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AMOEBIASIS: DIAGNOSIS AND PREVALENCE IN LEÓN NICARAGUA

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To Rosy, Byron Jr. and Heykel

ABSTRACT

About ten yearly cases of liver abscess are presented in University Hospital, León, Nicaragua. Based on serology most of them have been shown to be amoebiasis cases. This raised the question of *Entamoeba histolytica* prevalence in the population. Based on microscopy of stool specimens and serology, about 20% prevalence was found. With the new realization that pathogenic *E. histolytica* is morphologically indistinguishable from some common apathogenic species such as *E. dispar* (cysts detected by microscopy are reported as *E. histolytica*/*E. dispar*), the aim was to determine the true prevalence of *E. histolytica* using various tests designed to differentiate between pathogenic and apathogenic species.

In 480 apparently healthy individuals, the prevalence of *E. histolytica*/*E. dispar* was 12% (58/480) as determined by microscopy. Out of these 58 stool samples an *E. histolytica* specific PCR was positive in 5%; thus the prevalence *E. histolytica* was 0.6%.

In a group of 134 diarrhea patients, the most common finding was *E. histolytica*/*E. dispar* (24%) at the health center laboratory level. In the Microbiology Department *E. histolytica*/*E. dispar* was found only in 4.5%. With the Triage Parasite test, only one case of *E. histolytica*/*E. dispar* was found. By PCR, *E. dispar* was recognized in 10 (7.5%) and *E. histolytica* in two cases (1.5%). Over diagnosis was also confirmed in a quality control study where León health centers were examining 10 different stool samples. We found that the health center technicians continue to mix up *E. histolytica*/*E. dispar* with other amoebas. The consequences of the apparent widespread over diagnosis of *E. histolytica* were studied retrospectively in 100 records of patients with intestinal symptoms.

We found that all patients received treatment with metronidazole or related drugs. In 41% these treatments were not based on any laboratory findings at all. In 32 % *E. histolytica*/*E. dispar* were found. Other parasites (*Entamoeba coli*, *Giardia intestinalis*, *Endolimax nana*, *Enterobius. vermicularis*, *Iodamoeba bütschlii*) were seen in 27%.

To explain the high seroprevalence of anti-*E. histolytica* antibodies in sero-epidemiological surveys we considered two possibilities: cross reactivity due to the common intestinal apathogenic *E. dispar* and antibodies to ubiquitous free-living environmental amoebas. A study was undertaken to identify environmental amoebas and to determine cross-reactivity using antibodies from amoebiasis patients. Amoebas isolated from environmental water samples were characterized by morphological and immunohistochemical methods. In fresh water *Acanthamoeba* spp. were found in 21 %. Fifty three percent of tested wells in the geothermal area contained thermotolerant amoeboflagellates. *Naegleria* spp. was identified in 24 out of 39 (62 %) of isolated amoeboflagellates. Absorption studies did not support the idea that environmental free-living amoebas induce antibodies cross-reacting with *E. histolytica*. Antigenic cross reactivity between *E. dispar* and *E. histolytica* remains a possible explanation for the high seroprevalence in the population. This is supported by the observation that IF antibody titers in sera from healthy individuals are similar with both antigens. Also the ratio of antibody reactivity was similar when measured by a densitometric method. In contrast, sera from patients with invasive amoebiasis reacted preferentially with *E. histolytica*.

We conclude that amoebiasis is not a major problem in the community. Over-diagnosis and over-treatment of diarrhea patients thought to suffer from amoebiasis are serious problems. Thus there is an urgent need for education and quality assessment.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

- I. **Leiva, B.**, Lebbad, M., Téllez A., Winiiecka-Krusnell, J. Altamirano, I. and Linder E. **Overdiagnosis of *Entamoeba histolytica* and *Entamoeba dispar* in Nicaragua: A Microscopic, Triage Parasite Panel and PCR Study.** Archives of Medical Research 37 (2006) 529–534.
- II. **Leiva, B.**, Clasdorfer, E., Linder E. and Winiiecka-Krusnell J. **Free-living *Acanthamoeba* and *Naegleria* spp. amebae in water sources of León, Nicaragua.** Rev. Biol. Trop. 56 (2008): 439-446.
- III. **Leiva, B.**, Lebbad, M., Winiiecka-Krusnell, J., Argueñal, H. and Linder, E. **Infection with intestinal parasites and their relation to poverty in León, Nicaragua.** (Submitted)
- IV. **Leiva B.**, Winiiecka-Krusnell, J. and Linder E. **Amoebiasis in León, Nicaragua: over-diagnosis and over-treatment.** (Submitted)

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LIST OF ABBREVIATIONS

ALA	Amebic liver abscess patients
Co A	Concanavalin A
CNS	Central nervous system
DNA	Deoxy nucleic acid
ELISA	Enzyme-linked immunosorbent assay
FISH	Fluorescent in situ hybridization
Gal/GalNAc	N-acetyl-D-galactosamine
GAE	Granulomatous amebic encephalitis
GPI	Glucosephosphate isomerase
IFAT	Indirect fluorescent antibody test
K	Kappa value
Kd	Kilo Daltons
NPsp	Non pathogenic species
Nf-Pab	anti <i>N. fowleri</i> / <i>N. lovansensis</i> polyclonal antibodies
Nf-5D12u	anti- <i>N. fowleri</i> mouse monoclonal antibodies
Psp	Pathogenic species
PAHO	Pan-American Health Organization
PAM	Primary amoebic meningoencephalitis
PCR	Polymerase chain reaction
PGM	Phosphoglucomutase
QIAamp	Tissue kit spin columns
rRNA	Ribosomal ribonucleic acid
SAF	Sodium acetate-acetic acid-formalin
SREHP	Serin rich <i>E. histolytica</i> protein
ssrRNA	Small subunit ribosomal RNA
TPP	Triage parasite panel
WHO	World Health Organization

1 INTRODUCTION

1.1 *ENTAMOEBA HISTOLYTICA*

1.1.1 Overview

The clinical symptoms of amoebiasis caused by the protozoan parasites *Entamoeba histolytica* ranges from asymptomatic colonization to amoebic dysentery and invasive extra intestinal amoebiasis is most common in the form of liver abscess (Tanyuksel and Petri 2003). Invasive amoebiasis is one of the world's most prevalent and fatal infectious diseases. Primarily it is a problem of the developing world; around 500 million people are infected world wide while 75,000 die of the disease annually. Behind malaria and schistosomiasis it ranks third on the list of parasitic causes of death worldwide (Walsh 1986; Li and Stanley 1996). *E. histolytica* and *E. dispar* are currently recognized as distinct species, mostly based on genetic, biochemical, and immunological studies. It is therefore possible to obtain more reliable and correct epidemiological data using molecular, biochemical, and immunological features, and these allow better diagnosis and treatment (Diamond and Clark 1993).

Although only a minority of *E. histolytica* infections progress to develop intestinal diseases, such as diarrhea or dysentery, or extra intestinal diseases like amoebic liver abscess (ALA), the basis for this difference in clinical outcome remains unsolved. A recent report suggests that the parasite genotype plays a role in determining outcome of infection by *E. histolytica* (Ali, Mondal et al. 2007). Therefore, one of the current priorities in a post genomic era is to understand the genomic factors determining the outcome of an *E. histolytica* infection (Ali, Clark et al. 2008).

1.1.2 History

An excellent review at history of amoebiasis have been published by Jonathan I Ravdin (Ravdin 2000). In 1875, the Russian physician Fedor Lösch identified what he believed to be the causative agent (motile amoebas containing erythrocytes which he named *Amoeba coli*) of a case of dysentery, but he doubted its lone role in pathogenesis when it failed to produce disease in three of four dogs experimentally inoculated with it. In 1893 the German pair Quincke and Roos identified an important mode of transmission, when they described 15 diseased patients who all shared the same drinking water source (Guarner 1990).

In 1925, Brumpt proposed that humans can be infected by two morphologically identical species of *Entamoeba* producing quadrinucleate cysts measuring 10 µm or greater in diameter; the pathogenic organism was identified as *E. dysenteriae* and Brumpt named the non-pathogenic one *E. dispar*. It took almost 70 years and the advent of modern biochemical techniques to re-establish Brumpt's idea (Diamond and Clark 1993).

In 1973, pathogenic (P) and nonpathogenic (NP) species were differentiated by their relative agglutinability with the lectin Con A (Martinez-Palomo, Gonzalez-Robles et al. 1973). This was followed in 1978 by the discovery that pathogenicity could also be

correlated the enzymatic profile of the organism. Sargeant and colleagues used thin-layer starch-gel electrophoresis to separate isoenzyme variants of the glycolytic enzymes glucosephosphate isomerase (GPI), phosphoglucomutase (PGM) and L-malate:NADP oxidoreductase and classified 85 stocks of *E. histolytica* in four groups according to their enzyme profile. Enzyme Group II, which contained a faster migrating band of PGM, was found in all cases of clinical amoebiasis, but it was not found in any isolated obtained from asymptomatic individuals, although Sargeant pointed out that in a larger study such persons (in a preclinical state of disease) may be found (Lucas and Upcroft 2001). Diamond and Clark presented a host of data to confirm the existence of *E. histolytica* and *E. dispar*, and to honor the hypothesis put forward by Brumpt (Diamond and Clark 1993). Biochemically, they cited thousands of samples which correlated zymodeme class to pathogenicity (Ravdin 2000; Lucas and Upcroft 2001).

1.1.3 Biology of the parasite

E. histolytica exists in two forms: a motile and invasive trophozoite and an infective cyst.

The trophozoite varies in size; from 12 to 30 μm , although occasionally very large forms up to 90 μm may be seen. The pseudopods produced by an actively moving trophozoite are broad and finger-like and the parasite generally moves in one direction at a time. The nucleus is spherical and has a delicate nuclear membrane.

The cyst initially has a single nucleus but finally has 4 nuclei. It is then known as a mature cyst. *E. histolytica* cysts are round or slightly oval hyaline bodies 10 μm – 16 μm in diameter.

Life cycle. There are four stages in the life cycle of *E. histolytica*: the trophozoite, precyst, cyst and metacyst. Infection is normally initiated by the ingestion of fecally contaminated water or food containing *E. histolytica* cysts. The infective cyst form of the parasite survives passage through the stomach and small intestine. Excystation occurs in the bowel lumen, where motile and potentially invasive trophozoites aggregate in the intestinal mucin layer and form new cysts, resulting in a self-limited and asymptomatic infection. The trophozoite of *E. histolytica* can convert to a precyst form with a nucleus, and this form matures into a tetra-nucleated cyst as it migrates down and out the colon. In some cases, however, adherence to and lysis of the colonic epithelium, mediated by the galactose and N-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin, initiates invasion of the colon by the trophozoites (Petri 2002). Once the intestinal epithelium is invaded, extra intestinal spread to the peritoneum, liver, and other sites may follow. Factors controlling invasion, as opposed to encystations, most likely include parasites “quorum sensing” signaled by the (Gal/GalNAc)-specific lectin, interaction of amoebas with the bacterial flora of the intestine, and innate and acquired immune responses of the host (Haque, Mondal et al. 2003; Tanyuksel and Petri 2003).

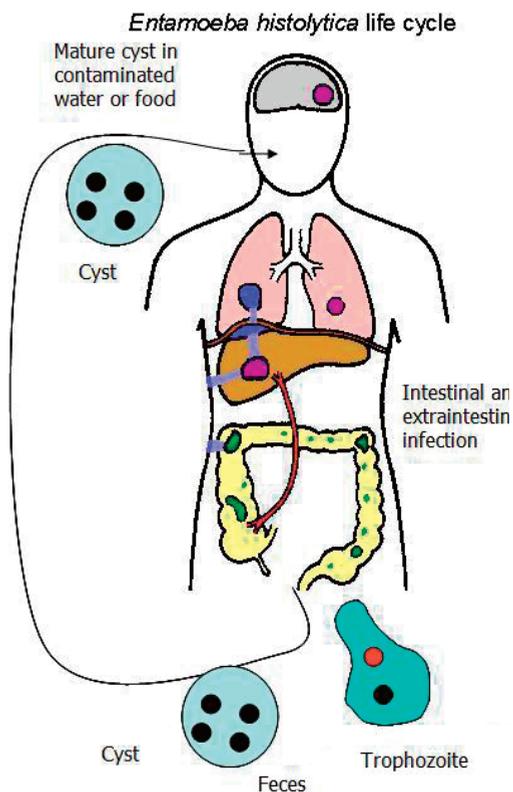


Figure 1

Life cycle of *Entamoeba histolytica*

Humans become infected by the oral route by the encysted parasite. Excystation takes place in the intestinal tract and causes local symptoms like diarrhea. The liberated trophozoite form may also invade the intestinal wall and extraintestinal tissues of the host. Severe disease is caused by tissue destruction e.g. in the liver and brain.

1.2 EPIDEMIOLOGY

In 1986 Walsh assessed the existing global prevalence data and concluded that in 1981, 480 million people harbored *E. histolytica* worldwide (Walsh 1986). She went on to extrapolate that 36 million develop clinically overt disease with 40-100.000 deaths per year, resulting in amoebiasis ranking third most common cause of death due to parasitic infection after malaria and shistosomiasis. As this data appeared well before the formal re-description of *E. histolytica* was published (Diamond and Clark 1993), the epidemiological estimations quoted did not distinguish between *E. dispar* and *E. histolytica*. Unfortunately, reliable epidemiological data on populations that do not have selection bias and from whom confirmed *E. histolytica* and *E. dispar* isolations have been made are rare. Such isolation studies from Durban, South Africa (Gathiram and Jackson 1985), have indicated the overall prevalence of both *E. histolytica* and *E. dispar* together to be 10%, with *E. histolytica* accounting proportionately for 1 % and *E. dispar* for 9 %. Consequently, global prevalence of *E. histolytica* could be 50 million and that of *E. dispar* 450 million. Revisions of the prevalence and incidence estimations are necessary while that of mortality would remain the same. Amoebiasis infections are endemic in most temperate and tropical climates in the developing world. In some tropical countries, antibody prevalence rates exceed 50% (Caballero-Salcedo, Viveros-Rogel et al. 1994; Tanyuksel and Petri 2003).

Epidemiology studies have showed that low socioeconomic status and unsanitary conditions are significant independent risk factor for infection. In addition people living in developing countries have a higher risk earlier age of infection than do those in developed regions. In Mexico, 11 % of the tested population aged 5 to 9 years was infected, with the prevalence of infection being higher in girls (9.34%) (Caballero-Salcedo, Viveros-Rogel et al. 1994).

The discovery of a third morphologically identical *Entamoeba* spp. further complicated our understanding of the epidemiology of *E. histolytica*. *Entamoeba moshkovskii* is another species of *Entamoeba* morphologically indistinguishable from *E. histolytica* and *E. dispar*. This species first described from Moscow sewage by Tshalaia in 1941 and was thereafter reported to occur in many different countries. *E. moshkovskii* was initially thought to be a free-living environmental strain. However, in 1961 an *E. histolytica*-like strain was isolated from a resident of Laredo, Texas. This strain was named the *E. histolytica* Laredo strain shares many biological features with *E. moshkovskii*. Both the Laredo strain and *E. moshkovskii* grow at room temperature and are resistant to emetine. These characteristics distinguished them from *E. histolytica* and *E. dispar*. Subsequent molecular studies have confirmed that the *E. histolytica* Laredo strain is a strain of *E. moshkovskii*. Although the early isolations of this species have been from sewage, recent studies have reported the recovery of *E. moshkovskii* from human feces (Fotedar, Stark et al. 2007; Ali, Clark et al. 2008; Pritt and Clark 2008).

1.3 PATHOGENESIS

The term “pathogenesis”, defined as the mechanisms involved in the initiation, evolution, and ultimate outcome of a disease process, relates to both host and parasite factors.

1.3.1 Early invasive lesion with superficial ulceration

Three main consecutive events occur at this stage: a focal superficial erosion of the mucosa, small glandular foci of micro invasion, and mild to moderate infiltration of the lamina propria.

Superficial epithelial erosion. Once the mucus barrier has been broken down *E. histolytica* reaches the luminal surface of enterocytes and initial contact produces a contact-dependent “hit-and-run” damage to the plasma membrane of effectors cells, phagocytosis, and intracellular degradation of ingested cells (Martinez-Palomo, Gonzalez-Robles et al. 1985).

1.3.2 Virulence factors

The adhesion and cytolytic events have been related to three types of molecules: lectins, amoebapores, and proteases.

1.3.2.1 *Gal-GalNac lectin.*

Excystation in the intestinal lumen produces trophozoites that use the galactose and N-acetyl-D-galactosamine (Gal/GalNac)-specific lectin to adherence to colonic mucins and thereby colonize the large intestine.

Adhesion of the parasite occurs mainly through a surface Gal/GalNac lectin which binds to exposed terminal Gal/GalNac residues of target cell glycoproteins (Petri, Chapman et al. 1989). Other molecules include a 220-kd lectin, a 112-kd adhesin and a surface lipophosphoglycan. It has been suggested that the Gal/GalNac lectin could be translocated to the basolateral surface of enterocytes before the parasite excretes its cytolytic effect, but the benefits of this action for the parasite remain unclear (Espinosa-Cantellano and Martinez-Palomo 2000).

Colitis results when the trophozoite penetrates the intestinal mucous layer, which otherwise acts as a barrier to invasion by inhibiting amoebic adherence to the underlying epithelium and by slowing trophozoites, which occurs only after the parasite lectin engages host N-acetyl-D-galactosamine on O-linked cell surface oligosaccharides (Petri 2002).

1.3.2.2 *Amoebapores.*

Secretion by the ameba of amoebapores, a 5-Kd pore forming protein, may contribute to killing. Structural modeling suggests a compact tertiary structure composed of four α -helical structures stabilized by three disulfate bonds. Thus, amoebapores are different from the much larger (65- to -70 kDa) perforins that contain three amphipathic segments, two α -helices and one β -sheet. Activation of human caspase 3, a distal effectors molecule in the apoptotic pathway, occurs rapidly after amoebic contact, and caspases are required for cell killing *in vitro* and for the formation of amoebic liver abscess *in vivo* (Huston, Houpt et al. 2000; Yan and Stanley 2001).

1.3.2.3 *Cysteine proteinase*

Interaction of the parasite with intestinal epithelium causes an inflammatory response marked by the activation of nuclear factor κ B and the secretion of lymphokines (Stanley 2001). The development of this epithelial response may depend on trophozoite virulence factors such as cysteine proteinase and leads to intestinal abnormalities through neutrophil-mediated damage. Neutrophils can also be protective, however, since activation of neutrophils or macrophages by tumor necrosis factor α or interferon γ kills amoebas *in vitro* and limits the size of amoebic liver abscess (Denis and Chadee 1989). In contrast to the intense inflammatory response typical of early invasive amoebiasis, inflammation surrounding well-established colonic ulcers and liver abscesses is minimal, given the degree of tissue damage.

In addition, other amoebic molecules such as lipophospho-peptidoglycan, perioxiredoxin, arginase, and lysine and glutamic acid-rich proteins are also implicated in the pathogenicity (Lejeune, Rybicka et al. 2009).

1.4 IMMUNITY

Immunity to infection with *E. histolytica* is associated with a mucosal IgA response against the carbohydrate-recognition domain of the Gal/GalNac lectin. Cell-mediated

responses have been described in patients with amoebic liver abscess, characterized by lymphocyte proliferation and lymphokine secretion that is amoebicidal in vitro. In patients with liver abscess, the prevalence of the class II MHC haplotype HLA-DR3 is increased by a factor of more than three, suggesting a role of CD4+ T-cell functions in the outcome of the disease (Arellano, Perez-Rodriguez et al. 1996).

1.4.1 Innate resistance mechanisms

Initial host resistance to *E. histolytica* is dependent on natural or innate resistance mechanisms prior to the development of acquired immunity. Amoebas encounter natural “barriers” to infection in both the gut and systematic circulation following tissue invasion. These innate defences may prevent or partially inhibit amoebic tissue invasion, although the level of protection afforded by innate resistance is difficult to quantify precisely. Mucins lining the colonic epithelium provide an important innate defence against tissue invasion by *E. histolytica*. Therefore, mucins serve as a first line of host defences in the gut by inhibiting amoebic attachment to the underlying mucosal epithelial cells. In the gut, other factors such as competition by intestinal bacteria for attachment to mucins may inhibit amoebic colonization (Ravdin 1989).

In the systematic circulation, *E. histolytica* is exposed to complement, another innate resistance mechanism that may act to decrease the numbers of invading amoebas. Amoebas activated both the alternative and classical complement pathways in normal serum (Calderon and Schreiber 1985).

1.4.2 T cell responses

A role for antigen-specific T cells in controlling invasive amoebiasis is evident from studies demonstrating that thymectomy or treatment with anti-T lymphocyte serum result in exacerbation of infection in animal models of the disease. T cells may participate in an immune response against *E. histolytica* in three distinct ways: 1 by directly lysing amoebas in a contact dependent manner; 2 by producing cytokines which activate macrophages (and possibly other effectors cells) for amoebicidal activity and; 3 by providing help for B cell antibody production (Ravdin 2000).

1.4.3 Humoral response

Seroepidemiologic studies indicate that 81% to 100% of patients with invasive amoebiasis develop specific circulatory antibodies against *E. histolytica* (Petri and Ravdin 1986).

Apart from serum and secretory IgA, the dominant class of antibodies produced against *E. histolytica* appears to be IgG. Both the Gal-lectin and SREHP molecules, for example, stimulated strong serum IgG responses in immunized animals. Serum IgM has been detected in human cases of intestinal and extra-intestinal disease, whereas IgE may be produced during amoebiasis but definitive reports are rare. How the humoral response relates to development of cellular mediated immunity (CMI) is unclear. Th1 and Th2-type responses are linked to production of specific antibody subclasses (Mosmann and Coffman 1989). The frequencies of different antibody subclasses in amoebiasis remain to be investigated. As part from a possible contribution to resistance

or control of infection, limited reports suggest that anti-amoebic antibodies may have deleterious consequences and exacerbate disease. Antibodies to the Gal-lectin have been shown to enhance of trophozoites to target cells, which may results in increased pathology due to elevated killing of host cells. In one study of amoebiasis patients, immune complexes, which may contribute to pathogenesis, were detected in patients colonic and liver tissue (Ravdin 2000).

1.5 CLINICAL FEATURES

1.5.1 Asymptomatic colonization

Asymptomatic infection with *E. histolytica* is defined as the presence of *E. histolytica* in stool in the absence of colitis or extra intestinal infection. Colonization with the morphologically identical parasite, *E. dispar* is three times more common in developing countries and at least 10 times more common in developed countries (Haque, Faruque et al. 1997).

1.5.2 Dysentery or colitis

Patients with amoebic colitis typically present with a several-week history of gradual onset of abdominal pain and tenderness, diarrhea, and bloody stools. In one series, patients with amoebic colitis had an average duration of pre-hospital illness of 21 days, compared with 4 days for patients with shigellosis. Because of the gradual onset, weight loss is a common finding. Surprisingly, fever is present in only the minority (8-38%) of patients with amoebic colitis. Colonic lesions can vary from mucosal thickening only to flask-shaped ulcerations to necrosis of the intestinal wall (Tanyuksel and Petri 2003).

1.5.3 Extra intestinal amoebiasis

The most common extra intestinal manifestation is amoebic liver abscess (ALA), which is associated with significant morbidity and mortality. This was a progressive and almost invariable fatal disease little more than a century ago, but since the introduction of effective medical treatment and rapid diagnosis, mortality has fallen to 1 - 3 %. Complications of extra intestinal amoebiasis include pleuropulmonary amoebiasis secondary to ALA rupture through the diaphragm, brain abscess, and genitourinary amoebiasis. Diagnosis of liver abscess is confirmed by a positive serological test, as amoebic serology is highly sensitive (> 94%) and highly specific (> 95%) for diagnosis. Abdominal ultrasound or computed tomography scan does not provide specificity for ALA. However, a positive serological test in combination with abdominal images are helpful for diagnosis where PCR is not routinely available (Tanyuksel and Petri 2003).

1.5.4 Clinical diagnosis

The differential diagnosis of an illness with diarrhea containing gross or occult blood should include infectious etiologies (including amoebiasis and infections with *Shigella* sp, *Salmonella*, *Campylobacter*, *Mycobacterium tuberculosis* and enteroinvasive and enterohemorrhagic *Escherichia coli*) and noninfectious causes (including inflammatory bowel disease, carcinoma, ischemic colitis, arteriovenous malformation, and

diverticulitis) (Fotedar, Stark et al. 2007). It can be difficult to make the diagnosis of amoebic colitis, since the presentation of the illness may be insidious or chronic, bleeding may occur without diarrhea, and fever is an unusual finding. Once the diagnosis can also be problematic, because a single stool examination for parasites is insensitive, histopathologic confirmation of infection on biopsy specimens may be difficult, and serological tests for antibodies to amoebas are not always positive. The best initial diagnostic approach at this time is detection of *E. histolytica* antigen in stool (Petri and Singh 1999). Unusual manifestations of amoebic colitis include acute necrotizing colitis, amoeboma (granulation tissue in colonic lumen mimicking colonic cancer in appearance), cutaneous amoebiasis, and rectovaginal fistulas. Acute fulminant or necrotizing colitis occurs in $\approx 0.5\%$ of cases, usually requires surgical intervention, and has a mortality of $>40\%$. Abdominal pain and distension and rebound tenderness are present in most patients with fulminant colitis, and identification for surgery includes perforation and persistence of abdominal distention and tenderness while undergoing anti-amoebic therapy. Partial or total colostomy with exteriorization of the ends is recommended over primary anastomosis, since anastomosis may fail because of the friable condition of the bowel (Aristizabal, Acevedo et al. 1991; Pritt and Clark 2008).

1.6 LABORATORY DIAGNOSIS

Laboratory diagnosis of amoebiasis is usually based on microscopy and serological methods including enzyme-linked immunosorbent assay (ELISA), indirect haemagglutination assay (IHA) and latex agglutination. During the last decade, there has been remarkable development in molecular biology-based diagnostic procedures to detect *E. histolytica*, to the point where today they are the preferred approach. Accurate diagnosis is important not just for patients with dysentery but also for the 90% of *E. histolytica* infections that are asymptomatic, because infection may easily be transmitted from person to person, especially in developing countries which have poor hygienic conditions and inadequate water treatment (Jackson, Gathiram et al. 1985).

1.6.1 Microscopical examination

Historically, light microscopy has been the method of choice to diagnose amoebiasis. Although the presence of haematophagous amoebic trophozoites (trophozoites that have ingested red blood cells) in a stool sample (feces) strongly suggests *E. histolytica* infection, such a finding is rarely seen (Gonzalez-Ruiz, Haque et al. 1994). In the absence of haemathophagous trophozoites, the sensitivity and specificity of microscopy is limited by its inability to distinguish between samples infected with *E. histolytica* and those infected with *E. dispar* (which is ≈ 10 times more common). Definitive diagnosis of intestinal amoebiasis requires high levels of skill and experience; inadequate training and diagnostic testing may lead to confusion between *E. histolytica*, other non-pathogenic amoebas (such as *Endolimax nana*, *Entamoeba coli*, *Entamoeba hartmanni* and *Elodamoeba bütschlii*), which with blood cells (leucocytes) in the feces frequently results in over diagnosis of amoebiasis (Kebede, Verweij et al. 2004). Delays in the processing of stool samples affect the sensitivity of light microscopy, which under the best of circumstances is only 60% of that of the stool culture methods (Haque, Neville et al. 1995; Fotedar, Stark et al. 2007).

Stool specimens can be examined either unstained or stained with Lugol's or D'Antoni's iodine. Iodine stains make the nucleus perfectly visible. Although several other stains, including Giemsa, methylene blue, Chlorazole black E, Wright's and iodine-trichrome, may be used successfully, Wheatley's trichrome staining or one of the modified iron hematoxylin stains for permanent smears has been suggested for routine use in the diagnosis of *E. histolytica*/*E. dispar* (Tanyuksel and Petri 2003).

1.6.2 Culture and isoenzymes

Boeck and Drbohlav first cultivated *E. histolytica* in a diphasic egg slant medium. Today, the National Institute of Health modification of Locke-egg medium has been used in some research laboratories. However, Robinson medium (Robinson 1968) and TYSGM-9 of Diamond (Diamond 1982) are more often used for xenic cultivation of *E. histolytica*. After being used successful axenic cultivation by Diamond, TYI-S-33 (Diamond, Harlow et al. 1978) is one of the most widely used axenic media. A method that involves the culture of stools samples followed by isoenzyme analysis can accurately distinguish *E. histolytica* and *E. dispar*, and is considered to be the "gold standard" for diagnosis. However, this method takes between one and several weeks to carry out and requires special laboratories facilities, making it impractical for use in the developing world, where most cases of amoebiasis occur. Classically, to differentiate "pathogenic and nonpathogenic" forms, isoenzyme patterns obtained from amoebic culture lysates were widely used (Tanyuksel and Petri 2003). A total of 24 different zymodemes, composed of 21 zymodemes from human isolates (9 *E. histolytica* and 12 *E. dispar*) and 3 zymodemes from experimental culture amoebic strains (Sargeant and Jackson 1985; Sargeant 1987), have been recognized.

1.6.3 Antibody detection

Most people with intestinal amoebic infection in areas of endemicity have been exposed to *E. histolytica* many times. Symptoms commonly attributed to *E. histolytica* may be absent in the majority of cases. This situation makes definitive diagnosis by antibody detection difficult because of the inability to distinguish past from current infection (Gathiram and Jackson 1985). Serological test are more helpful for the identifications, where *E. histolytica* infection is not common (Ohnishi and Murata 1997). Serum antibodies to *E. histolytica* can be detected in 75 to 85% of patients with symptomatic *E. histolytica* infection. Assays that have been used so far involve IHA (indirect haemagglutination assay), counterimmunoelectrophoresis (CIE), amoebic gel diffusion test, complement fixation, indirect fluorescent assay (IFA), latex agglutination and ELISA. The sensitivity of detection of specific antibodies to *E. histolytica* in serum is reported to be near 100%, which is promising for diagnosis of ALA. Serum anti-lectin immunoglobulin G (IgG) antibodies could be present within 1 week after the onset of symptoms of patients with amoebic colitis and ALA, with a value over 95%. Serological test results are some times false positive, and the test should be repeated if the result is doubtful. An additional problem is that the specificity of the antibodies detected against *E. histolytica* found in the population is unknown since the methods commonly used may at least partly have been induced by cross reacting immunogens present in other amoebas, such as *E. dispar* or free-living amoebas (Tanyuksel and Petri 2003).

1.6.4 Antigen assays for the diagnosis of amoebiasis

Two new test methods that involve ELISAs to detect antigens in stools samples are able to distinguish accurately between infection with *E. histolytica* and *E. dispar*. They are replacing microscopy and stool culture followed by isoenzyme analysis for both clinical and research purpose. Serological test methods that detect the presence of anti-amoebic antibodies also remain useful (Huston, Haque et al. 1999).

1.6.4.1 ELISA methods

The ELISA method might also prove useful for the detection of *E. histolytica* lectin antigen in the sera of patients with amoebic colitis and liver abscess. This test uses a monoclonal antibody against an amoebic adherence lectin that is inhibitable by Gal or GalNac. The lectin is conserved and highly immunogenic, and because of antigenic differences between the lectins of *E. histolytica* and *E. dispar*, it can be used to identify the pathogenic species. The *E. histolytica* TechLab kit I was designed in 1993 to detect specifically *E. histolytica* in feces (Haque, Neville et al. 1995). This kit was unable to specifically identify disease-causing *E. histolytica*. The level of detection of amoebic antigens is quite high, requiring approximately 1000 trophozoites well, and is limit testing of fresh or frozen samples. In Nicaragua, a study done in patients with diarrhea showed high sensitivity but low specificity. This kit has been discontinued by the manufacturer and has been replaced by second-generation TechLab *E. histolytica* II kit, which has been found to be sensitive (86 to 96 %) and specific (93 to 100%) comparing with microscopy (Haque, Ali et al. 1998). Other ELISA kits for antigen detection include the *Entamoeba* CELISA PATH kit (Ceballos, Brookvale, Australia), which uses a monoclonal antibody specific for lectin of *E. histolytica*, and ProSpectTEIA (Remel Inc.; Sunnyvale, CA), which detects *E. histolytica*-specific antigen in fecal specimens (Mirelman, Nuchamowitz et al. 1997; Fotedar, Stark et al. 2007).

1.6.4.2 Immunochromatographic assays

Triage parasite panel (TPP) (Biosite Diagnosis Inc., Sandiego, CA) is the first immunochromatographic assay for simultaneous detection of antigen specific for *Giardia intestinalis*, *E. histolytica*/*E. dispar*, *Cryptosporidium parvum*. The immunochromatographic strip used in this assay is coated with monoclonal antibodies specific for the 29-kDa surface antigen (*E. histolytica*/*E. dispar*), alpha-1-giardin (*G. intestinalis*), and protein disulfide isomerase (*C. parvum*). A high sensitivity (96% to 100%) and specificity (99.1% to 100%) of the TPP kit compared to microscopy for *E. histolytica*/*E. dispar* were reported (Sharp, Suarez et al. 2001).

1.6.5 PCR-based methods

Several PCR-based methods that amplify and detect *E. histolytica* DNA in stool samples have also been developed (Tannich and Burchard 1991). The sensitivity and specificity of PCR-based methods for the diagnosis of *E. histolytica* infection both approach those of stool culture followed by isoenzyme analysis. When used to detect the presence of trophozoites in culture, PCR amplification and detection of small, ribosomal RNA (rRNA) genes is ≈ 100 times more sensitive than the best currently available ELISA kit for the detection of *E. histolytica* antigens (Tanyuksel and Petri

2003). There is now a wide variety of PCR methods, targeting different genes, which have been described for detection and differentiation of the three *Entamoeba* species. The consistent genetic diversity detected between the 18S rDNAs of *E. histolytica* and *E. dispar* initiated the use of 18S rDNA as a target for differentiation of the two species (Fotedar, Stark et al. 2007).

Table 1. Sensitivity and specificity of different methods for diagnosis of amoebiasis.

Test	Colitis		Liver abscess
	Sensitivity	Specificity	Sensitivity
Microscopy (stool)	< 60%	10-50%	< 10%
Microscopy (abscess fluid)	Not available	Not available	< 25%
Culture with isoenzyme	Lower ELISA and PCR	“Golden standard”	< 25%
Stool antigen (ELISA)	> 90%	> 90%	Usually negative
Serum antigen (ELISA)	65% (early)	> 90%	~75% (late), ~100% 3 days
Abscess antigen (ELISA)	Not available	Not available	~ 100% (before treatment)
Salivary antigen detection	Not done	Not done	70%
PCR (stool)	> 70%	> 90%	Not done
Serum antibody (ELISA)	> 90%	> 85%	70-80% acute >90% later

(Tanyuksel and Petri 2003)

1.7 THERAPY

Therapy for invasive infection differs from therapy for noninvasive infection. Noninvasive infections may be treated with paramomycin. Nitroimidazoles, particularly metronidazole, are the mainstay of therapy for invasive amoebiasis (Powell, Newsome et al. 1994). Approximately 90 percent of patients who present with mild-to-moderate amoebic dysentery have a response to nitroimidazole therapy. In the rare case of fulminant amoebic colitis, it is prudent to add broad-spectrum antibiotics to treat intestinal bacteria that may spill into the peritoneum; surgical intervention is occasionally required for acute abdomen, gastrointestinal bleeding, or toxic megacolon. Parasites persist in the intestine in as many as 40 to 60 percent of patients who receive nitroimidazole. Therefore, nitroimidazole treatment should be followed with promomycin or the second-line agent diloxanide furoate to cure luminal infection (Blessmann and Tannich 2002; Pritt and Clark 2008).

1.8 SEROLOGICAL DIAGNOSTIC PROBLEMS IN AMOEBIASIS

Studies have shown that human serum contains antibodies to both *Naegleria* and *Acanthamoeba* species. The origin of these antibodies in the serum of people that have not been exposed to these amoebas is not known, but it has been suggested that they can rise as a result of exogenous exposition by cross-reacting antigens of other protozoan. Taking in account that *E. histolytica* is a parasitic protozoan widely distributed in developing countries it is possible that previous infections or exposition to *E. histolytica* stimulate the production of antibodies that recognize antigens of FLA via cross-reaction (Rodriguez, Bonilla et al. 2003).

1.8.1 Free living amoebas: Introduction

In contrast to gastroenteric infections caused by parasitic amoebas as represented by *E. histolytica*, a relatively small number of infections are caused by free-living amoebas, organisms normally found in soil or water. The genera included in this category are

Naegleria, *Acanthamoeba*, and more recently, *Balamuthia*. Representatives of these genera are pathogenic facultative parasites. They cause disease by being in the right place at the right time or by taking advantage of a host with impaired immune defenses (Martinez and Visvesvara 1997).

1.8.2 **Naegleria spp.**

Naegleria is associated with primary amoebic meningoencephalitis (PAM), a fulminating, rapidly fatal, infection of the central nervous system (CNS). *Naegleria fowleri* is the causal agent of most PMA infections, but other species of *Naegleria* having pathogenic potential have been described (*Naegleria australiensis* and *Naegleria italica*). The type habitat for *N. fowleri* is a natural or man-made lake, a thermal polluted body of water, or an inadequately chlorinated swimming pool where the amoebas can feed upon bacteria and proliferate. With respect to humans, mostly children, teenagers, and young adults in good health are infected by swimming or washing in such waters, where amoebas enter the nostril, migrate along the olfactory nerves to the cribriform plate, and gain access to the CNS. Amoebas proliferate rapidly and cause extensive damage to neural tissue.

Diagnosis is difficult and is dependent upon recognition of the amoebas in the microscope and they may not be dismissed as leukocytes. Because of the rapid onset, delayed diagnosis, destructive nature of the disease and lack of effective antimicrobial agents, death is an almost invariable consequence of infection when it takes place at the right time or has the advantage of a host with impaired immune defenses (Schuster and Visvesvara 2004). However, the common presence of thermotolerant *Naegleria* in water, especially *N. lovaniensis*, which is an indicator species for *N. fowleri*, suggests that also this pathogenic amoeba may pose a risk to public health in the area (Visvesvara, Moura et al. 2007).

1.8.3 **Acanthamoeba spp.**

Acanthamoeba spp. also infects the CNS, causing granulomatous amoebic encephalitis (GAE). Much more so than *Naegleria*, *Acanthamoeba* is ubiquitous in the environment, with amoebas being widely disseminated in soil and water. Unlike the healthy individuals acquiring *Naegleria* infections, persons contracting *Acanthamoeba* infections of the CNS are compromised host, suffering from concurrent diseases such as AIDS or other conditions such as alcoholism that predispose them to opportunistic infections. The portal of entry of amoebas can vary. It may be intranasal, allowing amoebas to migrate directly to CNS, or entry can be via a break in the skin or through the respiratory tract, with subsequent spread of amoebas to the CNS by hematogenous route. The disease assumes a chronic status, leading to slow deterioration.

Diagnosis is most often made by postmortem examination of brain tissue. Another major class of infection caused by *Acanthamoeba* spp. is amoebic keratitis. This condition was first noted in individuals suffering corneal trauma due to injury to the corneal surface that became infected with amoebas (Schuster and Visvesvara 2004; Visvesvara, Moura et al. 2007).

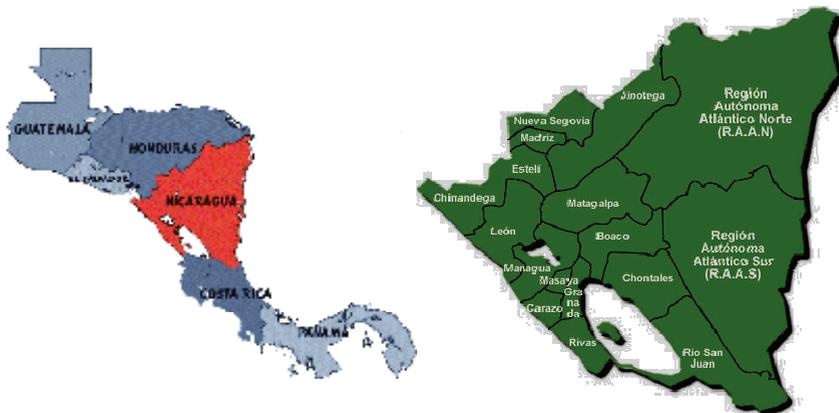
2 AIMS OF THE STUDY

2.1 GENERAL AIMS

To determine the true prevalence of *E. histolytica* and other *Amoebas* spp. using various tests designed to differentiate between pathogenic and apathogenic species.

2.2 SPECIFIC AIMS

1. To determine the true prevalence of *E. histolytica* in patients with diarrhea and normal population in León Nicaragua.
2. To assess the over-diagnosis and over-treatments of *E. histolytica*/*E. dispar* at primary attention level.
3. To evaluate the high seroprevalence.
4. To identify different free-living amoebas in different sources of environmental water in Nicaragua.



Central America and Nicaragua

3 MATERIALS AND METHODS

3.1 PREVALENCE OF INTESTINAL PARASITES

3.1.1 Study area

León department covers an area of 5 107 km² and has a population of 389 600 (2005 census). The capital city is León, has an extension of 1 114 km², which covers both urban and rural areas. The climate is warm and humid, with a mean temperature of 32°C. León is the second city in Nicaragua, with an estimated number of 200 000 inhabitants. Forty-eight percent of the population is less than 15 years old and 52 % are female. Eighty percent of the population is living in the urban area; only 48% have access to the sewage system. There are 5.3 persons in average per household (Pena, Wall et al. 2000).

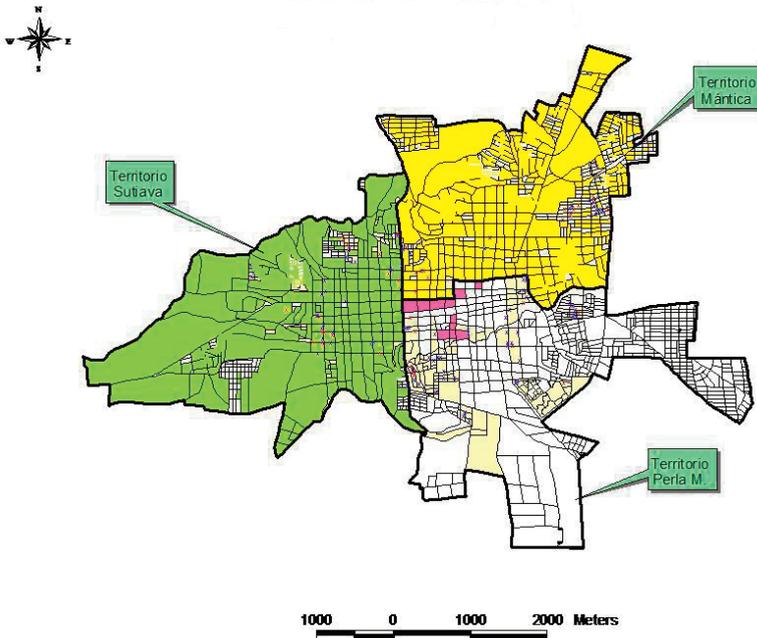


Figure 2
León city is divided in three areas (MINSa, Ministry of Health, Nicaragua)

3.1.2 Study group and Collection material

Paper I and III

Two study groups have been evaluated; 480 individuals from the surveillance system in León filled a form with socio-economical data. For evaluating living conditions, poverty index (PI) was estimated taking into consideration four dimensions or indicators. These dimensions are housing quality (HQ), school enrolment among minors (SE), dependency ratio (DR), and availability of sanitary services (ASS).

The second group 134 individuals, over two years old, presenting with diarrhea at three different health centers in León; this study was carried out from 2002 to 2003. In both study, patients had not used any anti-parasitic drugs one week before the sampling. The individuals were invited to participate in this study, and in case of children signed informed consent was provided by the children's parents before the study started. The study was approved by Ethical committee of Medicine Faculty.

Samples collected in the community and at the health centers were transported within 1 – 2 hrs to the Microbiology Department at the University of León. Immediately after delivery, samples were divided into different containers. One part of the specimen was fixed with 70% ethanol for DNA extraction, one part with SAF (Sodium acetate-acetic acid-formalin) fixative for staining methods, and the remaining unfixed fresh specimens were examined by direct microscopy of saline and iodine. In addition, formalin-ethyl acetate sedimentation technique was used for detection of cysts and eggs (Young, Bullock et al. 1979), iron haematoxylin staining for amoebas and flagellates and modified Ziehl Neelsen staining for detection of enteric coccidian (Garcia and Bruckner 1997).

Paper IV

A retrospective study was carried out in two health centers. In this study, we got permission from the head of the health center, and two person collected the information, they visited the health center, between June to November from 2004, the record of patients were given by nurses, the record were chosen at random in patients who showed intestinal symptoms, and over two years old. 100 patients were recorded, and a form from each patient was filled, with following date: abdominal pain, diarrhea, constipation, other symptoms, stools examination, sex, age.

An external quality assessment in coproparasitology was carried out from February to July of 2006 in fifteen laboratories at health centers, in León department, a questionnaire and a panel with ten plastic vials was previously prepared with different intestinal parasites such as: *E. histolytica/ E. dispar*, *Entamoeba coli*, *Endolimax nana*, *Giardia intestinalis*, *Entamoeba hartmanni* and helminths eggs such as: *Trichuris trichiura*, *Ascaris lumbricoides*, *Taenia sp.*, and one stool sample as negative control. Plastic bags, with ten vials were sealed and sent to each laboratory, and a form was filled in order to know the level of laboratory technicians and the laboratory condition.

Serum samples

Twenty-two serum samples from suspected of liver abscess patients in the León University Hospitals, and 25 samples from individuals in the community were used to evaluate the significance of high prevalence of anti-amoebic antibodies. Immunofluorescence and western blotting was used for detecting antibodies against different amoeba spp.

3.2 DIAGNOSTIC METHODS

3.2.1 Microscopical examination of fecal specimens

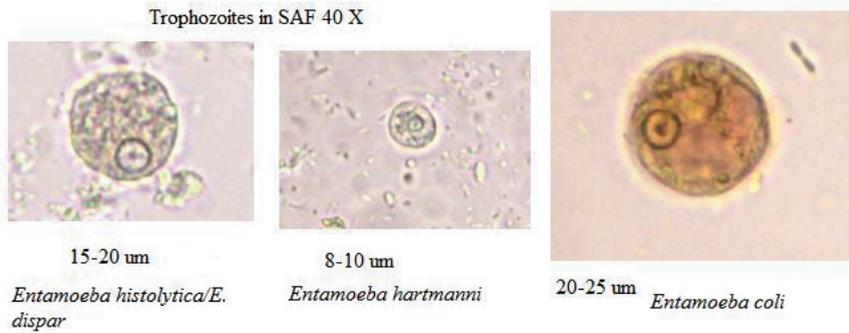


Figure 3. Three different *Entamoebas* spp. at the microscopic examination.

3.2.2 Antigen detection

3.2.2.1 *The Triage Parasite Panel (TPP)*

The Triage Parasite Panel (TPP) (Biosite Diagnostic, Inc, San Diego, Calif), an immuno-chromatographic assay for the detection of antigens specific for *G. intestinalis*, *E. histolytica/E. dispar*, and *Cryptosporidium parvum* in stool was used according to the procedure provided with the kit.



Figure 4.

The Triage Parasite Panel for the detection of potentially pathogenic protozoa in unfixed stools of patients with diarrhea. A color reaction indicates presence of *Giardia*, *Entamoeba histolytica/E. dispar* and *Cryptosporidium*.

3.2.3 PCR amplification

Genomic DNA extraction from stool samples was performed using the QIAamp tissue method (QIAamp, Germany). An 880 bp fragment of the *ssrRNA* gene of *E. histolytica* and *E. dispar*, respectively, was amplified using the forward primer Psp 3' and the reverse primer Psp 5' for *E. histolytica* and forward primer NPsp 3' and the reverse primer NPsp 5' for *E. dispar* described by Diamond and Clark 1993 (Clark and Diamond 1993). Positive controls consisting of DNA extracted from *E. histolytica* HM-1:IMSS nr 30459 and *E. dispar* SAW 937 R clone A nr 50698 were included in each run.

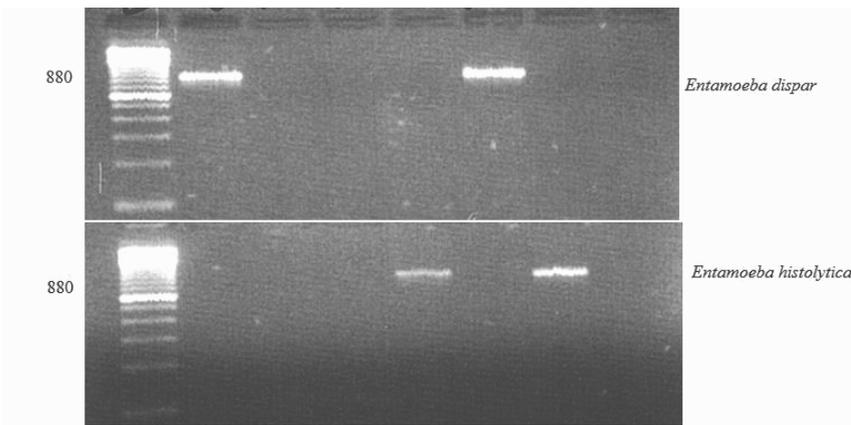


Figure 5. Detection of *E. histolytica* and *E. dispar* from stool samples by PCR. Lane 1 bp pattern. Lane 2 *E. dispar*, Lane 3 negative, Lane 4 negative, Lane 5 *E. histolytica*, Lane 6 Control *E. dispar* 880 bp. Lane 7 Control *E. histolytica* 880 bp, Lane 8 negative.

3.2.4 Antibody detection

To evaluate the significance of high prevalence of antibodies against *E. histolytica*, serum samples from liver abscess patient and normal population were used with other *Amoebas* spp.

3.2.4.1 Immunofluorescence.

Four dilutions were done, to each serum sample: 1/30, 1/90, 1/270, 1/810 and 1/2430 for IFAT. The relative intensity of fluorescence was measured from digital images using the NIH Image software. The images were inverted to give a “negative” image and the black background with the value 0.0. In addition, in each field, each cell was measured, to obtain a mean intensity value. The value obtained with *E. histolytica* as antigen was divided with that one obtained with *E. dispar* as antigen. Thus the relative intensity of serum reactivity was expressed as $I = [I \text{ } E. \text{ histolytica}] / [I \text{ } E. \text{ dispar}]$. Thus I values > 1 indicate that reactivity with *E. histolytica* was stronger than *E. dispar* and vice versa.

3.2.4.2 Western blotting

Antigen preparation. Trophozoites to a concentration of 10^6 cells of the different amoebas *E. histolytica*, *Acanthamoeba castellanii*, *Hartmannella vermiformis* and *E. dispar* were cultured. Cultured amoeba were washed in phosphate buffer saline pH 7,4 (PBS), three times per 10 min. at 1000 rpm and resuspended in PBS. Cells were disrupted by sonication, the quantity of protein in supernatant was measured by BCA protein Assay kit (PIERCE, Rockford IL, USA). SDS-PAGE was performed according to the protocol described elsewhere (Laemmli 1970). Dilutions of the serum (1/100) were done using washing solution. Conjugates of rabbit anti-human IgA, IgG, IgM were purchased from DAKO (Denmark). The position of the band was measured in order to calculate the molecular weight. Furthermore, absorption was made using the extract of *A. castellanii* and *H. vermiformis* and incubated with 1 ml of serum from suspected liver abscess patients. A dilution 1/100 was done and incubated at 4 °C in shaking for 2h. The solution was centrifuged 1500xg for 15 min. The supernatant was

utilized in the immuno-blotting assays as first antibody, against *E. histolytica*, *A. castellanii* and *H. vermiformis*.

3.3 DETECTION OF FREE LIVING AMOEBAS IN WATER SOURCES

3.3.1 Sample collection and cultivation of amoebas

Water samples were collected from León area in Nicaragua: 88 samples were from rivers and springs, 111 from wells, 74 from water taps and 21 from water tanks in urban and suburban León and from 3 nearby geothermal areas of San Jacinto, Posoltega and Tipitapa. Water samples were collected into sterile 50 ml tubes and transported to the laboratory at Microbiology Department, University of León. Amoebas were isolated from specimens filtered through 0.45 µm filters attached to sterile syringes. Filters were placed on non-nutrient agar plates covered with viable *Escherichia coli* K12 strain. Agar plates were incubated at 40 °C for the isolation of thermotolerant strains. Growing amoebas were repeatedly sub-cultured and cloned by limiting dilution.

3.3.2 Identification of isolates

Initial identification of amoebas was based on microscopical observation of living and fixed cysts and trophozoites. The presence of cellulose in cyst wall of *Acanthamoeba* was demonstrated using the cellulose-binding domain of cellulose essentially as described (Linder, Winiecka-Krusnell et al. 2002). Fluorescent *in situ* hybridization (FISH) was performed using 18S rRNA- targeted fluorescent oligonucleotide probes according to the protocols described before (Stothard, Hay et al. 1999; Grimm, Ludwig et al. 2001). Probes: GSP (genus-specific probe) for the detection of *Acanthamoeba* and NAEG1088 specific 18S rRNA probe for the detection of *Naegleria* were synthesized by Scandinavian Gene Synthesis AB, 73122 Köping, Sweden.

3.4 STATISTICAL ANALYSIS

The statistical analyses were performed using Epi Info software version 6.2 to calculate percentage and frequency of parasites and to determine the agreement between two methods: concordance was calculated using Cohen Kappa indices (κ), sensitivity and specificity using Youden's index in the Win Episcopy 2.0 program and SSPS program v 10 for X^2 and marginal odds ratio (ORs) and 95% confidence intervals (95% CIs) were used to measure the strength of association between risk factor and intestinal parasitic infection.

4 RESULTS AND DISCUSSION

4.1 TRUE PREVALENCE OF *E. HISTOLYTICA* AND *E. DISPAR* (PAPERS I AND III)

PAPER I (Diarrhea patients)

Among 134 diarrhea patients, the most common finding at the health center laboratory level, was *E. histolytica/E. dispar* (24%). At the Microbiology Department *E. histolytica/E. dispar* was found in 4.5 %. With the Triage Parasite test, only one case of *E. histolytica/E. dispar* was found. Upon confirmatory diagnostic analysis by PCR, *E. dispar* was recognized in 10 (7.5%) and *E. histolytica* in two cases (1.5%). PCR was capable to identify 2 *E. histolytica* and 10 *E. dispar* cases. The correlation between PCR results and microscopical examination was low ($\kappa=0.4$). This finding suggests that the true prevalence of *E. histolytica* in León is very low. This finding made us reassess the extent of the amoebiasis examination. There is a need to eliminate over diagnosis based on microscopy and to introduce a specific method for identification of *E. histolytica*, but in a poor country as Nicaragua it is difficult in this setting. Both education and training will be necessary for a good clinical manage and treatment of the patients. In this study, TPP test showed good sensitivity and specificity for *G. intestinalis* and high concordance with microscopy. However, the sensitivity for *E. histolytica/E. dispar* and *Cryptosporidium* spp. was lower than reported previously (Garcia, Shimizu et al. 2000; Sharp, Suarez et al. 2001). The observed lower sensitivity of the TPP could be explained by the relatively high sensitivity of PCR and the problems with specificity of microscopy discussed above. A renewed look at the local epidemiology of amoebiasis in León, Nicaragua demonstrates that the true prevalence of *E. histolytica* by PCR in individuals with and without diarrhea was relatively low.

PAPER III (Normal population)

In this study of prevalence intestinal parasites using different diagnostic methods, it was determined that one or more intestinal potential pathogenic parasites were found in 40% and 22% of people living in “poor” and “good” condition respectively. In contrast to the previous study 11 years ago, the prevalence of helminths has decreased. This could be due to de-worming campaigns, where the children between 2 to 12 year old were given albendazole, an anti-parasitic drug which is effective mainly for helminths (Keiser and Utzinger 2008). Correlation between protozoan parasites *E. histolytica/E. dispar* and *G. intestinalis* showed a different age distribution, similar to a study in León and reported by other authors (Ostan, Kilimcioglu et al. 2007). The prevalence of *Giardia* was found to be higher in younger children than in older people (Tellez, Morales et al. 1997).

Table 2. Prevalence of intestinal parasites, in patients with diarrhea (Paper I) and in normal population (Paper III) in León, Nicaragua 2003.

Parasites detected	Paper III		Paper I	
	n=480	(%)	n=134	(%)
Protozoan				
<i>Blastocystis hominis</i>	200	(42)	44	(33)
<i>Entamoeba coli</i>	126	(26)	14	(10)
<i>Giardia intestinalis</i>	100	(21)	22	(16)
<i>Endolimax nana</i>	91	(19)	23	(17)
<i>Entamoeba histolytica/E. dispar</i>	58	(12)	8	(6)
<i>Cryptosporidium sp.</i>	0	(0)	1	(0.7)
<i>Cyclospora cayetanensis</i>	0	(0)	3	(2.2)
<i>Isospora belli</i>	0	(0)	1	(0.7)
Helminthes				
<i>Trichuris trichiura</i>	11	(2.3)	7	(5.2)
<i>Hymenolepis nana</i>	5	(1.0)	8	(6.7)
<i>Ascaris lumbricoides</i>	3	(0.6)	5	(3.7)
<i>Hymenolepis diminuta</i>	2	(0.4)	0	(0)
Hookworm	1	(0.2)	0	(0)
<i>Taenia sp.</i>	1	(0.2)	0	(0)

Furthermore, this study shows that people living in poor conditions are directly related to potential pathogenic parasite infections ($P < 0.05$) as other authors have reported (Nematian, Nematian et al. 2004; Okyay, Ertug et al. 2004; Ugbomoiko, Dalumo et al. 2009). In the early study 11 years ago, there was not a good correlation.

In this study the prevalence of *E. histolytica* by PCR was relatively low 5% (3/58) as compared to *E. dispar* 34% and this result is similar to other results reported recently in other endemic areas (Verweij, Oostvogel et al. 2003; Pinheiro, Carneiro et al. 2004; Ben-Ayed, Ben et al. 2008). At the onset of studying amoebiasis in León several years ago, *E. histolytica* infection was thought to be common, since cysts of the organism were found in stool samples of about 20% of the population (Tellez, Morales et al. 1997).

Table 3. Detection of *E. histolytica/E. dispar* by microscopy and identified by Polymerase Chain Reaction (PCR), *E. histolytica* and *E. dispar*, in León, Nicaragua.

Species	Normal population		With diarrhea	
	Microscopy n=480	PCR n=58	Microscopy n= 134	PCR n= 134
<i>E. histolytica/E. dispar</i>	58 (12%)	37 (64%)	8 (6%)	12 (9%)
<i>E. dispar</i>	Not identifiable	34 (59%)	Not identifiable	10 (7.5%)
<i>E. histolytica</i>	Not identifiable	3 (5%)*	Not identifiable	2 (1.5%)

*If we take account all populations studied 3/480 (0.6%).

4.2 OVER DIAGNOSIS AND OVER TREATMENT (PAPER IV)

In the quality control assessment, 80 % of the technicians successfully identified the main parasites in the panel. The parasites identified correctly were *H. nana*, *A. lumbricoides* and the hardest to recognize were *E. hartmanni*, *Taenia sp.* and *E. nana*. All 15 laboratories found *E. histolytica* /*E. dispar* in the single sample where it was present. However, some laboratories falsely identified this protozoon in more than one sample, in total there were 20 cases. We found that the health center technicians continue to mix up *E. histolytica* /*E. dispar* with other amoebas causing false positive. The other reason for over diagnosis is that they were confusing vegetable matter with cysts (Kettelhut, Chiodini et al. 2003).

Furthermore, this report coincides with a previous study, where *E. histolytica*/*E. dispar* was overdiagnosed at the health center in León (Leiva, Lebbad et al. 2006). Also a fundamental problem, not restricted to the particular case of parasitological diagnostics, is that this type of routine diagnostics lacks the status of more sophisticated diagnostic procedures, an attitude which is possible to alter by paying attention to education, maintenance of equipment and laboratory routines. Education and training provided by e.g. university institutions, via its teaching arm, has played an important part in the solution of this problem (Kettelhut, Chiodini et al. 2003; Libman, Gyorkos et al. 2008). Novel tools for education and quality control, such as the Web Microscope for Parasitology (WMP) should contribute to an elimination of the problems of over diagnosis (Linder, Lundin et al. 2008).

Regarding the use of anti-amoebic drugs in symptomatic patients, 32% of patients were found with *E. histolytica*/*E. dispar* and the majority with cysts. On the other hand, no stool examination was not performed on 41 % of the patients. In summary 79% of those patients were treated with methronidazole. A rough calculation on sales of methronidazole and related drugs can be made based on the 100 individuals included in this study. The conclusion is that thousand of dollars are wasted due to over-treatment in a country with limited resources has been pointed out previously (Fotedar, Stark et al. 2007). Apart from the misuse of public and personal economic resources, the practice of over-treatment may hasten the development of drug resistance. Thus, over-treatment appears to be a widespread problem, which needs to be explored in detail (Bansal, Malla et al. 2006).

We conclude that over diagnosis resulting in over-treatment of amoebic dysentery is widespread in the León region of Nicaragua, and that it reflects a global problem affecting most seriously the poorest in developing countries.

4.3 FREE LIVING AMOEBAS IN WATER SOURCES (PAPER II)

We thought that antibodies against *E. histolytica* may be induced by other amoebas in the environment. Thus we wanted to test this possibility of cross-reaction by absorption and immunoblotting (WB) experiments and to look for the presence of free-living amoebas in the environment.

Free living amoebas isolated from the water samples were characterized by morphological and immunohistochemical methods. One hundred twenty five out of the 294 samples (42.5%) collected from different water sources yielded growth of FLA. Amoebas could be recovered from 75 out of 178 (42 %) water samples collected in the León municipality and an almost identical proportion was recovered from samples from the geothermal areas (50 out of 116, 43%). However, a detailed analysis showed some differences; the lowest prevalence of amoebas, 17 out of 74 (23%) was found in tap water in León municipality and the highest prevalence in wells from geothermal areas, 25 out of 36 (69%).

Table 4. Identification of *Naegleria* isolates by different methods.

	Enflagellation	FISH ¹	≥42 °C	IFAT ²	
				Nf-Pab ³	Nf-5D12u ⁴
León Municipality	17	8	8	3	0
Geothermal areas	22	16	16	9	0
Total	39	24	24	12	0

¹FISH = Fluorescent in situ hybridization, ²IFAT = Immunofluorescence antibody test, ³Nf-Pab = anti *N. fowleri* /*N. lovaniensis* polyclonal antibodies, ⁴Nf-5D12u = anti *N. fowleri* mouse monoclonal antibodies.

There were significantly higher levels of amoeboflagellates from wells in geothermal areas as compared to wells in the León municipality, 53% as opposed to 15%. The common presence of thermotolerant, potentially pathogenic amoebas, may pose a risk to public health in the community.

In thermal areas we found significantly more *Naegleria* than *Acanthamoeba*. Such environmental conditions occur in geothermal areas globally, but may occur also in the environment, which has been heated by other means. Raised temperatures during the hot summer months or warm water from power plants facilitate the growth of *N. fowleri* (Martinez and Visvesvara 1997). However, in the cyst form these protozoa are extremely resistant and may occur in dust (da Silva and da Rosa 2003). Thus the information provided in this study may serve as baseline for future studies on the role of free-living amoebas e.g. in waterborne-disease outbreaks in the region. Among such potentially important enteropathogens are *Vibrio cholerae*, *E. coli* 0157, and *Helicobacter pylori* (Winiecka-Krusnell and Linder 2001; Brown, Smith et al. 2002).

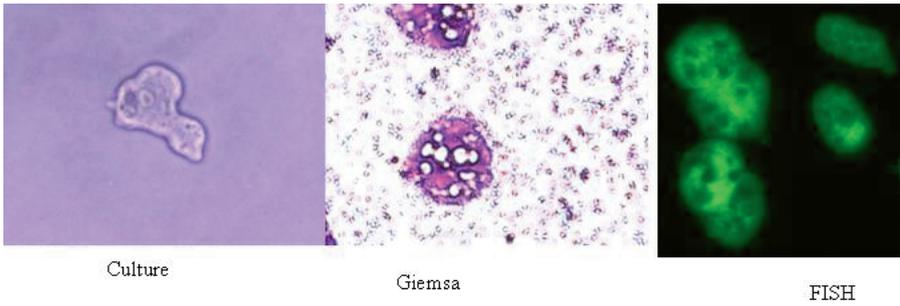


Figure 6. Diagnostic methods for free living amoebas: Phase contrast image seen in culture (Culture), the appearance of amoebas in fixed monolayers stained by the Giemsa stain and in fluorescence microscopy using Fluorescent *in situ* hybridization (FISH).

Liver abscess patient sera showed reactivity against *Acanthamoeba* and *Hartmannella*. Some sera reacted strongly with numerous protein components in WB. The results suggest that exposure to ubiquitous protozoa may account for the majority of antibodies produced during invasive amoebiasis and that assays for specific anti-*E. histolytica* antibodies are needed not only for prevalence studies but also for diagnosis. Also could be reflecting cross-reactivities, since *E. histolytica* is closely related to *Acanthamoeba* and *E. dispar* (Walochnik, Obwaller et al. 2001; Rodriguez, Bonilla et al. 2003).

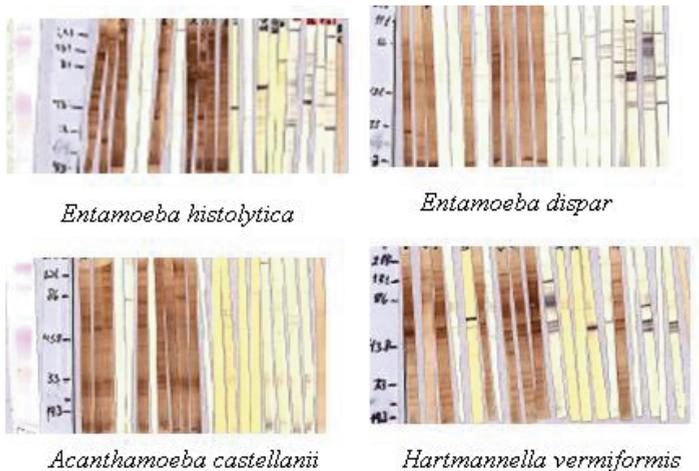


Figure 7. Reactivity of amoebiasis patient sera with different *Amoebas* spp.

The hypothesis was that common antigenic components could be present in *E. histolytica* and free-living amoebas. Contact with free-living amoebas could therefore give rise to cross reactive antibodies seen in the healthy population as anti-*E. histolytica* antibodies. The purpose was to study if antibody reactivity with free-living amoebas could be identified in amoebiasis patient sera. We also wanted to see if antibodies in amoebiasis patient sera could be absorbed with free-living amoebas.

In the example shown here predominant immuno-reactive components recognized by amoebiasis patient sera are highly variable. We also could not neutralize antibodies against *E. histolytica*: Absorption with free-living amoebas did not neutralize antibodies against *E. histolytica*. No predominant parasite components were recognized by the amoebiasis patient sera tested. This suggests that immunological cross reactivity between *E. histolytica* and free-living amoeba is not important in explaining the high seroprevalence of antibodies against *E. histolytica* in the population.

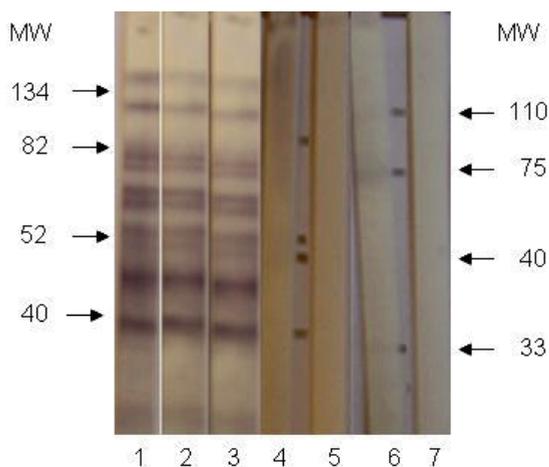


Figure 8. Evaluated tree antigens: *E. histolytica*, *A. castellanii* and *H. vermiformis* and absorbed serum sample with *A. castellanii* and *H. vermiformis*, from liver abscess patients. Line 1 Serum against *E. histolytica* without absorption, show band of 182, 126, 82, 75, 52, and 33. Line 2 Serum against *E. histolytica* and absorbed with *A. castellanii* showed the same bands. Line 3 Serum against *E. histolytica*, and absorbed with *Hartmannella* showed the same bands. Line 4 Serum against *Acanthamoeba* without absorption showed band 98, 63, 61, 40 kd. Line 5 Serum against *Acanthamoeba* and absorbed with *Acanthamoeba* showed no band. Line 6 Serum against *Hartmannella*, without absorption showed band of 110, 75 and 33 kd. Line 7 Serum against *Hartmannella* and absorbed with *Hartmannella* showed no band.

4.4 SIGNIFICANCE OF HIGH SEROPREVALENCE (UNPUBLISHED)

High titers of antibodies against *E. histolytica* are accepted diagnostic markers for invasive amoebiasis. However antibodies can also be detected in about 20% of uninfected individuals. Our questions was it such antibodies are induced by *E. dispar* which about 10 times more abundant than *E. histolytica*? In order to evaluate the degree of cross reactivity of antibodies against *E. histolytica* with *E. dispar*, we compared the relative intensity of reaction by indirect immunofluorescence (IFAT) with *E. histolytica* and *E. dispar* in individual sera from patients with suspected liver abscess.

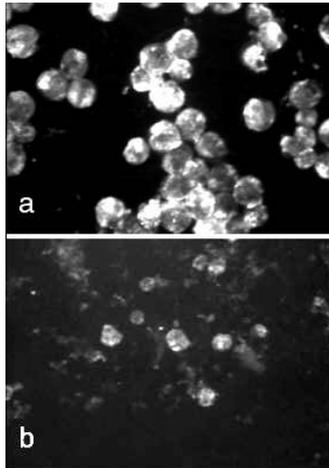


Figure 9. Comparative immunofluorescence assay for the demonstration of antibodies against *Entamoeba histolytica* (a) and *E. dispar* (b) in the same patient serum. Patient serum in dilution corresponding to end-point dilution using *E. dispar* as antigen gives stronger fluorescence on *E. histolytica*. Note non-homogenous staining patterns.

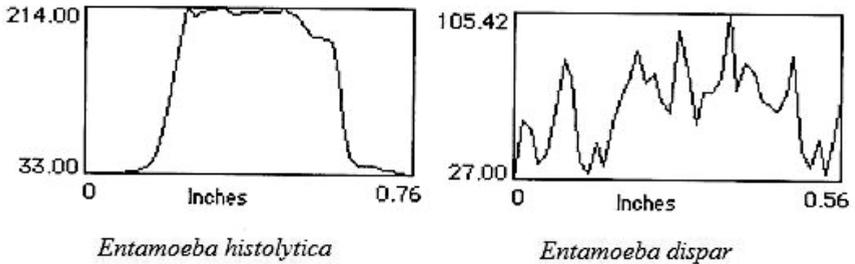


Figure 10. The relative intensity of antibodies reactions with the two antigens parasites can be calculated based on densitometry of amoebas stained by indirect immunofluorescence. A negative image (the highest intensity of staining being black in a grey-scale image) of stained monolayers was analyzed using the computer program (NIH image <http://rsb.info.nih.gov/nih-image/>). The size of *E. histolytica* cell corresponds to about 0.5 inches. *E. dispar* is smaller and more irregular stained. The maximum densitometric value on the y axis was used to calculate the ratio of fluorescence intensity.

Comparing the relative intensity of antibody with *E. histolytica* and *E. dispar* we found that amoebiasis patients usually had intensity ratios above 1, mean range ± 5.3 react more intensity with *E. histolytica*, but also the reverse was observed in healthy population had intensity ratios below 1, mean range ± 0.72 . However we were unable to test our hypothesis by absorption experiments because of lack of cultured *E. dispar*.

Table 5. Anti- amoebic antibodies, measurement of intensity of immunofluorescence by NIHimage computer program and end point of 20 liver abscess patients.

Serum	<i>Entamoeba histolytica</i>	<i>Entamoeba dispar</i>	Intensity**
N°	End point*	End point	Ratio
1	1/2430	1/810	4,88
2	1/810	1/270	0,95
3	1/810	1/270	0,95
4	1/810	1/270	0,95
5	1/810	1/270	0,95
6	1/810	1/270	0,95
7	1/810	1/270	0,95
8	1/810	1/270	0,95
9	1/810	1/270	0,95
10	1/810	1/270	0,95
11	1/270	1/90	1,89
12	1/90	1/90	2,03
13	1/2430	1/270	4,74
14	1/270	1/90	0,83
15	1/810	1/90	3,51
16	1/90	1/90	0,79
17	1/810	1/270	3,73
18	1/270	1/270	1,48
19	1/2430	1/810	2,43
20	1/90	1/90	0,66

* Four fold serial dilution ** Intensity of fluorescence at the end point dilution as measured by densitometry of a negative image. (see legends to Figs 6 and 7)

Table 6. Anti-amoebic antibodies in healthy individuals have similar end-point titers. The fluorescence intensity, however, is different. This can be seen as a difference in ratio.

N°	<i>Entamoeba histolytica</i>	<i>Entamoeba dispar</i>	Ratio
	End point	End point	
1	1/90	1/90	0,95
2	1/90	1/30	0,8
3	1/90	1/90	1,04
4	1/90	1/90	0,36
5	1/90	1/90	0,57
6	1/90	1/90	1,15
7	1/90	1/90	0,83
8	1/90	1/90	0,97
9	1/90	1/30	0,83
10	1/90	1/90	0,99
11	1/90	1/90	0,49
12	1/90	1/90	0,48
13	1/270	1/90	0,76
14	1/90	1/90	1,03
15	1/90	1/90	1,29
16	1/90	1/90	1,03
17	1/90	1/90	0,62
18	1/90	1/90	1,29
19	1/90	1/90	0,85
20	1/90	1/90	1,2
21	1/90	1/90	0,99
22	1/90	1/90	1,2
23	1/90	1/90	1,55
24	1/30	1/30	0,87
25	1/90	1/30	1,15

Antibodies reacting with *E. histolytica* and *E. dispar* in sera from the healthy population may have been induced by either or both parasites. However, the specificity of antibodies could not be completed due to difficulties in obtaining pure antigens for immunoblotting and absorption experiments. Thus the hypothesis that the observed seroepidemiological findings reflect exposure to *E. dispar*, and not *E. histolytica* could not be tested.

4.5 FUTURE PERSPECTIVES

Over diagnosis appears to constitute a severe problem in León, Nicaragua with consequent over-treatment. To avoid microscopy - based over diagnosis is the priority. At health centers, we intend to continue with further education, as well as carrying out quality controls in all laboratories nationwide. This knowledge has made the Ministry

of Health aware of the problem. We also intend to conduct educational work with primary health care physicians, to update their knowledge about amoebiasis and the problem of over treatment.

There is a novel method allowing an internet-based distribution and viewing of identical parasite containing specimens to an unlimited number of viewers. To determine the feasibility in an area with varying internet access speeds was registered the connection speed from educational institutions in Central America.

Free-living amoebas are potential problem in environments such as hospitals and agricultural areas could be a public health problem, both as pathogens and as reservoirs carrying pathogenic bacteria.

5 CONCLUDING REMARKS

The prevalence study in normal population and individuals with diarrhea for *E. histolytica* determined by PCR was relatively low. Alternative detection methods were insensitive. Triage Parasite Panel and microscopy finding were not correlated with PCR. At the health center laboratories we found an over diagnosis of *E. histolytica/E. dispar*, with the realization that pathogenic *E. histolytica* and morphologically indistinguishable from some common apathogenic species such as *E. dispar* (and therefore named *E. histolytica/E. dispar*).

In the study of infection with intestinal parasites, the prevalence of helminths decreased compared to a previous study, and the presence of intestinal parasites was related with poor living conditions. In a quality control study in 15 laboratories, we found miss diagnosis and over diagnosis for *E. histolytica/E. dispar*. Furthermore in the retrospective study around 41 % of patients were treated without any evidence of presence of parasites. This is a problem with clinical management and treatment of the patients; it entails an economical problem affecting most seriously the poorest.

Serology is a useful tool for the diagnosis of invasive amoebiasis. The use of serology to study the seroepidemiology of *E. histolytica* infection appears to be as problematic as the use of microscopy to demonstrate *E. histolytica* in fixed specimens.

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