscopic differentiation is impossible (1-6).

Entamoeba histolytica releases specific antigens into the intestine during its life cycle. These antigens are excreted with the faeces of infected persons. The antigen detection by enzyme immunoassay can serve as specific and easy to perform alternative to microscopy.

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2 INTENDED USE

The **Entamoeba histolytica Ag ELISA** is an *in-vitro*-diagnostic device for direct detection of *Entamoeba histolytica* specific antigen in faecal samples.

3 PRINCIPLE OF THE TEST

Entamoeba histolytica Ag ELISA is a fast enzymometric two-step immunoassay based on polyclonal peptide antibodies recognizing two different epitopes of the serine-rich 30 kD membrane protein (SREHP) of *Entamoeba histolytica*.

Diluted stool specimens as well as positive and negative controls react in the first incubation step of 60 minutes at room temperature with the solid phase bound antibodies. In the following washing step unbound components are removed from the wells.

In the next step horseradish-peroxidase (HRP) labelled antibodies react with solid phase bound antibody-antigen-complexes within a reaction time of 30 minutes at room temperature. Non-bound material is separated from the solid-phase immune complexes by a subsequent washing step.

HRP converts the subsequently added colorless substrate solution of 3,3’,5,5’-tetramethylbenzidine (TMB) into a blue product. The enzyme reaction is terminated by sulphuric acid dispensed into the wells after 10 min incubation at room temperature turning the solution from blue to yellow.

The optical density (OD) of the solution read at 450/620 nm is directly proportional to the specifically bound amount of *Entamoeba histolytica* antigen.

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4 TEST COMPONENTS

			For 96 Wells
1	MT PLATE	**Microtitration plate** coated with polyclonal anti-SREHP- antibodies (rabbit)	12 single breakable 8-well strips colour coding light brown vacuum-sealed with desiccant
2	WASH BUF 10x	**Wash buffer** 10-fold	100 mL concentrate for 1000 mL solution white cap
3	DIL	**Sample diluent**	100 mL · ready to use coloured yellow black cap
4	CONTROL +	**Positive control** SREHP peptide	2.0 mL · ready to use coloured blue red cap
5	CONTROL –	**Negative control** *Entamoeba histolytica* negative sample	2.0 mL · ready to use coloured blue green cap
6	CONJ HRP	**HRP-conjugate** HRP labelled, polyclonal anti-SREHP-antibodies (Sheep)	15 mL · ready to use coloured green brown cap
7	SUB TMB	**Substrate** 3,3',5,5'-Tetramethylbenzidine and hydrogen peroxide	15 mL · ready to use blue cap
8	STOP	**Stop solution** 0.25 M sulphuric acid	15 mL · ready to use yellow cap

5 PREPARATION AND STORAGE OF SAMPLES

5.1 Collection and storage

Stool samples should be stored at 2 °C - 8 °C immediately after collection and processed within 72 hours. Longer storage is possible at -20 °C. Repeated freezing and thawing of samples should be avoided.

Stool samples already diluted with the sample diluent [DIL] can be stored for 72 h at 2 °C - 8 °C before testing.

Faecal samples that are already diluted in transportation media should not be used.

5.2 Preparation

Warm samples to room temperature and mix well.

Pipette **1000 µL** of sample diluent into a clean tube. Using a disposable stirring rod transfer about **200 mg** (diameter about 4-6 mm) of faeces if solid or **200 µL** if liquid into the tube and suspend thoroughly. If necessary, sediment floating particles by a centrifugation step with a micro centrifuge for one min at maximum speed.

6 MATERIALS REQUIRED BUT NOT PROVIDED

- micropipettes
- multi-channel pipette or multi-pipette
- reagent container for multi-channel pipette
- 8-channel wash comb with vacuum pump and waste bottle or microplate washer
- microplate reader with optical filters for 450 nm for measurement and ≥ 620 nm for reference
- distilled or de-ionized water
- glassware
- tubes (2 mL) for sample preparation

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7 PREPARATION AND STORAGE OF REAGENTS

7.1 Kit size and expiry

One kit is designed for 96 determinations.

The expiry date of each component is reported on its respective label, that of the complete kit on the outer box label.

Upon receipt, all test components have to be kept at 2 °C - 8 °C, preferably in the original kit box.

After opening all kit components are stable for at least 2 months, provided proper storage.

The ready to use wash buffer solution is stable for at least one month when stored at 2 °C - 8 °C.

7.2 Reagent preparation

Allow all components to reach room temperature prior to use in the assay.

The microtitration plate is vacuum-sealed in a foil with desiccant. The plate consists of a frame and strips with breakable wells. Allow the sealed plate to reach room temperature before opening. Unused wells should be stored refrigerated and protected from moisture in the original cover carefully resealed.

Prepare a sufficient amount of wash solution by diluting the 10-fold concentrated wash buffer 1 + 9 with distilled or de-ionized water.

For Example:

10 mL wash buffer concentrate *(2)* + 90 mL distilled or deionized water.

8 ASSAY PROCEDURE

- Dilute samples with sample diluent *(3)* 1:6,
  e.g. 200 mg or 200 µL faeces + 1.0 mL sample diluent *(3)*
- Avoid any time shift during dispensing of reagents and samples.
- Make sure the soak time of the wash buffer in the wells is at least 5 seconds per wash cycle and that residual fluid is completely drained in every single wash cycle.
- Avoid light exposure of the TMB substrate solution.

8.1 Working Steps

1. Warm all reagents to room temperature (RT) before use. Mix gently without causing foam.
2. Pipette:
   100 µL *CONTROL +* positive control (4)
   100 µL *CONTROL -* negative control (5)
   100 µL diluted sample
3. Cover plate and incubate for **60 min** at RT.
4. Decant, then wash each well **5x** with **300 µL** wash solution (diluted from ***(2)***) and tap dry onto absorbent paper.
5. Dispense **3 drops (or 100 µL) *CONJ HRP (6)*** per well
6. Cover plate and incubate for **30 min** at RT.
7. Decant, then wash each well **5x** with **300 µL** wash solution (diluted from ***(2)***) and tap dry onto absorbent paper.
8. Dispense **3 drops (or 100 µL) *SUBSTR TMB*** substrate (7) per well.
9. Incubate for **10 min** at RT protected from light.
10. Dispense **3 drops (or 100 µL) *STOP*** stop solution (8), mix gently.
11. Read OD at 450 nm / ≥ 620 nm with a microplate reader within 30 min after reaction stop.

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9 RESULT INTERPRETATION

9.1 Qualitative evaluation

Cut-off determination: OD negative control + 0.20

Samples with absorbances equal to or higher than the cut-off value are considered *positive*, samples with absorbances below the cut-off value are considered *negative* for Entamoeba histolytica antigen.

10 REFERENCE VALUES

Entamoeba histolytica Ag ELISA	
Positive	≥ Cut-off
Negative	< Cut-off

It is recommended that each laboratory establishes its own normal and pathological reference ranges as usually done for other diagnostic parameters, too. Therefore, the above mentioned reference values provide a guide only to values which might be expected.

10.1 Test validity

The test run is valid, if:

- the mean OD of the negative control is

  ≤ 0.20 (manual performance)

  ≤ 0.30 (automatic performance)

- the mean OD of the positive control is:

  ≥ 0.80

If the above mentioned quality criteria are not met, repeat the test and make sure that the test procedure is followed correctly (incubation times and temperatures, sample and wash buffer dilution, wash steps etc.). In case of repeated failure of the quality criteria contact your supplier.

10.2 Limitations of the procedure

There is no correlation between measured absorbance and seriousness of the infection.

It is also not allowed to correlate absorbencies of the samples with that of the positive control.

Cross contamination of reagents and samples can produce false positive results. Incorrect dilutions and not sufficiently homogenized samples can cause false results.

A negative ELISA result does not exclude an *Entamoeba histolytica* infection, because the number of excreted cysts can decrease below the detection limit of the assay in invasive amoebiasis. Thus additional investigations (e.g. detection of specific antibodies or ultrasound) should be performed in case of a negative ELISA result but clinical suspect. Clinical findings have to be considered for a final result interpretation.

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Transfer Pipettes
2. Graduated Cylinder
3. Reagent grade (DI) water
4. ELISA plate reader with 450 and 620-650 nm filters

WARNINGS AND PRECAUTIONS FOR USERS

For *In Vitro* Diagnostic Use

1. Do not use solutions if they precipitate or become cloudy.
Exception: Wash concentrate may precipitate during refrigerated storage, but will dissolve upon warming.
2. Do not add azides to the samples or any of the reagents. Controls and some reagents contain Thimerosal as a preservative.
3. Treat all reagents and samples as potentially infectious materials. Use care to prevent aerosols and decontaminate any spills of samples.
4. Stop solution is a 5% solution of phosphoric acid in water. If spilled on the skin, wash with copious amounts of water. If acid gets into the eyes, wash with copious amounts of water and seek medical attention.
5. Persons who are color blind or visually impaired may not be able to read the test visually and should use spectrophotometric readings to interpret results.

STORAGE CONDITIONS

Reagents, strips and bottled components:
Store between 2 - 8ºC.
Bottle containing diluted wash buffer may be stored at room temperature.

COLLECTION OF STOOL (FAECES)

No modification of collection techniques used for standard microscopic O&P is needed. Stool samples may be used as unpreserved or frozen, or in preservation media of 10% formalin, SAF or MF.
Unpreserved samples should be kept at 2 - 8º C and tested within 24 hours of collection. Samples that cannot be tested within this time should be frozen at -20º C or lower until used. Freezing does not adversely affect the test.
Formalized, SAF and MF preserved samples may be kept at room temperature (15-25º C) and tested within 18 months of collection. DO NOT freeze preserved samples.
All dilutions of unpreserved stools must be made with the Dilution Buffer provided.

PREPARATION OF SAMPLE

Fresh/Frozen Stools

Thaw sample if needed. Prepare a 1:4 dilution in tubes using 0.3 ml of Dilution Buffer and one swab of fecal specimen (approximately 0.1 g). Coat swab with specimen and transfer into the Dilution Buffer, expressing as much liquid as possible and mix well. For watery specimens, add 0.1 ml of sample to 0.3 ml Dilution Buffer in tubes. The Faecal Preparation Tube K6998SAS, Clindia Benelux can be used for sample preparation. For automatic ELISA devices it is advised to centrifuge the samples before use.

Preserved Stools (Formalin, SAF and MF)

Mix contents thoroughly inside collection container. No further processing is required.

ASSAY PROCEDURE

General remarks:

1. Use a separate disposable tip for each sample transfer to avoid cross-contamination.
2. All reagents must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
3. Once the assay has been started, all steps should be completed without interruption.

Reconstitution of the Reagents:

Wash Buffer - Remove cap and add contents of one bottle of Wash Concentrate to a bottle containing 950 ml of DI water. Swirl to mix.
CAUTION: Crystals may form when the concentrated washing solution is stored at 2-8 °C These crystals can easily be dissolved when bringing the vials to room temperature or by placing them in a water bath at 37 °C.

Assay Procedure:

1. Break off the required number of wells needed (number of samples plus 2 for controls) and place in holder.
2. Add 100 µl of negative control to well # 1 and 100µl of positive control to well # 2.*
3. Add 50µl of dilution buffer to each sample well. DO NOT add dilution buffer to control wells.
4. Add 50µl of sample to each well with dilution buffer.
5. Incubate for 60 minutes at room temperature (15-25º C), then wash**. After last wash slap the wells out on a clean absorbent towel to remove left over wash buffer.
6. Add 100µl of Enzyme Conjugate to each well.
7. Incubate for 30 minutes at room temperature (15-25º C), then wash**. After last wash slap the wells out on a clean absorbent towel to remove left over wash buffer.
8. Add 100µl of Chromogen to each well.
9. Incubate 10 minutes at room temperature (15-25º C). For automatic ELISA devices incubate 8 minutes at roomtemperature.
10. Add 100µl of Stop Solution to each well. Mix wells by gently tapping the side of the strip holder with index finger.
11. Read results visually or at 450/620-650 nm.

** Controls must be included each time the kit is run.*
*** Washings consist of vigorously filling each well to overflowing and decanting contents seven separate times. For automatic ELISA devices the washing consists of seven wash steps using a volume of 400 µl.*

Only one set of controls is required per run.
Read results within 4 hours from addition of Stop Solution.
All incubations are done at room temperature (15-25 °C).

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RESULTS

Interpretation of Results - Visual

Reactive: Any sample well that is obviously more yellow than the negative control well.
Non-reactive: Any sample well that is not obviously more yellow than the negative control well.

NOTE: The negative control, as well as some samples, may show some slight color. A sample well must be obviously darker than the negative control well to be called a positive result. Please refer to the enclosed visual read card for color comparisons.

Interpretation of Results - ELISA Reader

Read all wells at 450/620-650 nm.
Reactive: Absorbance reading of 0.08 OD units and above indicates the sample contains *Giardia* antigen.
Non-reactive: Absorbance reading less than 0.08 OD units indicates the sample does not contain detectable levels of *Giardia* antigen.

Expected Values

Normal healthy individuals should be free of *Giardia* and should test negative. A positive reaction indicates that the patient is shedding detectable amounts of *Giardia* antigen. Certain populations, such as homosexual men and children in day care settings, have shown higher rates of infection with *Giardia* than the normal population. Please refer to the Summary section for references.

LIMITATION OF PROCEDURE

Test results should be used as an aid in diagnosis and should not be interpreted as diagnostic by themselves.
DO NOT concentrate stool samples. Assay will not give accurate results on a concentrated sample.
A negative result can occur from an antigen level lower than the detection limits of this assay. Multiple samples over time may be indicated for those patients that are suspected of being positive for *Giardia*.

QUALITY CONTROL

The use of a positive and negative control allows easy validation of kit stability. For a valid test, the positive control must have an absorbance of at least 0.5 OD units and the negative control must be less than 0.08 OD units. Should the value fall outside these limits, the kit should not be used.

REPRODUCIBILITY

The intra-assay (well to well) CV was calculated using 4 positive and 4 negative samples assayed 24 times in a single run. The mean CV was 3.67% with the highest being 6.18%.

The inter-assay (run to run) CV was calculated using 4 positive and 4 negative samples assayed on three separate days. The mean CV was 4.08% with the highest being 11.61%

CROSS-REACTIVITY

No cross-reactions were seen with the following organisms:
*Entamoeba hartmanni, Endolimax nana, Entamoeba histolytica/dispar, Entamoeba coli, Blastocystis hominis, Dientamoeba fragili*s, *Chilomastix mesnili, Strongyloides stercoralis, Cryptosporidium, Ascaris lumbricoides, Enterobius vermicularis, Diphyllobothrium species*, *Hymenolepis nana, Clonorchis sinensis, Enteromonas hominis, Trichuris trichiura, Iodamoeba buetschlii, Hookworm, Schistosoma mansoni, rotavirus*, *Taenia eggs*, *Fasciola eggs*, *Isospora belli, Entamoeba polecki*, *adenovirus, &* 33 bacterial species (list available on request).

TROUBLESHOOTING

Problem: Negative control has substantial color development.
Correction: Washings were insufficient. Repeat test with more vigorous washings.

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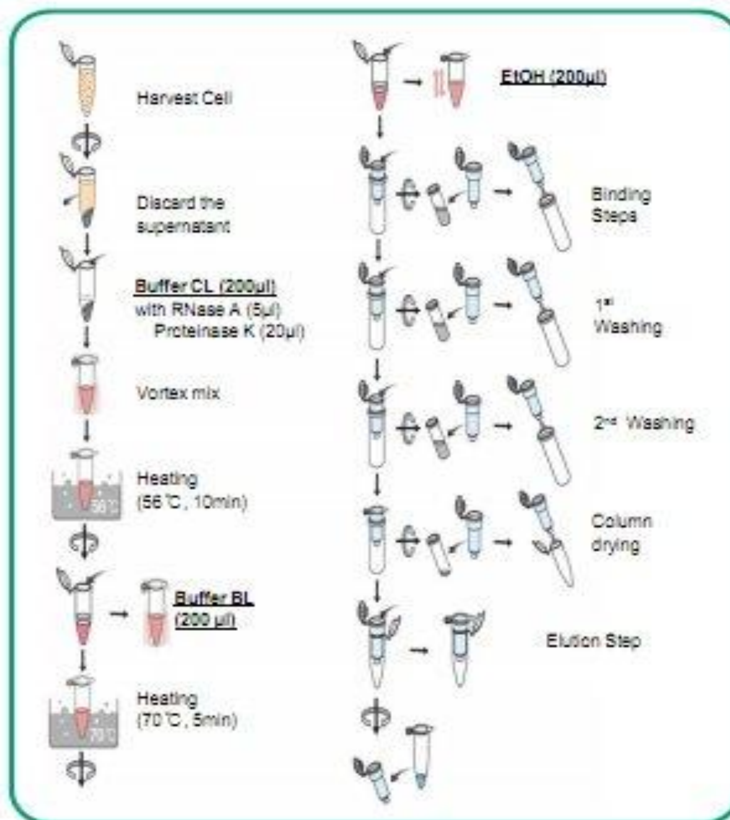
G-spin™ Total DNA Extraction Kit

The single kit solution for *all* your genomic DNA requirements. The G-spin™ Total DNA Extraction Kit provides efficient methods for purification of total DNA from cultured animal cells, animal tissue, rodent tails, fixed tissue, animal hair, gram negative bacteria and blood samples for reliable PCR and Southern blotting. Purified DNA can be up to 50kb in size, with fragments of 20-30kb predominating.

Protocol



Description

- **G-spin Total DNA Extraction Kit yields pure DNA ready for direct amplification in 20-30 minutes.**
  - Purification does not require phenol/chloroform extraction or alcohol precipitation
  - DNA is eluted in buffer or water, ready for direct addition to PCR or other enzymatic reactions
  - DNA can also be stored at -20°C for later use
  - DNA is free of protein, nucleases and inhibitors
  - Fragments range from 20kb up to 50kb
- **Specific protocols provided for:**
  - Type A Protocol: blood, body fluids
  - Type B Protocol: tissues, rodent tail
  - Type C Protocol: cell, buffy coat

Maxime PCR PreMix Kit (*i*-Taq)

for 20µl rxn

Cat. No. **25025** (96 tubes); **25026** (500 tubes)

DESCRIPTION

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution. *Maxime* PCR PreMix Kit (*i*-Taq) is the product what is mixed every component. *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20°C; under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

CONTENTS

- *Maxime* PCR PreMix (*i*-Taq; for 20µl rxn) 96 (500) tubes.

Component in 20 µl reaction	
i-Taq™ DNA Polymerase(5U/µl)	2.5U
dNTPs	2.5mM each
Reaction Buffer(10x)	1x
Gel Loading buffer	1x

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp.	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94℃	2min	2min	2min
30-40 Cycles	Denaturation	94℃	20sec	20sec	20sec
	Annealing	50-65℃	10sec	10sec	20sec
	Extension	65-72℃	20-30sec	40-50sec	1min/Kb
Final extension		72 ℃	Optional. Normally, 2-5min		

PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (*i*-Taq).
   Note 1 : Recommended volume of template and primer : 3µl~5µl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/µl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20µl.

Example Total 20µl reaction volume

PCR reaction mixture	Add
Template DNA	1 ~ 2µl
Primer (F : 10pmol/µl)	1µl
Primer (R : 10pmol/µl)	1µl
Distilled Water	16 ~ 17µl
Total reaction volume	**20 µl**

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

Note : The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this product is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

Note : This CYCLING PARAMETERS serves as a guideline for PCR amplification. optimal reaction conditions such as PCR cycles, annealing temperature, extension temperature and incubation times, may vary and must be individually determined.

EXPERIMENTAL INFORMATION

- *Comparison with i-Taq™ DNA Polymerase and i-Master mix PCR PreMix*

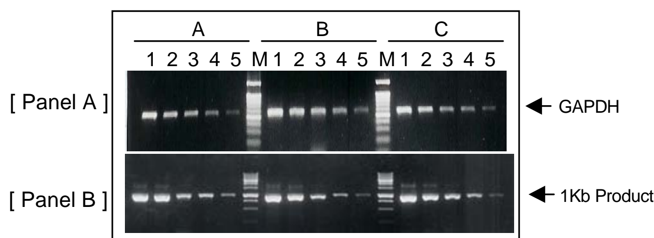


Fig.1. [Panel A] RT-PCR amplification at the Indicating cDNA diluted mixtures.
Total RNA was purified from mouse cells using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed with *i-Taq*™ DNA Polymerase, *i*-Master mix PCR Kit and *Maxime* PCR PreMix (*i*-Taq).
A, *i*-Master mix PCR Kit; **B**, *i*-Taq™ DNA Polymerase; **C**, *Maxime* PCR PreMix (*i*-Taq)
Lane M, 100bp Ladder DNA Marker; **lane 1,** undiluted cDNA; **lane 2,** 1/2 diluted cDNA; **lane 3,** 1/4 diluted cDNA; **lane 4,** 1/8 diluted cDNA; **lane 5,** 1/16 diluted cDNA
[Panel B] PCR amplification
Comparison with *i*-Taq™ DNA Polymerase, *i*-Master mix PCR PreMix Kit and *Maxime* PCR PreMix (*i*-Taq) by amplifying 1Kb DNA fragment from variable amounts of λDNA
Aliquots of 5µl in 20µl reaction are loaded on 1% agarose gel.
Lanes M, 1Kb ladder; **lanes 1,** 200 pg; **lane 2,** 20 pg; **lane 3,** 2 pg; **lane 4,** 200 fg; **lane 5,** 20 fg

- *Comparison with different company kit*

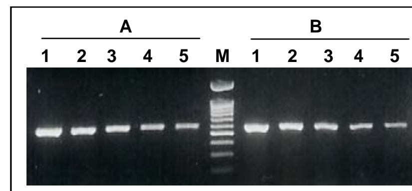


Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
A, Company A; **B**, iNtRON's *Maxime* PCR PreMix (*i*-Taq)
Lane M, 100bp Ladder DNA Marker; **lane 1,** undiluted cDNA; **lane 2,** 1/2 diluted cDNA; **lane 3,** 1/4 diluted cDNA; **lane 4,** 1/8 diluted cDNA; **lane 5,** 1/16 diluted cDNA

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Optimizing Restriction Endonuclease Reactions

Protocols.io also provides an interactive version of this protocol.

There are several key factors to consider when setting up a restriction endonuclease digest. Using the proper amounts of DNA, enzyme and buffer components in the correct reaction volume will allow you to achieve optimal digestion. By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes. This enzyme : DNA : reaction volume ratio can be used as a guide when designing reactions. However, most researchers follow the "typical" reaction conditions listed, where a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity. NEB offers the following tips to help you to achieve maximal success in your restriction endonuclease reactions.

A "Typical" Restriction Digest

Restriction Enzyme	10 units is sufficient, generally 1µl is used
DNA	1 µg
10X NEBuffer	5 µl (1X)
Total Reaction Volume	50 µl
Incubation Time	1 hour*
Incubation Temperature	Enzyme dependent

* Can be decreased to 5-15 minutes by using a Time-Saver™ Qualified enzyme.

Enzyme

- Keep on ice when not in the freezer
- Should be the last component added to reaction

- Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube. Follow with a quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.
- In general, we recommend 5–10 units of enzyme per µg DNA, and 10–20 units for genomic DNA in a 1 hour digest.
- NEB has introduced a line of High-Fidelity (HF®) enzymes that provide added flexibility to reaction setup.

DNA

- Should be free of contaminants such as phenol, chloroform, alcohol, EDTA, detergents or excessive salts. Extra wash steps during purification are recommended.
- Methylation of DNA can inhibit digestion with certain enzymes. For more information about methylation, Effect of CpG Methylation on Restriction Enzyme Cleavage and Dam and Dcm Methylases of *E.coli*

Buffer

- Use at a 1X concentration
- Supplement with SAM (S-Adenosyl methionine) to the recommended concentration if required.

Reaction Volume

- A 50 µl reaction volume is recommended for digestion of 1 µg of substrate
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- Additives in the restriction enzyme storage buffer (e.g., glycerol, salt) as well as contaminants found in the substrate solution (e.g., salt, EDTA, or alcohol) can be problematic in smaller reaction volumes. The following guidelines can be used for techniques that require smaller reaction volumes.

	Restriction Enzyme*	**DNA**	**10X NEBuffer**
10 µl rxn**	1 unit	0.1 µg	1 µl
25 µl rxn	5 units	0.5 µg	2.5 µl
50 µl rxn	10 units	1 µg	5 µl

- * Restriction Enzymes can be diluted using the recommended diluent buffer when smaller amounts are needed.
  ** 10 µl rxns should not be incubated for longer than 1 hour to avoid evaporation.

Incubation Time

- Incubation time is typically 1 hour
- Can often be decreased by using an excess of enzyme, or by using one of our Time-Saver Qualified enzymes.
- It is possible, with many enzymes, to use fewer units and digest for up to 16 hours. For more information, visit Extended Digests with Restriction Endonucleases.

Stopping a Reaction

If no further manipulation of DNA is required:

- Terminate with a stop solution (10 µl per 50 µl rxn) [1x: 2.5% Ficoll®-400, 10mM EDTA, 3.3mM Tris-Hcl, 0.08% SDS, 0.02% Dye 1, 0.001% Dye 2, pH 8.0@25°C] (e.g., NEB #B7024)

When further manipulation of DNA is required:

- Heat inactivation can be used
- Remove enzyme by using a spin column or phenol/chloroform extraction

Storage

- Storage at -20°C is recommended for most restriction enzymes. For a few enzymes, storage at-70°C is recommended for periods longer than 30 days. Please refer to the enzyme's technical data sheet or catalog entry for storage information.
- 10X NEBuffers should also be stored at -20°C

Stability

- All enzymes are assayed for activity every 4 months. The expiration date is found on the label.
- Exposure to temperatures above -20°C should be minimized whenever possible

Control Reactions

If you are having difficulty cleaving your DNA substrate, we recommend the following control reactions:

- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing.