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The association between helminthes infestation and IBD in the context of *NOD2/CARD15* gene polymorphism and serum levels of IL1Beta and IL10.

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
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العلاقة بين الاصابة بالديدان الطفيلية ومرضى المعي الالتهابي في التغاير الوراثي للجين *NOD2/CARD15* ومستويات الحركيات الخلوية IL1Beta,IL10 .

اطروحة مقدمة
الى مجلس كلية الطب/ جامعة القادسية
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من قبل

عباس غافل عباس الوائلي

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بإشراف

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الخلاصـــــــــــــــــــــــــــه

تم جمع (50)عينه من دم وخروج المرضى المصابين بمرض المعي الالتهابي خلال الفترة من كانون الثاني 2018 ولغاية تموز 2018 معظم هؤلاء المرضى يراجعون مركز الجهاز الهضمي والكبد في مستشفى الديوانيه التعليمي ،الكثير منهم يعانون من نزف شرجي دموي ،الام في البطن ،نقص في الوزن .وعند اجراء الفحص بواسطة الناظور تبين ان (19)منهم يعانون من مرض كرون و(31)منهم يعانون من التهاب القولون التقرحي كما تمت مطابقة النتائج مع (50)شخص من الاصحاء كمجموعة سيطرة.

تم سحب (5)مل من الدم ل(50)مريض مصاب بمرض المعي الالتهابي و(50)شخص من الاصحاء كمجموعة سيطرة ،(2)مل من الدم تم استخدامها لغرض استخلاص المادة الوراثية لغرض تحديد التغاير الوراثي في الجينات باستخدام تقنية تفاعل البلمرة المتعدد ،(3)مل لقياس مستوى الحركيات الخلوية باستخدام تقنية الاليزا.

كان عدد الذكور في المرضى لهذه الدراسة (38) وعدد الاناث(12)اعمارهم تتراوح بين (12-63)سنة.

كذلك تم اخذ عينة خروج من كل شخص وتم استخلاص DNA لغرض التحري عن وجود بعض الديدان المعوية بطريقتين: الاولى الطريقة التقليدية القديمة والثانية طريقة تفاعل سلسلة البلمرة المتعدد، أظهرت النتائج وجود الديدان التالية:

Strongyloides stercoralis(28%) ,Ascaris lumbricoides(12%) ,Enterobious vermiculari(34%).

وقد لوحظ عدم ظهور الاصابه ب ***Trichuris trichiura***

كما بينت الدراسة ان النسبة الاعلى من المرضى المصابين بمرض المعي الالتهابي تراوحت اعمارهم بين(20-40) سنه عددهم 28 (56%)كما بينت النتائج ان 34 (68%) من المصابين يسكنون المدينة و16 (32%) منهم في المناطق الريفية.

تم قياس مستوى السايتوكينات (IL1B,IL10) في مصل مجاميع المرضى والاصحاء حيث كان مستوى IL-10اعلى مما تم تسجيله في مرضى كرون ومجموعة السيطرة وكان مستوى IL1B اعلى في مرضى كرون مما في مرضى التهاب القولون التقرحي ومجموعة السيطرة .

اظهرت الدراسة ان التوزيع للجينات الخاصة ب *NOD* كان كالتالي CC(68%) مما تم تسجيله في مجموعة السيطرة(74%)،CT (32%)ومجموعة السيطرة (18%) ,TT(0%) ومجموعة السيطرة (8%) .

معدل الاعمار في هذه الدراسة (36.68) ومجموعة السيطرة (37.22)اكثر عمر يحدث فيه المرض هو اربعين سنه فما فوق (56.%)بالنسبة لمرضى المعي الالتهابي .كما بينت الدراسة ان لم يكن هناك فرق معنوي بين الرجال والنساء لمرضى المعي الالتهابي ، هناك علاقة بين الإصابة بالمرض والديدان كانت نسبة الاصابة بالأصحاء اكثر من المرضى الذين يعانون من مرض المعي الالتهابي.

اظهرت نتائج التتابع الجيني Gene Sequence ان تسلسل ***Ascaris lumbricoide*** ذو تطابق 100 % مع سلالات M74584.1 في قواعد البيانات العالمية. في حين ظهر تسلسل ***Enterobious vermicularis*** ذو تطابق 100% مع سلالات HQ646164.1. كما ظهر تسلسل,***Strongyloides stercoralis*** ذو تطابق 100% مع سلالات AB453316.1 على التوالي في قواعد البيانات العالمية NCBI global databases . لم تسجل نتائج للاصابه ب ***Trichuris trichiura***.

Summary

Fifty clinical samples of blood and feces were collected from patients with IBD and control groups during January2018 to July 2018 some of these patients regularly attending the endoscopy unit of Aldiwanya teaching hospital, suffers from rectum bleeding, abdominal pain ,loss of weight in endoscopy been 19 suffer from crohn's disease and 31 suffer from ulcerative colitis and an ethnically matched apparently healthy control groups(N=50).

Five ml of blood samples obtained from 50 patients with IBD and compare to 50 healthy control groups ,2ml of blood sample were utilized for genomic DNA extraction for detection of single nucleotide polymorphism of *NOD* gene by PCR restriction fragment length polymorphism technique

Three ml of blood samples were assessed for serum measurement of cytokines using enzyme linked immunosorbent assay technique. Number of males in this study 38 and females 12,their ages ranging between 12-63 years.

Also 50 clinical samples of feces were collected from each patient and control ,performed DNA extraction for investigation of some intestinal helminthes such as ***Strongyloides stercoralis ,Ascaris lumbricoides ,Enterobious vermicularis.*** By two methods first general stool examination ,second PCR .the mean of ages in this study of patients was 36.68 years and in control 37.22 years old.

The highest age of incidence ranging between 20-40 years old ,and the result showed 34(68%)from patients in urban areas and 16 (32%)lived in rural areas. There are relation between the infection with disease and

helminthes ,was rate of infection in control groups more than the patients which suffer from inflammatory bowel disease.

In those infestation with helminthes is ***Strongyloides stercoralis*** was established(**28%),*Ascaris lumbricoides(*12%) ,*Enterobious vermicularis* (34%),** and don't record any result with ***Trichuris trichiura.***

Measuring cytokines levels(IL10,IL1B) in serum of all patients and control ,mean of IL10 was higher in UC than CD and control ,while in IL1B was higher in CD than UC and control respectively.

In *NOD2* genotype was frequency distribution in patients with IBD and control groups as following :CC68% ,in control74% . CT32%,in control 18% . TT 0% in control 8%.

The gene sequence results were showed ***Ascaris lumbricoides*** identity 100% with M74584.1, ***Enterobious vermicularis*** identity 100% with HQ646164.1,***Strongyloides stercoralis*** identity with 100% AB453316.1 in NCBI databases.

1.1: Introduction:

Inflammatory bowel disease (IBD)is a chronic relapsing inflammatory disorder of the bowel, consisting mainly of Crohn's disease (CD) and ulcerative colitis (UC). The incidence of IBD has been rising not only in Western countries, but also in Asia (Yang *et al*., 2008).

The distinct difference between being that Crohn's disease can affect any part of the gastrointestinal tract, from mouth to anus, with most beginning in the terminal ileum; while Ulcerative Colitis is restricted to the colon. The exact causes of both are still a mystery. Recently, research has achieved a better understanding of the pathogenesis of this disease through advances in three areas: IBD-specific genes, environmental factors, and the immunopathology (Xavier and Podolsky, 2007).

IBD is found in greater proportions in industrialized countries and rates are rising in developing countries (Baumgart and Carding, 2007and Loftus *et al*., 2007). In North America, the prevalence rates of Crohn's disease are highest for Caucasians, followed by African-Americans, Asians, and Hispanics (Bernstein *et al*., 2010).

It is generally believed that chronic IBD occurs in genetically predisposed individuals who are exposed to unknown environmental and microbial triggers. However, the genetic links provide only a partial explanation for disease development as the majority of patients with IBD have neither a family history nor a known genetic defect (Kaser *et al*., 2010). Although IBD occurs worldwide, its epidemiologic and clinical characteristics vary depending upon the geographic location and the ethnicity of the population. Identifying the characteristic features of IBD in populations living in different geographical locations and with

different ethnicities may provide significant clues about its etiology and pathophysiology(Kim and Won, 2010).

Aim of the study

The aim was realized by the following objectives**:**

1- Identification of different species of helminthes and the relationship with development of IBD.
2- Studying of polymorphism in *NOD2/CARD15* gene and their association to IL10 and IL1B in IBD patients.
3- Estimation of serum levels of IL10 and IL1B by ELISA technique in patients with IBD.
4- Understanding the correlation between parasitic infections and autoimmune disorders may be helpful in prediction, early identification and conceivably the prevention of these diseases
5- Identifying children at risk so that treatment can begin much earlier in the course of the disease (normally diagnosis is made after considerable damage has been done).
6- Helping differentiate Crohn's disease from ulcerative colitis patients.

1.2 Literatures Review

1.2.1 Inflammatory Bowel Diseases

Inflammatory Bowel Disease (IBD) comprises those conditions which tend to be chronic or relapsing immune activation and inflammation within gastrointestinal tract (GIT). Ulcerative Colitis (UC) and Crohn's disease are the two major forms of this disease with unidentified etiopathology (Sands, 2002).

The UC and Crohn's disease are chronic, idiopathic, inflammatory diseases of the GIT that share common symptoms such as diarrhea, abdominal pain, fever, and weight loss. Ulcerative colitis involves all or part of the colon, whereas, Crohn’s disease commonly involves the terminal ileum and proximal colon (Mc Quaid, 2005; Budarf *et al*., 2009).

The two major forms of IBD share many clinical and epidemiological characteristics, suggesting that underlying causation may be similar. Yet, UC & Crohn's disease are distinct syndromes with divergent treatment and prognosis (Sands, 2002).

In the majority of cases, the characteristics manifestations permit to distinguish between the two entities, but in 10 % of cases it remains difficult, and there is need for further diagnostic tools. Even when it is possible to differentiate the diagnosis, age at onset, extent of disease, familial occurrence of disease, extra intestinal manifestations, response to treatment, and the natural disease course may represent clinical subgroups that suggest different a etiology(Loftus, 2004).

The incidence of IBD has been rising not only in Western countries, but also in Asia, including Korea (Yang *et al*., 2008). The characteristics of Western and Asian IBD patients differ in epidemiology,

phenotype and genetic susceptibility (Inoue *et al*., 2002; Leong *et al*., 2004).

There has been a sharp increase in the incidence of IBD in the late 1900s in Western countries as well as in some Eastern parts of Europe and North America (Farrokhyar *et al*., 2001; Lakatos *et al*., 2004). Both Crohn's disease and ulcerative colitis stem possibly from a common mechanism with an exact etiology that remains obscure (Lakatos and Lakatose 2003; Hugot, 2004).

1.2.1.1: Ulcerative Colitis

Ulcerative colitis was first described as early as 1859 by Samuel Wilks as 'the morbid appearance of the intestine of misses Banks', who recognized it as an entity distinct from bacillary dysentery and distinguished it from ulcerations caused by Mercurial poisoning, Diphtheritic inflammation, and congestion (Wilks, 2002), while the first complete description of this disorder might be of Sir Arthus Hurst, who described an associated sigmoidoscopic appearance and support a clear differentiation from bacillary dysentery, and considered the latter as a predisposing factor (Hurst, 1999).

The main symptom of UC is diarrhea, which is generally bloody and may be associated with abdominal pain; loss of appetite and subsequent weight loss are common, as is fatigue. In cases of severe bleeding, anemia may also occur. The symptoms of ulcerative colitis do tend to come and go, with fairly long periods between flare-ups, in which patients may experience no distress at all. Characteristic histological findings are acute and chronic inflammation of the mucosa by polymorph nuclear leukocyte and mononuclear cells, crypt abscesses, distortion of the mucosal glands and goblet cells depletion (Hendrickson *et al*., 2002).

Contrary to Crohn's disease, UC only involves the colon. Typically, the inflamed area starts at the level of the rectum. In the mildest form of UC, only the distal part of the large intestine is involved, referred to as proctitis. In more severe cases it can extend to the left side of the colon, or extend throughout the entire colon as a pancolitis. The inflammation seen in UC involves only the mucosal layer of the bowel wall, and is histologically characterized by large infiltrates of lymphocytes, plasma cells, and polymorphonuclear granulocytes, accompanied by depletion of goblet cells and crypt hyperplasia. Endoscopic examination of the colon shows a tender colon, which is easily damaged, swollen and hyperemic with superficial ulcerations, which can be deeper in the more severely affected patients. Furthermore, pseudopolyps are commonly found (Demirsoy *et al*., 2010).

Treatment is aimed at controlling the inflammatory reaction and restoring losses of fluid, salts, and blood. Treatment of UC involves anti-inflammatory and immunosuppressive drugs including aminosalicates and corticosteroids. Treatment with azathioprine/6-mercaptopurine and cyclosporine issued in the more severely affected patients. If there is no improvement, surgery may become necessary. Since UC is restricted to the colon, complete resection of the colon will be curative, although disease may manifest in the rectal stump (proctitis), or in the case of ileoanal anastomosis in the newly formed ileoanal pouch (‘pouchitis’) (Baumgart and Sandborn, 2007).

Complications of the disease include perforation of the colon. This may be preceded by severe inflammation of the entire colon with accompanying weakening and ballooning of the intestinal wall, in which case the dilated colon becomes at risk of rupturing. Similar to Crohn’s

disease, patients with UC, have an increased risk of developing colon cancer (Charles *et al*., 2001).

1.2.1.2: Crohn's Disease

Crohn's disease was coined after the description by Burrill B. Crohn, Leon Ginsberg and Gordon D. Oppenheimer in 1932 as a disease of the terminal ileum, affecting mainly young adults, characterized by a sub- acute or chronic necrotizing and cicatrizing inflammation (Crohn *et al*.,1932) . Although Crohn gave his name to the disease, it was already described in detail some 20 years before, by Dalziel T., as chronic interstitial enteritis (Dalziel, 1913).

When patients with this disease were first reported to their physician, most of them suffered from abdominal pain, diarrhea, weight loss, and fatigue. Disease can manifest anywhere in the GIT, with predilection sites in the terminal ileum of the colon and the per-anal region (Charles *et al*., 2001).

In children, the incidence of Crohn's disease appears to have risen above that of UC. Crohn's disease and UC can follow an active and remitting course and their response to therapies can be highly variable (Levine *et al*., 2007).

Crohn's disease encompasses a multisystem group of disorders with specific clinical and pathological features characterized by focal, asymmetric, transmural, and, occasionally, granulomatous inflammation primarily affecting the GIT (Lakatos, 2006).

This multisystem disorder with potential for systemic and extra intestinal complications can affect any age group, but the onset is most common in the second and third decades (teenagers and young

adults).The incidence and prevalence of Crohn's disease in the United States are rising for reasons that are unclear. The incidence and prevalence of Crohn's disease in the United States are similar to other "Westernized" countries, and estimated to be 5/100,000 and 50 / 100,000, respectively (Sands, 2004).

Endoscopically the tissue appears swollen, with a narrowed lumen of the bowel. Histologically, the inflammation extends throughout the entire bowel wall, affecting the mucosa and the underlying muscular and serosal layers of the intestine (Loftus, 2004).

The inflammation is characterized most typically by non caseating granulomas, and so called 'skip lesions' in which affected parts of the intestine are segmented by healthy patches, which may result in a cobble stone aspect of the mucosa. In a subgroup of patients, the upper gastrointestinal tract is affected. The typical non-caseating granulomas can be found in any area of the upper GIT, from the esophagus to the duodenal bulb. Some patients even have disease manifestations in the oral cavity, and may present with a swollen upper lip or mucocutaneous lesions. In addition to the gastrointestinal symptoms, there can be extra intestinal manifestations, including inflammation of the joints, eyes, skin, and liver (Signe *et al*., 2010).

So far, there is no curative treatment for Crohn's disease. Conventional treatment of the disease involves anti-inflammatory and immunosuppressive medication including aminosalicates and corticosteroids, sometimes combined with antibiotics (Ahn *et al*., 2006).

More severely affected patients are also being treated with immunomodulatory agents including azathioprine/6-mercaptopurine, and methotrexate. More recently, cytokine modulating agents, in particular

anti-tumor necrosis factor (TNF) (Sandborn *et al*., 2005). Patients with longstanding CD have increased risk for carcinomas in the affected areas (Charles *et al.*, 2001). The concept of a defect in the intestinal barrier as a key defect in the pathogenesis of Crohn's disease has emerged (Schreiber *et al*., 2005), table (1.1).

Table(1.1):**The differentiation between the ulcerative colitis and Crohn's diseases (Loftus, *et al*., 2007).**

	Ulcerative Colitis	**Crohn's Disease**
Anatomic location	Colon	Entire GI T (ileum/colon), perianal disease
Distribution	Continuous from rectum proximally	Skip areas, patchy
Granulomas	Absent	May be present
Inflammation	Mucosal	Transmural
Fistula, Abscess, Stricture	Absent	May be present
Serologies	pANCA	ASCA, OmpC, CBir1
Smoking	May be protective	Increases risk, worsens disease
Appendectomy	May be protective	May increase risk
Clinical presentation	Small volume bloody diarrhea, tenesmus, urgency	Variable: abdominal pain, diarrhea, weight loss, fever
Surgery	Curative (total proctocolectomy)	Not curative
Monozygotic Twin concordance	15-20%	20-50%

1.2.1.3: Indeterminate colitis (IC)

In approximately ten to fifteen percent of patients with colitis, no definitive diagnosis of either ulcerative colitis or Crohn's disease can be made by colonoscopy or histological examination. This type of IBD is called indeterminate colitis(IC), after introduction of the term (Price, 1978).

Most cases of indeterminate colitis are characterized by fulminate colitis, a condition in which the classic features of ulcerative colitis or Crohn's disease may be obscured by severe ulceration with early superficial fissuring ulceration, transmutable lymphoid aggregates, and relative rectal sparing, Currently, it is not clear whether these patients represent a distinct entity within IBD. The ability to discern if IC is in fact a predecessor for Crohn's disease or UC is specifically important in patients that need to undergo surgery in which a pouch needs to be formed, since the risk of developing pouchitis is three to four times higher in patients that prove to have CD (Odze, 2003).

The trend to use IC for patients who seem to have IBD but who cannot be readily called UC or Crohn's disease is even more marked in children than in adults, and further complicated by the fact that upper GI (gastric or duodenal disease or both but lacking granulomas on biopsy) can be seen in patients in whom UC seems to be the most likely diagnosis clinically. However, the upper GI pathology tends to resolve, being less common in adults (6%–12%) compared with children (20%–75%) (Parente *et al*., 2000; Castellaneta *et al*., 2004).

1.3 Predisposing factor of IBD

The exact etiology of IBD remains to be elucidated. So far, general consensus has come to the recognition that IBD is an ongoing and unwanted perpetuating activation of the immune system directed towards the normally non-pathogenic intestinal flora in a genetically susceptible host. The exact mechanism, by which these factors come together and result in IBD however, largely remains to be determined. Notwithstanding intensive IBD research, the etiology of this condition remains unknown; however, it is thought to result from a combination of genetic predisposition and environmental factors that may be channeled through an abnormality in gut-barrier function (Blumberg and Strober, 2001).

The pathogenesis of IBD seems to be multifactorial that include genetic and microbial predisposition triggered by environmental and behavioral factors leading to disruption of gastrointestinal mucosal barrier, then luminal antigen-mediated activation of mucosal immune system occurred, ending in tissue damage and clinical manifestations of IBD (Martins and Peppercorn, 2004).

1.4 Etiology

The etiology of IBD is not yet known but is likely to be multifactorial. The pathogenesis of the disease is largely determined by environmental and immunological factors on a genetically predisposed host (Bing *et al*., 1998).

1.4.1: Environmental and exogenous factors

The increasing incidences of IBD in western countries and in non-Caucasian populations in developing countries (Desai and Gupte, 2005) suggest that environmental factors play an important role and are related to modern way of living with alterations of the diet, improved sanitation, fewer infectious diseases, and perhaps an increased stress level (Vind *et al*., 2005).

Virtually each environmental factor one can think of has been implied as an etiological factor for IBD. These include climate, dietary factors, (personal) hygiene and life-style habits like smoking. The diet has been studied as an obvious environmental factor in the etiology of IBD. However, although some IBD patients find that they do not tolerate certain food-substances, and benefit from excluding these substances from their diet, there are no convincing data that any food substances would be causative for IBD, or that certain diets would influence the cause of disease. One exception to this thesis may however be a high fat intake (Reif *et al*., 1997).

Another environmental factor that, in epidemiological studies, has been implicated as a risk factor for IBD, in particular Crohn's disease, is the use of oral contraceptives. The use of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown in some studies to aggravate the disease activity of IBD (Danese *et al*., 2004). On the contrary, the risk for developing UC is approximately 2.2 times lower in smokers as compared to non-smokers, and smoking has been associated with a positive effect on disease severity (Birrenbach and Bocker, 2004).

1.4.1.1: Microbiological factors

When thinking of the pathogenesis of IBD, one has to keep in mind that the gut lumen belongs to the milieu exterior, which is in direct contact with the outside world. In fact, it is a highly specialized environment, that houses approximately 100 trillion (10^{14}) bacteria, 10 times more than the number of cells that make up the human body. Several of these bacteria are necessary for the breakdown of certain food substances that cannot be processed by the human digestive system, as well as for the production of certain essential amino acids and vitamins. In addition, the presence of non-pathogenic bacteria prohibits colonization with pathogenic bacteria to which the GIT is frequently exposed. In this respect microbiological entities are obvious candidates as causative agents in any inflammatory disease like IBD (Guarner and Malagelada, 2003).

1.4.2: Infectious agents

Several micro-organisms, including *Listeria monocytogenes*, *Escherichia coli*, Cytomegalovirus, and *Saccharomyces cerevisiae*, have been proposed in the etiology of IBD (Kishore *et al*., 2004). This concept of a pathogenic microorganism causing IBD is further correlated by the demonstration of the ability of E. *coli* to colonize and cause inflammation (Liu *et al*., 1995) and higher prevalence of adhesive E. *coli* strains in Crohn's disease patients (Darfeuille-Michaud *et al*., 2004).

1.4.3: Host factors

The immune system has a pre-set manner of responding to many different stimuli. The strength and type of this response is not only dependent on the type of stimulus, but is at least in part also determined by the genetic make-up of the host (Annes,*et al*.,2006).

1.4.3.1: Mucosal Immunology

The most striking property of the intestinal immune system is its ability to discern between the myriad of antigens present in the gut lumen. Against the potentially dangerous antigens from pathogenic organisms a sound and strong immune response is mounted, whereas under normal conditions, there is no response against harmless food antigens or the commensal flora. This process has been found to involve several different mechanisms, including energy and deletion as well as active suppression through specialized T-cells, known as regulatory cells (Kraus and Mayer, 2005).

As in all immune responses the mucosal immune response in the GIT consists of two main pillars, the innate and adaptive immune response. The innate immune system is comprised of cells and mechanisms that defend the host from infection by other organisms in a non-specific manner (Medzhitov and Janeway, 2000).

The adaptive immune system can grossly be divided in Th1 type responses on the one hand, and Th2 type responses on the other. Immunologically, Crohn's disease is found to be a Th1mediated disease, in which the inflamed area extends throughout the entire bowel wall, including the muscular and serosa layers of the intestine. Typical for Th1 type responses is the high expression of IL-12 by antigen presenting cells (APCs) after antigen recognition, and indeed, high levels of IL-12 are produced by macrophages of Crohn's disease patients (Monteleone *et al*., 1997).

Clearly, there is an important role for Th1 and Th2 cytokines in CD and UC, respectively. Cytokines are being recognized as promising therapeutic targets. The best-known example of cytokine modulating

therapy in IBD is directed towards TNF- γ. This typical pro-inflammatory cytokine is present in large amounts in Crohn's disease patients, and is also found in elevated levels in UC patients (Locksley *et al*., 2001).

1.4.4: Inheritance and IBD

There are multiple lines of evidence that suggest a genetic contribution to the pathogenesis of IBD. These include epidemiologic data on racial and ethnic differences in disease prevalence, familial aggregation and twin studies as well as the association of IBD with recognized genetic syndromes, including psoriasis, eczema, and ankylosing spondylitis. The most striking support for the contribution of both genetics and environmental factors in the pathogenesis of IBD is provided by twin concordance studies, especially for Crohn's disease. However, in IBD the concordance rates for monozygotic twins are significantly less than 100%, (42% to 58% for Crohn's disease, and 6% to 17% for UC). From these studies it has become evident that IBD is not inherited in a purely Mendelian fashion, but rather has a complex genetic etiology, with an intricate interaction with environmental factors (Vermeire and Rutgeerts, 2005; Bonen and Cho, 2003).

Studies on comparing the prevalence of IBD among different ethnic groups suggest genetic tendencies. IBD is seen two to four times greater in the Jewish population as compared with other ethnic groups. Ashkenazi Jews have the greatest risk within the Jewish population. Other epidemiologic studies have shown higher rates in whites, lower rates in African Americans, and the lowest rates in Asians (Ahmad *et al.*, 2001). The increased familial risk is similar to other diseases including type 1 and type 2diabetes, schizophrenia, and celiac disease; the genetic risk ratio is higher for Crohn's disease than UC (Halme *et al*., 2006).

1.4.4.1: Genetic aspects

Many genes have been implicated in the etiology of IBD, all with different levels of significance. In 2001, the groups of Hugot *et al*., (2001) and Ogura *et al*., (2001) described 3 different mutations in the Nucleotide-binding Oligomerization Domain *(NOD2)* gene that are associated with a highly increased susceptibility to Crohn's disease. This gene maps to the locus on chromosome 16 previously identified as a susceptibility locus for IBD in a genome-wide scan. Individuals homozygous for these mutations, either as the consequence of the same mutation on each chromosome, or because of the combination of 2 different mutated alleles (so-called compound heterozygosity) leads to a 20-40 fold higher chance of developing Crohn's disease, whereas the risk of developing the disease was found to be approximately four times higher in individuals that are heterozygous (Bonen and Cho, 2003).

1.4.5: Immunopathogenesis of IBD

The pathogenesis of IBD seems to be multifactorial that include genetic and microbial predisposition triggered by environmental and behavioral factors leading to disruption of gastrointestinal mucosal barrier, then luminal antigen-mediated activation of mucosal immune system occurred, ending in tissue damage and clinical manifestations of IBD (Martins and Peppercorn, 2004). A schematic diagram shows the pathogenesis of Crohn's disease and UC shown in figure (1.1).

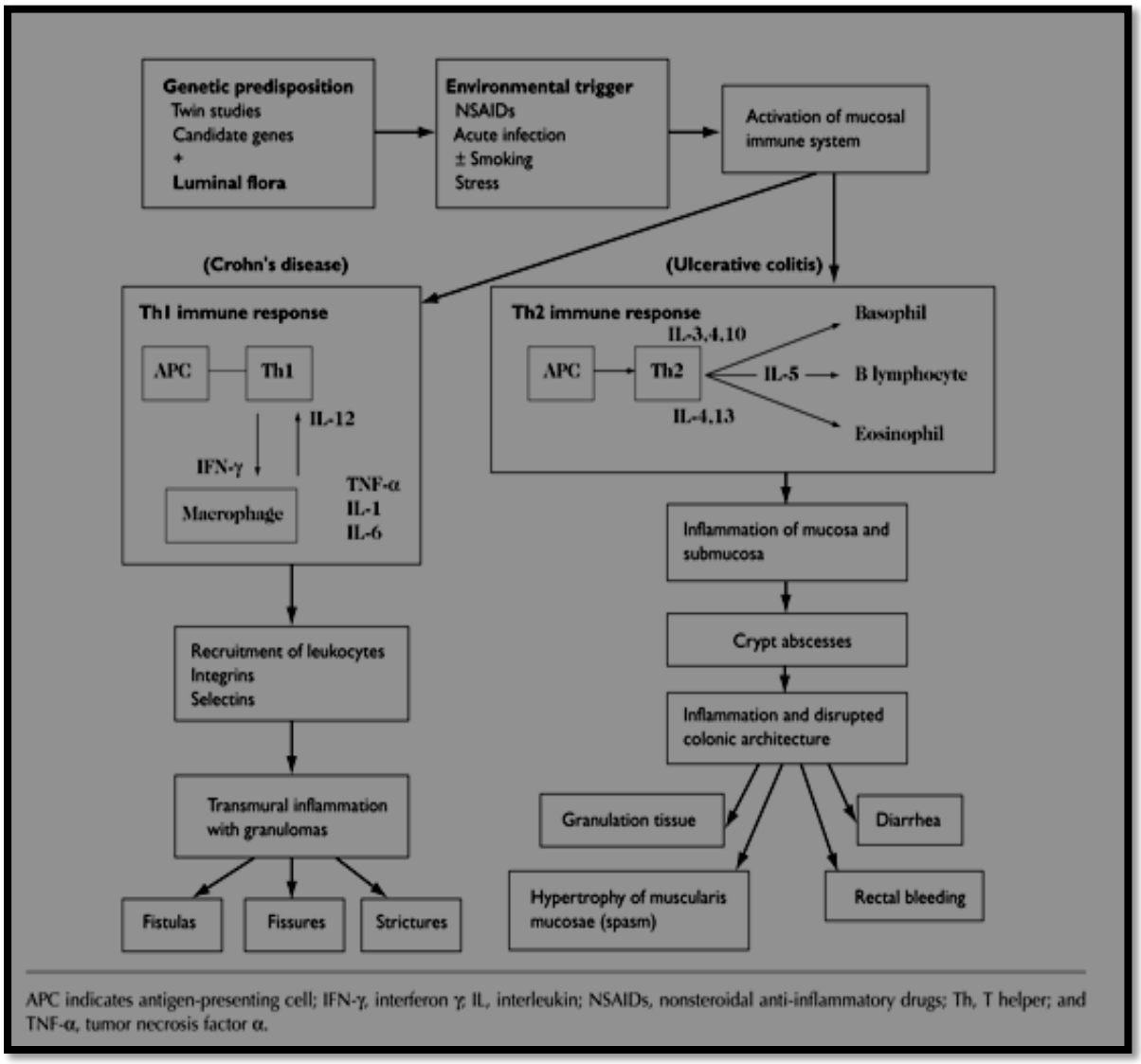


Figure (1.1): A schematic diagram shows the pathogenesis of CD and UC (Al-Hilaly, 2006).

1.5: Diagnosis and Lab. Findings of IBD

Because of the mimicry between IBD manifestations and many other enteric pathogens infections include: *Salmonella*, *Shigella*, *Campylobacter*, *Aeromonas, Plesiomonas, Yersinia, Eschericha coli* O157:H7, *Clostridium difficile*, *Giardia lamblia, Histoplasma spp*, *Mycobacterium tuberculosis*, and *Entamoeba histolytica*, these pathogens should be excluded before confirming the diagnosis of IBD. The diagnosis of IBD is confirmed by clinical evaluation and a combination

of biochemical, endoscopic, radiological, histological, or nuclear medicine based investigations (Sands, 2004).

In the case of UC the diagnosis should be made on the basis of clinical suspicion supported by appropriate macroscopic findings on sigmoidoscopy or colonoscopy, typical histological findings on biopsy, and negative stool examinations for infectious agents. For Crohn's disease the diagnosis depends on demonstrating focal, asymmetric, and often granulomatous inflammation but the investigations selected vary according to the presenting manifestations, physical findings, and complications (Sands, 2004).

Regarding the diagnosis of IBD, two factors should be considered: first, distinguishing IBD patients from alternative inflammatory disease patients and secondly, correctly differentiating between UC and Crohn's disease. Before establishing a diagnosis of IBD, other various forms of intestinal inflammation with identifiable causes should be excluded using relevant history, careful physical examination, prudent laboratory tests, and thorough review of the radiographic, endoscopic and pathologic data (Sands, 2004). There are several diagnostic criteria available for IBD including Mendel off's criteria (Calkins *et al*., 1984) and Lennard-Jones criteria (Lennard-Jones, 1989). The tests aids in the diagnosis of IBD are listed in table (1-2).

There is no single gold standard for the diagnosis of IBD. All diagnostic criteria use an integrating assessment of the clinical presentation, endoscopic, radiographic, and pathologic findings for diagnosis. More recently, serologic assays, utilizing antineutrophil cytoplasmic antibody (ANCA) and anti-Saccharomyces cerevisiae

antibody (ASCA) have been added as diagnostic tools with an adjunctive role in differentiating UC and Crohn's disease (Vermeire *et al.*, 2001).

Table (1.2): Tests used to diagnose IBD (Hendrickson, *et al*., 2002).

Test	Finding
Complete blood counts	Microcytic anemia, leukocytosis with band forms, thrombocytosis
Acute-phase reactants	Elevated sedimentation rate and serum orosomucoid, and C-reactive protein levels
Chemistries	Low serum iron level, hypoalbuminemia, elevated liver enzyme levels
Special serologic tests	pANCA, ASCA
Stool examinations	Exclude bacterial pathogens, ova and parasites, occult blood, and fecal leukocytes
Endoscopic evaluation	Esophagogastroduodenoscopy with biopsy, colonoscopy with biopsy
Radiologic evaluation	Upper gastrointestinal tract with small bowel follow-through, enteroclysis (as indicated), barium enema

The diagnosis of IBD in children is often delayed because of atypical intestinal and extraintestinal symptoms at the time of presentation (Seidman, 2000). There is currently no standardized method for evaluation of a child with suspected IBD (Zholudev *et al*., 2004; Zholudev *et al*., 2006). Some studies have shown that routine laboratory tests (erythrocyte sedimentation rate [ESR], platelet count, and hemoglobin) used in evaluation for IBD have varying sensitivity and specificity (Canani *et al*., 2004; Cabrera-Abreu *et al*., 2004). These investigations are useful markers of inflammation, anemia, nutritional deficiency, or intestinal damage.

1.6 Helminthes:

1.6.1 *Strongyloides stercoralis* :

Strongyloides stercoralis is a soil-dwelling nematode which alternates between the free-living and the parasitic stage, and affects an estimated 100-200 million individuals worldwide (Tefé-Silva*et al*., 2012).

Warm moist temperatures, lower socioeconomic status and poor sanitation leading to fecal contamination of soil have contributed to the increased prevalence of strongyloidiasis in the tropics (Keiser and Nutman, 2004).

Several risk factors have been associated with strongyloidiasis, including immunosuppressive therapy for rheumatic disease, malignancy (especially lymphomas), transplants, human immunodeficiency virus (HIV) infection, HTLV-1 infection, diabetes, hypochlorhydria, alcoholism, tuberculosis, impaired bowel motility, malnourishment, chronic obstructive pulmonary disease, leprosy and chronic renal failure (Carvalho and Da Fonseca Porto 2004; Hachim *et al*.,2005andMarcos *et al.*,2008).

The overwhelming autoinfective cycle can lead to a life-threatening illness due to hyperinfection or disseminated syndrome, with large numbers of larvae affecting the gastrointestinal tract, peritoneum and lungs. Sepsis, another complication of strongyloidiasis, occurs when enteric bacteria gain access into the extraintestinal sites, either by carriage on the surface of the filariform larvae or by entry through mucosal ulcers induced by the larvae.

It is a fatal complication reported to occur in 12-60% of the cases (Fardet*et al*., 2007). Blood cultures from these patients have grown

Escherichia coli, Klebsiellapneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Enterococcus faecalis, Streptococcus epidermidis, Streptococcus bovis and *Streptococcus pneumonia* (Marcos *et al.*, 2008)

Strongyloides stercoralis can be distinguished from other worms in one feature which is its capability of replicating within the host. Rhabditiform larvae in the bowel lumen turn into filariform larvae before secretion and invade the intestinal wall or the perianal skin, letting ongoing cycles of autoinfection which is an important feature of strongyloidiasis(Concha*etal*.,2005).

1.6.2 Clinical sign and pathogenicity of strongyloidiasis

Most infected people may keep symptomless but some other patients might have clinical symptoms like blood in the stool (occult blood), loss of appetite, nausea,alternate bouts of diarrhoea and constipation, abdominal pain, epigastric pain, post prandial fullness of stomach, bloating and some complaining heartburns(Genta, 1992; Siddiqui*et al*., 2000).

Parasites presence in lungs can induce dry cough and frequent sore throat. Parasite motion under the skin has called as maggot currents that causes erythematous raised skin rashes (utricaria, serpiginous maculo popular rash), it is usually involving on the legs, thighs, dough regions and the buttocks (Montes*etal*.,2008).

Chronic persistent infection is caused by auto-infection and among immunocompromise individuals, *S.stercoralis* can be the reason of hyper infection syndrome and distributed strongyloidiasis (Arsic*et al*., 2005).

It has also been observed that patients who suffer from chronic strongyloidiasis may appear with arthritis signs and symptoms related to

chronic malabsorption, symptoms of asthma, nephritic syndrome, duodenal obstruction and cardiac arrhythmias. It was noticed that using mild to moderate eosinophilia in order to be the most common laboratory hematological test among the patients who were infected (Armignacco*et al*., 1989; Al Maslamani*et al*., 2009).

Through clinical symptoms that involve obstruction ,vomiting, ileus, intestinal ulceration, bowel edema, appendicitis and hemorrhage, peritonitis, secondary bacterial sepsis and nephritic syndrome can diagnose hyper infection syndrome (Csermely*et al*.,2006).It can be seen that respiratory tract symptoms related with hyper infection for instance; wheezing, dyspnea, Pneumonitis, hemoptysis, acute respiratory distress syndrome (ARDS) and respiratory failure (Newberry *et al*,2005; Copelovitch *et al.*, 2010).It has been noted pulmonary hemorrhage secondary to disseminated *S.stercoralis* infection in some patients with systemic lupus erythematous, this refers to the co-morbidities significance in the human strongyloidiasis management (Plata *et al*., 2015).

Clinical presentation that includes aseptic / bacterial meningitis can be shown when larval migration occurs in the brain and also chronic and persistent diarrhea, cachexia, anorexia and failure to thrive might be demonstrated in children with malnutrition infection with *S.stercoralis* . It has importantly noticed hyper infection and serious consequences (severe morbidity and mortality) when there has an initiation of immunosuppressive therapy in clinically asymptomatic carriers (Reddy and Swarnalata, 2005).

It has recently detected in a study on the CD4+ T cell subsets activities including the Th1, Th2 and Th17 during chronic infection with

S.stercoralis that it was an increasing in the frequency of non-functional and dual functional Th2 cells against the specific *S.stercoralis*antigen.It has also confirmed that upon treatment initiation it was found decreasing frequency of Th2 cells (Weatherhead and Mejia, 2014; Anuradha*et al*., 2015).

Many researchers have focused on the screening importance for chronic Strongyloides infection as it is the one that can be responsible for complications arising from other associated infections. It was reported that a pediatric patient from Dominican Republic who suffered from recurrent pruritic rash, eosinophilia that occurred with acute onset bacterial meningitis. Later on, it was found during the investigation the suffering was from chronic strongyloidiasis. It should be taken in to consideration that this case is the best example explaining the fact that, chronic infection with *S.stercoralis* could be a predisposing factor for the development of hyper infection syndrome under immunocompromised conditions/glucocorticoid therapy/ immunosuppressive treatment (Pukkila*et al*., 2014).

The infection with human strongyloidiasis among patients who suffer from ulcerative colitis, strongyloidiasis associated with malignant pleural effusion, disseminated strongyloidiasis has been studied in a diabetic patient, respiratory hyper infection in a patient with kidney failure. Acute respiratory distress syndrome (ARDS) recognized to infection with *S.stercoralis* infection in a patient with non-Hodgkin's lymphoma and disseminated strongyloidiasis in acquired immunodeficiency syndrome (AIDS) patients, these are some clinical demonstrations in human strongyloidiasis which require many heavy studies and research (Ghoshal*et al*., 2006).

1.6.3 Diagnosis of *Strongyloides stercoralis*

The basis of Strongyloidiasis diagnosing is on suspicious patients with clinical marks and symptoms of the disease; however in some cases, it may be difficult to diagnosis though a low intestinal worm load and larval secretion in the feces (Rajapurkar*et al*., 2007).The classic triad of urticaria, abdominal pain and diarrhea is suggestive of a diagnosis of strongyloidiasis. Parasites are usually found in the feces and they are sometimes seen in other body fluids or in tissue samples (Basile*et al*., 2010). After feces test and some diagnostic methods that are used to detect *S. stercoralis* ,the parasitological diagnosis is commonly done, stool examination, a modified Baermann technique, and stool culture on a blood agar plate are also involved. Enzyme-linked immunosorbent assay (ELISA) are used for serological diagnosis and are worthy in detecting both symptomatic and asymptomatic strongyloidiasis infection, with a high qualitative for detecting IgG antibodies to *S. stercoralis* (Basile*et al*., 2010).The diagnosis is comparatively natural in the patients who suffer from expanded infection due to the high numbers of existed larvae in the stool and usually, in the sputum. WBC numbers may be high. Some studies have demonstrated that the eosinophilia absence cannot eliminate a diagnosis of strongyloidiasis in spite of the increasing in eosinophil during infection (Krishnan *et al.*, 2006).

It has been reported that there is a possibility to diagnosis through imaging as some chest radiographs on patients have displayed infiltrate consistent with Loffler's syndrome. Procedures, like bronchoalveolar lavage and sputum culture are applied to diagnose disseminated strongyloidiasis (Williams *et al.*, 1988, Yassin*et al*., 2010). When Strongyloides parasite is to be detected, it is required to duodenal fluid

aspiration and intestine biopsy or the use of Enterotest (Yassin*et al.*, 2010).

1.7 Enterobius *vermicularis* :

Enterobius vermicularis commonly referred to as the pinworm or seat worms, belonged to nematode worm, with the largest geographic range of any helminthes (Kucik *et al*.,2004). The oldest known pinworm ova have demonstrating that been found in human coprolites specimen (dried fecal specimens) ,the findings from Dakhlen oasis in Egypt and Danger Cave, Utah (Fry *et al.*, 1969).

(Ferreira *et. al*, 1997 and Hugot *et al*, 1999) established pinworms as an example of an inherited parasite, meaning it is a host-specific parasite that has a long history of co-evolution with ancient human ancestors dating back to Africa before human dispersion across the continents. The organism was first identified in 1758 by karlLinnaeus, who named it *Oxyuyris vermicularis*, It is normally confined to humans, although related species can infect other animals including chimpanzees, gibbons, and marmosets.

Epidemiology study of this parasite very important because this parasite has such broad scope both historically and geographically, that it would be difficult to find a time in human history when pinworm was not present, also this parasite is spread mainly at the community and household levels, transmission of it also occurs between regions (global transmission) and between communities (regional transmission),it is suspect the parasite is more common in areas that are more or less developed and participate in frequent trade with countries in temperate regions.(CDC,1993;CDC,1999).At the community level, pinworm infection occurs more frequently in school-aged children (ages 5-14) than in the average population (Song, Cho, Kim and Choi, 2003). Pinworm

infection is linked to age being most common in children of school age, followed by preschool children, adults are the last common age-group to experience Enterobiasis, with the exception of mothers whose children are infected(CDC,2006).

1.7.1 stages of infection and transmission

The infection by pinworm, occur when the eggs are the infective stage are accidentally ingested in a contamination environment (kang *et al* 2006) **.**

Humans is only host of pinworm, infections occur in one of four ways: (1) retro infection ,when hatched larvae migrate back into the large intestine: (2) self-infection ,when the patient is reinfected by hand-to-mouth transmission: (3)Cross-infection when infective eggs are ingested, either with contaminated food or from fingers that have been in contact with contaminated surface or body parts from infected humans; and (4) inhalation of airborne eggs (Burton *et al*,2005).Enterobiasis presents an interesting case at the household level, when one family member becomes infected, there is a high probability that all other family members will become infected(Juckett,1995).Because the high level of cross-infection is lead to an enormous number of eggs being produced in a short period of time and these egg may be remain viable for two to three weeks on clothes and bedding , facilitating easy spread among family members and groups of children (Goldman and Wilson;1997).Fecal-oral route and self-reinfection is common when the hand touches the perianal area and carries the infection to the mouth (Yoon *et al*., 2000).Inhaled eggs may not enter the lung ,due to their weight and size ,but may be deposited in the orapharynx ,where they easily swallowing ,where simply

breathing the air in infected children room may produce infection (Schmidt and Roberts,2000).

The vegetable such as lettuce (*lactuca sativa*) ,celery (*Apium graveolew*),leek (*Allium porrum*) and Garden cress(*Cardamineamara*) from vegetable that carry the pinworm to human(Rahma *et al*.,2008).finger nials play major role in transmission of *E.vermicularis* ,known that persons who infected by Entrobiasis may scratch the contaminated perianal skin and transfer the eggs on fingers and under fingernails to the mouth(Herrstrdm *et al*.,1997).

1.7.2 Pathogenesis

Human infection occur when the eggs in the infective stage are accidentally ingested in a contaminated environmental(kang *et al*.,2006). Most significant pathologic conditions are produced by gravid females that migrate to anus to the perianal skin to ovipositor, causing a crawling sensation and purities. This condition results in scratching the area, eosinophilia accompany this infection . *E.vermicularis* is one of the most common of all parasitic infection in the world , practically common in children . A gravid female migrates out of the anus at night and lays numerous sticky eggs on the anal skin. Sometimes female bursts and releases all the eggs on the skin (which member about 10,000) , and this cause intense itching (Ralph and John , 2002).

1.7.3 Laboratory diagnosis

A. Microscopic diagnosis

Microscopic examination is useful in parasitological diagnosis only when the recovered specimens are of good quality (Reinhard, 1990; Araujo and Ferreira, 2000). Diagnosis is dependent on accurate identification of adult worms or eggs, or both - which can be visualized in the perianal region for *E. vermicularis* (Chai *et al.*,1997). Individual eggs

are invisible to the naked eye, but they can be seen using a low-power microscope (Caldwell,1982).There are still laboratories in some countries where the prevalence of *E.vermicularis* is investigated directly from the stool of patients. concentration method used it not to detection ova ,but also to male and female of worm(Totkova *et al*,2003).

B. Cellophane -tape

The most popular methods of detecting the eggs are to use a piece of Scotch tape, which is then stuck to a slide and examined, the clear-cellulose-tape preparation is the most widely used procedure for the detection of human pinworm infection (Gillepie and Pearson ,2001).The first use of this procedure by Graham(1941),when Cut a10cm strip of cellophane, or similar, and press middle 3-5cm firmly against the right and left perianal folds**.**

This test is most successful if done every morning for several days, because the females do not lay eggs every day, and the number of eggs vary(Caldwell,1982) .the most efficient one is the cellophane tape technique, by which 99 % of positive cases can be detected.

1.8 *Ascaris lumbricoides*:

Ascariasis, a soil-transmitted infection, is the most common human helminthic infection. Current estimates indicate that more than 1.4 billion people are infected worldwide. In the United States, there are an estimated 4 million people infected, primarily in the southeastern states and among immigrants Kightlinger,(1998).The etiologic agent , *Ascaris lumbricoides,* an intestinal roundworm, is the largest nematode to infect humans. The adult worm lives in the small intestine and can grow to a length of more than 30 cm. The female worms are larger than the males. Important factors associated with an increased prevalence of disease include socio-economic status, defecation practices and cultural

differences relating to personal and food hygiene as well as housing and sewage systems. Most infections are subclinical; more severe complications occur in children who tend to suffer from the highest worm burdens (Bottomley,2006).

1.8.1 Clinical Manifestations

Although most individuals infected with *Ascaris lumbricoides* are essentially asymptomatic, the burden of symptomatic infection is relatively high as a result of the high prevalence of infection on a worldwide basis. Symptomatic disease is usually related to either the larval migration stage and manifests as pulmonary disease, or to the intestinal stage of the adult worm (Petney,1998).

The pulmonary manifestations of ascariasis occur during trans pulmonary migration of the organisms and are directly related to the concentration of larvae. Thus, symptoms are more pronounced with higher burdens of migratory worms. The trans pulmonary migration of helminthes larvae is responsible for the development of a transient eosinophilic pneumonitis characteristic of Loffler's syndrome with peripheral eosinophilia, eosinophilic infiltrates and elevated serum IgE concentrations. Symptoms usually develop 9-12 days after ingestion of the eggs, while the larvae reside in the lung(Despommier2005). Affected individuals often develop bronchospasm, dyspnea and wheezing. Fever, a persistent, nonproductive cough and, at times chest pain, can also occur. Hepatomegaly may also be present. In some areas of the world such as Saudi Arabia where transmission of infection is related to the time of the year, seasonal pneumonitis has been describe (Bethony, 2006).

A potential consequence of the intestinal phase of the infection relates to the effect it may have on the nutritional health of the host. Children heavily infected with *Ascaris* have been shown to exhibit

impaired digestion and absorption of proteins and steatorrhea (Brooker, S.*et al* 2004). Heavy infections have been associated with stunted growth and a reduction in cognitive function. However, the role of *Ascaris* in these deficiencies is not clearly defined. Some of these studies were done in developing countries where additional nutritional factors cannot be excluded. There is also a high incidence of co-infection with other parasites that can affect growth and nutritional status. Interestingly, a controlled study done in the southern United States failed to demonstrate significant differences in the nutritional status of *Ascaris* infected and uninfected individuals (Williams-Blangero,1999).

1.8.2 Hepatobiliary and Pancreatic Symptoms

Hepatobiliary symptoms have been reported in patients with Ascariasis and are due to the migration of adult worms into the biliary tree. Affected individuals can experience biliary colic, jaundice, ascending cholangitis, calculus cholecystitis and perforation of the bile duct.Pancreatitis may develop as a result of an obstruction of the pancreatic duct. Hepatic abscesses have also been reported (Martin, 1983).

Ninety-eight percent of the patients presented with abdominal pain, 16%developed ascending cholangitis, 4% developed pancreatitis and 1% developed obstructive jaundice. Both ultrasonography,as well as endoscopic retrograde cholangio pancreatography (ERCP) have been used as diagnostic tools for biliary or pancreatic ascariasis (deSilva, 2003).

1.8.3 Diagnosis

The diagnosis of ascariasis is made through microscopic examination of stool specimens. *Ascaris* eggs are easily recognized, although if very few eggs are present the diagnosis may be easily missed. Techniques for concentrating the stool specimen will increase the yield of diagnosis

through microscopy. Occasionally an adult worm is passed via rectum. Eosinophilia may be present, especially during the larval migration through the lungs. In very heavily infected individuals a plain X-ray of the abdomen may sometimes reveal a mass of worms (WHO 1992) .

1.9 Trichuriasis

Trichuris trichiura is an intestinal nematode affecting an estimated 795 million persons worldwide. Also known as whipworm due to its characteristic shape, *Trichuris* can be classified as a soil-transmitted helminthes because its life cycle mandates embryonic development of its eggs or larvae in the soil. It is the second most common nematode found in humans, behind *Ascaris* (Bethony,2006).

Trichuriasis is more common in areas with tropical weather such as Asia, Sub-Sarahan Africa and the Americas, particularly in impoverished regions of the Caribbean. It is also more common in poor rural communities and areas that lack proper sanitary facilities with easily contaminated food and water. A large number of individuals who are infected actually harbor fewer than 20 worms and are asymptomatic; those with a larger burden of infection (greater than 200worms) are most likely to develop clinical disease (Lustigman, 2012). School age children tend to be most heavily infected. There is no reservoir host for *Trichuris*. Transmission occurs when contaminated soil reaches the food, drink, or hands of a person and is subsequently ingested. Therefore, poor sanitary conditions is a major risk factor. It is noteworthy that patients are often co infected with other soil-transmitted helminthes like *Ascaris* and hookworm due to similar transmission modalities (Bundy,1989).

1.9.1 Disease Signs and Symptoms

Frequently, infection with *Trichuris* is asymptomatic or results only in peripheral eosinophilia. Clinical disease most oft en occurs in children, as it is this population that tends to be most heavily-infected and presents

as *Trichuris* colitis. In fact, this is the most common and major disease entity associated with infection. Acutely, some patients will develop *Trichuris* dysentery syndrome, characterized by abdominal pain and diarrhea with blood and mucus (Chan,1994).With severe dysentery, children develop weight loss and become emaciated. Anemia is common and results from both mucosal bleeding secondary to capillary damage and chronic inflammation. The anemia of trichuriasis is not as severe as that seen with hookworm. *Trichuris* infection of the rectum can lead to mucosal swelling. In that case, tenesmus is common and if prolonged can lead to rectal prolapse, especially in children. Adult worms can be seen on the prolapsed mucosa. Chronic trichuriasis oft en mimics inflammatory bowel disease. Physical symptoms include chronic malnutrition, short stature and finger clubbing. These symptoms are oft en alleviated with appropriate anthelminthic treatment. Rapid growth have been reported in children following deworming with an ant helminthic agent. Deficits in the cognitive and intellectual development of children have also been reported in association with trichuriasis (Bundy,1988).trichuriasis, like other nematode infections, has modest immunomodulatory effects (Quinnell,2003).

1.10 *NOD2/CARD15* gene

NOD2/CARD15 gene situated at chromosome 16q12 within the inflammatory bowel disease (IBD) region and their association to CD has been a major step forward in understanding the disease pathophysiology (Hugot *et al* 2001). *NOD2/CARD15* is known to act as an intracellular receptor in monocytes for bacterial components triggering activation of NF kappa-B and thus leading to subsequent activation of the inflammatory response. Within the *NOD2/CARD15* gene, there are three independent mutations recently found to be associated with CD: two

missense mutations (Arg702Trp and Gly908Arg) and one frame shift mutation (3020insC)Ahmed *et al* 2002). The 3020insC mutation results in a truncated protein leading to an altered stimulation of NF kappa-B after bacterial activation. Although these mutations fit very well into the present pathogenic hypothesis, they are only found in approximately 30% of CD patients. Therefore genotype-phenotype correlations were conducted to find out if *NOD2/CARD15* mutations are associated with a distinct clinical subtype of CD. Mutations within the *NOD2/ CARD15* gene and their correlation to clinical data have been performed by others with differing results. Associations were found to ileal involvement, fibrostenotic or fistulizing behavior and younger age at diagnosis,(Salkic *et al* 2015) whereas other investigators failed to notice a relationship to the subgroups of the Vienna classification. However, up to now, no clinically relevant role for the *NOD2/CARD15* mutations has been described. Thus, an accurate genotype/phenotype analysis is required to elucidate the role of mutations in the *NOD2/CARD15* gene and to characterize in more detail their clinical contribution to the course of CD (Ogura *et al* 2001). We therefore investigated the three common *NOD2/CARD15* mutations (Arg702Trp, Gly908Arg, 3020insC) in 180 patients with CD and related the results to the demographic and disease phenotype.

Studies investigating the frequency of reoperations in patients with mutations in the *NOD2/CARD15* gene are very limited.Recently (Henckaert *et al* 2009),described a patient homozygous for the 3020insC mutation who had three bowel resection in agreement with other observation. findings appear to be in contrast to the results reported by Ahmad *et al* 2002 who could not identify *NOD2/CARD15* mutations to be associated with a higher frequency of surgical reoperations. However,

they analyses ileal stenotic reoperations without subdividing into reoperations at the neoterminal site and other reoperations within the small bowel In contrast, in a large Japanese cohort of CD patients, none of the three *NOD2/ CARD15* mutations were found (Cheny *et al* 2013). This strengthens the importance of regional differences regarding the contribution of *NOD2/CARD15* mutations to CD.

1.10.1 Association of *NOD2/CARD15* With Crohn's Disease

In early 2001, two groups independently published back-to-back articles in Nature identifying the gene *NOD2* (also called *CARD15*) as the first susceptibility gene for Crohn disease (Hugot *et al* 2001).Three mutations within the leucine-rich repeat region were associated with CD, and these and other studies have demonstrated an approximately 2-fold risk for CD in *NOD2* heterozygotes and an approximately 20-fold risk for CD in *NOD2* homozygotes or complex heterozygotes(Lu *et al* 2012).

1.10.2 Possible Clinical Role of *NOD2/CARD15* in Crohn's Disease

The identification of *NOD2* raised the prospect of its potential role in clinical practice. Could *NOD2* be used as part of the diagnostic workup for suspected cases of Crohn's disease Unfortunately not, as the vast majority of people who carry *NOD2* mutations or are even homozygotes for *NOD2* mutations will not develop CD. This is true of all known genetic variants associated with inflammatory bowel disease and there is no role for genetic testing in CD at this point in time(Frank 2010).

Nevertheless, the question has been raised as to whether there might be some benefit to knowing *NOD2* status. *NOD2* mutations are associated with small bowel CD and, in some studies, with structuring disease. This anatomical association negates the use of *NOD2* in distinguishing CD from ulcerative colitis, as further diagnostic tests are not needed to

distinguish small bowel from colonic inflammation. Parameters that helped distinguish Crohn's colitis from ulcerative colitis would, however, be extremely useful on certain occasions, such as when patients were being considered for colectomy(Henckaert 2009).Specifically,CD patients who carry *NOD2* mutations are more likely to require small bowel surgery, and there is evidence of a gene-dose effect: *NOD2* homozygotes, on average, require surgery sooner than do heterozygotes, who, in turn, require surgery earlier than do wild-type homozygotes. However, this effect is not strong enough alone to influence clinical practice. It may be that a combination of genetic profiles is needed to have a sufficiently large effect, but further work is needed in additional cohorts(Lichtenstein 2011). One study has suggested an additive effect of *NOD2* variation and IBD-related serology and the development of more severe CD; however, this finding requires validation.

1.10.3 *NOD2/CARD15* gene in Ulcerative Colitis

Inflammatory bowel disease, comprising ulcerative colitis (UC) and Crohn's disease (CD), is a common, chronic and relapsing inflammatory disease affecting the gastrointestinal tract(Yang 2008). The etiology of the disease remains unknown, but is postulated to result from an aberrant mucosal immune response to as yet unidentified factors in genetically susceptible individuals (Fiocchi 1998). Epidemiological investigations have consistently shown familial clustering of disease and a higher concordance of inflammatory bowel disease in monozygotic when compared with dizygotic twins, supporting a genetic influence (Ozen *et al* 2006).Among the various putative inflammatory bowel disease-susceptible regions identified by genome-wide screening, *NOD2 /CARD15* gene on chromosome 16q11-12 (IBD1) has been found to be

consistently associated with inflammatory bowel disease, in particular CD (Hampe *et al* 2001).These associations with CD have been replicated variant across Caucasian populations, although both type of and the degree of association vary between populations (Hugot *et al* 2007). Some studies also suggest more modest associations between these variants and ulcerative colitis of particular interest are three coding single nucleotide polymorphisms (SNPs) within the gene, designated SNP8, SNP12, and SNP13, that are strongly and independently associated with CD in various populations(Guo *et al* 2004). SNP8 and SNP12 cause amino acid substitutions in the leucine-rich region (LRR), namely C14772T (Arg702Trp) and G25386C (Gly908Arg). SNP13 is a C-insertion 32629insC (1007insC), which leads to a frame shift that causes a truncated protein missing the final 33 amino acids (Inoue *et al* 2002). A multivariate analysis has shown that SNP13 is the main carrier of the genetic signal (Yamazaki *et al* 2002). Experimental studies have shown that these three disease-susceptibility SNPs [disease susceptibility variant (DSV)] alter bacterial recognition and cause inappropriate activation of the NF-[kappa] B system contributing to the chronic inflammation characteristic of the disease (Bonen *et al* 2003).The risk for CD is increased by two- to three fold among heterozygotes and by 10- to 44-fold among homozygotes ⁄ compound heterozygotes (Hugot 2001). It is known that the three DSVs, i.e. SNP8, SNP12 and SNP13 between themselves, represent 82% of the NOD2-mutated chromosomes,and that these polymorphisms account for about 18% of the genetic risk of CD in the Caucasian population(Hugot *et al* 2007).

1.10.4 The role of Cytokines in pathogenicity of IBD:

Cytokines are small proteins, signaling molecules, approximately (8-80kDa) that usually act in an autocrine or paracrine manner. They are extremely potent, acting at Picomolar and sometime even Femtomolar levels 1 picogram(10-12g), was able to protect one million cells from ten million virus particles in tissue culture experiment. Cytokines function as part of a larger inter-related system of proteins and signaling cascades, the cytokine network, direct cell-cell interaction and local production if soluble mediators, control communication between cells of the immune systems. Cell – cell interaction involves a number of different classes of molecules like Major Histocompatibility Complex(MHC), accessory molecules, integrins, co-stimulatory molecules and membrane forms of cytokines (Al-Obaidi, 2007).

IL-1β is primarily produced by activated macrophages as well several cells include the mononuclear endothelial, keratinocytes, astrocytes, synovial cells, glial cells, osteoblasts, neutrophils, and numerous other cells, there are variant agent mediate stimulation of IL-1β production, like endotoxins, microorganisms, antigens as well as other cytokines (Awasthi 2004).IL-1β is well known that cytokines are considered to play a key role in the inflammation process, it is important pro inflammatory cytokine that on the one hand mediate in secretion of nitric oxide as well as activate monocytes, macrophages, and neutrophils, and on the other hand induce Th1andTh17adaptive cellular responses (Kim and Amar, 2006, Netea *et al*, 2010).IL-10 is an anti-inflammatory cytokine that inhibits both antigen presentation and subsequent release of pro-inflammatory cytokines, thereby attenuating mucosal inflammation. The pivotal role played by IL-10 within the mucosal immune system has been extensively

studied in the chronic ileo-colitis that develops in gene-targeted IL-10 knockout mice and by its therapeutic efficacy in several animal models of colitis (Wirtz S, Neurath MF.2007). An inactivation of IL-10 in mice results in an increased production of IL-12 and IFN-γ (Rennick, Fort .2000). Inflamed tissues and granulomas of CD show low IL-10(Melgar *et al* 2003) reported a highly significant increase in IL-10 mRNA levels in T lymphocytes and in IL-10-positive cells in the colons of UC patients. Recently production of IL-10 by regulatory T cells has been implicated as important issue in IBD (Latinne, Fiasse 2006). Other regulatory cells that may participate in UC through the production of IL-10 are a regulatory B cells subtype called Bregs. The importance of IL-10 production by B cells has been evidenced in IBD models and in humans(Goetz ,*et al* 2006) (Mizoguchi *et al* 2006) showed that Bregs can be responsible for the suppression and/or recovery form acquired immune mediated inflammations by mechanisms that include IL-10 and TGF-β1 in IBD(Mizoguchi,Bhan2006). IL-10 is an immunoregulatory cytokine that strongly down regulates the production of proinflammatory cytokines and T helper 1 cytokines of particular interest are the data on IL-10 deficient mice by(Kuhn *et al* 1993),showing chronic noninfectious intestinal inflammation This finding corroborates the action of IL-10 against chronic stimulation of the intestinal immune system. It has also been demonstrated that an elevated production of proinflammatory cytokines by IBD mononuclear cells could be down regulated in vitro by external IL-10, and also in vivo with topical IL-10 enema preparations . This indicates that the intrinsic intestinal bioactivity of IL-10 may be insufficient to control intestinal inflammation. Therefore, recognition of this cytokine will provide a new dimension to understanding the pathophysiology of IBD (Schreiber1995).

2- Materials and methods.

2.1 Materials.

2.1.1 Equipment and Instruments

Table (2-1): Equipment and Instruments used and their company:

No.	Equipment & instrument	Company
1	Digital camera	Samsung/ china
2	Disposable syringe 10 ml, 5ml and 3ml	Sterile EO. / China
3	Eppendorf tubes	Bioneer/ Korea
4	Exispin centrifuge	Bioneer/ Korea
5	Gel electrophoresis	Shandod Scientific/ UK
6	High Speed Cold centrifuge	/Germany
7	Incubator	Mammert/Germany
8	Micropipettes 5-50, 0.5-10, 100-1000µl	CYAN/ Belgium
9	Microscope	BioRad USA
10	Refrigerator	Concord /Lebanon
11	Sensitive Balance	Sartorius/Germany
12	Sterile test tube	Superestar/ India
13	Thermocycler PCR	BioRad/ USA
14	UV Transilluminator	ATTA/ Korea
15	Vortex	CYAN/ Belgium
16	Water Bath	Mammert/Germany

2.1.- Kits

Table (2-2): The kits used in this study with their companies and countries of origin:

No.	Kit	Company	Country
1	**Stool DNA extraction Kit**	Bioneer	Korea
	Proteinase K		
	Stool Lysis buffer (SL)		
	Binding buffer (ST) Washing buffer 1 (W1) Washing buffer 2 (W2) Elution buffer (E)		
	GD column		
2	**gSYAN Blood DNA Extraction Kit**	**Geneaid**	**USA**
	GST buffer		
	GSB buffer W1 buffer Wash buffer Elution buffer		
	GD column Collection tube 2ml Proteinase K 11mg		
3	**Maxime PCR PreMix**	**INtRON**	**Korea**
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP,		

	dTTP)		
	Tris-HCl pH 9.0, KCl, $MgCl_2$		
	Stabilizer and Tracking dye		

1. Human IL-1β (Interleukin 1 Beta) ELISA Kit.

Human IL-1β (Interleukin 1 Beta) ELISA Kit was used in this study for quantitative determination of IL-1β concentrations in serum of human blood samples and done according to company instruction as following:

A. ELISA kit components:

Reagent	**Quantity**
Micro ELISA Plate	8 wells ×12 strips
Reference Standard	2 vials
Reference Standard & Sample Diluent	1vial 20mL
Concentrated Biotinylated Detection Ab	1vial 120µL
Biotinylated Detection Ab Diluent	1vial 10mL
Concentrated HRP Conjugate	1vial 120µL
HRP Conjugate Diluent	1vial 10mL
Concentrated Wash Buffer (25×)	1vial 30mL
Substrate Reagent	1vial 10mL
Stop Solution	1vial 10mL

1. Human IL-10 (Interleukin 10) ELISA Kit.

Human IL-10 (Interleukin 1 0) ELISA Kit was used in this study for quantitative determination of IL-10 concentrations in serum of human blood samples and done according to company instruction as following:

2.1.3 Primers

The PCR primers that used for direct detection of some intestinal infection helminths were designed in this using NCBI-Genbank Sequence database and primer 3 plus. Wherase, PCR RFLP primers for identification of NOD2 gene polymorphism primers were designed by (Zelinkova *et al.*2017). All these primers were provided from Macrogen Company, Korea as following tables:

Table (2-3): **Helminthes PCR primers:**

Helminthes	Sequence (5'-3')		Product Size
Ascaris lumbricoides	F	GCCGTTGTTGCACTTAACGT	520bp
	R	GCGTTTCAAACTTGACGCCT	
Enterobius vermicularis	F	ACAAACGGGGGCATTCGTAT	407bp
	R	GCACCACCAACCACCAAATC	
Trichuris trichiura	F	CAGTCACCGGTACCTGTTCC	361bp
	R	CAAGTGATCCACCGTTCGGA	
Strongyloides stercoralis	F	AACGGCTACCACATCCAAGG	692 bp
	R	TTGCAACCATACTTCCCCCG	

Table (2-4): ***NOD2* gene polymorphism primers**

Gene	Sequence (5'-3')		Product Size
NOD2 gene R702W	F	TTTGGAGGAAAAGTGGAAGA	170bp
	R	AACATTCCATACATCCTGGC	

2.1.4 Restriction enzyme

Table (2-5): The restriction enzymes were used in PCR RFLP assay with their company and country of origin:

Restriction enzymes	SNP	Polymorphism	Company/Country
MspI	R702W	C/T	New England Biolabs. UK

2-1-5- Chemicals

Table (2-6): All the chemicals materials that used in this stud with their company and country of origin:

No.	Chemical	Company and Origin
1	Absolute Ethanol	Scharlau (Spain)
2	Agarose	BioBasic (Canada)
3	TBE buffer 10X	BioBasic (Canada)
4	Ethidium Bromide	BioBasic (Canada)

5	Ladder 100bp	Bioneer (Korea)
6	Free nuclease water	Bioneer (Korea)

2-2- Methods

2-2-1- Samples collection

After disinfectant the area with alcohol (70%),blood sample (5ml) was collected from cubital fossa vein from IBD patients and control groups.

Collected sample was transferred immediately in to two tubes as follows:

A. Two milliliter of blood in 5 ml tube (EDTA tube) used for PCR technique to detect *NOD2/CARD15* gene polymorphism

B. Three milliliter of blood in a gel tube (serum tube),then the blood samples were centrifuged at (4700 rpm for 5 min) to obtain serum then frozen at -20 C for screening of IL1B,IL10 cytokines levels .

2.2.2 Study Groups:

Two groups were included in this study;

A- Patients Group : A total of fifty patients from Al-Diwaniyah province (males38 and females12) with inflammatory bowel disease; 31 patients with Ulcerative Colitis and 19 with Crohn's Diseases patients, who have been diagnosed by specialist physicians in Al-Diwaniyah Teaching Hospital for Gastrointestinal Tract and Hepatic diseases unit, depending on clinical features, biopsy for histopathology, and endoscopy. All were regularly attending the consultant clinic for treatment and follow-up during the period from January 2018 to July 2018.

B- Control Group; A total number of fifty individuals, who were apparently healthy (males 33 and females 17) were involved as a control group. They matched the patients group regarding sex, and age and had no history of / or clinical features of IBD, no obvious abnormalities, none of them had an acute or chronic diseases.

2.2.3: Study Protocol and Sampling : Members of the two groups were subjected to the questionnaire form (Appendix I); all were subjected to the following assays;

1- GSE.

Stool examination for parasites

1. Saline wet mount: It is used to detect worms, bile stained eggs, larvae, protozoan trophozoites and cysts. In addition, it can reveal the presence of RBCs and WBCs.

2. Iodine wet mount: It is used to stain the glycogen and nuclei of the cysts. A cyst is appreciated better in an iodine preparation, but the motility of the trophozoite is inhibited in the iodine preparation.

Procedure

• Place a drop of saline on the left half of the slide and one drop of iodine on the right half.

• With an applicator stick, pick up a small portion of the specimen (equivalent to the size of a match head) and mix it with a saline drop.

• Similarly, pick up a similar amount and mix with a drop of iodine.

• Put the cover slip separately on both and examine under the microscope.

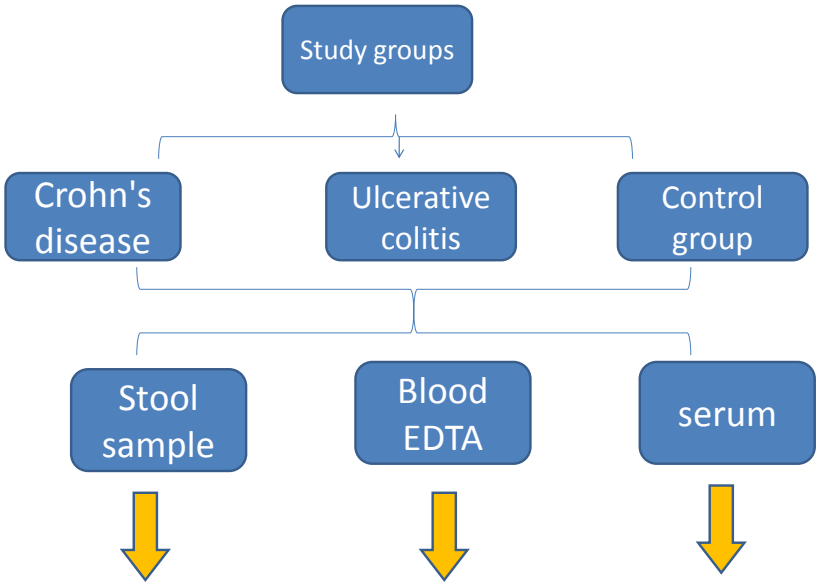
• The ova, cysts, trophozoites and adult worms can be identified as per their characteristic features.

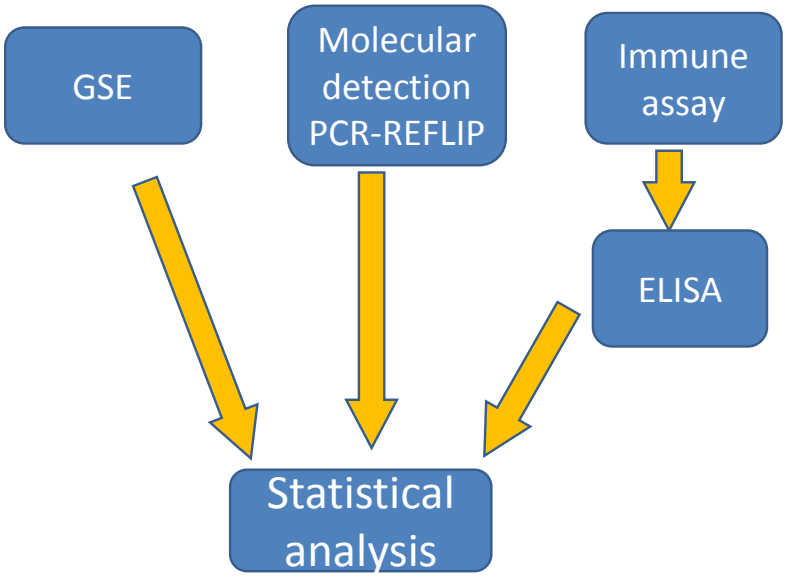
2-4 Screening the bellow (study design):

. EDTA tube for blood extraction and then PCR- RFLP.

.Gel tube for investigate IL1B, IL10.

.Tube for stool extraction and PCR.





Figures (2.1) flowchart illustrate study design

B. Assay procedure:

Before beginning the assay, all kit reagents and samples were bring at room temperature.

1. **Add Sample:** 100µL of Standard,or Sample were added per micro ELISA plate well. The blank well is added with Reference Standard and Sample diluent. After that solutions mixed gently and cover the plate with sealer, and then incubated for 90 minutes at 37℃.then washing.

2. **Biotinylated Detection Ab:** The liquid of each well were removed, and immediately 100µL Biotinylated Detection Ab working solution was added to each well and covered with the plate sealer and then incubated for 1 hour at 37°C.

3. **Wash:** All plate wells were aspirated and washed, and repeated the process three times. The wash done by filling each well with Wash Buffer (approximately 350µL) using a squirt bottle.

4. **HRP Conjugate:** 100µL of HRP Conjugate working solution was added to each well and covered with the plate sealer. And then incubated for 30 minutes at 37°C.

5. **Wash:** The wash process was repeated for five times as conducted in step 3.

6. **Substrate:** 90µL of substrate Solution was added to each well and covered with a new Plate sealer, then incubated for about 15 minutes at 37°C.

7. **Stop**: 50µLof Stop Solution was added to each well. Then, the color turns to yellow immediately.

8. **OD Measurement**: for determine the optical density (OD value) of each well at once, used a micro-plate reader set at 450 nm.

C. Calculation of results:

The ELISA results were calculate depend on the average of the duplicate readings for each standard and samples optical density. Then create a standard curve by plotting the mean OD value for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph.

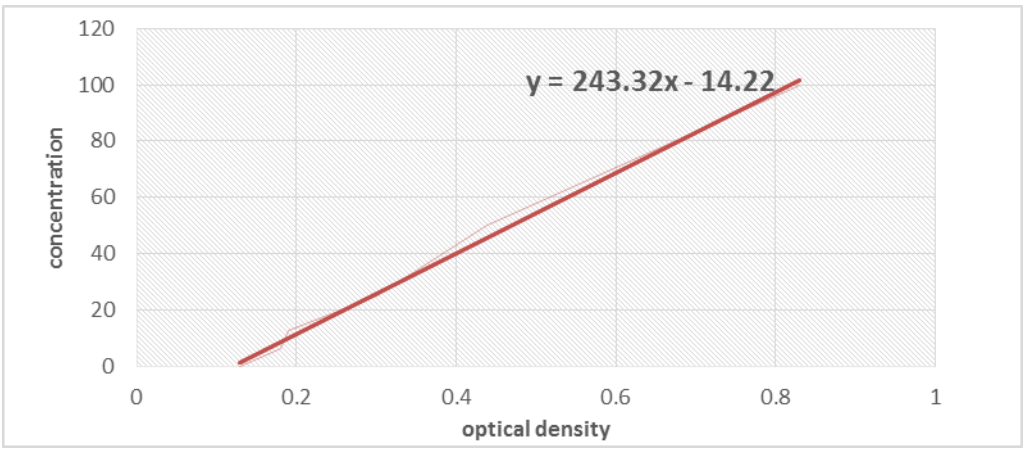


Figure (2-1): The standard curve of IL 1 beta.

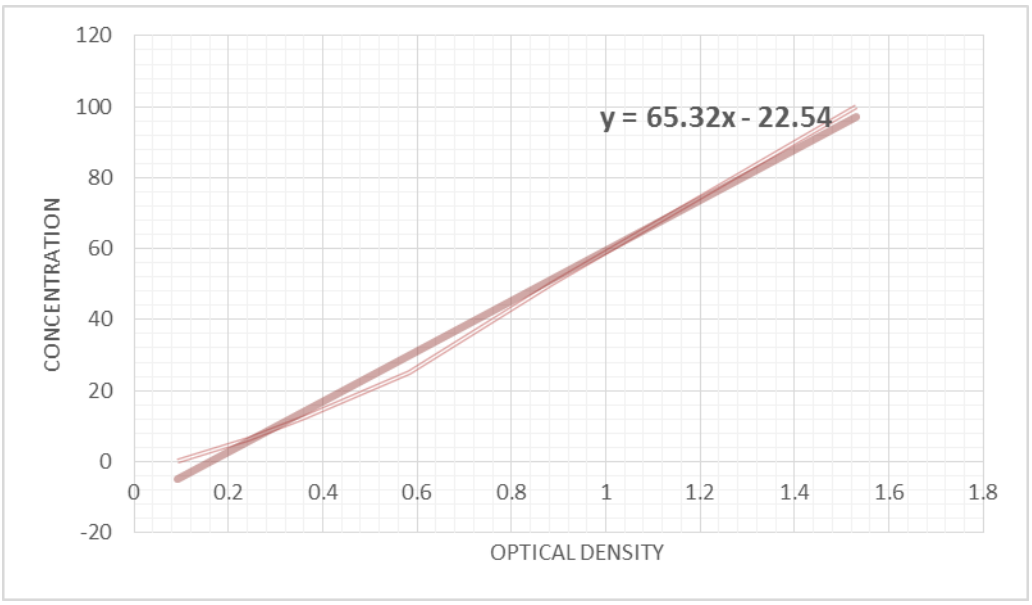


Figure (2-2): The standard curve of IL 10.

2-6 Polymerase chain reaction (PCR):

The PCR technique was performed for detection some intestinal infection helminthes (*Ascaris lumbricoides*, *Enterobious vermicularis*, *Trichuris trichiura*,*Strongyloides stercoralis*) from stool of IBD human patient samples. This method was carried out according to method described by (Zelinkova *et al*, 2017) as following steps:

2.7 Stool genomic DNA Extraction

Genomic DNA from stool samples was extracted by using AccuPrep® stool DNA Extraction Kit, Bioneer. Korea, and done according to company instructions as following steps:

1. A 200 mg of the stool sample was transferred to sterile 1.5ml microcentrifuge tube, and then 20µl of proteinase K and 400 µl Stool lysis buffer (SL) were added mixed by vortex. And incubated at 60 C for 10 minutes.
2. After 10 mins, the tube placed in centrifuge at 12,000rpm for 5 mins.
3. The supernatant was transferred in to a new tube and 200µl Binding buffer was added to each tubes.
4. The tubes were incubated again for 10 min at 60 C.
5. 100 µl isopropanol was added and the samples mixed by lightly vortex for about 5 seconds, then spin down for 10 seconds to down the liquid clinging to the walls and lid of the tube.
6. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 8000rpm for 5 minutes. And the 2 ml collection tube containing the flow-through was discarded and placed the column in a new 2 ml collection tube.
7. 500µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collection tube.

8. 500µl W2 Buffer (ethanol) was added to each column. Then centrifuged at 8000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collection tube.

9. All the tubes were centrifuged again for 1 minute at 12000 rpm to dry the column matrix.

10. The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre-heated elution buffer were added to the centre of the column matrix.

11. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

2.8 Genomic DNA estimation

The extracted genomic DNA was estimated by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.
3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of DNA was added to measurement.

2.9 PCR master mix preparation

PCR master mix was prepared by using (**Maxime PCR PreMix Kit**) and this master mix done according to company instructions as following table (2-7)

PCR Master mix	Volume
DNA template	5µL
Forward primer (10pmol)	1µL
Reverse primer (10pmol)	1µL
PCR water	13µL
Total volume	20µL

After that, these PCR master mix component that mentioned in table above placed in standard **PCR PreMix Kit** that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (T100 Thermal cycler. BioRad USA).

2.9.1 PCR Thermo cycler Conditions

PCR thermocycler conditions by using convential PCR thermocycler system as following table (2-8)

PCR step	Temp.	Time	repeat
Initial Denaturation	94ºC	5min	1
Denaturation	94 ºC	30sec.	30 cycle
Annealing	58 ºC	30sec	
Extension	72 ºC	1min	
Final extension	72 ºC	5min	1
Hold	4 ºC	Forever	-

2.9.2 PCR product analysis

The PCR products of was analyzed by agarose gel electrophoresis following steps:

1- 1% Agarose gel was prepared in using 1X TBE and melting in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µL of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature,

then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 10µl of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt.

5- PCR products were visualized by using UV Transilluminator.

2.10 DNA sequencing method

DNA sequencing method was performed for confirmative detection of PCR positive intestinal infection helminths and study of phylogenetic relationship tree analysis and NCBI-Blast submission local related helminths as well as submission in NCBI-GenBank at first time in Iraq. The PCR positive products were sent to Macrogen Company in Korea in ice bag by DHL for performed the DNA sequencing by AB DNA sequencing system.

The DNA sequencing analysis was conducted by using Molecular Evolutionary Genetics Analysis version 6.0. (Mega 6.0) and Multiple sequence alignment analysis of the partial subunit ribosomal RNA gene based ClustalW alignment analysis and The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method.

The genetic Sequences identity was Estimates by NCBI-BLAST. Then submitted into NCBI-GenBank submission using Bankit submission tool.

2.11 PCR RFLP method

PCR RFLP was performed for identification of NOD2 (R702W) gene polymorphism in CD and in healthy control blood samples. This method was carried out according to described by (Zelinkova *et al.*, 2017) as following steps:

2.11.1 Blood genomic DNA Extraction

Blood genomic DNA from blood samples were extracted by using gSYAN DNA kit extraction kit (Frozen Blood protocol) Geneaid. USA, and done according to company instructions as following steps:

1. A 200µl of frozen blood was transferred to sterile 1.5ml microcentrifuge tube, and then added 20µl of proteinase K and mixed by vortex. And incubated at 60℃ for 15 minutes.

2. After that, 200µl of GSB cell lysis buffer was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 70℃ for 15 minutes, and inverted every 3 minutes through incubation periods.

3. 200µl absolute ethanol were added to lysate and immediately mixed by shaking vigorously.

4. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000rpm for 5 minutes. And the 2 ml collection tube containing the flow.through was discarded and placed the column in a new 2 ml collection tube.

5.400µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow through was discarded and placed the column back in the 2 ml collection tube.

6.600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow. Through was discarded and placed the column back in the 2 ml collection tube.

7. All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.

8. The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of preheated elution buffer were added to the center of the column matrix.

9. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

2.11.4 PCR Thermo cycler Conditions

PCR thermocycler conditions were done for each NOD2 (R702W) gene as following tables(2-10):

PCR step	Temp.	Time	Repeat
Initial denaturation	95°C	5min.	1
Denaturation	95°C	30 sec.	35cycle
Annealing	55°C	30 sec.	

Extension	72°C	30 sec.	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

2.11.6 RFLP master mix preparation

RFLP master mix was for NOD2 (R702W) gene polymorphism was prepared by using *MspI* restriction enzyme (New England Biolabs. UK) and this master mix done independent according to company instructions as following table(2-11):

Table (2-11): **RFLP master mix *NOD2* (R702W)**

RFLP-PCR Master mix	Volume
PCR product	10µl
MspI Restriction enzyme buffer 10X	2 µl
MspI (10 unit)	1 µl
Free nuclease water	7 µl
Total volume	20 µl

After that, this master mix placed in Exispin vortex centrifuge at 3000rpm for 2 minutes, then transferred into incubation at 37°C for

overnight. After that, RFLP product was analysis by 2.5% agarose gel electrophoresis methods that mention in PCR product analysis. The (CC) wild type homozygote, the product digested by restriction enzyme 70 and invisible 54, 46bp band. The (C/T) heterozygote, the product digested by restriction enzyme into 124, 70 and invisible 54 and 46bp bands. The (TT) mutant type homozygote that shows digested by restriction enzyme into 124bp and invisible 46bp bands.

2.12 Statistical analysis

Data were collected, summarized, analysed and presented using statistical package for social sciences (SPSS) version 23 and Microsoft Office Excel 2010. **Qualitative (categorical) variables** were expressed as number and percentage, whereas, **quantitative (numeric) variables** were first evaluated for normality distribution using **Kolmogorov-Smirnov** test, and then accordingly normally distributed numeric variables were expressed as **mean** (an index of central tendency) and **standard deviation** (an index of dispersion), while those numeric variables that are not normally distributed were expressed as **median** (an index of central tendency) and **inter-quartile range** (an index of dispersion).

The following statistical tests were used:

1. **Chi-square test** was use to evaluate association between any two categorical variables provided that less than 20 % of cells have expected count of less than 5. However, **Fischer exact test** was used instead when chi-square test was not valid (in case that more than 20 % of cells have expected count of less than 5).
2. **Independent samples t-test** was used to evaluate the difference in mean of numeric variables between any two groups provided that

these variables were normally distributed; otherwise **Mann Whitney U test** would be used instead if those variables were not normally distributed.

3. **One way analysis of variance (ANOVA)** was used to evaluate difference in mean of numeric variables among more than two groups provided that these numeric variables were normally distributed; but **Kruskal Wallis test** was chosen in case of non-normally distributed variables. **One way ANOVA** was followed by **pos hoc LSD** test to evaluate individual differences in mean values between any two groups among groups tested primarily using **one way ANOVA**; whereas, **Kruskal Wallis test** was followed by **Mann Whitney U** test for the same purpose in case of non-normally distributed numeric variables.
4. **Spearman correlation** was used to evaluate the correlation between any 2 numeric variables and the results were expressed as correlation co-efficient (*r*) and the level of significance (*P*).
5. In order to detect the cutoff value that predict a positive finding, **receiver operator characteristic (ROC) curve analysis** was used with its corresponding **area under the curve (AUC)**, **accuracy level**, **sensitivity, specificity** and level of significance (*P*).

The level of significance was considered at *P*-value of equal or less than 0.05. The level of high significance was considered at *P*-value of equal or less than 0.01. (Field, 2005).

3.Results:

3.1 Demographic characteristics of patients and control individuals

The current study included 50 patients with idiopathic inflammatory bowel disease (IBD), 31 with Ulcerative colitis (UC) and 19 with Crohn's disease (CD), serving as study group, and 50 apparently healthy subjects serving as control group. Demographic characteristics of control and study groups are shown in tables 3.1 through 3.5.

3.1.1 Distribution of patients and control subjects according to age and gender

The mean age of patients with IBD was 36.68 years and the range was 12 – 63 years, whereas the mean age of control subject was 37.22 years and he range was 12 – 63 years. Indeed, there was no significant difference in mean age between patients and control subjects (P = 0.828), table 3.1. On the other hand, most of the patients enrolled in the present study were between 20 and 40 years of age, as shown in table 3.1. The lack of statistical difference in mean age in this study is mandatory in such case control study to avoid any possible bias in the results attributed to age factor.

According to gender, patients included 38 males (76 %) and 12 females (24 %) while control subjects included 33 males (66 %) and 17 females (34 %); the difference in the distribution of patients and control subjects was statistically insignificant (P = 0.978), table 3.1. this finding, that is the lack of significant difference in distribution of individuals in both groups according to gender, is also a pre-requisite in order to avoid bias related to gender in such case control study.

Table(3.1):Distribution of patients with IBD and control subjects according to age

Age (years)	**IBD** ***n* = 50**	**Control** ***n* = 50**	***P***
Mean ± SD	36.68 ±12.24	37.22 ±12.56	0.828 † NS
Range	12 – 63	12 – 63	
<20 years, *n* (%)	4 (8 %)	4 (8 %)	0.978 ¥ NS
20-40 years, *n* (%)	28 (56 %)	27 (54 %)	
> 40 years, *n* (%)	18 (36 %)	19 (38 %)	

IBD: inflammatory bowel diseases; *n*: number of cases; SD: standard deviation; †: independent samples t-test; ¥: Chi-square test; NS: not significant at $P \leq 0.05$

Table(3.2): Distribution of patients and control subjects according to gender

Gender	**IBD** ***n* = 50**		**Control** ***n* = 50**		χ^2	***P***
	n	**%**	***n***	**%**		
Male	38	76	33	66	1.214	0.271¥ NS
Female	12	24	17	34		

IBD: inflammatory bowel diseases; *n*: number of cases; ¥: Chi-square test; NS: not significant at $P \leq 0.05$

3.1.2 Distribution of patients and control subjects according to residency

Patients from urban areas constituted 68 % (34 out of 50) whereas, those originating from rural areas accounted for 32 % (16 out of 50). On the other hand control group included 35 (70 %) and 15 (30 %) persons from urban and rural areas, respectively. Moreover, there was no statistical significance difference in the distribution of patients with IBD and control subjects with respect to residency ($P = 0.829$), as shown in table 3.3.

Table(3.3):Distribution of patients and control subjects according to residency

Region	IBD *n* = 50		Control *n* = 50		χ^2	*P*
	n	%	*N*	%		
Urban	34	68	35	70	0.047	0.829 ¥ NS
Rural	16	32	15	30		

IBD: inflammatory bowel diseases; *n*: number of cases; ¥: Chi-square test; NS: not significant at $P \leq 0.05$

3.1.3 Distribution of patients and control subjects according to bad habits (smoking and alcoholism)

Alcoholism was seen neither in control group nor in patients group, table 4.4; however, smoking was observed in 14 % of patients (7 out of 50) and in 12 % of control subjects (6 out of 50); the difference in distribution of patients and control subjects according to smoking was statistically insignificant ($P = 0.766$), as shown in table 3.4.

Table(3.4):Distribution of patients and control subjects according to smoking and alcoholism

Bad habits	IBD *n* = 50		Control *n* = 50		χ^2	*P*
	n	%	*N*	%		
Smoking	7	14	6	12	0.088	0.766 ¥ NS
Alcoholism	0	0	0	0	---	---

IBD: inflammatory bowel diseases; *n*: number of cases; ¥: Chi-square test; NS: not significant at $P \leq 0.05$

3.1.4 Distribution of patients and control subjects according to family history of IBD

Positive family history was seen in 8 patients out of 50 (16 %) and in 6 out of 50 control subjects (12 %); there was no statistical significant difference in the distribution of patients with IBD and control subjects with respect to family history of idiopathic inflammatory bowel disease, as shown in table 3.5.

Table(3.5):Distribution of patients and control subjects according to family history

Family history	IBD *n* = 50		Control *n* = 50		χ^2	*P*
	n	%	*n*	%		
Positive	8	16	6	12	0.332	0.564 ¥ NS
Negative	42	84	44	88		

IBD: inflammatory bowel diseases; *n*: number of cases; ¥: Chi-square test; NS: not significant at $P \leq 0.05$

3.2 Comparison of conventional stool light microscopy and conventional PCR methods in the detection of helminthes

The comparison between the conventional microscopic examination of stool specimen with conventional PCR detection of parasite in stool specimen is shown in tables 3.6,3.7 and 3.8 for control group, patients group and both groups collectively, respectively. Regarding control group, table 3.6, conventional light microscopic examination showed sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 76.9 %, 100.0 %, 100.0 %, 92.5 % and 94.0 % for *A. lumbricoides*. In addition, conventional light microscopic examination showed sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 88.0 %, 100.0 %, 100.0 %, 89.3 % and 94.0 % for *E. vermicularis*. Moreover, conventional light microscopic examination showed sensitivity, specificity, positive predictive value, negative predictive value

and accuracy level of 52.6 %, 100.0 %, 100.0 %, 77.5 % and 82.0 % for *S. stercoralis*. All over, conventional light microscopic examination showed sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 75.0 %, 78.6 %, 90.0 %, 55.0 % and 76.0 % for total parasite infestation.

Table(3.6):Light microscopy versus PCR in control group

Parasite	Microscope	PCR			
		Positive		Negative	
		n	**%**	***N***	**%**
A. lumbricoides	Positive	10	20.0	0	0.0
	Negative	3	6.0	37	74.0
E. vermicularis	Positive	22	44.0	0	0.0
	Negative	3	6.0	25	50.0
S. stercoralis	Positive	10	20.0	0	0.0
	Negative	9	18.0	31	62.0
Total	Positive	27	54.0	3	6.0
	Negative	9	18.0	11	22.0
Results	**Sensitivity %**	**Specificity %**	**PPV %**	**NPV %**	**Accuracy %**
A. lumbricoides	76.9	100.0	100.0	92.5	94.0
E. vermicularis	88.0	100.0	100.0	89.3	94.0
S. stercoralis	52.6	100.0	100.0	77.5	82.0
Total	75.0	78.6	90.0	55.0	76.0

Regarding patient group, table 3.7, conventional light microscopic examination showed sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 83.3 %, 100.0 %, 100.0 %, 97.8 % and 98.0 % for *A. lumbricoides*. In addition, conventional light microscopic examination showed sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 88.2 %, 100.0 %, 100.0 %, 94.3 % and 96.0 % for *E. vermicularis*. Moreover, conventional light microscopic examination showed

sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 50.0 %, 100.0 %, 100.0 %, 83.7 % and 86.0 % for *S. stercoralis*. All over, conventional light microscopic examination showed sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 84.6 %, 100.0 %, 100.0 %, 85.7 % and 92.0 % for total parasite infestation.

Table(3.7): Light microscopy versus PCR in IBD group

Parasite	Microscope	PCR			
		Positive		Negative	
		N	%	*N*	%
A. lumbricoides	Positive	5	10.0	0	0.0
	Negative	1	2.0	44	88.0
E. vermicularis	Positive	15	30.0	0	0.0
	Negative	2	4.0	33	66.0
S. stercoralis	Positive	7	14.0	0	0.0
	Negative	7	14.0	36	72.0
Total	Positive	22	44.0	0	0.0
	Negative	4	8.0	24	48.0
Results	**Sensitivity %**	**Specificity %**	**PPV %**	**NPV %**	**Accuracy %**
A. lumbricoides	83.3	100.0	100.0	97.8	98.0
E. vermicularis	88.2	100.0	100.0	94.3	96.0
S. stercoralis	50.0	100.0	100.0	83.7	86.0
Total	84.6	100.0	100.0	85.7	92.0

Regarding both groups, table 3.8, conventional light microscopic examination showed sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 78.9 %, 100.0 %, 100.0 %, 95.3 % and 96.0 % for *A. lumbricoides*. In addition, conventional light microscopic examination showed sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 88.1 %, 100.0 %, 100.0 %, 92.1 % and 95.0 % for *E. vermicularis*. Moreover, conventional light microscopic examination showed

sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 51.5 %, 100.0 %, 100.0 %, 80.7 % and 84.0 % for *S. stercoralis*. All over, conventional light microscopic examination showed sensitivity, specificity, positive predictive value, negative predictive value and accuracy level of 79.0 %, 92.1 %, 94.2 %, 72.9 % and 84.0 % for total parasite infestation.

Table(3.8): Light microscopy versus PCR in both groups collectively

Parasite	Microscope	PCR			
		Positive		Negative	
		N	%	*N*	%
A. lumbricoides	Positive	15	15.0	0	0.0
	Negative	4	4.0	81	81.0
E. vermicularis	Positive	37	37.0	0	0.0
	Negative	5	5.0	58	58.0
S. stercoralis	Positive	17	17.0	0	0.0
	Negative	16	16.0	67	67.0
Total	Positive	49	49.0	3	3.0
	Negative	13	13.0	35	35.0
Results	**Sensitivity %**	**Specificity %**	**PPV %**	**NPV %**	**Accuracy %**
A. lumbricoides	78.9	100.0	100.0	95.3	96.0
E. vermicularis	88.1	100.0	100.0	92.1	95.0
S. stercoralis	51.5	100.0	100.0	80.7	84.0
Total	79.0	92.1	94.2	72.9	84.0

3.3 Association between parasitic infestation and inflammatory bowel disease

3.3.1 The distribution of patients and control subjects according to parasitic infestation

The distribution of patients with inflammatory bowel disease and control subjects according to presence and type of parasitic infestation is demonstrated in table 4.9. First of all, the helminths (round worms) that were detected in the current study included *Ascaris lumbricoides, Enterobius vermicularis,* and *Strongyloides stercoralis.*

Ascaris lumbricoides was seen in 12 % and 26 % of patients and control subjects, respectively, that is it is more common in control subjects than in patients with IBD; however, the difference did not reach statistical significance (*P* = 0.074); in terms of risk the odds ratio was 0.43 which means that patient with *Ascaris lumbricoides* are less liable to get IBD by a fraction of 0.57 and the preventive fraction was 0.30, table 3.9.

Enterobius vermicularis was seen in 34 % and 50 % of patients and control subjects, respectively, that is it is more common in control subjects than in patients with IBD; however, the difference did not reach statistical significance (*P* = 0.105); in terms of risk the odds ratio was 0.52 which means that patient with *Enterobius vermicularis* are less liable to get IBD by a fraction of 0.48 and the preventive fraction was 0.28, table 3.9.

Strongyloides stercoralis was seen in 28 % and 38 % of patients and control subjects, respectively, that is it is more common in control subjects than in patients with IBD; however, the difference did not reach statistical significance (*P* = 0.288); in terms of risk the odds ratio was 0.63 which means that patient with

Strongyloides stercoralis are less liable to get IBD by a fraction of 0.37 and the preventive fraction was 0.20, table 3.9.

Total parasite burden was seen in 52 % and 62 % of patients and control subjects, respectively, that is it is more common in control subjects than in patients with IBD; moreover, the difference was statistically significance (P = 0.039); in terms of risk the odds ratio was 0.42 which means that patient with round worm infestation are less liable to get IBD by a fraction of 0.58 and the preventive fraction was 0.37, table 3.9.

Table(3.9):Association between parasitic infestation and inflammatory bowel disease

Parasite	IBD n = 50		Control n = 50		*P* ¥	OR	95 % CI		PF
	N	%	*N*	%			Lower	Upper	
Total parasite	26	52	36	62	0.039 S	0.42	0.18	0.97	0.37
Ascaris lumbricoides	6	12	13	26	0.074 NS	0.43	0.15	1.26	0.30
Enterobius vermicularis	17	34	25	50	0.105 NS	0.52	0.23	1.15	0.28
Strongyloides stercoralis	14	28	19	38	0.288 NS	0.63	0.27	1.47	0.20

IBS: inflammatory bowel diseases; *n*: number of cases; ¥: Chi-square test; NS: not significant at $P \leq 0.05$; OR: odds ratio; CI: confidence intervals; PF: preventive fraction

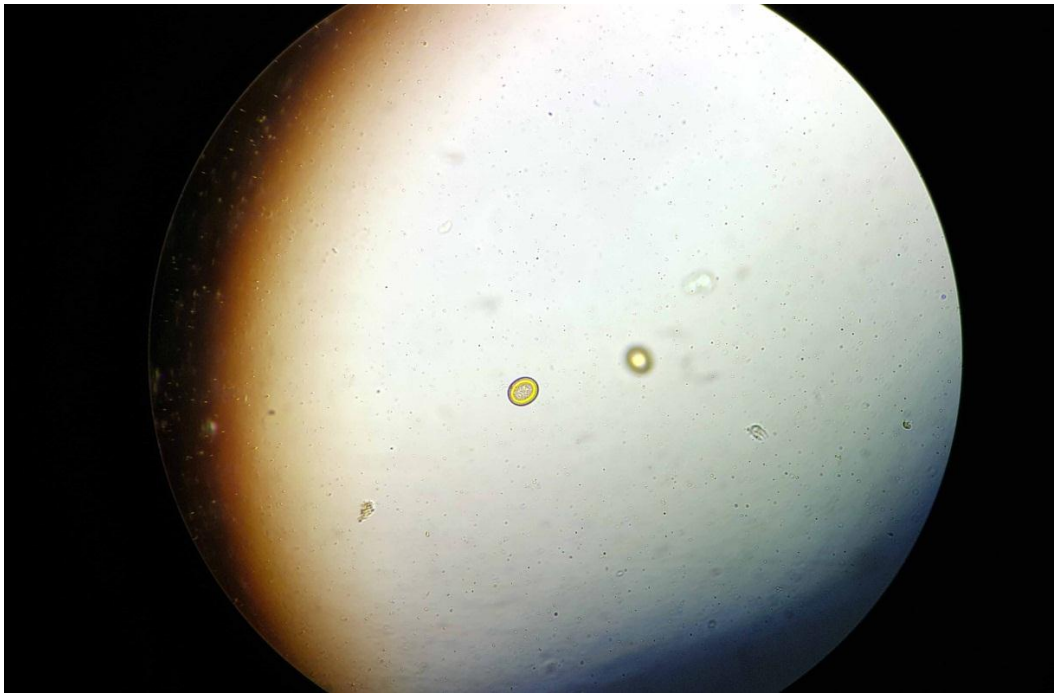


Fig.(3.1): *Ascaris lumbricoides* egg.

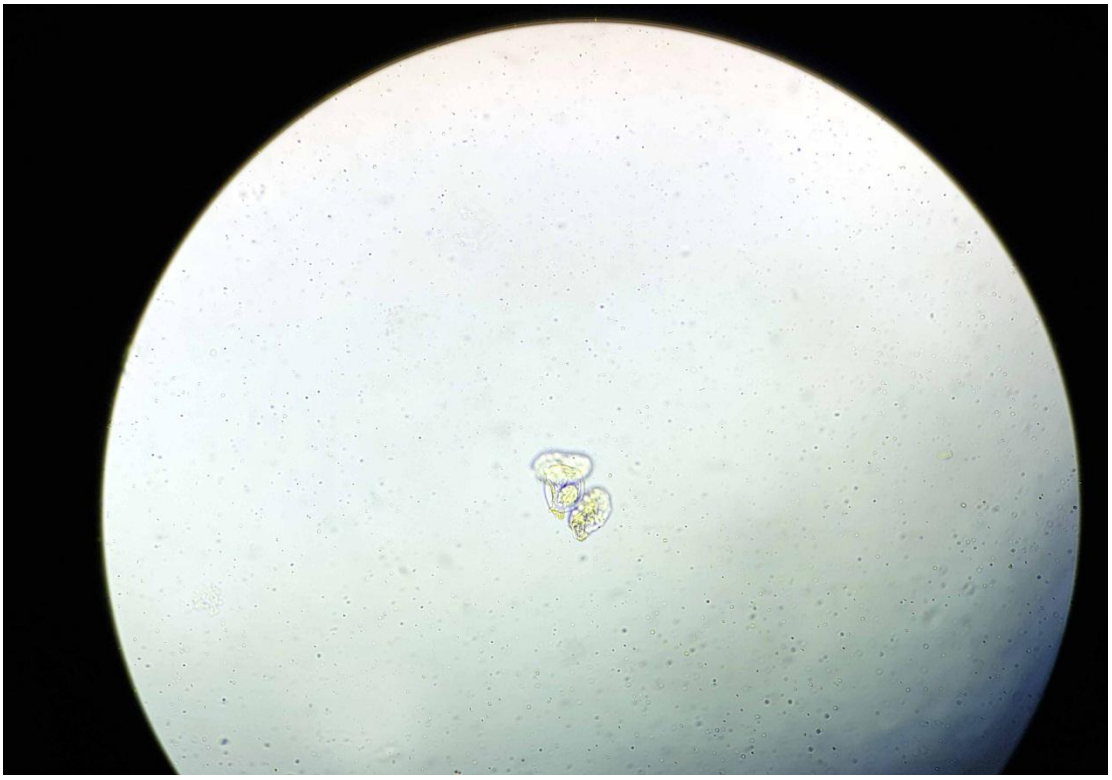
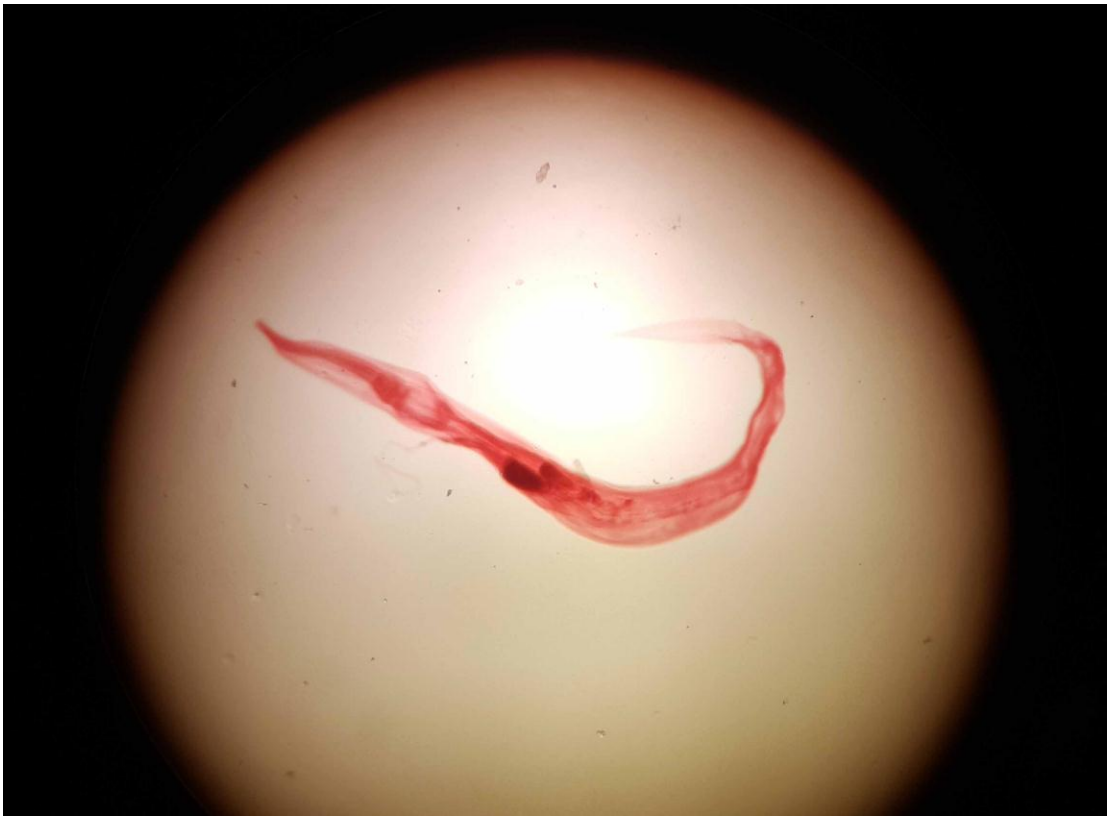


Fig. (3.3) : *Enterobius vermicularis* egg.



Fig(3-4): *strongyloides stercoralis* (filariform larvae).

3.3.2 Distribution of patients and control group according to parasite infestation by residency (urban versus rural)

Evidence of round worm infestation was seen in a total of 62 out of 100 individuals enrolled in the current study; regarding control group, in urban area the prevalence rate of round worm infestation was 77.1 % whereas in rural areas the prevalence rate of round worm infestation was 60.0 %; the difference was statistically insignificant (P = 0.372), as shown in table 4.10. Regarding UC group, in urban area the prevalence rate of round worm infestation was 63.6 % whereas in rural areas the prevalence rate of round worm infestation was 55.6 %; the difference was statistically insignificant (P = 0.990), as shown in table 3.10. regarding CD group, in urban area the prevalence rate of round worm infestation was 75.0 % whereas in rural areas the prevalence rate of round worm infestation

was 42.9 %; the difference was statistically insignificant (*P* = 0.364), as shown in table 3.10. Regarding all enrolled subjects, in urban area the prevalence rate of round worm infestation was 63.8 % whereas in rural areas the prevalence rate of round worm infestation was 58.1 %; the difference was statistically insignificant (*P* = 0.587), as shown in table 3.10.

Table(3.10):Distribution of patients and control group according to parasite infestation by residency (urban versus rural)

Group	Parasite	Total		Urban		Rural		χ^2	*P*
		n	%	*N*	%	*N*	%		
Control	Positive	36	72.0	27	77.1	9	60.0	0.798	0.372 † NS
	Negative	14	28.0	8	22.9	6	40.0		
UC	Positive	19	61.3	14	63.6	5	55.6	0.001	0.990 † NS
	Negative	12	38.7	8	36.4	4	44.4		
CD	Positive	7	36.8	3	25.0	4	57.1	0.825	0.364 † NS
	Negative	12	63.2	9	75.0	3	42.9		
Total	Positive	62	62.0	44	63.8	18	58.1	0.295	0.587 ¥ NS
	Negative	38	38.0	25	36.2	13	41.9		

n: number of cases; †: Yates correction for continuity; ¥: Chi-square test; NS: not significant at $P \leq 0.05$

3.4 Serum interleukin levels in patients and control subjects.

3.4.1Comparison of serum IL-10 and IL-1β according to type of inflammatory bowel disease:

Serum levels of the anti-inflammatory cytokine IL-10 and of the pro-inflammatory cytokine IL-1 β were measured for all participants and results are shown in table 3.11. Median serum level of IL-10 in control group was 16 pg/ml, while that of patients with UC was 18 pg/ml and those with CD was 11 pg/ml. Hence serum level of IL-10 was significantly lower in patients with CD than both control group and UC group ($P < 0.05$), table 3.11 and figure 3.1. Moreover, here

was no significant difference in the serum level of IL-10 between patients with UC and control group ($P > 0.05$), table 3.11 and figure 3.1.

Serum IL-1 β was significantly ($P < 0.05$) highest in patients with CD followed by patients with UC and then by control group, 232 pg/ml, 65 pg/ml and 59.5 pg/ml, respectively, as shown in able 3.11 and figure 4.2; in addition there was no significant difference in serum Il-1 β level between patients with UC and control group ($P > 0.05$), table 3.11 and figure 3.2.

Table(3.11): Serum interleukin levels in patients and control subjects

Serum level	Statistic	Control *n* = 50	UC *n* = 31	CD *n* = 19
IL-10	Median (IQR)	16.00 (20.00) A	18.00 (23.00) A	11.00 (12.00) B
	Range	4.00 -56.00	3.00 -54.00	2.00 -65.00
IL-1β	Median (IQR)	59.50 (116.25) B	65.00 (222.00) B	232.00 (278.00) A
	Range	23.00 -653.00	3.00 -876.00	4.00 -866.00

UC: ulcerative colitis; CD: Crohn's disease; *n*: number of cases; IQR: inter-quartile range; Capital letters (A and B) where used to indicate significance level following Mann Whitney U test; similar letters indicate no significant difference at $P \leq 0.05$; different letters indicate significant difference at $P \leq 0.05$

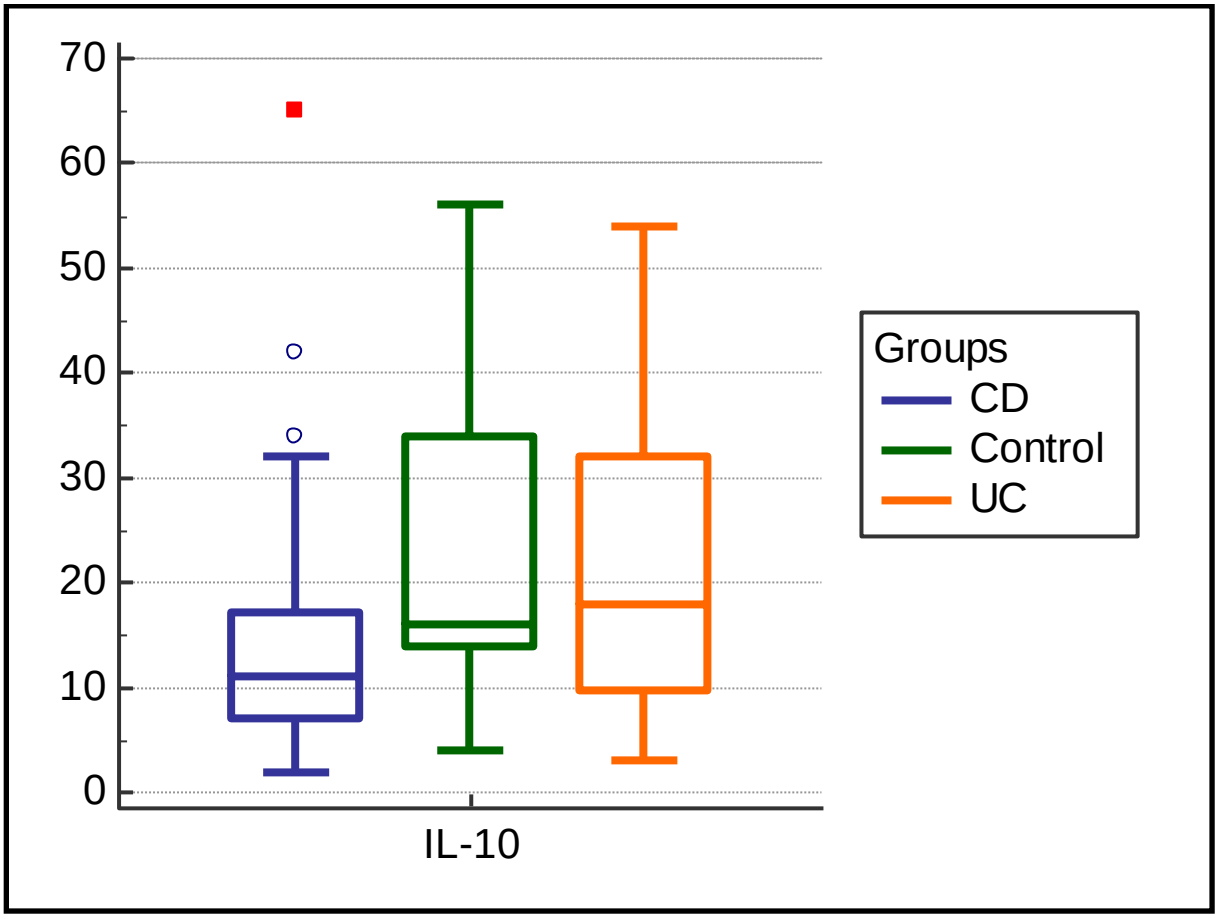
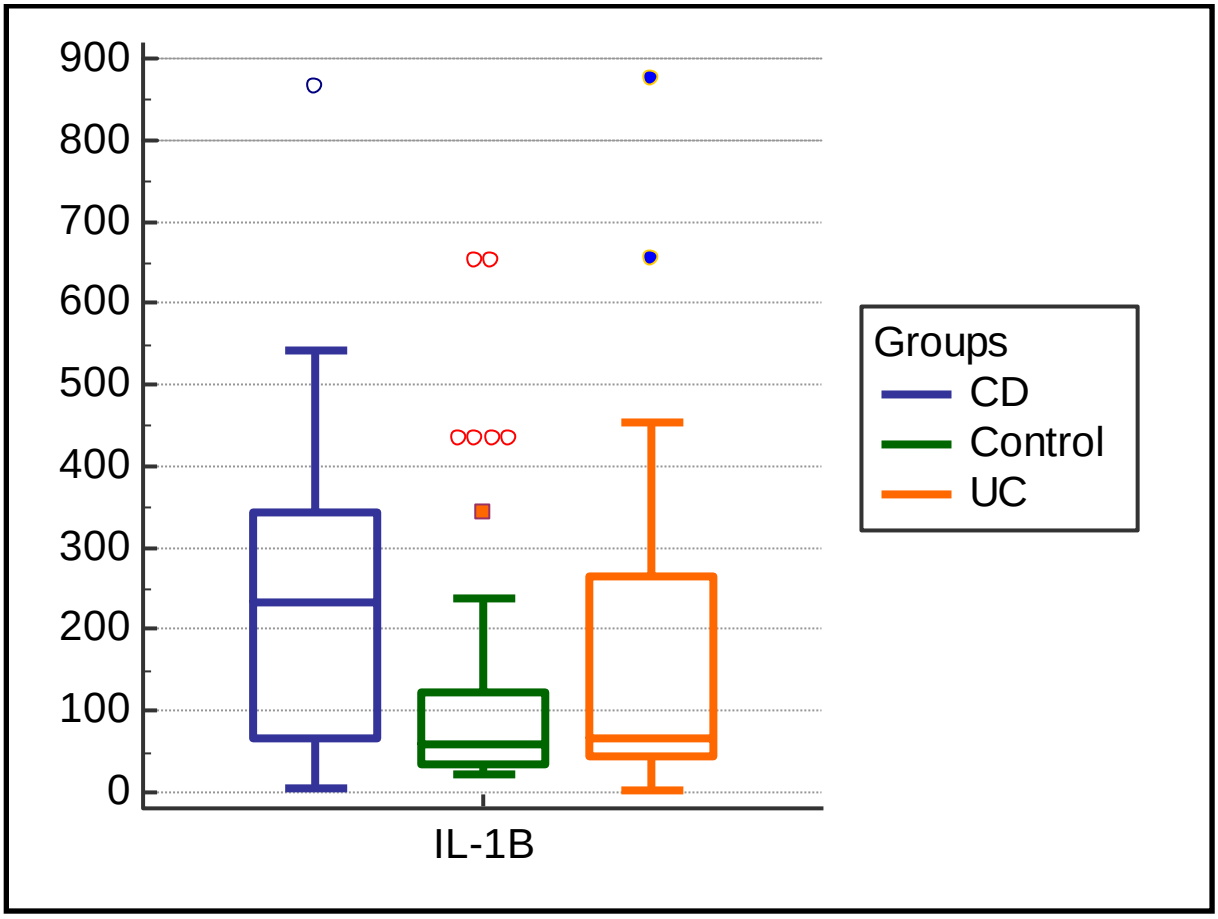


Figure 3.1: Box plot showing comparison of median serum IL-10 level among patients with Ulcerative colitis (UC) and Crohn's disease and control subjects



Figure(3.5): Box plot showing comparison of median serum IL-1β level among patients with Ulcerative colitis (UC) and Crohn's disease and control subjects

3.4.2 Comparison of serum IL-10 and IL-1β according to presence or absence of parasitic infestation

In control group, median serum IL-10 level was significantly higher in those having helminthes infestation than those who are free of parasite, 17 versus 14 pg/ml, respectively (P = 0.002), as shown in table 3.9 and figure 3.5 . In addition, there was no significant difference in median serum IL-1 β those having helminth infestation and those who are free of parasite, 59.5 versus 59.5 pg/ml, respectively (P = 0.633), as shown in table 3.9 and figure 3.5.

In patients with UC, median serum IL-10 level was significantly higher in those having helminthes infestation than those who are free of parasite, 31 versus 8 pg/ml, respectively ($P < 0.001$), as shown in table 4.10 and figure 4.3. In addition, median serum IL-1 β was higher in those who are free of parasite in comparison with those having helminthes infestation, 222 versus 54 pg/ml; however, the difference did not reach statistical significance ($P = 0.172$), as shown in table 3.10 and figure 3.3.

In patients with CD, median serum IL-10 level was significantly higher in those having helminthes infestation than those who are free of parasite, 32 versus 7.5 pg/ml, respectively ($P = 0.001$), as shown in table 3.10 and figure 3.3. In addition, median serum IL-1 β was lower in those who are free of parasite in comparison with those having helminthes infestation, 132 versus 321 pg/ml; however, the difference did not reach statistical significance ($P = 0.290$), as shown in table 3.11 and figure 3.3.

Table(3.12):Serum IL-10 and IL-1β according to presence or absence of parasitic infestation in control

Serum IL	Statistic	Parasite infestation		*P* €
		Positive *n* = 36	Negative *n* = 14	
IL-10	Median (IQR)	17.00 (26.50)	14.00 (5.50)	0.002 HS
	Range	10.00 -56.00	4.00 -18.00	
IL1B	Median (IQR)	59.50 (168.50)	59.50 (127.25)	0.633 NS
	Range	23.00-653.00	23.00 -653.00	

n: number of cases; IQR: inter-quartile range; €: Mann Whitney U test; NS: not significant at $P \leq 0.05$; HS: highly significant at $P \leq 0.01$

Table(3.13):Serum IL-10 and IL-1β according to presence or absence of parasitic infestation in UC:

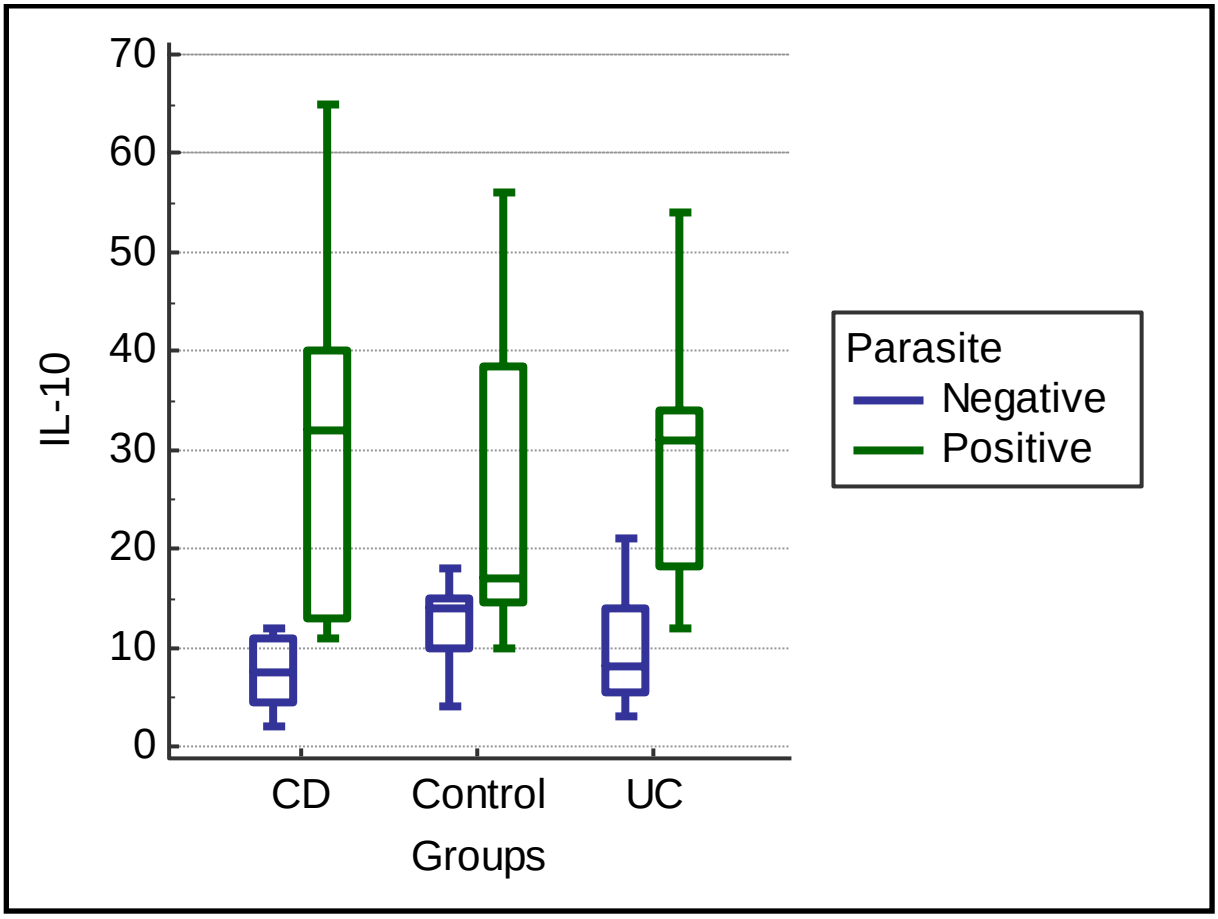
Serum IL	Statistic	Parasite infestation		*P* €
		Positive *n* = 19	Negative *n* = 12	
IL-10	Median (IQR)	31.00 (16.00)	8.00 (9.75)	<0.001 HS
	Range	12.00 -54.00	3.00 -21.00	
IL1B	Median (IQR)	54.00 (217.00)	222.00 (257.75)	0.172 NS
	Range	3.00 -655.00	8.00 -876.00	

n: number of cases; IQR: inter-quartile range; €: Mann Whitney U test; NS: not significant at $P \leq 0.05$; HS: highly significant at $P \leq 0.01$

Table(3.14):Serum IL-10 and IL-1β according to presence or absence of parasitic infestation in CD

Serum IL	Statistic	Parasite infestation		*P* €
		Positive *n* = 7	Negative *n* = 12	
IL-10	Median (IQR)	32.00 (31.00)	7.50 (7.75)	0.001 HS
	Range	11.00 -65.00	2.00 -12.00	
IL1B	Median (IQR)	321.00 (445.00)	123.00 (269.50)	0.290 NS
	Range	4.00 -543.00	12.00 -866.00	

n: number of cases; IQR: inter-quartile range; €: Mann Whitney U test; NS: not significant at $P \leq 0.05$; HS: highly significant at $P \leq 0.01$



Figure(3.6): Box plot showing comparison of median serum IL-10 level among patients with Ulcerative colitis (UC) and Crohn's disease and control subjects according to presence or absence of parasitic infestation

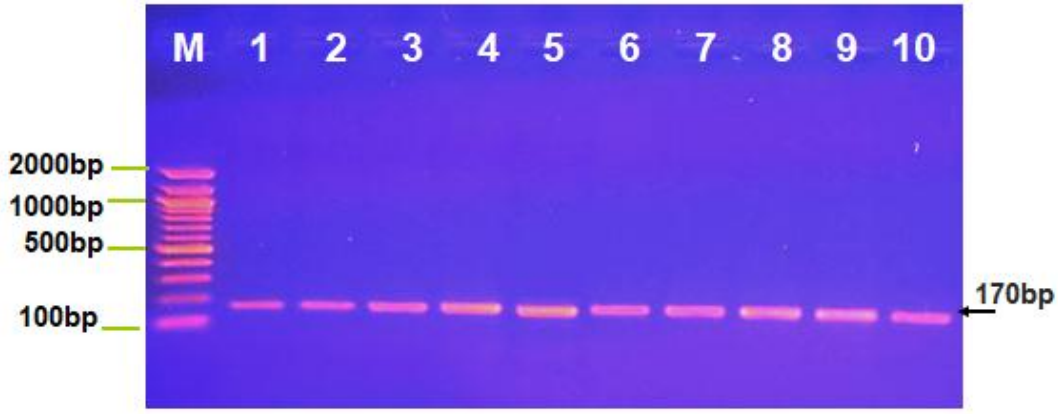
3.5 *NOD2* Genotype

Genotyping of *NOD2* gene was performed in the current study using RFLP-PCR technique. The results of the PCR targeting a 170-bp region of the *NOD2* gene revealed the presence of this gene in the blood samples of the IBD patients as shown in figures 3.7.

3.6 Molecular identification

3.6.1 PCR

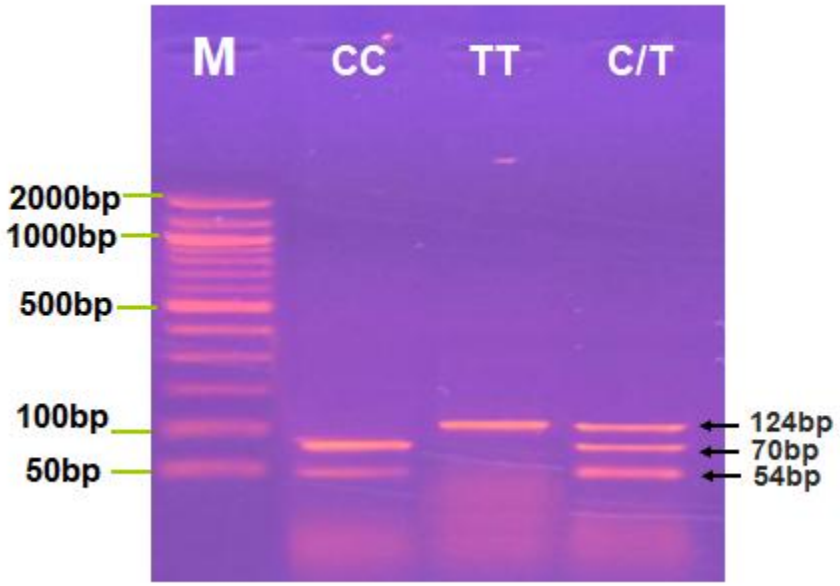
The results of the PCR targeting a 170-bp region of the *NOD2* gene, figure 3.7, revealed the presence of this gene in the blood samples of the IBD patients.



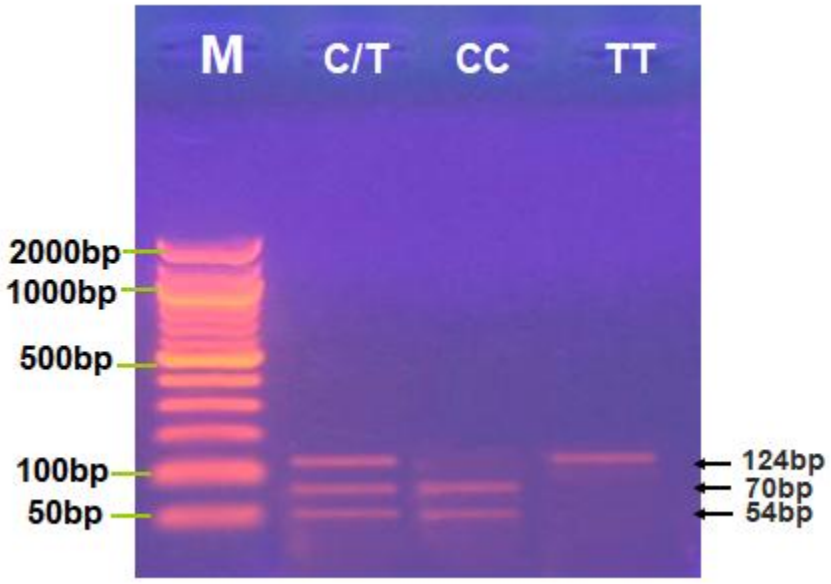
Figure(3-7):Agarose gel electrophoresis image that show the PCR product analysis of ***NOD2* gene** from blood patient samples and healthy control sample. Where M: marker (2000-50bp), lane (1-10) positive *NOD2* gene amplification at 170bp PCR product size.

3.6.2 PCR-RFLP

The results of the RFLP-PCR analysis of the polymorphisms regarding the *NOD2* gene (R702W) revealed the presence of the wild type (homozygote) digested by *MspI* into 70bp, 54bp, and invisible 46bp bands. The analysis also showed the occurrence of the mutant type (homozygote) digested by *MspI* into 124bp and invisible 46bp. The result of the current analysis also recorded the incidence of the mutant heterozygote digested by *MspI*into 124bp, 70bp, 54bp, and invisible 46bp, figure 3.8.



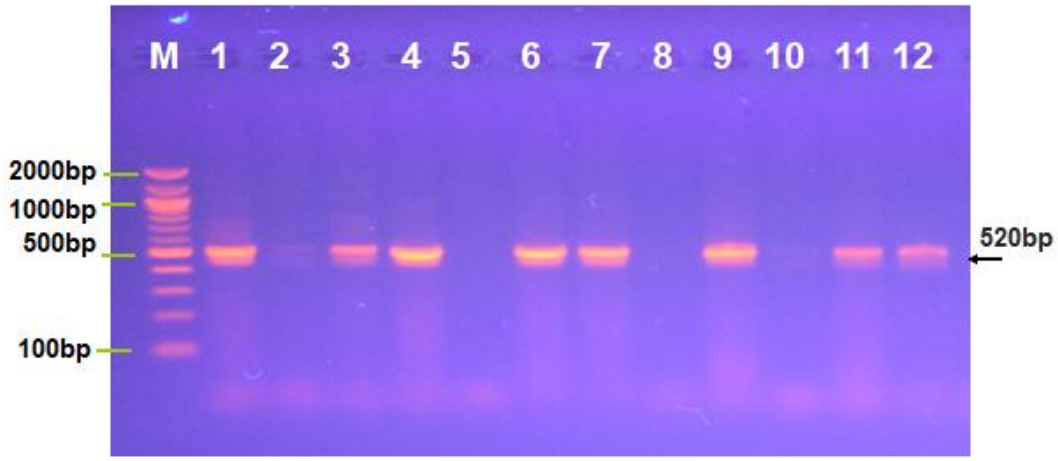
Figure(3-8):Agarose gel electrophoresis image that show the RFLP-PCR product analysis of ***NOD2*** **gene (R702W)** polymorphism in patients samples by using *MspI* restriction enzyme in 2% agarose. Where M: marker (2000-50bp). Lane (CC) wild type homozygote that show digested by restriction enzyme into 70bp, 54bp and invisible 46bp band, lane (TT) mutant type homozygote, the product digested by restriction enzyme into 124bp and invisible 46bp, and lane (C/T) heterozygote, the product digested by restriction enzyme into 124bp, 70bp, 54bp and invisible 46bp.



Figure(3-9):Agarose gel electrophoresis image that show the PCR-RFLP product analysis of ***NOD2*** **gene (R702W)** polymorphism in healthy control samples by using *MspI* restriction enzyme in 2% agarose. Where M: marker (2000-50bp). Lane (C/T) heterozygote, the product digested by restriction enzyme into 124bp, 70bp, 54bp and invisible 46bp, lane (CC) wild type homozygote that show digested by restriction enzyme into 70bp, 54bp and invisible 46bp band, Lane (TT) mutant type homozygote, the product digested by restriction enzyme into 124bp and invisible 46bp.

3.6.3 rRNA-gene-dependent PCR for *Ascaris lumbricoides*

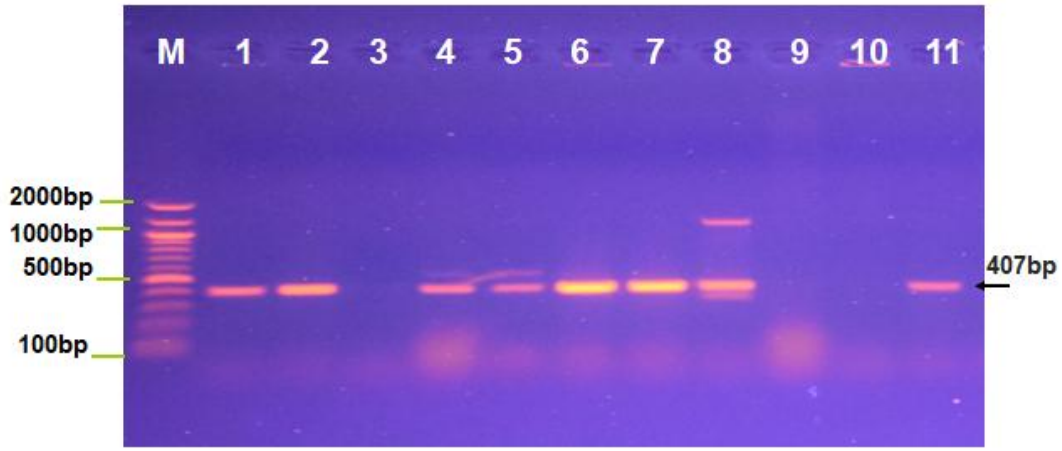
The results of the small subunit rRNA gene analysis in *Ascaris lumbricoides* showed the amplification of the gene at a 520-bp region, figure 3.10.



Figure(3-10):Agarose gel electrophoresis image that show the PCR product analysis of small subunit ribosomal RNA gene in *Ascaris lumbricoides* from Human stool samples. Where M: Marker (2000-100bp), lane (1-12) showed some positive *Ascaris lumbricoides* samples at 520bp PCR product size.

3.6.4 rRNA-gene-dependent PCR for *Enterobius vermicularis*

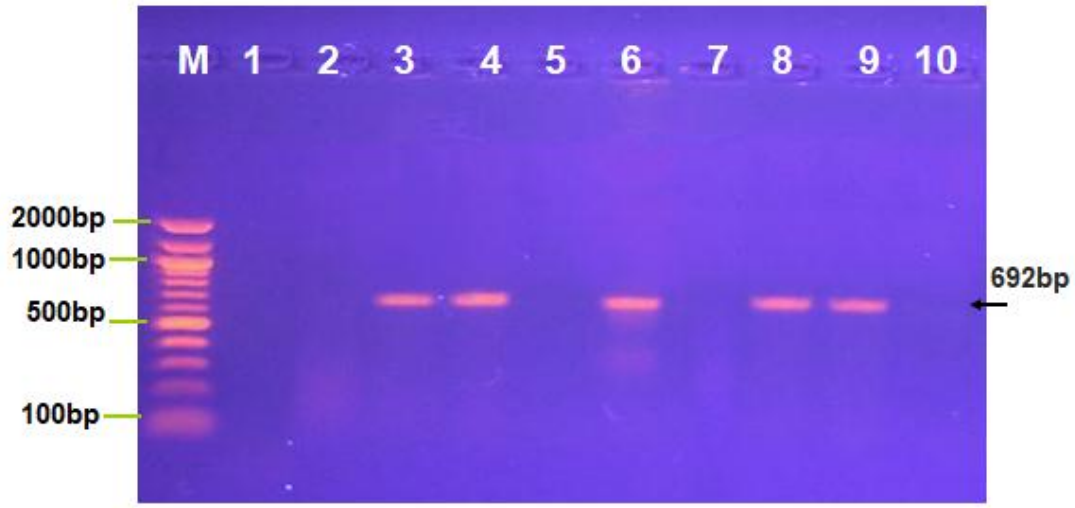
The results of the small subunit rRNA gene analysis in *Enterobius vermicularis* showed the amplification of the gene at a 407-bp region, figure 3.11.



Figure(3-11):Agarose gel electrophoresis image that show the PCR product analysis of small subunit ribosomal RNA gene in *Enterobius vermicularis* from Human stool samples. Where M: Marker (2000-100bp), lane (1-11) showed some positive *Enterobius vermicularis* samples at 407bp PCR product size.

3.6.5 rRNA-gene-dependent PCR for *Strongyloides stercoralis*

The results of the small subunit rRNA gene analysis in *Strongyloides stercoralis* showed the amplification of the gene at a 692-bp region, figure 3.12.



Figure(3-12):Agarose gel electrophoresis image that show the PCR product analysis of small subunit ribosomal RNA gene in *Strongyloides stercoralis* from Human stool samples. Where M: Marker (2000-100bp), lane (1-10) showed some positive *Strongyloides stercoralis* samples at 692bp PCR product size.

3.6.6 RRNA-gene-dependent PCR for *Trichuris trichiura*

The results of the small subunit rRNA gene analysis in *Trichuris trichiura* showed the amplification of the gene at a 361-bp region, figure 3.13.

Figure(3-13):Agarose gel electrophoresis image that show the PCR product analysis of small subunit ribosomal RNA gene in *Trichuris trichiura* from Human stool samples. Where M: Marker (2000-100bp), lane (1-12) showed no positive *Trichuris trichiura* samples at 361bp PCR product size.

Hardy Weinberg equation was applied to node genotypes, CC, CT and TT, distribution within the control group and results are shown in table 3.15. The homozygous wild genotype CC was encountered in 37 out of 50 control subjects; the heterozygous CT genotype was seen in 9 out of 50 control subjects and the homozygous mutant TT genotype was seen in 4 out of 50 control subjects, table 3.15. The observed distribution of control subjects according to NOD2 genotypes was significantly different from the expected one (P = 0.010), as shown in table 3.15.

Table(3.15): Hardy Weinberg equation

Genotypes	Observed	Expected	χ^2	*P* ¥
Homozygote reference CC	37	34.5	6.558	0.010 S
Heterozygote CT	9	14.1		
Homozygote variant TT	4	1.4		

¥: Chi-square test; S: significant at $P \leq 0.05$

Table(3.16): *NOD*2 Genotype frequency distribution in patients with IBD and control group

Genotype	IBD *n* = 50	Control *n* = 50	χ^2	*P* ¥
CC	34 (68 %)	37 (74 %)	6.087	0.048 S
CT	16 (32 %)	9 (18 %)		
TT	0 (0 %)	4 (8 %)		

n: number of cases; ¥: Chi-square test; S: significant at $P \leq 0.05$

Table(3.17): *NOD*2 allele frequency distribution in patients with IBD and control group

Allele	IBD *n* = 100	Control *n* = 100	*P*	OR	95 % CI		EF	PF
					Lower	Upper		
C	84	83	0.849 ¥ NS	1.08	0.51	2.27	0.04	
T	16	17		0.93	0.44	1.96		0.04

IBS: inflammatory bowel diseases; *n*: number of cases; ¥: Chi-square test; NS: not significant at $P \leq 0.05$; OR: odds ratio; CI: confidence intervals; EF: etiologic fraction; PF: preventive fraction

Table(3.18): The level of serum IL-10 and IL-1 β in control group

Interleukin	Statistic	CC *n* = 37	CT *n* = 9	TT *n* = 4	*P* €
IL-10	Median (IQR)	16.00 (20.00)	17.00 (19.00)	14.00 (3.75)	0.179 NS
	Range	5.00 -56.00	4.00 -56.00	10.00 -15.00	
IL-1β	Median (IQR)	54.00 (89.00)	75.00 (194.50)	54.00 (478.00)	0.860 NS
	Range	23.00 -653.00	23.00 -434.00	23.00 -653.00	

n: number of cases; IQR: inter-quartile range; €: Kruskal Wallis test; NS: not significant at $P \leq 0.05$

Table(3.19): The level of serum IL-10 and IL-1 β in patients with IBD

Interleukin	Statistic	CC *n* = 34	CT *n* = 16	TT *n* = 0	*P* €
IL-10	Median (IQR)	12.00 (13.25)	15.50 (28.50)	---	0.950 NS
	Range	2.00 -65.00	3.00 - 54.00	---	
IL-1β	Median (IQR)	105.00 (282.75)	95.50 (253.00)	---	0.700 NS
	Range	3.00 -876.00	8.00 - 532.00	---	

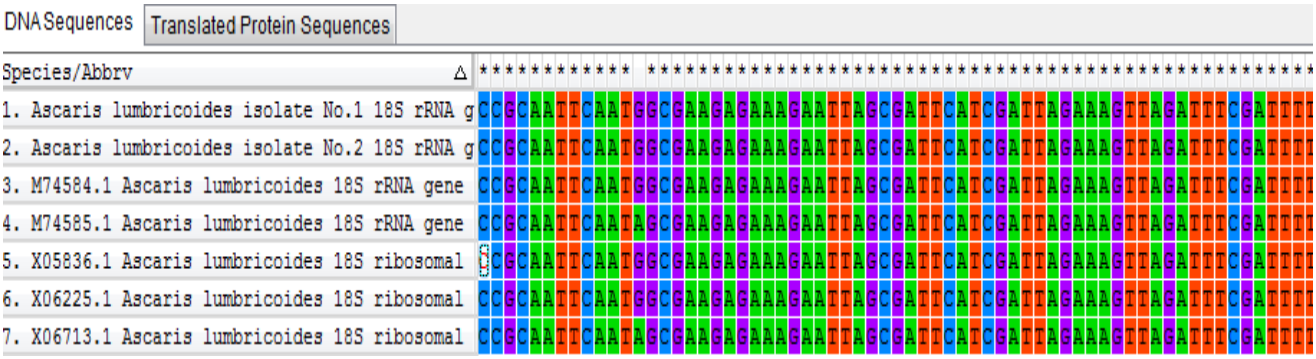
n: number of cases; IQR: inter-quartile range; €: Kruskal Wallis test; NS: not significant at $P \leq 0.05$

Table(3.20):Association between parasitic helminthes infestation and NOD2 genotypes in all subjects enrolled in the current study

Genotype	Parasite		χ^2	*P* ¥
	Positive *n* = 62	Negative *n* = 38		
CC	49 (79.0 %)	22 (57.9 %)	9.07	0.011 S
CT	13 (21.0 %)	12 (31.6 %)		
TT	0 (0.0 %)	4 (10.5 %)		

n: number of cases; ¥: Chi-square test; S: significant at $P \leq 0.05$

3.7 DNA sequencing:



Figure(3-14): Multiple sequence alignment analysis of the partial 18S ribosomal rRNA gene sequence in local *Ascaris lumbricoides* isolates and NCBI-Genbank *Ascaris lumbricoides* based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis similarity (*) and differences in 18 ribosomal rRNA gene nucleotide sequences.

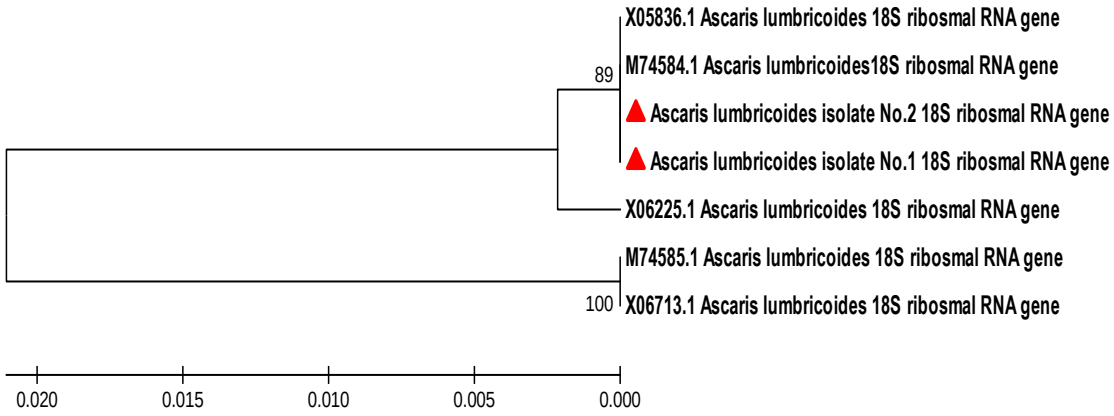
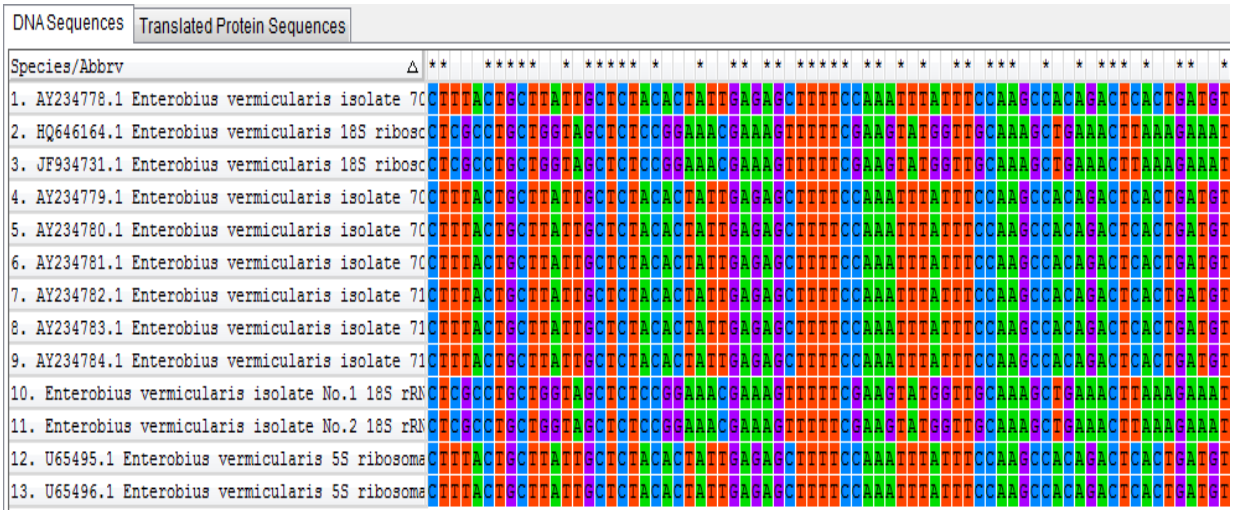


Figure (3-15): Phylogenetic tree analysis based on the partial sequence of 18S ribosomal rRNA gene in local *Ascaris lumbricoides* isolates that used for genetic comfirmative detection analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA species genetic type (MEGA 6.0 version). The local *Ascaris lumbricoides* No.1-No.2 isolates were show closed related to NCBI-Blast Ascaris lumbricoides (M74584.1) at total genetic change (0.005-0.020).

Table (3-21): **NCBI BLAST Homology sequence identity**

Local isolates	Genbank accession numbers	NCBI-BLAST Homology sequence identity (%)	
		Genbank isolates	Identity
***Ascaris lumbricoides* isolate No.1**	**MH893632**	**M74584.1**	**100%**
***Ascaris lumbricoides* isolate No.2**	**MH893633**	**M74584.1**	**100%**



Figure(3-16): Multiple sequence alignment analysis of the partial 18S ribosomal rRNA gene sequence in local *Enterobius vermicularis* isolates and NCBI-Genbank *Enterobius vermicularis* based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis similarity (*) and differences in 18 ribosomal rRNA gene nucleotide sequences.

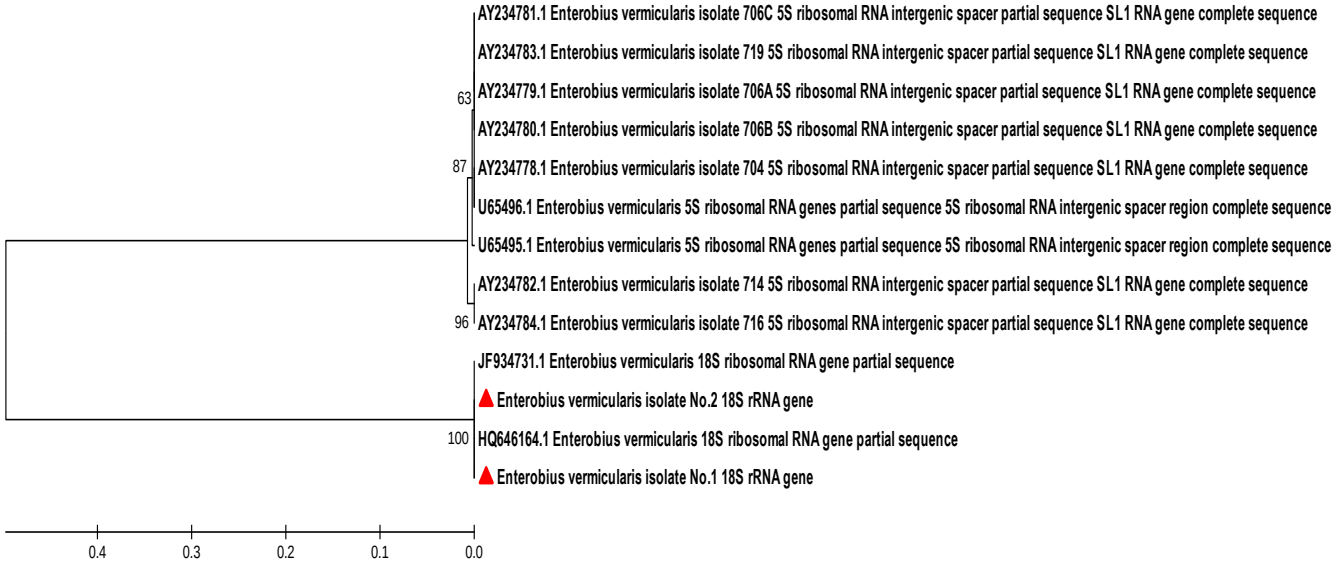
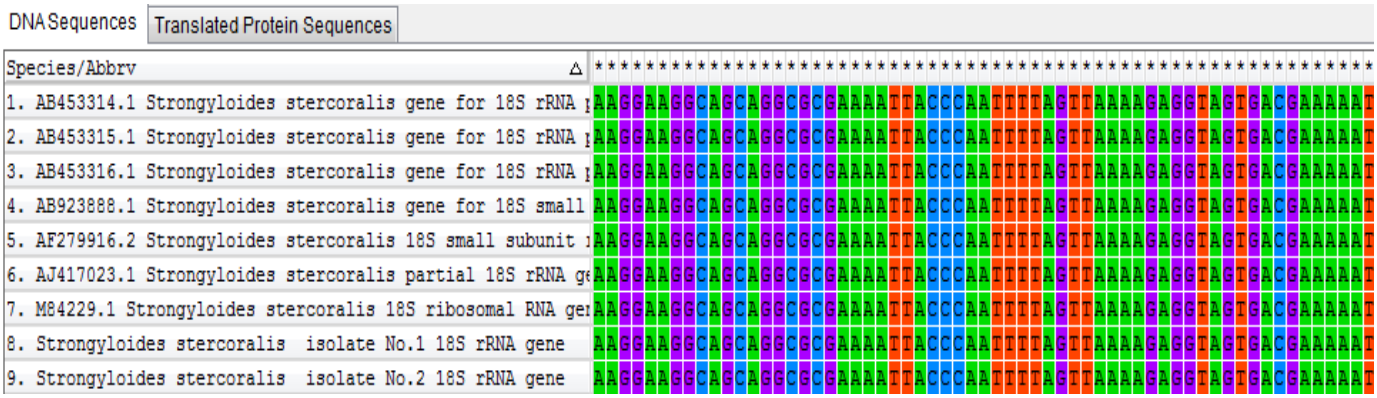


Figure (3-17): Phylogenetic tree analysis based on the partial sequence of 18S ribosomal rRNA gene in local *Enterobius vermicularis* isolates that used for genetic comfirmative detection analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA species genetic type (MEGA 6.0 version). The local *Ascaris lumbricoides* No.1-No.2 isolates were show closed related to NCBI-Blast *Enterobius vermicularis* (HQ646164.1) at total genetic change (0.1-0.4).

Table (3-22): **NCBI BLAST Homology sequence identity**

Local isolates	Genbank accession numbers	NCBI-BLAST Homology sequence identity (%)	
		Genbank isolates	Identity
***Enterobius vermicularis* No.1**	**MH893634**	**HQ646164.1**	**100%**
***Enterobius vermicularis* No.2**	**MH893635**	**HQ646164.1**	**100%**



Figure(3-18): Multiple sequence alignment analysis of the partial 18S ribosomal rRNA gene sequence in local *Strongyloides stercoralis* isolates and NCBI-Genbank *Strongyloides stercoralis* based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis similarity (*) and differences in 18 ribosomal rRNA gene nucleotide sequences.

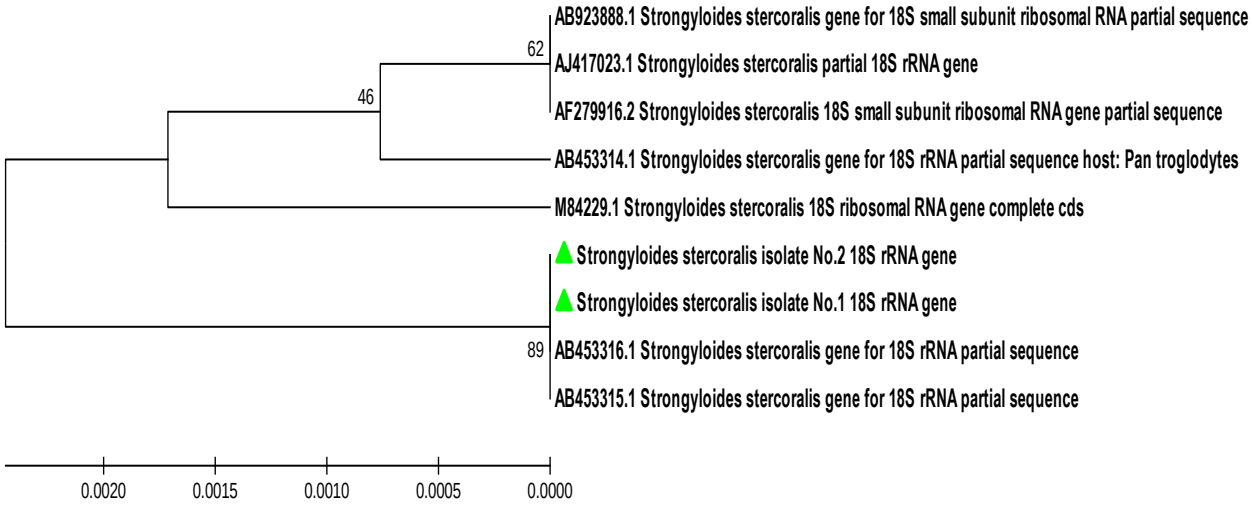


Figure (3-19): Phylogenetic tree analysis based on the partial sequence of 18S ribosomal rRNA gene in local *Strongyloides stercoralis* isolates that used for genetic comfirmative detection analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA species genetic type (MEGA 6.0 version). The local *Strongyloides stercoralis* No.1-No.2 isolates were show closed related to NCBI-Blast *Strongyloides stercoralis* (AB453316.1) at total genetic change (0.1-0.4).

Table (3-23): **NCBI BLAST Homology sequence identity**

Local isolates	Genbank accession numbers	NCBI-BLAST Homology sequence identity (%)	
		Genbank isolates	Identity
***Strongyloides stercoralis* No.1**	**MH893636**	**AB453316.1**	**100%**
***Strongyloides stercoralis* No.2**	**MH893637**	**AB453316.1**	**100%**

4.1 Overview:

Following the discovery of microscopical organisms as causes of diseases, nowadays known as infectious diseases, people worldwide has gain growing attitude to keep clean and personal hygiene has become the rule to avoid the acquisition of such pathogens. Therefore, during the last 50 years, children have been taught to wash their hands to avoid transmission of microbial agents to their bodies aiming at preventing infectious disorders. The use of excess antiseptics also provided opportunity to housewives to keep their home environment as clean as possible and nearly devoid of microbial agents. Thanks to the inventions of vaccines and anti-microbial agents that dramatically reduced the rate of infectious disorders. The big picture in the developed world can be summarized by two main trends. The first tread is that infectious disorders such as mumps, rubella, T.B, pneumonia, meningitis, etc., have reached very low incidence rates in these developed countries; the second trend however; on the other hand is that a number of disorders such type 1 diabetes mellitus, hay fever, celiac disease, asthma, Crohn's disease and ulcerative colitis have witnessed marked increase in incidence rate particularly when compared with their incidence rates in developing countries. It has been suggested that early "abstinence" of children immune system from exposure to a threshold of various microbial agents has resulted in malfunction immune system with subsequent rise in the incidence rate of auto-immune as well as allergic disorders. This hypothesis is nowadays very well-known under the title excessive hygiene hypothesis (Scudellari, 2017).

Epidemiologic studies have shown that the prevalence rates of idiopathic inflammatory bowel disease, Crohn's disease and ulcerative colitis, are higher in developed countries such as USA and Western Europe than in underdeveloped and developing countries. On the other hand, the prevalence rate of parasitic infestation

with round worms, helminthes, is significantly lower in developed countries in comparison with developing countries. Based on these epidemiologic data, a number of authors has suggested a link between high incidence rate of Crohn's disease and ulcerative colitis and low incidence rate of helminthes infestation in developed countries and has hypothesized that under exposure to children in their early lives to helminthes infestation resulted in maldevelopment of their immune system with subsequent predisposition to autoimmunity that may manifest itself in the form of either Crohn's disease of ulcerative colitis (Sýkora *et al*., 2018; M'Koma, 2013).

However; because of the lack of clear consensus about this suggestion and because of the high prevalence of helminthes infestation in our community in Iraq (Saheb *et al*., 2017), the present study was planned and conducted to investigate the possible association among the immune system function, the prevalence of helminth infestation and the prevalence and pathogenesis of idiopathic inflammatory bowel diseases.

4.2 Demographic characteristics of patients with inflammatory bowel disease

The present study showed that the mean age of patients with IBD (ulcerative colitis and Crohn's disease) was 36.68 years and the range was from 12 to 63 years. This finding agrees to the findings of several other studies which described a mean age ranging from 35 to 37 (Prelipcean *et al*., 2014); this study also agree with Velonias *et al.* (2017), who found that the mean age of majority of patients with ulcerative colitis and Crohn's disease was 36 years. In the current study, majority of cases were male patients and female patients constituted a minority, 75 % versus 24 %. This finding agrees with Baars *et al*., (2012), who found the majority of patients with inflammatory bowel disease were males (64 %); also agrees with the finding of Yang *et al*., (2014) who found that male patients constituted 66.7 % out

of all patients with Crohn's disease; however, the result of the current study disagree with Gasparini *et al*., (2018) who found that inflammatory bowel disease was more common in female patients (59.7 %). On the other hand, some authors described no sex predilection for inflammatory disease (Shah *et al*., 2018). Collectively, the results of the present study, in addition to previous studies indicated the lack of clear consensus on sex predilection of inflammatory disease; this may explain the absence of factors related to either gender in the predisposition to inflammatory bowel disease, namely hormonal influences.

In the current study, majority of cases with inflammatory bowel disease were urban areas in comparison with rural areas, 68 % versus 32 %. This result agrees with the majority of studies as Soon *et al*. in 2012 described 40 studies in their meta-analysis that support the finding that both ulcerative colitis and Crohn's disease are more common in urban than in rural areas. Increased urbanization is one hypothesis for the rising incidence of IBD. Urban residence is associated with higher incidence of both Crohn's disease (CD) and ulcerative colitis (UC) (Benchimol *et al*., 2017). The mechanism by which rurality protects against IBD is uncertain, and may include dietary and lifestyle factors, environmental exposures, or segregation of individuals with different genetic risk profiles (Benchimol *et al*., 2017). However, the higher prevalence rate of helminthes infestation in early childhood in individuals with rural inhabitant in comparison with those linning in urban areas may partly explain this protective effect through the immune modulation effect of these parasites .

The current study showed no significant association between smoking and inflammatory bowel disease since rates of smoking were 14 % versus 12 % in IBD patients and control group respectively. The finding of this study agrees with Ng *et al*. (2015) who found no significant association with smoking whereas it disagrees

with Wang et al., (2018) who found that smoking increases the risk of Crohn's disease. Several other studies have assessed the association between smoking and inflammatory bowel disease; however, the results were controversial (Niu *et al*., 2016; Moon *et al*., 2014; Reif *et al*., 2000).

In the present study the prevalence rate of positive family history was 16 %; however, there was no significant association between the IBD and family history of IBD. In agreement with the present study, it was found the rate of positive family history in patients with IBD approaches 13 % (Childers *et al*., 2014). Some authors found no significant association between family history of IBD and occurrence of inflammatory bowel disease (Gupta *et al*., 2017; Chung *et al*., 2014), in agreement with the findings of the current study. However, some studies found significant association between inflammatory disease and positive family history (Torres *et al*., 2016).

4.3 The sensitivity and specificity of conventional light microscopic examination in comparison with conventional PCR in identification of parasitic helminthes infestation

In the present study, conventional PCR was more sensitive in detecting parasite infestation than conventional light microscopy; however, the specificity of light microscopy was 100 %. Moreover, the sensitivity of light microscopy was 83.3 %, 88.2 % and 50.0 % for *A. lumbricoides*, *E. vermicularis and S. stercoralis*, respectively. These findings agrees with O'Connell and Nutman (2016) who found that for *A. lumbricoides*, microscopy had a sensitivity of 70–88% while molecular tests had a sensitivity of 85–100%; for *S. stercoralis*, sensitivity of microscopy was 16–50%.

4.4 The distribution of patients and control subjects according to parasitic infestation

The current study has shown that apparently healthy control individuals have higher rates of round helminthes infestation than patients with inflammatory bowel disease since *Ascaris lumbricoides* was seen in 12 % and 26 % of patients and control subjects, respectively, that is it is more common in control subjects than in patients with IBD; *Enterobius vermicularis* was seen in 34 % and 50 % of patients and control subjects, respectively, that is it is more common in control subjects than in patients with IBD; *Strongyloides stercoralis* was seen in 28 % and 38 % of patients and control subjects, respectively, that is it is more common in control subjects than in patients with IBD. In addition, we found that the total burden of parasite was significantly higher in control subjects than in patients with IBD.

These findings suggested a protective role for helminthes against development of inflammatory bowel disease. The authors of the current study were able to find that multiple parasitic infestations were significantly protective against both ulcerative colitis and Crohn's disease with an estimated odds ratio of 0.42 (95 % confidence interval of 0.18-097). Similar observations have been recorded by a number of cross sectional studies. In a case control study from South Africa, childhood exposure to helminthes was protective against both CD and UC development (adjusted OR of 0.2 [95% CI 0.1–0.4] for CD and adjusted OR of 0.2 [95% CI 0.1–0.6] for UC) (Chu *et al*., 2013).

It is known that environmental factors, including helminthes exposure and smoking, show close association with the risk of developing IBD, (Cosnes *et al*., 2011; Weinstock and Elliott, 2009) despite 18.8% and 50% of contribution from genes to UC and CD, respectively (Halfvarson *et al*., 2003). For instance, in sub-Saharan Africa where helminthes of intestinal infestation were frequent, the

prevalence of IBD was surprisingly low in these local black populations; however, the incidence of these diseases is approaching to white populations when African people immigrated in USA and UK, which cannot be explained only by genetic factors (Fiasse and Latinne, 2006). In other words, helminthes are seemed to be inversely associated with the development of IBD. So far and in view of these data obtained from the present study, supported by the cross sectional study of Chu *et al*. (2013), the hypothesis that helminthes infestation is protective against inflammatory bowel disease, namely ulcerative colitis and Crohn's disease becomes epidemiologically supported.

Ten clinical trials indicate that controlled, low-dose helminthic therapy is safe in IBD and related GIT diseases, with some trials showing statistically significant efficacy at endpoint. In 2003, an open-label phase 1 trial examined safety by exposing CD and UC patients to pig whipworm ova (Summers *et al*., 2003). Four patients with active CD and three patients with UC were given a single oral dose of live eggs. Patients were routinely monitored using multiple disease and quality of life indexes over a period of 12 weeks. The trial found that all patients improved clinically without any adverse events. While patients improved for a mean duration of approximately 8 weeks, three patients experienced remission relapse 12 weeks after single helminthic therapy. The study suggested that multiple doses may be required to prolong the benefit of treatment. The study also found that there were no significant clinical complications when patients received multiple doses of live eggs at 3-week intervals for 30 weeks. The group followed up with a placebo-controlled trial of 54 UC patients. The pig whipworm arm received an oral dose of live ova at 3-week intervals for 12 weeks (Summers et al., 2005). Again, whipworm therapy produced no adverse events. Between the treatment and placebo groups, statistically significant efficacy was observed at 12 weeks in two separate indices in *post hoc* analysis. One limitation of pig

whipworm therapy is that humans are not the natural host and repeated dosing is required to maintain ongoing infection. In addition, given the larvae are invasive, site of infection is unpredictable with potential migration into the lymphatics and/or small blood vessels (Van Kruiningen and West, 2005). The problems of repeated inoculation and unpredictable migration motivated an alternative modality. In 2006, a proof-of-concept study explored human hookworm for the treatment of CD (Croese *et al*., 2006). While both hookworm and whipworm possess parasite lifecycles that require development in the external environment and therefore unable to proliferate directly in the host; the hookworm is adapted to survive in humans and establish a chronic infection that can last for years from a single inoculation. This makes human hookworm an attractive therapeutic, as a defined dose can be controlled and eliminated *via* anthelmintic therapy (Mortimer *et al*., 2006). CD patients with longstanding but mostly inactive disease were inoculated with 25 or 50 live hookworm larvae in an initial and reinoculation trial. Disease index for CD patients was unchanged until week 17. After 20 weeks, clinical scores improved and five patients were in remission at week 45.

Two recent human hookworm clinical trials explored the safety and efficacy of hookworm therapy in celiac disease (Croese *et al*., 2015; Daveson *et al*., 2011). The first double-blind, placebo-controlled study inoculated patients twice with 15 live hookworm larvae followed by an aggressive oral gluten challenge after patient intestinal infection was established (Daveson *et al*., 2011). Experimental infection proved to be safe but did not result in clinical benefit following gluten challenge. Interestingly, follow-up immunological analysis found that hookworm infection altered cellular immunity (McSorley *et al*., 2011), through decreasing basal levels of IFNγ and IL-17 in the intestine and altering $CD4^+$ T cell immunity both in the intestine and, interestingly the circulatory system. The second study combined live hookworm larvae inoculation (20 larvae per individual) with desensitization,

specifically a sustained gluten micro challenge (Croese *et al*., 2015). Of note, no uninfected controls were used in the study. Escalating gluten challenges were well tolerated and resulted in stabilization or improvement across all tested indices of gluten toxicity. IFNγ-producing intestinal T cells were observed to decrease, while Treg numbers in the epithelium increased significantly.

Although, the present study has no experimental data, the authors believes that randomized control clinical trials on human volunteers is justified in the present time in view of the above mentioned data.

4.5 Distribution of patients and control group according to parasite infestation by residency (urban versus rural)

In the current study, Evidence of round worm infestation was seen in a total of 62 out of 100 individuals enrolled in the current study; regarding control group, in rural area the prevalence rate of round worm infestation was 77.1 % whereas in urban areas the prevalence rate of round worm infestation was 60.0 %; the difference was statistically insignificant (P = 0.372). In one study, it was found that the prevalence rate of helminthes infestation was more common in urban than in rural area, supporting the finding of the present study (Phiri *et al*., 2000); but the difference was significant; however, in another study, the difference in prevalence rate between rural and urban areas was statistically insignificant (Nkurunungi *et al*., 2019); this agrees with the current study. On the other hand, the prevalence rate of helminthes infestation was significantly higher in rural than in urban areas in some studies (Oninla *et al*., 2007). So far, the result of the present study and previous studies showed no clear consensus about the association between urbanization and the risk of helminthes infestation and that the prevalence rate may be affected by factors other than the residency such as hygiene, cultural differences, sanitation and other unknown factors.

4.6 Serum interleukin levels in patients and control subjects and immunological role associated with helminthes infestation

In the present study, serum level of IL-10 was significantly lower in patients with CD than both control group and UC group ($P < 0.05$). Moreover, there was no significant difference in the serum level of IL-10 between patients with UC and control group ($P > 0.05$). The current study has shown that patients with concomitant inflammatory bowel disease and parasitic infestation have well defined immune modulation response when compared to those patients with inflammatory bowel disease and devoid of parasitic infestation. The immune modulation was that the level of anti-inflammatory interleukin 10 was higher and the level of the pro-inflammatory interleukin 1 beta was lower favoring immune suppression.

Immune regulation by parasites compromises immunity but also protects the host from damaging immunopathological reactions to the presence of parasites (McSorley and Maizels, 2012).In gastrointestinal nematode infections, particularly infections by the highly prevalent *Ascaris*, hookworm, and *Trichuris* species, the pathology is less intense, and long-term infestation is common. Infection is associated with a regulatory set of cells and cytokines, as IL-10 and TGF-β are significantly linked with hypo responsiveness and susceptibility (Figueiredo *et al*., 2010; Turner JD, *et al*. 2008).

In support for the current study observation, Turnner *et al*. in 2008 have shown enhanced production of the anti-inflammatory cytokines IL-10 and TGF-β1. Turnner et al. (2008) have demonstrated that constitutive levels of the regulatory cytokines IL-10 and TGF-β1 are enhanced in direct relation to the intestinal worm burden and provide evidence that this elevation in anti-inflammatory cytokine secretion in peripheral blood induces immunological hypo responsiveness. This observation is striking when considering the plethora of other microorganisms

encountered and the many other environmental, dietary, and lifestyle factors that are typically varied in the setting of a natural human infection, all of which could potentially induce or suppress IL-10 and/or TGF-β1 output. We infer from our data, therefore, that chronic gut-worm infection plays an important role in driving human immunoregulatory networks.Thus,findings provide a mechanism for the observed lower incidence of autoimmune disease, allergies, and asthma in communities where gut worms are endemic (Stene and Nafstad, 2001; ISAAC, 1998).

It has been hypothesized that worm-driven immunoregulatory networks, exemplified by the induction of IL-10 and or TGF-β secreting T_{reg} cells, represent a parasite survival strategy that enables suppression of an effective immune response (Maizels *et al*., 2004). Gut helminthes are generally considered to benefit from the modulation of Th2-like responses because data generated in model systems clearly demonstrate that Th2 cytokines drive effector mechanisms at the site of infection that lead to parasite expulsion (Cliffe *et al*., 2005). Furthermore, we and others have observed that Th2 cytokines and antibody responses linked to Th2 activity are inversely associated with human intestinal helminthes infection (Turner et al., 2003) and with reinfection following chemotherapeutic intervention (Jackson *et al*., 2004). Our data are consistent with this hypothesis; accumulations of secreted IL-10 and TGF-β1 are inversely associated with Th2 (IL-4) recall responses to parasite antigens. However, in the case of TGF-β1, we have concluded that this suppressive cytokine is also associated with diminished Th1 responsiveness to bacterial antigen and a nonspecific stimulus. This may be a consequence of gut worm–driven regulatory activity spilling over onto unrelated adaptive cellular responses in heavily polyparasitized individuals. Alternatively, given that TGF-β1 expression increases during wound healing and has thus been implicated in the regulation of such responses (Kulkarni *et al*., 2002), the suppression of Th1

responses by TGF-β may be a consequence of tissue repair responses to damage of the gut mucosa, liver, or lungs by heavy worm infection and continuous larval reinvasion. A further possibility is that enteric bacterial infections triggered by worm-mediated disruption of the gut mucosa might also up-regulate counter inflammatory cytokine expression.

Dissection of the mechanism by which gut worms promote secretion of IL-10 and TGF-β1 is valuable in understanding why allergies and autoimmunity are increasing in the developed world and may present new avenues for treating or preventing such disorders. In fact, gut worms have already been demonstrated to have therapeutic value in the treatment of inflammatory bowel disease (Thompson and Weinstock, 2005). Furthermore, considering the frequency with which intestinal worm infections occur in humans (approximately 1 billion people are infected with at least 1 species) (Bethony *et al*., 2006) and their overlapping geographical distribution with devastating diseases (Borkow *et al*., 2000).

Since the etiology of IBD is still unknown and causative therapies aren't available, the patients 'exposure to helminthes appeared to be a novel and promising approach in the treatment of colitis. During helminthes infection, pronounced Th2 immune responses as well as an activation of B cells, basophils, mast cells, dendritic cells, and eosinophil are evoked in the host to control and expel the parasites. By the expansion of regulatory cells such as alternatively activated macrophages, $CD4^+$ and $CD8^+$ Tregs or regulatory B cells, and the consequent induction of anti-inflammatory cytokines, e.g. IL-10 or TGF-β, helminthes constitute immunoregulatory conditions to ensure their survival (Allen and Maizels, 2011). This immunomodulatory state was suggested to limit intestinal inflammation in IBD. However, utilization of helminthes in different human

studies and animal experiments of colitis highlighted controversial results. First results of clinical trials of *Trichuris suis* ova (TSO) therapy in UC and Crohn`s disease patients showed a reduction of the disease activity index (Summers et al., 2005a; Summers et al., 2005b).

In the present study it was shown that the level of the pro-inflammatory cytokine IL-1 beta was lower in patients with parasite infestation. The mechanism of this may be attributable to the increased level of the anti-inflammatory IL-10. The exact mechanism of the interaction between these two cytokines has been extensively studies by a number of authors and the down regulation of IL-1 beta mediated by TL-10 has been shown in a number of experimental studies (Jenkins *et al*., 1994).

The current study also contributed to some extent in the explanation of the varying severity of inflammation between ulcerative colitis and Crohn's diseases. It is well documented that Ulcerative colitis causes an inflammation that is mainly seen in the mucosa and sub-mucosa whereas, Crohn's disease causes more sever inflammation that involves all intestinal layers starting form mucosa reaching the serosa, termed by pathologist as transmural inflammation. In the current study, the serum level of IL-10, anti-inflammatory cytokine, was higher, and the level of IL-1 beta, pro-inflammatory cytokine, was lower, in ulcerative colitis than in Crohn's disease. This may provide clues to the different pathogenic pathways involved in the two inflammatory bowel disorders, as well as provide some explanation for less sever, and limited inflammatory response in ulcerative colitis in comparison with Crohn's disease. To the best of our knowledge, this is the first study that has brought insight toward the role of variation in cytokine level expression in relation to severity of intestinal inflammation.

4.7 The role of *NOD2* genotype polymorphism in the pathogenesis of inflammatory bowel disease

The current study has shown no role for *NOD2* genotype polymorphism in the pathogenesis of inflammatory bowel disease. Nucleotide-binding oligomerization domain1/ caspase activation recruitment domain 4 *(NOD2/CARD15)* is a member of the Nod-like receptor family, which is phylogenetically conserved (Fritz *et al.*, 2006). It is constitutively expressed in epithelial cells throughout the gastrointestinal tract (Hysi *et al.*, 2005). *NOD2/CARD4*15contains leucine-rich repeat (LRR) domain and NOD domains and has only one CARD domain (Ting et al., 2006).

Polymorphism in LRR domain of the *NOD2/CARD15* gene showed association with disease severity of UC in North Indian patients. This might be due to disruption of the LRR region critical for NOD1-mediated bacterial sensing. Haplotype-based approach showed that GTTG haplotype carriers were over represented in UC patients which could increase the risk of the disease (Verma *et al*., 2012).

Initially, it was suggested that there is association of the deletion variant of *NOD2/CARD15* +32656 (complex intronic insertion-deletion polymorphism) with susceptibility to IBD using a combination of transmission disequilibrium testing (TDT) and case-control analysis (McGovern *et al*., 2005). However this variant was not associated with a strong effect on susceptibility to IBD in children and adults in a Northern Europe study cohort (Van Limbergen *et al*., 2007). Similar results have been found in the East Anglia IBD cohort, where no association was found between *NOD1* +32656 and IBD and also no heterogeneity between UC and CD (Tremelling *et al*., 2006).

4.8 DNA Sequence results:

The DNA sequencing analysis of 18S ribosomal RNA intergenic spacer, partial sequence gene in two isolates of some positive gastrointestinal helminthes parasites isolates (*Ascaris lumbricoides,Enterobius vermicularis, and Strongyloides stercoralis*) were showed closed related to NCBI-Blast (*Ascaris lumbricoides , Enterobius vermicularis, and Strongyloides stercoralis*) in Genbank accession number (M74584.1, HQ646164.1, and AB453316.1) respectively. According to phylogenetic tree analysis using (Mega 6.0).

The Homology sequence identity between local gastrointestinal helminthes parasites isolates (*Ascaris lumbricoides , Enterobius vermicularis, and Strongyloides stercoralis)* and NCBI BLAST *Ascaris lumbricoides , Enterobius vermicularis, and Strongyloides stercoralis* isolates were showed (99-100%) homology identity by using (NCBI-BLAST analysis).

Sequencing of the internal transcribed spacers ITS 1 IN 18S ribosomal RNA gene provides the best reliability in detection of close phylogenetic distances. This method generally is used for accurate confirmative detection with related taxon (Egger et al. 1993).

The phylogenetic tree analysis was concluded using the UPGMA method (Sneath and Sokal , 1973). The UPGMA tree is the simplest method for constructing trees that depend on rate of mutations is constant over time and for all lineages in the tree. This is called a 'molecular clock hypothesis . The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura and Kumar , 2004). are recorded the units of the number of base substitutions per site in final dataset of Evolutionary analyses were conducted in MEGA6 Tamura K., **(**Stecher et al., 2013).

Conclusions

1. The present study provided significant observation evidence that infestation with round worm (helminthes) provided protection against acquisition of inflammatory bowel disease.
2. The current study also showed immunological evidence that helminthes can modulate host immune response to prevent and minimize the inflammatory response in cases of IBD through increasing the level of anti-inflammatory cytokines and decreasing the level of pro-inflammatory cytokines.
3. The combined infestation with multiple intestinal helminthes has a better protective role than single parasite in protection against inflammatory bowel disease.
4. The genetic polymorphism concerning NOD2 gene plays no role in the etiology and immune response associating inflammatory bowel disease both Crohn's disease and ulcerative colitis.
5. The best method for diagnosis of helminthes infestation in terms of sensitivity and specificity was conventional PCR.

Recommendations

1. A randomized control clinical trial is advised to be carried on volunteers already affected by inflammatory disease through induced helminthes infestation to assess the role of this approach as a mode of treatment.
2. A larger sample size multi-centric study for longer duration is advised to validate the results of the current study.
3. Experimental studies on lab animals can be carried out to see the effect of induced helminthes infestation on chemically induced colitis.
4. Applying same study with an objective to assess the correlation among helminthes infestation, inflammatory bowel disease and other cytokines such as IL-4, IL-6 and TNF-alpha.
5. Study the association between helminthes infestation and colorectal carcinoma through case control or longitudinal studies.
6. Study the controversial role of helminthes in protection against ischemic heart disease by a case control or a cohort study.

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