EPIDEMIOLOGY, ZOONOTIC POTENTIAL, HAEMATOLOGY AND CONTROL OF AMOEBIASIS IN DOGS AND HUMANS

MUHAMMAD AZHAR ALAM
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IN THE NAME OF ALLAH, THE MERCIFUL, THE COMPASSIONATE

DEDICATIONS

I dedicate this humble effort to

My Father,

Mother,

Brother, Sisters,

Dearest Api,

and

Teachers

Who inspired and encouraged

me to higher ideals of life

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(MUHAMMAD AZHAR ALAM)

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CHAPTER 1
INTRODUCTION

Entamoeba histolytica, the causative agent of a protozoan disease called Amoebiasis, belongs to the genus *Entamoeba*. Discovered in 1875 by a Russian scientist named Losch, the disease is distributed worldwide, especially among humans and other primates (Suzuki et *al*., 2008). This protozoan mainly infects the large intestines. The pathogenesis of *Entamoeba histolytica* are not well understood, however, it is believed to involve competition with intestinal flora ii) target cell lysis by direct attachment iii) Release of toxins iv) phagocytic action of target cells (Devinder *et al.,*1996). Disease can occur from asymptomatic carrier state to severe symptomatic case, depending on the strain virulence of *Entamoeba histolytica,* host immunity as well as nutritional status and the presence of host intestinal pathogen (Stanley. 2001). Many cases are asymptomatic, however, in symptomatic cases; the signs include severe diarrhea along with blood, mucous, abdominal pain and sudden bowel evacuation. The organism can cause ulcerative and necrotizing colitis and hepatic abscess in man, captive, non-human primates, dogs and cats (Marguez *et al.,* 1991; Shinada*et al.,* 1992). About 50 million people are infected by *Entamoeba histolytica* with causality rates 40,000-100,000 per year.

With the increase in trend to keep dogs as pet / guard animals, there is more contact tendency between dog and dog's owners. The protozoa, *Entamoba histolytica,* have a fecal-oral life cycle comprising of infectious cysts (passed in the feces) and trophozoites (replicating and infecting the large intestine). Infection through the ingestion of cysts with contaminated food or water and the risk factors are similar to the diseases transmitted by fecal-oral route. Furthermore, the protozoan is common in those areas where socioeconomic conditions are very low with poorly adopted a hygienic strategy (Ravdin, 1995; Walsh, 1986). Together, this increases the chances of getting exposure to intestinal parasite of public health significance. The exposure may involve either direct contact or ingestion of

contaminated food and water (Horenzini et al., 2007). Considerable prevalence also found in institutions like orphanages, prisons and mental hospitals due to problems like over-crowding and unhygienic conditions are prevailing.

Incidence of disease is increasing day by day in Pakistan. However, no work has been done so far on the epidemiology of this disease. Keeping in view the importance of disease in terms of public health significance and implications, the present study has been conducted to record the prevalence of *E. histolytica* infection in dogs and dog's owners. Various diagnostic techniques such as Triple fecal test and stool antigen ELIZA have been compared in their diagnostic efficiency. Furthermore, therapeutic trials have been analyzed by using various herbal and allopathic drugs. The proposed research has been done with the following aims:

1- To record the prevalence of amebiasis in human and dogs, according to month, season, age and sex
2- To determine the zoonotic potential of disease in the studied area
3- To evaluate the various diagnostic techniques used for the diagnosis of amoebiasis
4- To compare the efficacy of different herbal and allopathic drugs against amoebiasis

CHAPTER 2
REVIEW OF LITERATURE

Entamoeba histolytica is a protozoan parasite that causes liver abscess and amebic dysentery in humans. The genus *Entamoeba* contains many species, from which six (*Entamoeba histolytica, Entamoeba coli, Entamoeba dispar, Entamoeba polecki, Entamoeba hartmanni, Entamoeba moshkovskii)* are mostly found in the intestinal lumen of human. *E. histolytica, Entamoeba moshkovskii* and *Entamoeba dispar* are morphologically identical, but are different genetically. *E. histolytica* is the only pathogenic species, while all other species are non-pathogenic. *E. histolytica* is considered as a leading cause of human mortality due to parasitic infection.

Amoebiasis caused by *E. histolytica* was first described by Hippocrates, who described it as a deadly disease characterized with fever and dysentery (460-377 B.C.). With the advancement of biological sciences tremendous knowledge has been achieved regarding its diagnosis, natural history and epidemiology. However, amoebiasis remains a permanent important health problem in tropical countries where hygiene and sanitary conditions are poor (Ximenez *et al.,* 2009). Clinical features of the amoebiasis range from asymptomatic colonization to amoebic diarrhea and dysentery that may lead to extra-intestinal invasive amoebiasis causing liver abscesses (Fotedar *et al.,* 2007). Current data of WHO shows that amoebiasis is the cause of approximately 100,000 deaths annually, second to malaria in mortality (Stanely 2003; Ravdin 2005; WHO 1997). *Entamoeba moshkovskii* has also been isolated from the individuals inhabiting in the endemic areas of amoebiasis (Ali *et al.,* 2003, Fotedar *et al.,* 2007, Khairnar *et al.,* 2007, Parija and Khairnar, 2005). The re-classification of three morphologically identical species has made complex the epidemiology of amoebiasis because they cannot be differentiated microscopically which is mostly used for the diagnosis in tropical countries due to limited resources. For the differentiation of these morphologically identical species highly sensitive and specific techniques like ELISA and PCR have been

used (Ackers, 2002). The mechanism and the exact prevalence and incidence of infection caused by *E. histolytica* are still unknown.

E. histolytica trophozoites reside in the lumen of the large intestine of human beings and use starches and mucosa secretions for their nutrition. *E. histolytica* also interacts metabolically with the host gut bacteria as well. Trophozoites starts tissue invasion by hydrolyzing mucosal cells and absorbs predigested food particles to meet their dietary provisions. Filopodia are the tiny cytoplasmic projections that are believed to play a role in pathogenicity. Other factors that may also influence the invasiveness of *E. histolytica* are the oxidation-reduction potential and pH of gut contents. Once the trophozoites invade the intestinal wall, they reach the sub mucosa and the underlying blood vessels. From the blood, trophozoites travel to different organs like liver, lungs and skin. The parasites at this stage are considered as dead-end organisms because they cannot encyst and leave the host and cause infection in other hosts. The normal formation of cysts takes place in the intestinal lumen and cysts are passed out to the environment through feces. These cysts are resistant to a variety of environmental physical factors. Trophozoites cannot survive outside the human body. The life cycle of *E. histolytica* comprises of two stages, infective cyst stage and multiplying trophozoite stage. Mature cysts are released from the large intestine of infected human in large numbers and these cysts can remain viable and infective in cool, moist environment for at least 12 days. In water these cysts can live up to 30 days. These cysts are killed at temperature below 5°C and above 40°C. Mature cysts residing in contaminated water are ingested and they are passed to the small intestine unharmed. In the small intestine, the pH is alkaline and these cysts give eight motile trophozoites by nuclear division. These motile trophozoites then settle in the large intestine where they multiply by binary fission and feed on host cells, bacteria and food particles.

According to some studies conducted in African countries, almost 6 to 75% of the total population is carriers of the parasite (Alonzo *et al.,* 1993, Molback *et al.,* 1994, Njoya *et al.,* 1999, Roche *et al.,* 1999). These studies were conducted using simple microscope and give a rough estimate of the distribution of the Entamoeba histolytica –like parasites in these populations. They cannot be used to estimate the epidemiology of clinical disease because it is not possible to microscopically differentiate *E. histolytica* from *E. dispar*. Prevalence studies, like these require confirmation by techniques that clearly differentiate pathogenic *E. histolytica* with nonpathogenic *E. dispar*. For example, in Mexico, the incidence rate of ameobiasis from 1995 to 2000 was reported between 1000 to 5000 cases/ 100,000 inhabitants annually, while the incident values from 2002 to 2006 were 1128.8 to 615.85 cases inhabitants annually. The most affected group of people in developing countries is those under 15 years of age, with a noticeable increase in children aged 5 to 9 years (Ximenz, 2009). In Aracaju, it is demonstrated that *E. histolytica* was found in 1% of cases, while *E. dispar* was found in 13% of the cases. In Ecuador, the reported infection rates of *E. histolytica* and *E. dispar* were 18.9% and 70.3% respectively by using isoenzyme analysis report (Gatti *et al.,* 2002). The prevalence rate of intestinal amoebiasis among hospitalized patients in the Indian subcontinent was found to be around 11.7% using simple microscopy. While using molecular biology tools such as PCR, *E. histolytica* was found to be 3.5% of those infected (Khairnar *et al.,* 2007). In Bangladesh, the prevalence of *E. histolytica,* using ELISA antigen detection kits was found to be 4.2% among the children living in the urban slum of Dhaka (Haque *et al.,* 2006). Different studies have been conducted in different parts of the world, but the most concerned region, i.e. Africa, remains unexplored, therefor the epidemiology of amebiasis remains uncertain especially in Africa.

E. histolytica causes intestinal and extraintestinal amoebiasis based on the site of infection. Though most infections do not harm the host (asymptomatic infections),

establishment in the colonic mucosa via the Galactose/N-acetyl Galactosamine inhibit able lectin (Gal-lectin) is a pre-requisite for the disease (Chadee *et al.,* 1987). Pathogenic forms of the parasite are known to secrete enzymes that facilitate their invasion into the mucosa and sub-mucosa causing deep-flask shaped ulcers and in some cases entering the circulation and reaching internal organs like the liver, lungs, skin, etc. The disease in the colon is the most common with acute diarrhea and dysentery accounting for 90% of the clinical amebiasis cases (Espinosa-Cantellano and Martínez-Palomo, 2000) and only 1% involve the liver (Haque *et al.,* 2003).

Asymptomatic infections are characterized by the parasite living in perfect harmony within the host. *E. histolytica* trophozoites have developed elusive tactics to prevent them from being purged from the host. By modulating signals by intestinal epithelial cells (IEC), trophozoites direct anti-inflammatory host responses leading to a tolerogenic/hyporesponsive immune state favorable to their survival (Kammanadiminti and Chadee, 2006). Furthermore, products secreted by non-pathogenic *E. histolytica* strains normally disrupt and suppress NFNjB signaling and as a result diminish pro-inflammatory responses that are normally detrimental to the parasite (Artis, 2008). Interleukin 10 (IL-10), an anti-inflammatory cytokine, has been shown to play a significant role in maintaining this hyporesponsive state. On the other hand, a deficiency of IL-10 more often than not predisposes the host to develop the clinical amoebiasis (Hamano *et al.,* 2006).

After an incubation period of 1-4 weeks, the parasite invades the colonic mucosa, producing characteristic ulcerative lesions and a profuse bloody diarrhea (amoebic dysentery). Amoebic invasion through the mucosa and into the submucosa is the hallmark of amoebic colitis. Contact of the trophozoites via the Gal/GalNAc lectin triggers a signaling cascade initiating the death of the host cell through different mechanisms such as phagocytosis, cytotoxicity and caspase activation that instigate the invasive (intestinal and/or

extra-intestinal) stages of the disease. Other molecules involved in the disease process include: a serine-rich *E. histolytica* protein (SREHP), amoebapores, and cysteine proteases (Boettner *at al.*, 2002; Mortimer and Chadee, 2010). Activation of damaging inflammatory and non- inflammatory responses following contact of the trophozoites to the gut wall induce a massive neutrophil infiltration across the epithelium into the underlying tissues, resulting in weakening of epithelial cells and the mucous layer and allowing trophozoites to invade the intestinal epithelium and disseminating to other body sites (Ackers and Mirelman, 2006). The ulcers formed may be generalized involving the whole length of the large intestine or they may be localized in the ileo-cecal or sigmoido-rectal regions. Ulcers are normally disconnected with sizes varying from pinhead size to more than 2.5 cm in diameter. They may be deep or superficial. The base of the deep ulcers is generally formed by the muscularis layer. Nonetheless, superficial ulcers do not extend beyond the muscularis layer. A large number of fatalities result from perforated colons with concomitant peritonitis. *E. histolytica* also causes amoebomas. These are pseudo-tumoral lesions, whose formation is associated with necrosis, inflammation and oedema of the mucosa and submucosa of the colon. These granulomatous masses may obstruct the bowel.

While the serine rich *E. histolytica* protein (SREHP) have been shown to promote adhesion of the trophozoites to host cells, cysteine proteases (CP), are known for their virulence in other protozoa as well as in tumor metastasis. Five *E. histolytica* proteins (EhCP1, 2, 3, 5 and 112) have been identified. All are alleged to play a role in the destruction of host cells, phagocytosis, together with the recruitment of neutrophils and macrophages and the induction of intestinal inflammation (Mortimer and Chadee, 2010). Moreover, EhCP5 has also been shown to perform a variety of functions such as evasion of the host complement and immune system by preventing the activation of the classical complement system via the inactivation of IgG and the degradation of IgA (Laughlin and Temesvari, 2005).

The phagosome-associated proteins play an important role in the pathogenesis and virulence of *E. histolytica*. Many have been identified and their function in endocytosis and pathogenesis has been established. Examples include: EhRacA, EhRacG, EhPAK, actin and several Rab7-related GTPases (Laughlin and Temesvari, 2005). Cytokines such as IL-1, , IL-8 and TNF are suspected of aggravating the disease process and driving the immuno-pathogenesis mechanism (Kammanadiminti *et al.,* 2003). Although neutrophils are known to cause intestinal tissue damage they are nevertheless critical for controlling the infection. Nonetheless, host and/or parasite factors normally play a role in determining whether the parasite is cleared or the disease becomes established (Asgharpour *et al.,* 2005).

Although most intestinal invasions heal following an acute inflammatory response, *E. histolytica* evades destruction in a modest number of individuals and a chronic state is established. This chronic state is associated with the development of a non-protective adaptive immune response. Human data, *in vitro* and *in vivo* models support a paradigm that Th1 responses in the gut clear *E. histolytica*, while Th2 responses through the production of IL-4 are anti-protective, likely through suppressing IFN-DŽ. It is not yet clear what to signals drive an anti-protective Th2 immune response instead of an effective protective Th1 response towards the infection. It has been suggested that genetics, the MHC restriction, nutrition and bacterial flora might play a role in directing the immune response towards *E. histolytica* infection e.g. the MHC class II allele DQBl0601 was reported to be associated with resistance to *E. histolytica* (Mortimer and Chadee, 2010). Susceptibility to ALA has been found to be associated with HLA-DR3 and complotype SC01 in some Mexican populations; this association has not been reported for amoebic colitis or asymptomatic colonization with *E. histolytica* (Stanley, 2003).

About 5% of individuals with intestinal amoebiasis develop extra intestinal amoebiasis, 1-3 months after the disappearance of the dysenteric attack. Once in the blood,

the parasite uses many different strategies to avoid elimination by the host and reaches other sites in the body, such as the liver, lungs, brain, etc. The most common extra intestinal site affected by the parasite is the liver and an amoebic liver abscess (ALA) is the most common manifestation, predominantly seen in adult males. This chronic stage of ALA is characterized by defective cell-mediated immunity and the suppression of T cells and their defective proliferative responses (Campbell *et al.,* 1999). *E. histolytica* trophozoites reaching the liver create their unique abscesses, which are well circumscribed regions of cytolysed liver cells, liquefied cells, and cellular debris. The lesions are surrounded by connective tissue enclosing few inflammatory cells and trophozoites. Parenchymal cells adjacent to the lesion are often unaffected. However, lysis of neutrophils by *E. histolytica* trophozoites might release mediators that lead to the death of liver cells, and extend the damage to hepatocytes not in direct contact with the parasite. Studies have shown that in ALA in mice, most hepatocytes die from apoptosis, but necrosis is also present. In ALA from humans, the small numbers of amoebas relative to the size of the abscess suggest that *E. histolytica* can kill hepatocytes without direct contact (Stanley 2003). From the liver, *E. histolytica* trophozoites may enter into the general circulation and reach other organs.

The diagnosis of amoebiasis depends on the demonstration of the *E. histolytica* trophozoites or cysts in the stool or colonic mucosa of patients. For many years direct examination of smear with the help of simple microscope is being used which may need repeated stool sample examination. The presence of haematophagous amoebic trophozoites in a stool sample suggests *E. histolytica* infection (Gonzalez-Ruiz, A. *et al.,* 1994). Nonetheless, the specificity of this finding was further reduced when it was demonstrated that in some patients *E. dispar* also contains RBCs (Fotedar *et al.,* 2007). Moreover, because of high frequency of *E. dispar* in many areas, dysentery due to entities such as shigellosis and

Campylobacter will probably be misdiagnosed as amoebic colitis if microscopy is the sole diagnostic criteria (Stanley 2003).

However, in the absence of haematophagous trophozoites, the sensitivity of microscopy is limited by its ability to distinguish between samples infected with *E. histolytica* and the morphologically identical *E. dispar* and *E. moshkovskii*. Confusion between *E. histolytica*, other non-pathogenic amoeba and white blood cells, such as macrophages and polymorphonuclear cells in feces frequently result in the over diagnosis of amoebiasis. Delays in the processing of stool samples affect the sensitivity of light microscopy, which under the best circumstances is only 60% of that of the stool culture method followed by iso-enzyme analysis (Krogstad *et al.,* 1978).

Stool culture technique followed by iso-enzyme analysis has been considered as the "gold standard" for many years. This method has been used to distinguish between *E. histolytica* and *E. dispar*. Culture of *E. histolytica* can be performed from fecal specimens, rectal biopsy specimens, or liver abscess aspirates. However, the process usually takes between 1-4 weeks to perform and requires sophisticated laboratory equipment, making it not feasible as a routine procedure especially in the developing world where *E. histolytica* is rampant. The rate of success of *E. histolytica* culture in reference laboratories has been reported to be between 50 and 70%. Moreover, isoenzyme (zymodeme) analysis is labor intensive, costly and often produces false-negative results for many microscopy positive stool specimens (Strachan *et al.,* 1988).

Serological methods may be useful diagnostically to detect infections with *E. histolytica* in developed countries where infections are not as common as in endemic developing nations (Ohnishi *et al.,* 1997). In developing countries individuals are constantly exposed to *E. histolytica* making serological tests unable to definitively distinguish past from current infections (Caballero *et al.,* 1994). Amoebic serology is highly sensitive and specific

for the diagnosis of ALA (Zengzhu *et al.,* 1999). Conversely, a study of asymptomatic individuals living in an *E. histolytica* endemic area of Vietnam revealed that about 83% of infected individuals had detectable anti-amoebic antibodies (Blessmann *et al.,* 2002). Several assays for the detection of antibodies to *E. histolytica* infections have been developed. These include: indirect hemagglutination (IHA), latex agglutination, immunoelectrophoresis, counter immunoelectrophoresis (CIE), the amebic gel diffusion test, immunodiffusion, complement fixation, indirect immunofluorescence assay (IFA), and enzyme-linked immunosorbent assay (ELISA). With the exception of ELISA, all the other tests have been either costly to perform (Complement fixation), less sensitive and nonspecific (IHA and Latex agglutination test), time consuming (immunodiffusion) or requires skills in culture and antigen preparation (IFA) (Fotedar *et al.,* 2007).

ELISA is a reliable, easy to perform and rapid method for the diagnosis of *E. histolytica* infections, especially in developing countries. It has been used widely for the study of the epidemiology and diagnosis of symptomatic amoebiasis (intestinal and/or extraintestinal). An ELISA to detect antibodies to *E. histolytica* has been shown to be 97.9% sensitive and 94.8% specific for detection of *E. histolytica* antibodies in ALA patients in a non-endemic country (Hira *et al.,* 2001). Unlike IgG, immunoglobulin M (IgM) is short lived and does not remain in the serum for longer periods, making it a very useful marker for the detection of current *E. histolytica* infections. An ELISA for the detection of serum IgM antibodies to the amoebic Gal or GalNAc-inhibitable adherence lectin has been reported. In this study, conducted in Egypt, anti-lectin IgM antibodies in the serum were detected in 45% of patients who had been suffering from acute colitis for <1 week (Abd-Alla *et al.,* 1998). Since there is no cross-reaction with other non-*E. histolytica* parasites (Goncalves *et al.,* 2004), the use of ELISA thus seems to be an excellent choice for the routine laboratory diagnosis as well as the surveillance and control of amoebiasis in the developing world.

The newer methods available to distinguish between *E. dispar* and *E. histolytica* have suggested that the actual number of infections may be closer to 50 million rather than the commonly accepted figure of 500 million infections worldwide. PCR and monoclonal antibody techniques are now available to distinguish between these three species in fresh and preserved stool samples, including those with mixed infections. Several investigators have developed ELISAs that detect antigens in fresh stool samples with sensitivity closer to that of stool culture methods and PCR. These ELISAs are usually easy and rapid to perform. Copro-antigen based ELISA kits specific for *E. histolytica* exploit monoclonal antibodies against the Gal/GalNAc-specific lectin of *E. histolytica* (*E. histolytica* II; Tech Lab, Blacksburg, VA) or against serine-rich antigen of *E. histolytica* (Optimum S kit; Merlin Diagnostika, Bornheim-Hersel, Germany). Other ELISA kits include the *Entamoeba* CELISA PATH kit (Cellabs, Brookvale, Australia) and the ProSpecT EIA (Remel Inc.; previously manufactured by Alexon-Trend, Inc., Sunnyvale, CA) (Fotedar*et al.,* 2007). Tech Lab introduced an ELISA kit for the specific detection of *E. histolytica* in feces during last decade of 20th century. This antigen detection test capture and detects the parasite's Gal/GalNAc lectin in stool samples. It can also be used for the detection of the lectin antigen in the serum and liver abscesses in patients with invasive intestinal amoebiasis and ALA (Haque *et al.,* 2000). However, the diagnosis of ALA normally relies on the identification of liver lesions and positive anti-*E. histolytica* serology but these both techniques does not provide conclusive results for ALA. The Gal/GalNAc lectin is conserved and highly immunogenic, and because of the epitopic differences in the lectins of *E. histolytica* and *E. dispar*, the test enables specific identification of *E. histolytica* (Haque *et al.,* 1993; Mirelman 1997). Because of some disadvantages observed with the TechLab ELISA kit, a newer, more sensitive and specific version, Tech Lab *E. histolytica* II kit, was produced. This second –generation *E. histolytica* II kit has demonstrated good sensitivities and specificities when compared to real-time PCR (71% to

79% and 96% to 100%, respectively) (Roy *et al.,* 2005; Visser *et al.,* 2006). Other studies, however, have reported a lesser sensitivity (14.3%) and specificity (98.4%) in comparison to stool culture and isoenzyme analysis (Gatti *et al.,* 2002). Cross reactivity has been another concern with the use of the assay, since it seems that *E. dispar* positive samples by means of PCR may sometimes give false-positive outcomes (Furrows *et al.,* 2004). Accordingly, accurate detection of *E. histolytica*, *E. dispar* and *E. moshkovskii* could be helpful for diagnostic and epidemiological studies in places where it is impractical and expensive to use molecular assays and where amoebiasis is most prevalent, such as in the developing countries. An antigen detection kit for the specific identification of *E. dispar* and *E. moshkovskii* is yet to be developed.

Several PCR-based techniques that amplify and detect *E. histolytica* DNA is currently used for the clinical and epidemiological studies in non-endemic rich countries (Acuna-Soto *et al.,* 1993; Katzwinkel-Wladarsch *et al.,* 1994; Calderaro *et al.,* 2006; Hamzah *et al.,* 2006). The sensitivity and specificity of PCR-based methods for the diagnosis of *E. histolytica* infection approaches those of stool culture followed by iso-enzyme analysis. PCR methods can be used to detect *E. histolytica* in stool, tissues and liver lesion aspirates. Of all the different gene targets used to identify *E. histolytica*, the small-subunit rRNA gene (18SrDNA) is believed to be more sensitive than the best antigen detection method used and performs equally well compared to stool culture (Mirelman *et al.,* 1997). Several groups have developed a variety of excellent conventional PCR assays, targeting different genes, for the direct detection and differentiation of *E. histolytica*, *E. dispar*, and E. moshkovskii DNA in clinical specimens such as stool and liver abscess samples (Tanyuksel and Petri Jr., 2003; Paul *et al.,* 2007). Of all the targeted genes, assays amplifying the 18SrDNA genes are the ones in wide use as they are present in multiple copies on extra-chromosomal plasmids, thus making them easily detectable than a single copy genes (Battacharya *et al.,* 1989). Other gene

targets used in PCR to study the epidemiology of *E. histolytica* include: the serine-rich *E. histolytica* protein (SREPH) gene (Stanley *et al.,* 1990), cysteine proteinases gene and actin genes (Freitas *et al.,* 2004). The SREHP is also used to study the genotypes of *E. histolytica* in human populations. However, it is now being replaced by the use of PCR amplification of tRNA gene-linked short tandem repeats, which in addition to providing details of the epidemiology of *E. histolytica*, it also provides a tool to predict the outcome of the infection (Ali *et al.,* 2005).

A nested-multiplex PCR method was developed by many groups. This method has the added advantage of increasing the sensitivity and specificity of the test whilst simultaneously detecting and differentiating *E. histolytica* and *E. dispar* from DNA extracted from microscopy-positive stool specimens (Evangelopoulos *et al.,* 2000; Hung *et al.,* 2005; Nunez *et al.,* 2001). A nested PCR method for the identification of E. moshkovskii in fecal samples was developed as a nested 18S rDNA PCR followed by restriction endonuclease digestion (Ali *et al.,* 2003). The method exhibited a high sensitivity and specificity (100%).

Real time PCR is another type of PCR that is more sensitive than the conventional PCR. It is faster than the conventional PCR and characterized by the elimination of gel analysis and other post-PCR analysis, thus reducing the risk of contamination and cost (Klein 2002). However, its application in developing countries is limited to research only. Real-time PCR allows specific detection of the PCR product by binding to one or two fluorescence-labeled probes during PCR, thereby enabling continuous monitoring of the PCR product formation throughout the reaction. Furthermore, real-time PCR is a quantitative method and allows the determination of the number of parasites in various samples (Fotedar *et al.,* 2007). Despite being used for the successful identification of *E. histolytica*, *E. dispar* and E. moshkovskii, the use of PCR methods is still confined to research institutes in the developing world where

amoebiasis is endemic. PCR-based diagnosis in low-income societies is hindered by difficulties such as cost, and time to perform the test.

A new platform for the detection of pathogens has been developed known as loop-mediated isothermal amplification (LAMP) and was developed in 2000 by Notomi and colleagues. This method uses a set of two specifically designed inner primers and two outer primers that recognize six distinct regions of the targeted DNA. The reaction is performed under isothermal conditions and simple incubators, such as a water bath or heat block, are adequate for the specific amplification of the desired genetic material. Considering these advantages, the LAMP assay could be a useful and valuable diagnostic tool particularly in developing countries where most of the infections are common in hospital laboratories. Recently this method was developed specifically for the detection of *E. histolytica* (Liang *et al.,* 2009). The efficiency of the developed method was compared to that of existing PCR methodology and was similar in terms of sensitivity and specificity. This method needs further evaluations to be used in local conditions in Africa in order to improve the understanding of amebiasis in the continent as well as other parts of the world.

The outcome of an infection depends upon several factors among which the genetic characteristics of the specific pathogen have been identified as an important one. Few polymorphic genetic loci have been identified and targeted to aid in the study of the population structure of *E. histolytica* strains and their possible relationships with the parasite's virulence and disease outcome (Clark, 2006; Paul *et al.,* 2007). Examples of these genetic markers include protein coding genes (serine-rich *E. histolytica*protein, [SREHP] and Chitinase) and non-coding DNA (Strain Specific Gene and tRNA gene linked short tandem repeats [STR]) of PCR-amplified genes (Haghighi *et al.,* 2003; Samie *et al.,* 2008). In a study in Bangladesh, the tRNA-linked STR genotyping system has provided evidence that the parasite genome does influence the outcome of infection. tRNA-linked STR genotyping was

also behind the recent observation of the differences between parasite genotypes in the intestine and the liver abscess of the same patients (Ali *et al.,* 2007). Few studies, albeit inconclusive, using the polymorphic SREHP marker have indicated that certain SREHP profiles might be responsible for the presentation of intestinal amoebic symptoms (Ayeh-kumi *et al.,* 2001; Samie *et al.,* 2008). All studies with SREHP marker did support previous findings of extensive genetic diversity among *E. histolytica* isolates from the same geographic origin (Ayeh-kumi *et al.,* 2001; Simonishvili *et al.,* 2005; Samie *et al.,* 2008; Tanyuksel *et al.,* 2008). Thus, it seems that the parasite genotype does play a role in the outcome of infection in humans thus linking parasite diversity and virulence. Other approaches, such as SNP identification coupled with microarray-based analysis of gene expression or proteomic comparisons among parasites will be needed to identify the actual genes responsible for these results and to help us understand the mechanism of parasite virulence and pathogenesis (Ali *et al.,* 2008).

Till date, there is not much known about the species prevalence rates in different regions of the world, particularly in the African continent where very few studies have been conducted using molecular methods. In order to address this limitation, there is a need to implement species-specific diagnosis of *E. histolytica*, *E. dispar* and E. moshkovskii, particularly in countries where these organisms are endemic. Based on the limited information available, it appears that molecular and genomic studies still need to be combined to molecular epidemiology studies in order to advance our understanding of amoebiasis. The currently available genome sequence is very useful in better understanding the biology of the parasite, however, genome of *E. histolytica* strains from Africa still needs to be sequenced. Comparative genomics will probably allow the understanding of the pathogenicity of some strains of *E. histolytica* compared to non-pathogenic strains as well as better understanding of *E. dispar* in relation to *E. histolytica*. Further collaborations between

scientists from developing countries and those from developing countries is essential in answering questions on the epidemiology, pathogenesis and biochemistry of *E. histolytica* which is the causing agent of amoebiasis.

CHAPTER 3
MATERIALS AND METHODS

3.1: COLLECTION OF SAMPLES

During a period of one year, from December 2008 to November 2009, blood (n = 600) and fecal samples (n = 600) were collected aseptically from veterinary hospitals (Thoker Niaz Baig and Jallo area) and Pet center at the University of Veterinary and Animal Sciences, Lahore. Fecal samples of human (n = 600) were collected from medical hospitals (named as Children, Mayo and Jinnah) as well as from the houses where dogs were kept as a pet animal. The water samples comprised of tap (n = 300) and sewerage (n = 300) was collected from houses located in different areas of the Lahore city. Likewise, soil samples (n =600) were also collected from different areas of the city, Lahore.

3.1.1: COLLECTION OF FECAL SAMPLES FROM DOGS AND DOG OWNERS

During one year, approximately 50 fecal samples/month were collected from dog and their owners. Fecal samples were collected in three aliquots in the sterile bottles; two bottles were added with sodium acetate acetic acid formalin fixative (SAF) and one without fixative. Sodium acetate acitic acid formaline fixative was prepared as. Sodium acetate 1.5g, glacial acid 2.0 ml, Formaldehyde 37-40% solution 4.0ml and Distilled water 92.0 ml. The collection was done on three consecutive days; day 1 and 3 in the fixative, whereas, day 2 collection was added without fixative. The collected samples were processed at the parasitology laboratory of the University of Veterinary and Animal Sciences, Lahore, Pakistan where the samples were examined by the triple fecal test and the stool antigen ELISA. A brief history regarding age, sex, and other necessary information was also recorded.

3.1.2: COLLECTION OF WATER SAMPLES

Approximately 25 samples (20 mL) each comprised of tap water and sewerage per month were collected from houses and different drains in the city Lahore. The collected samples were brought to the Parasitology laboratory at the University of Veterinary and Animal Sciences, Lahore. These samples were examined through zinc sulphate concentration floatation method. (Foreyt, 2001)

3.1.3: COLLECTION OF SOIL SAMPLES

During the study 600 soil samples (50/ month) consisting 20gm each. Samples were collected from parks. These samples were brought to the Parasitology laboratory, University of Veterinary and Animal Sciences, Lahore and were processed by zinc sulphate concentration floatation method. (Foreyt, 2001)

3.1.4: COLLECTION OF BLOOD SAMPLES FROM DOGS AND DOG OWNERS

Six hundred (600) blood samples each from dogs and dog owners (5ml each) were collected.

Blood collection was done either from the cephalic or sepenous vein in dogs and from wrist vein in dog owners described as, the collection areas was shaved first and then apply the antiseptic. The collection was done with 5ml syringe and was added to venoject (Terumo Europe, Belgium) coated with anticoagulant (K-EDTA)

3.2: EXAMINATION OF FECAL SAMPLES

3.2.1: TRIPLE FECAL TEST

The triple fecal test was performed according to method as described by Van Gool et al. (2003). Briefly, fixative added samples were screened with iodine staining for cysts and trophzoites of *Entamoeba histolytica* and positive samples were examined by permanent staining with chlorazole black stain. The unpreserved samples were treated with fromalin-

ether concentration method (Reference) and were examined for cysts and trophozoites of *Entamoeba histolytica*.

3.2.2: Stool Antigen ELISA

The unpreserved samples were used for detection of specific antigen of *Entamoba histolytica* in feces and stool samples by using the commercially available ELISA kit (Techlab, Blacks burg, Virginia, USA). Briefly, the diluted stool and fecal samples were added to polyclonal anti-*Entamoeba histolytica* adhesion antibodies added microtitre ELISA plates. The binding of antigen and antibody was detected by the addition of horseradish peroxidase (HRP) enzyme. The intensity of developed color was recorded by an ELISA reader (Thermolectron, Finland).

EXAMINATION OF WATER AND SOIL SAMPLES

ZINC SULPHATE CONCENTRATION METHOD

Briefly, 10mL of water sample and 10 gram of soil samples was mixed with a floatation solution (Zinc Sulphate) was strained and centrifuged at 1500 RPM for 10 minutes separately. The centrifuge tube was then filled with $ZnSo_4$solution upto the top of centrifuge tube and a cover slip was put on the top of the solution. After some time, the cover slip was removed from the centrifuge tube and put on the slide for examination under microscope for the cyst of *Entamoeba histolytica*. Same procedure was replicated taking 3-5 gram of soil and mixing together with $ZnSO_4$solution.

3.3: HAEMATOLOGICAL EXAMINATION OF BLOOD FROM DOGS AND HUMAN

3.3.1: COMPLETE BLOOD COUNT (CBC)

The complete blood count (CBC) was done through hematological analyzer (Abacus, Austria).

3.3.2: LIVER ENZYMES AND ELECTROLYTE ANALYSIS

The collected blood was centrifuged at 4000rpm for 10min, plasma was separated and stored at 20^{o}C till further use. The concentration of the liver enzymes such as serum glutamic pyruvic transaminase (SGPT) and Glutamic oxcalacetic transaminase (SGOT) was evaluated using commercially available (Sigma Chemicals, MO, USA), whereas, the lactate dehydrogenase (LDH) concentration was observed using other commercially available kit (Quinicaclinicaaplicada, Amposta, Spain). The samples were processed as per manufacturer's recommendations. The color intensity was read through the serum chemistry analyzer (Metrolab, Argentina). Similarly, serum samples were further analyzed for sodium (Na), potasium (K), Calcium (Ca) and Magnesium (Mg) using commercial kits (RandoxLaboratries Ltd. UK). The color intensity was read through the serum chemistry analyzer (Metrolab, Argentina).

3.4: THERAPEUTIC TRIALS

A total of 120 dogs (110 infected and 10 healthy) of age from 1-3 years and of either sex were included during late summer and autumn. All these dogs were kept under similar feeding and managmental conditions throughout the course of treatment. Detailed history and treatment of each dog was recorded on a prescribed Performa.

3.4.1: GROUPING OF DOGS

One hundred and ten dogs were randomly divided into five groups viz; A,B,C,D and E. The first 3 groups were further subdivided into 3 subgruops A1, A2, A3, B1, B2, B3, C1, C2, and C3 having 10 dogs in each group while 10 dogs each were kept in group D and E.

Table 3.1: Grouping of dog according to drug used and mode of treatment

Name of drug	**Groups of dogs**	**Sub-groups**	**Dose rate (mg/Kg of body weight)**
Nigella sativa (Kalwangi)	A	A1	60
		A2	70
		A3	80
Sausserea lappa (Qust-e-shireen)	B	B1	60
		B2	70
		B3	80
Allium cepa (Onion)	C	C1	60
		C2	70
		C3	80
Metronidazole®	D	-	11.5
Positive Control	E	Ocyst infection only	
Mock	F	No drug and infection	

3.4.2: PREPARATION AND ADMINISTRATION OF HERBAL DRUGS

Methanol extract was prepared by using soxhlet's apparatus. The extract obtained was evaporated till the complete removal of alcohol and administered to dogs. The methanol extracts were suspended in 2%gum tragacanth solution for the oral administration of the animals. Thus 2% of w/v aqueous solution were prepared and stored in the refrigerator at $4^{O}C$. At the time of medication, calculated amount of the powdered drugs was weighed according to dosage levels and was given orally by using a drenching gun. Qust-e-sheireen (*Saussurea lappa*) was suspended in 300mL gum solution because of its low solubility. The three dosage levels of herbal drugs were selected on the basis of preliminary trials in animals and keeping in mind the doses used in human beings as traditional anthelmintics.

3.4.3: PARASITOLOGICAL EVALUATION OF EXPERIMENTAL DOGS

Post administration, the fecal samples were collected on 0, 3^{rd}, 7^{th}, and 18^{th} day. The animals which were positive even on 18^{th} day were given a second dose of respective drug and feces were re-examined on 21^{st} and 28^{th} day post treatment.

The floatation method was used for the fecal diagnosis of *Entamoba histolytica*, which has the potential to identify amoebic trophozoites or cysts (Barreto 1962).The amoebic cysts and Amoebic trophozoites were seen by the following procedure.

a) A 15ml centrifuge tube was filled with $ZnSO_4$ solution (1.18 specific gravity) and poured into glass dish.

b) Using a tonque depressor, the faeces were pushed (2 to 3 grams, a piece the size of a grape) through the strainer into the $znso_4$ solution in the dish.

Precaution was taken that the sieve must be in the liquid in order for the feces to pass through.

c) If more the feces were used, the more likely would be able to find eggs, which are present in low numbers.

d) By using a funnel, $ZnSO_4$ fecal mixture was poured back into the centrifuge tube.

e) Feces were centrifuged for 2 minutes at high speed (1500-2000 RPM).

f) Using a headed-rod or loop, a sample was removed from the surface of the solution and placed on a microscope slide. One drop of iodine was added (to skin the cysts and ova) and a cover slip. The slide was examined at a 10X power of the microscope.

3.4.5: EFFICACY OF DRUGS

Efficacy of drug was calculated on the basis of reduction in the fecal egg count after treatment and by controlling method described by Moskey and Harwood (1941).

a. Percent efficacy = a – b (100)/egg per gram of feces before treatment

b. Egg per gram of feces after treatment

3.4.6: BIOCHEMICAL ANALYSIS PROCEDURE

Blood samples were collected from each animal of all groups before and after treatment on 0, 18th day and 28th day. The clean non homolized sera was prepared after blood coagulation and kept in a clean vial at 20^{o}C until used. The serum was used for quantitative determination of serum glutamic oxalocetictransminase (SGOT), serum glutamic pyruvic transaminase (SGPT) serum alkaline phophatare (SAP) and complete blood count (CBC) and electrolyte i.e Na, Ca, Mg, , K analysis.

CHAPTER 4
RESULTS

4.1: PREVALENCE OF AMOEBIASIS IN DOGS

From December 2008 to November 2009, the prevalence of *Entamoeba histolytica* was determined in dogs (n = 600) using the triple fecal test as well as commercially available ELISA kit (Cellab, USA). Compared to ELISA, triple fecal test showed higher positivity or prevalence of *Entamoeba histolytica*. Of the total samples processed, only 94 (15.66%) fecal samples were found positive by the triple faecal test, whereas, using ELISA, the positivity to *Entamoeba histolytica* in fecal sample was 66 (11.0%). Only 66 samples were found positive through ELISA.

4.1.1: PREVALENCE OF *ENTAMOEBA HISTOLYTICA* BY MONTH

Based upon triple fecal test, the prevalence of *Entamoeba histolytica* was highest in August (30.0%) followed by July (26.0%), whereas, it was the lowest in January, April and November (12.0% each) (P = 0.130). Likewise, based upon ELISA, prevalence of *Entamoeba histolytica* was highest in August (22.0%) followed by July (20.0%), whereas, it was the lowest in November (6.0%) (P = 0.168) (Table 4.1).

4.1.2: PREVALENCE OF *ENTAMOEBA HISTOLYTICA* BY SEASON

Season-wise, using the triple fecal test, the prevalence of *Entamoeba histolytica* was highest in summer (21.0%) followed by autumn (15.0%), whereas, it was the lowest (12.0%) during the winter (P = 0.000). Similarly, based upon ELISA, the prevalence was highest in summer (15.5%) followed by autumn (10.0%), whereas, it was the lowest in winter (8.0%) (P = 0.090) (Table 4.2).

Table 4.1: Month-wise prevalence of *Entamoeba histolytic*a in dogs by triple fecal test and ELISA

Month	**Number examined (N)**	**Triple Fecal Test**			**ELISA**		
		Positive (n)	**Percent infection (%)**	**P value**	**Positive (n)**	**% infection**	**P value**
December – 2008	50	7	14.0	0.130	5	10	0.168
January – 2009	50	6	12.0		4	8.0	
February – 2009	50	5	10.0		4	8.0	
March – 2009	50	7	14.0		5	10	
April – 2009	50	6	12.0		4	8.0	
May – 2009	50	5	10.0		3	6.0	
June – 2009	50	9	18.0		7	14.0	
July – 2009	50	13	26.0		10	20.0	
August – 2009	50	15	30.0		11	22.0	
September – 2009	50	8	16.0		6	12.0	
October – 2009	50	7	14.0		4	8.0	
November – 2009	50	6	12.0		3	6.0	
Total	**600**	**94**	**15.66**		**66**	**11.0**	

Table 4.2: Season-wise prevalence of *Entamoeba histolytica* in dogs by triple fecal test and ELISA

Season	No. examined (N)	Triple Fecal Test			ELISA		
		Positive (n)	% infection	P value	Positive (n)	% infection	P value
Winter	200	24	12	0.000	16	8.0	0.090
Spring	100	13	13		09	9.0	
Summer	200	42	21		31	15.5	
Autumn	100	15	15		10	10.0	
Total	**600**	**94**	**15.66**		**66**	**11.0**	

Table 4.3: Age-wise prevalence of *Entamoeba histolytica* in dogs by triple fecal test and ELISA

Age group	No. examined (N)	Triple Fecal Test			ELISA		
		Positive (n)	% infection	P Value	Positive (n)	% infection	P Value
0 – 6 Month	170	28	16.47	0.001	19	11.2	0.0012
7 Month – 1 year	145	36	24.82		27	18.6	
1 – 4 year	285	30	10.52		20	7.0	
Total	**600**	**94**	**15.66**		**66**	**11.0**	

4.1.3: PREVALENCE OF *ENTAMOEBA HISTOLYTICA* BY AGE

Using the triple fecal test, the prevalence was highest in dogs of age group of one month to 1 year (24.82%) than 1-4 years (10.52%) (P = 0.001). Likewise, using ELISA, the prevalence was highest in 7 months-1 year age group (18.6%), whereas, it was the lowest in 1-4 years group (7.0%) (P = 0.0012) (Table 4.3).

4.1.4: PREVALENCE OF ENTAMOEBA HISTOLYTICA BY SEX

With respect to sex, using the triple fecal test, less difference in prevalence was seen between male and female dogs (16.0% and 15. 1%), respectively (P = 0.768). Contrary to this, using ELISA, a difference in prevalence was seen where frequency of *Entamoeba histolytica* was more in male (12.4%) than females (8.8%) (P = 0.167) (Table 4.4).

Table 4.4: Sex-wise prevalence of *Entamoeba histolytica* in dogs by triple faecal test and ELISA

Sex	No. examined (N)	Triple Fecal Test			ELISA		
		Positive (n)	% infection	P value	Positive (n)	% infection	P value
Male	362	58	16.0	0.768	45	12.4	0.167
Female	238	36	15.12		21	8.8	
Total	**600**	**94**	**15.66**		**66**	**11.0**	

4.2: PREVALENCE OF *ENTAMOEBA HISTOLYTICA* IN DOG OWNERS

Of the total stool sample examined during a period of one year (n = 600 and 50/month), using the triple fecal test, *Entamoeba histolytica* was detected only in 135 (22.5%) samples. However, the analysis of stool sample via ELISA detected *Entamoeba histolytica* among 101 samples (16.8%). Compared to ELISA, triple fecal test detected higher prevalence of *Entamoeba histolytica*.

4.2.1: PREVALENCE OF ENTAMOEBA HISTOLYTICA BY MONTH

In human, based upon triple fecal test, the prevalence of *Entamoeba histolytica* was highest in August (42.0%) followed by July (38.0%), whereas, it was the lowest in January, April and November (16.0%) (P = 0.017). Likewise, based upon ELISA, prevalence of *Entamoeba histolytica* was highest in August (34.0%) followed by July (30.0%), whereas, it was the lowest in January (10.0%) (P = 0.021) (Table 4.5).

4.2.2: PREVALENCE OF ENTAMOEBA HISTOLYTICA BY SEASON

Season-wise, using the triple fecal test, the prevalence of *Entamoeba histolytica* was highest in summer (31.0%), whereas, it was the lowest (17.0%) during the winter (P = 0.005). Similarly, based upon ELISA, the prevalence was highest in summer (24.0%) followed by spring (13.0%), whereas, it was the lowest in winter (12.5.0%) (P = 0.010) (Table 4.6).

4.2.3: PREVALENCE OF *ENTAMOEBA HISTOLYTICA* BY AGE

Using the triple fecal test, the prevalence was highest in human of age group of 15-20 year (31.96%), whereas, it was lowest in 36 years of age (17.25%) (P = 0.009). With respect to age, using ELISA, the prevailing pattern of Entamoeba histolytica was similar to that observed with triple fecal tests. It was highest in age group of 15-20 years (24.6%), whereas, it was lowest in age group of 36 and above (11.7%) (P = 0.011) (Table 4.7).

4.2.4: PREVALENCE OF ENTAMOEBA HISTOLYTICA BY SEX

With respect to sex, using the triple fecal test, the prevalence was slightly higher in males (22.72%) than females (17.85%) (P = 0.547). Contrary to this, using ELISA, a difference in prevalence was seen where the frequency of *Entamoeba histolytica* was more in male (18.7%) than females (14.3%) (P = 0.712) (Table 4.8).

Table 4.5: Month-wise prevalence of *Entamoeba histolytica* in dog owners by triple faecal test and ELISA

Month	No. examined	Triple Fecal Test			ELISA		
		Positive (n)	% infection	P value	Positive (n)	% infection	P value
December – 2008	50	9	18	0.017	7	14.0	0.021
January – 2009	50	8	16		5	10.0	
February – 2009	50	9	18		6	12.0	
March – 2009	50	10	20		8	16.0	
April – 2009	50	8	16		6	12.0	
May – 2009	50	10	20		7	14.0	
June – 2009	50	12	24		10	20.0	
July – 2009	50	19	38		15	30.0	
August – 2009	50	21	42		17	34.0	
September – 2009	50	10	20		7	14.0	
October – 2009	50	11	22		7	14.0	
November – 2009	50	8	16		6	12.0	
Total	**600**	**135**	**22.50**		**101**	**16.83**	

Table 4.6: Season-wise prevalence of *Entamoeba histolytica* in dog owners by triple faecal test and ELISA

Season	No. examined (N)	Triple Fecal Test			ELISA		
		Positive	% infection	P value	Positive	% infection	P value
Winter	200	34	17	0.005	25	12.5	0.010
Spring	100	18	18		13	13.0	
Summer	200	62	31		48	24.0	
Autumn	100	21	21		15	15.0	
Total	**600**	**135**	**22.5**		**101**	**16.8**	

Table 4.7: Age-wise prevalence of *Entamoeba histolytica* in dog owners by triple faecal test and ELISA

Age group	No. examined (N)	Triple Fecal Test			ELISA		
		Positive (n)	% infection	P value	Positive (n)	% infection	P value
15 – 20 years	122	39	31.96	0.009	30	24.6	0.011
21 – 35 years	281	62	22.06		48	17.1	
36 and above	197	34	17.25		23	11.7	
Total	**600**	**135**	**22.5**		**101**	**16.8**	

Table 4.8: Sex-wise prevalence of *Entamoeba histolytica* in dog owners by triple faecal test and ELISA

Sex	No. examined (N)	Triple Fecal Test			ELISA		
		Positive (n)	% infection	P value	Positive (n)	% infection	P value
Male	572	130	22.72	0.547	97	18.7	0.712
Female	28	5	17.85		4	14.3	
Total	**600**	**135**	**22.5**		**101**	**16.8**	

4.3: PREVALENCE OF *ENTAMOEBA HISTOLYTICA* INFECTION IN SEWERAGE AND TAP WATER

A total of 600 samples comprised sewerage (n = 300) and tap water (n = 300) was examined $ZnSO_4$ concentration method. During each month of studying year, 25 samples each were collected and analyzed. Of the total sewerage samples, only 95 (31.66%) were found positive to *Entamoeba histolytica.* The prevalence was highest in august (64.0%) followed by July (60.0%), whereas, it was lowest in December (8.0%) (P = 0.000). For tap water, on the other hand, only 16 (5.33 %) were found positive to *Entamoeba histolytica*. The prevalence, in equal percentage (12.0%), was noted during the months of June and August, whereas, the months of December, January and November were found to be devoid of detection of *Entamoeba histolytica* (P = 0.527) (Table 4.9). With respect to season, in sewerage water samples, the prevalence was highest during summer (53.0%), whereas, it was the lowest in winter (14.0%) (P = 0.000). Contrary to this, in tap water, the prevalence was more frequent in summer (10.0%) followed by spring (6.0%), whereas, it was the lowest in winter (1.0%) (P = 0.041) (Table 4.10).

Table 4.9: Month-wise prevalence of *Entamoeba histolytica* in sewerage and tap water

Month	No. examined (N)	Sewerage water			Tap water		
		Positive (n)	% infection	P value	Positive (n)	% infection	P value
December 2008	25	2	8	0.000	0	0	0.527
January – 2009	25	4	16		0	0	
February – 2009	25	5	20		1	4	
March – 2009	25	5	20		1	4	
April – 2009	25	7	28		2	8	
May – 2009	25	9	36		2	8	
June – 2009	25	13	52		3	12	
July – 2009	25	15	60		2	8	
August – 2009	25	16	64		3	12	
September – 2009	25	10	40		1	4	
October – 2009	25	6	24		1	4	
November – 2009	25	3	12		0	0	
Total	**300**	**95**	**31.66**		**16**	**5.33**	

Table 4.10: Season-wise prevalence of *Entamoeba histolytica* in sewerage and tap water

Season	No. examined (N)	Sewerage water			Tap water		
		Positive (n)	% infection	P value	Positive (n)	% infection	P value
Winter	100	14	14	0.000	1	1	0.041
Spring	50	12	24		3	6	
Summer	100	53	53		10	10	
Autumn	50	16	32		2	4	
Total	**300**	**95**	31.7		16	5.3	

4.4: PREVALENCE OF *ENTAMOEBA HISTOLYTICA* IN SOIL

Of the total of 600 (50/month) soil samples examined, only 65 (10.8%) were found positive. A higher frequency of *Entamoeba histolytica* was seen during August (20.0%) followed by July (18.0%), whereas, it was lowest in December (4.0%) (P = 0.082) (Table 4.11). With respect to season, the highest prevalence was observed in summer (12.5%) followed by autumn (16.0%), whereas, it was the lowest during winter (7.5%) (P = 0.112) (Table 4.12).

Table 4.11: Month-wise prevalence of *Entamoeba histolytica* in soil samples

Month	**No. examined**	**No. positive**	**% infection**	**P value**
December – 2008	50	2	4	0.082
January – 2009	50	3	6	
February – 2009	50	3	6	
March – 2009	50	5	10	
April – 2009	50	4	8	
May – 2009	50	3	6	
June-2009	50	3	6	
July – 2009	50	9	18	
August – 2009	50	10	20	
September – 2009	50	8	16	
October – 2009	50	8	16	
November – 2009	50	7	14	
Total	**600**	65	10.83	

Table 4.12: Season-wise prevalence of *Entamoeba histolytica* in soil samples

Season	**No. examine**	**No. positive**	**% infection**	**P value**
Winter	200	15	7.5	0.112
Spring	100	9	9	
Summer	200	25	12.5	
Autumn	100	16	16	
Total	**600**	**65**	**10.8**	

4.5: THERAPEUTIC TRIALS AND AMOEBIASIS

The results of therapeutic trials with herbal drugs, including *Nigella sativa, Saussurea lappa, Allium cepa* and commercially available allopathic drug i.e. Metromidazole® were as follows;

4.5.1a: *NIGELLA SATIVA*

Compared to day 0, different dose treatment and subsequent evaluation for cyst shedding showed a significant difference at various intervals of days (P = 0.000). While comparing different dose concentrations of *Nigella sativa* used, 80mg/Kg of body weight was found to be more effective than both 60mg/Kg and 70mg/Kg. The general body condition of dogs was improved after treatment which remained healthy with no clinical toxic effects observed. Nevertheless, the efficacy of Metronidazole® at the recommended dose level was better than *Nigella sativa* (Table 4.13).

4.5.1b: *SAUSSUREA LAPPA* (Qust-e-shireen)

All the doses (60, 70 and 80 mg/kg body weight) of *Saussurea lappa* caused a significant decreased in cyst shedding from 7 day post treatment onward (P = 0.000). On 28th day, the efficacy of *Saussurea lappa* was 50.72%, 53.73% and 54.79% using 60, 70 and 80 mg/Kg of dose. The efficacy of all the doses of *Saussurea lappa* was comparable with almost similar results. Nevertheless, the efficacy of Metronidazole® was still better than *Saussurea lappa* (Table 4.14).The general body condition of all the three treated subgroups with *Saussurea lappa* improved gradually with no clinical toxic effects.

Table 4.13: Effect of *Nigella sativa* against *Entamoeba histolytica* on different days of treatment

Dosage level (mg/kg)	Efficacy in percentage on different days				P value
	0 day	7th day	18th Day	28th Day	
60	335	255(23.88%)	210 (37.31%)	115 (65.67%)	0.000
70	375	285 (24.0%)	225 (40.0%)	120 (68.0%)	
80	345	260 (24.63%)	195 (43.47%)	105 (69.56%)	
Metronidazole® (25 mg/kg)	350	200 (42.85%)	100(50%)	0	

Table 4.14: Effect of Saussurea *lappa* against *Entamoeba histolytica* on different days of treatment

Name of drug	Efficacy in percentage on different days				P value
Dose (mg/Kg)	0 day	7th day	18th day	28th day	
60	345	270 (21.73%)	220 (23.36%)	170 (50.72%)	0.000
70	335	255 (23.83%)	205 (38.80%)	155 (53.73%)	
80	365	275 (24.65%)	220 (39.72%)	165 (54.79%)	
Metronidazole® (25 mg/kg)	350	200 (42.85%)	100(50%)	0	

4.5.1c: *ALLIUM CEPA* (Onion)

Of the different dose rate applied, followed by subsequent evaluation of its efficacy via cyst shedding, dose (80mg/Kg of the body weight) was found to be more effective than others (P = 0.006). On the 28th day of the drug administration, the efficacy of *Allium cepa* was found to be 43.66%, 45.33% and 50.68% of the doses 60mg/Kg, 70 mg/Kg and 80mg/Kg, respectively. Like other two drugs, the efficacy of Metromidazole® was still better than *Allium cepa* (Table 4.15). The general body condition improved after treatment and no clinical toxic effect of *Allium cepa* was seen.

Table 4.15: Effect of *Allium cepa* against *Entamoeba histolytica* on different days of treatment

Dose (mg/kg)	**Efficacy in percentage on different days**				**P value**
	0 day	**7th day**	**14th day**	**28th day**	
60	355	305 (14.08%)	250 (29.57%)	200 (43.66%)	0.006
70	375	315 (16%)	255 (32%)	205 (45.33%)	
80	365	305 (16.43%)	235 (35.61%)	180 (50.68%)	
Metronidazole® (25 mg/kg)	350	200(42.85%)	100(50%)	0	

4.6: EFFECT OF HERBAL AND ALLOPATHIC DRUGS ON BLOOD PROFILE, SERUM ENZYMES AND ELECTROLYTES

From the results, it is evident that *Nigella Sativa,* at all the three dosage levels, showed good effect in keeping the normal blood chemistry profile. In this regards, dose rate of 80mg/Kg showed a considerable effect not only in treatment against *Entamoeba* but also in keeping the blood profile of dogs as normal (Table 4.16). Before and after treatment with *Saussurea lappa*, the results indicated that *Saussurea lappa* at all the three dosage levels showed somewhat notable effects on the blood picture. At the dose rate of 80 mg/kg body weight, it had a slight effect on the haemoglobin, neutrophils, eosinophils, basophils, lymphocytes and monocytes levels (Table 4.17). Similarly, with *Allium Cepa*, a very poor effect on all three dosage levels on the blood chemistry profile animals was seen (Table 4.18). For *Metronidazole*®, results showed good effects in maintaining the normal blood chemistry profile against *Entamoeba histolytica* infection.

The highest dosage of *Nigella sativa* showed good effect on the liver function test (Table 4.19). *Saussurea lappa,* at different dosage levels, had a slight effect on the liver function test even at the higher dosage 80 mg/kg body weight (Table 4.20). Similarly, *Allium cepa* at different dosage levels, showed very little change in parameters of liver function test even at the highest dosage level (Table 4.21). In the present study, there found a decrease in Sodium (Na) and Potassium (K) level in *Entamoeba histolytica* positive cases in all groups of dogs. After treatment, there levels become normal, whereas, Magnesium (Mg) and Calcium (Ca) remains normal before and after treatment. (Table 4.22, 4.23 and 4.24).

Table 4.16: Blood profile of dogs before and after treatment with *Nigella sativa* and *Metronidazole*®

Dose (mg/Kg)	**Effect**	**Haemoglobin (g/100 ml)**	**Neutrophils (%)**	**Eosinophils (%)**	**Basophils (%)**	**Lymphocytes (%)**	**Monocytes (%)**	**P value**
60	Before	10	120	1420	4.0	5400	980	0.303
	After	10.6	155	1400	3.8	5200	930	
70	Before	10.6	155	1400	3.8	5200	930	0.290
	After	11	190	1370	3.2	4800	860	
80	Before	11	190	1370	3.2	4800	860	0.270
	After	13.4	225	1320	2.1	4400	790	
Metronidazole		15	260	980	1.2	2700	620	
Normal Values		12.1-20.3	0-300	0-1200	0-1	690-4500	0-840	

Table 4.17: Blood profile of dogs before and after treatment with *sauusureea lappa* and *Metronidazole*®

Dose (mg/Kg)		**Haemoglobin (g/100 mL)**	**Neutrophils (%)**	**Eosinophils (%)**	**Basophils (%)**	**Lymphocytes (%)**	**Monocytes (%)**	**P value**
60	Before	9	110	1380	3.8	4900	920	0.270
	After	9.2	121	1370	3.7	4850	905	
70	Before	9.2	121	1370	3.7	4850	905	0.260
	After	9.7	137	1355	3.4	4750	875	
80	Before	9.7	137	1355	3.4	4750	875	0.320
	After	10.4	165	1330	2.9	4500	850	
Metronidazole		15	260	980	1.2	2700	620	
Normal Values		12.1-20.3	0-300	0-1200	0-1	690-4500	0-840	

Table 4.18: Blood profile of dogs before and after treatment with *Allium cepa* and *Metronidazole*®

Dose (mg/Kg)		Haemoglobin (g/100 mL)	Neutrophils (%)	Eosinophils (%)	Basophils (%)	Lymphoetyes (%)	Monocytes (%)	P value
60	Before	10.1	135	1395	3.6	5200	895	0.72
	After	10.3	141	1390	3.6	5200	890	
70	Before	10.3	141	1390	3.6	5200	890	0.27
	After	10.9	150	1379	3.4	5130	875	
80	Before	10.9	150	1365	3.4	5130	875	0.30
	After	11.1	163	1350	3.0	4900	850	
Metronidazole		15	260	980	1.2	2700	620	
Normal Values		12.1-20	0-300	0-1200	0-1	690-4500	0-840	

Table 4.19: Liver function test in dogs before and after treatment with *Nigella sativa* and *Metronidazole*®

Dose Rate	Treatment	Glucose (mg)	Total Protein (g)	Albumin (g)	A/G	SGOT (Imu/I)	Alkaline Phosphatase (Imu/I)	P value
60 mg/kg BW	Before	125	3.1	2.1	0.6	62	25	0.39
	After	125	3.3	2.3	0.7	61	35	
70 mg/kg BW	Before	125	3.3	2.3	0.7	61	35	0.65
	After	120	3.8	2.8	0.9	57	52	
80 mg/kg BW	Before	120	3.8	2.8	0.9	57	52	0.44
	After	117	5.2	3.5	1.3	52	90	
Metronidazole		105	5.7	3.2	1.6	42	130	
Normal Value		70-138	5.0-7.4	2.7-4.4	0.8-2.0	5-55	10-150	

Table 4.20: Liver function test in dogs before and after treatment with *Saussurea lappa* and *Metronidazole®*

Dose Rate (mg/Kg)	Treatment	Glucose (mg)	Total Protein (g)	Albumin (g)	A/G	SGOT (Imu/I)	Alkaline Phosphatase (Imu/I)	P value
60	Before	120	3.4	1.9	0.8	60	45	0.31
	After	121	3.5	2.0	0.8	60	57	
70	Before	121	3.5	2.0	0.8	60	57	0.49
	After	119	3.8	2.2	0.9	59	68	
80	Before	119	3.8	2.2	0.9	59	68	0.36
	After	121	4.5	2.8	1.1	56	85	
Metronidazole		105	5.7	3.2	1.6	42	130	
Normal Value		70-138	5.0-7.4	2.7-4.4	0.8-2.0	5-55	10-150	

Table 4.21: Liver function test in dogs before and after treatment with *Allium cepa* and *Metronidazole®*

Dose	Treatment	Glucose (mg)	Total Protein (g)	Albumin (g)	A/G	SGOT (Imu/I)	Alkaline Phosphatase (Imu/I)	P value
60	Before	135	3.9	2.0	0.7	62	46	0.50
	After	133	4.0	2.1	0.7	62	54	
70	Before	133	4.0	2.1	0.7	62	54	0.33
	After	134	4.2	2.3	0.8	61	61	
80	Before	134	4.2	2.3	0.8	61	75	0.38
	After	130	4.5	2.6	1.0	60	175	
Metronidazole		105	5.7	3.2	1.6	42	130	
Normal Value		70-138	5.0-7.4	2.7-4.4	0.8-2.0	5-55	10-150	

Table 4.22: Electrolytes in normal (A), *Entamoeba histolytica* positive (B) after treatment with *Nigella sativa* (C) and *Metronidazole*®

Dose (mg/Kg)	Sodium (mEq/L)				Potassium (mEq/L)				Magnesium (mEq/L)				Calcium (mg/dL)			
	A	B	C	P value	A	B	C	P value	A	B	C	P value	A	B	C	P value
60	140-151	120	127	0.000	3.4-5.4	2.8	3.0	0.13	0.7-1.1	0.6	0.7	0.013	9.5-12.0	9.9	10.1	0.33
70	140-151	127	134		3.4-5.4	3.0	3.4		0.7-1.1	0.7	0.9		9.5-12.0	10.1	10.6	
80	140-151	134	142		3.4-5.4	3.4	4.1		0.7-1.1	0.9	1.0		9.5-12.0	10.6	11	
Metronidazole	140-151	125	146		3.4-5.4	3.1	4.2		0.7-1.1	0.7	1.2		9.5-12.0	9.8	10.7	

Table 4.23: Electrolytes in normal (A), *Entamoeba histolytica* positive (B) and after treatment with *Saurrurea lappa* (C) and *Metronidazole*®

Dose (mg/Kg)	**Sodium (** mEq/L**)**				**Potassium (** mEq/L**)**				**Magnesium (** mEq/L**)**				**Calcium (** mg/dl**)**			
	A	B	C	P value	A	B	C	P value	A	B	C	P value	A	B	C	P value
60	140-151	125	129	0.000	3.4-5.4	2.9	2.9	0.805	0.7-1.1	0.7	0.7	0.013	9.5-12.0	10.1	10.3	0.19
70	140-151	129	135		3.4-5.4	2.9	3.0		0.7-1.1	0.7	0.8		9.5-12.0	10.3	10.7	
80	140-151	135	139		3.4-5.4	3.0	3.2		0.7-1.1	0.8	1.1		9.5-12.0	10.7	11	
Metronidazole	140-151	125	146		3.4-5.4	3.1	4.2		0.7-1.1	0.7	1.2		9.5-12.0	9.8	10.7	

Table 4.24: Electrolytes in normal (A), *Entamoeba histolytica* positive (B) and after treatment with *Allium cepa* (c) and *Metronidazole*®

Dose (mg/Kg)	**Sodium (**mEq/L**)**				**Potassium (**mEq/L**)**				**Magnesium (**mEq/L**)**				**Calcium (**mg/dL**)**			
	A	B	C	P value	A	B	C	P value	A	B	C	P value	A	B	C	P value
60	140-151	127	127	0.000	3.4-5.4	2.9	2.9	0.805	0.7-1.1	0.6	0.6	0.011	9.5-12.0	10.1	10.2	0.276
70	140-151	127	128		3.4-5.4	2.9	3.0		0.7-1.1	0.6	0.7		9.5-12.0	10.2	10.4	
80	140-151	128	132		3.4-5.4	3.0	3.2		0.7-1.1	0.7	1.0		9.5-12.0	10.4	10.9	
Metronidazole	140-151	125	146		3.4-5.4	3.1	4.2		0.7-1.1	0.7	1.2		9.5-12.0	9.8	10.7	

CHAPTER 5
DISCUSSION

Entamoeba histolytica infects dogs which can act as a reservoir of infection for humans. Dogs have great associations with human beings that is why humans used to keep dogs as a pet animal. Dogs roam everywhere and try to eat everything which came in front of them. They can also feed on human excreta. They should also eat the dead bodies of animals, so they become a carrier of many intestinal problems.

Due to low living standard many dog owners cannot give the proper attention to dog health. So dogs become infected and cause problems for human health. In the present study a total of 600 dog fecal samples was examined for the prevalence of *Entamoeba histolytica*. Of these 94 samples were found positive by the triple fecal test. The prevalence was 15.7 % in this study, this is in accordance with Adejinmi *et al.,* (2001), Sarger *et al.,* (2006), Lorenini *et al.,* (2007), Martinez *et al.,* (2007), Umair *et al.,* (2008), Chalobof (2010). Lower prevalence was observed in dogs from the oceanic climatic area of Thailand by Saovanee *et al.,* (2009). Whereas the slightly higher prevalence than the present study was reported by Adejinmi *et al.,* (2004). The variation in the rate of prevalence may be attributed to environmental, management and sanitary conditions and use of antiprotozoan agents. It was noted that infection was the highest (21%) during summer season followed by autumn (15%) then spring (13%) whereas the lowest (12%) during winter. These findings are closely related to those of Adejinmi *et al.,* (2001) and Sarger *et al.,* (2006). In the present study an increased prevalence of amoebiasis in young (pups) has been reported. The findings agreed with the reports of Adejinmi *et al.,* (2001) and Lorenini *et al.,* (2006).

In the present study prevalence in relation to sex indicated that in male dogs prevalence was 16 percent whereas in female dogs it was 15.12 per cent. Nearly similar results were also reported by Lorenini *et al.,* (2007), Martinez-Moreno *et al.,* (2007). In the present study the prevalence of *Entamoeba histolytica* infection in dogs by ELISA was 70.2 per cent which were closely related to the results by Adejinmi *et al.,* (2001), Lorenini *et al.,* (2007), Martinez-Moreno et al. (2007). Prevalence in 0-6 months pups were 67.9 per cent, in 7 months-1 year old dogs were 75 per cent and in 1-4 years old, dogs was 66.7 per cent by ELISA which was in agreement with the results reported by Adejinmi *et al.,* (2001), Johnson *et al.,* (2010). In male dogs the prevalence was 77.6 per cent and in female dogs the prevalence was 58.33 percent in the present study the highest (73.8%) prevalence was reported during the summer followed by Spring (69.23%) then in Autumn whereas the lowest (66.6%) during the winter season which is in accordance to adejinmi *et al.,* (2001) , Johnson *et al.,* (2010).

As regards the age wise prevalence it was 31.96 per cent in 15-20 years age group followed by 21.06 per cent in 21-35 years and 17.25 per cent in 36 years and above age group which are comparable to the Zahida Tasawer *et al.,* (2010). Sex wise prevalence indicated that it was 22.72 per cent in males and 17.85 per cent in females. These results are in close agreement with the findings of Zahida Tasawer *et al.,* (2010).

Pakistan being the 3rd world country and people is facing the problem of GIT due to *Entamoeba histolytica*. The living standard and prevailed hygienic conditions of the country are very poor. Also the sanitary conditions are not upto the mark that is why the prevalence of *Entamoeba histolytica* is very high. The trend of keeping dogs as pet animal in houses is increasing day by day. But due to unawareness with sanitary conditions the prevalence of *Entamoeba histolytica* in dog owners is also high. The occurrence of amoebiasis in an area is

influenced by multifactorial system that is composed of hosts, parasitic agents, transmission process and environmental effects. In natural life of amoebiasis, the protozoa, environment and their host form an association of a potentially epidemiological danger and it is important that the existence and localization of such an association should be recognized before time, so that disease can be controlled easily. In the present study epidemiological data on amoebiasis due to *Entamoeba histolytica* was collected from various hospitals of Lahore and dog owners. Similar species were also detected by Ashok *et al,* (1995) and Chaudhary *et al.*(2004).

In the present study the overall results obtained from various medical hospitals and dog owners of Lahore indicated a total of 600 human stool samples were examined, of these 135 (22.50%) were found positive by the Triple fecal test. From these 135 positive samples 101 (74.81%) were found positive by ELISA. The prevalence rate of infection is comparable to the 21 per cent found by Aza *et al.,* (2003) in Malaysia, Fumarola *et al.,* (2007),Pritt *et al.,* (2008) and Tasawar *et al.,* (2010) .Slightly a lower infection 15.6 per cent was found by Ali *et al.,* (2003), shetty *et al.,* (1990), Waqar *et al.,* (2003), Sayyari *et al.,* (2005) in children at Karachi, Pakistan. It appears that infection rate in present study is nearly similar to above mentioned workers in various countries of the world. However Ashok *et al.,* (1995) 1.4 per cent from India, Hussain *et al.,* (1997) 8 per cent from Pakistan, Chaudhary *et al.,* (2004) 5.9 per cent from Kashmir and Siddique *et al.,* (2002) 48.8 per cent from Karachi, Pakistan, Kaur *et al.,* (2002) 11 per cent in India recorded prevalence in different parts of the world. Slightly higher prevalence was reported by Siddique *et al.,* (2002), Doorn et al (2005), Ali *et al.,* (2008),Ayed *et al.,* (2008),Jose *et al.,* (2008), Araujo *et al.,* (2008) than the present study.The difference may be due to different geographical regions and varied environmental conditions and also use of antiprotozoan agents.

It was reported that infection was highest (42%) during August followed by July (38%) then June (24%) whereas the lowest (16%) during January, April and November. These findings are closely related to those of findings of Maur *et al.,* (2002), Haidari and Rokni (2003). An increased incidence of amoebiasis in children has been reported in present study. The findings agreed with reports of Ali *et al.,* (2003), Maur *et al.,* (2002) and Aza *et al.,* (2003).

It was reported that that infection was slightly higher in Males (22.72%) than Females (17.85%) as was also reported by Aza *et al.,* (2003), Okafor and Azubike (1992), Sharma *et al.,* (2004), Hamze *et al.,* (2004), Saovanee *et al.,* (2009).

When the data on monthly and seasonal incidence of amoebiasis was analyzed, it was observed that higher incidence of amoebiasis occurred in the month of August (42%).It was also reported that the two important factors influencing the incidence of amoebiasis are adequate temperature and moisture in the environment, which helped in hatching of Oocysts and development of rapid life cycle stages. It was also noted that there are two seasonal periods in which the temperature and moisture are favorable for the rapid propagation of the parasitic life cycle. During these periods i.e rainy and post rainy seasons infection of amoebiasis was very high as was also reported by Aza *et al.,* (2003), Saovanee *et al.,* (2009).

Prevalence of *Entamoeba histolytica* in sewerage and tap water

Amoebiasis an important water borne protozoan infection is considered a reamerging threat. Many studies on the epidemiology of human amoebiasis have been carried out in developed countries but no data is available on the occurance of this disease in Pakistan.The objective of the present study was to investigate the presence of *Entamoeba histolytica* in various water samplesin Lahore,Pakistan.A total of 600 samples were examined,300 from municipal water supply (tap water),300 from sewerage (untreated)water.Cysts of *Entamoeba histolytica*

were detected by using floatation method.Of these 18 per cent were found positive (sewerage water 30.66% and tap water 5.33%).Results of the present study are closely related to Clark and Diamond (1992),Marshall et al (1997).It was also noted that in developing countries water borne gastrointestinal pathogens especially *Entamoeba histolytica* is frequently associated with morbidity particularly in children. This parasite is the most common cause of the infection worldwide (Tanyuksel et al,.2001).In developed countries out break of amoebiasis has been reported by sewerage contaminated water (Barwick et al,.1999).

Entamoeba histolytica infection was found to be very high in dog owners that means infection is being transferred by drinking contaminated tap water and use of contaminated food. In the transmission flies and cockroaches played a role as mechanical vectors. Dogs, Monkeys and Rodents serve as animal reservoirs for some amoebic species. It was also found that in some areas of Lahore people use to drink partially treated sewerage water. In rural areas of Lahore normally alloy pipes are used as drinking lines and sewerage lines and both run in close proximity. Due to breakage in these pipes, there might be the possibility of mixing of drinking water with sewerage water and become contaminated. Another cause is that the dog owners after handling dogs donot wash hands and eat food with contaminated hands and this is the common source of infection of amoebiasis. In the present study it was noted from the history and hospitals data that most of the patients are facing gastrointestinal tract infection due to *Entamoeba histolytica* because living standard and prevailed hygienic conditions of country are very poor and also the sanitary conditions are not upto mark. It was noted that in Lahore (study area) the trend of keeping dogs as pet animals in houses is increasing day by day, this increase the chances of spreading the infection in Pakistan. It was noted that prevalence of amoebic infection depend on socio-economic status, sanitation and overcrowding. According to WHO, approximately 10%

of the world population is infected by Entamoeba spp. About 50 million cases of invasive disease occur each year, causing upto 100000 deaths. In endemic area nearly 100% of examined persons are carriers of amoeba with or without clinical manifestation.

For the effective therapy and strategic chemoprophylaxis of amoebiasis, a safe drug is required with high activity against all stages of *E. histolytica*. Modern drugs are effective but most of them possess adverse effects (Levine, 1982). Thus the development of newer, safer and economical drug has remained an active area of research. The antiparasitic activity of indigenous drugs including Nigella Sativa seeds (Kalongi), Saussurea Lappa (Qustshireen) and Allium Cepa(onion) at different dosage levels were evaluated. The efficacy of these herbal drugs were compared with each other and with allopathic drug i.e. metronidazole.

Nigelle Sativa (kalongi) at 60, 70, 80 mg/kg body weight was 37.31 , 40 and 43.47 percent effective respectively on 18th day post treatment at one dose level whereas at two dose level it was 65.67,68 and 69.56 per cent on 28th day post treatment respectively.

These findings are in agreement with Jahangir et al. (2001), Khan et al. (2012), Githiori et al. (2005,2006) Maqbool et al. (2004), Waller et al. (2001), Itty et al. (1997), Kalilani et al.(1995), Sohni YR et al.(1995) and Akhtar and Javed (1991) against bovine fasciolosis, ovine paramphistomosis and ovine monieziasis. Akhter and Farah (1986) and Nath (1983) have reported that Nagella sativa contain Nigelle metabarbain, Melanthin Sapginin and melanthiginin etc. It also contains Volatile oil (15.1%), 2 methyl 4 isopropyl-p-guinine and fixed oil (37.5%) as active ingredients. The maximum efficacy was 69.56 per cent on 28th days post treatment. As Nigella sativa was used for the first time against amoebiasis so we are unable to get any reference regarding its use against E. histolytica infection in dogs. Saussurea lappa at 60, 70, and 80 mg/kg body weight was 23.36, 38.80 and 39.72 percent respectively effective against

amoebiasis on post treatment 18th day. After the administration of 2nd dose it was 50.72, 53.73 and 54.79 percent respectively effective on 28th day post treatment. From the results it was noted that there was no significant difference between three levels of drug. it was concluded the saussurea lappa equally effective at all the three dosage levels. These findings are in agreement with the findings of Kailani et al. (1995),Sohni et al.(1995), Itty et al. (1997), waller et al. (2001), Jahangir et al. (2001), Maqbool et al. (2004), Githiori et al. (2005, 2006), Stafford et al. (2007) and khan et al. (2012).

Allium Cepa at 60, 70, and 80 gm/kg body weight was 29.57, 32 and 35.61 per cent respectively effective against amoebiasis on post treatment 18th day. After administration of second dose on 18th day the drug efficacy was 43.66, 45.33 and 50.68 per cent respectively on 28th day. These results are in agreement with the findings of Akhter and Javed (1985,1991), Akhter and Aslam (1989), Kailani et al. (1995), Sohni YR et al.(1995), Itty et al. (1997), Waller et al. (2001), Maqbool et al. (2004), Githiori et al. (2005-2006), Stafford et al. (2007) and Khan et al. (2012) against different parasitic diseases.

The allopathic drug i.e metronidazole was given at recommended dose 25 mg/kg body weight and showed efficacy50 per cent on 18^{th} day. After administration of 2^{nd} dose on 18th day the efficacy was 100 percent on 28th day. These results are in agreement with the findings of H.steinitz (1972), Munnich and Molnar (1972), Legua (1997),Freeman *et al.,* (1997), Qin *et al.,* (2000), Galindo *et al.,* (2002), Fujishima *et al.,* (2010).

Blood picture, serum enzymes and serum electrolytes were studied in children effected with amoebiasis. There was drop in haemoglobin contents, erythrocytic number and neutrophils whereas an increase in basophils, monophils, eosinophils and lymphocytes numbers. As regard with serum enzyme study indicated that there was drop in all serum enzymes.Serum electrolyte

study indicated that there was decrease in Sodium (Na) and Potassium (K) levels whereas there was no change in Calcium (Ca) and Magnesium (Mg) levels as was reported by Raisinghani *et al.,* (1981).

The main change in blood picture before and after treatment with Nigella sativa, Saussurea lappa, Allium Cepa and Metronidazole indicated that dogs suffering from amoebiasis exhibited a significant drop in Neutrophils Esinophils, Basophils, Lymphocytes monocytes and increase in Hemoglobin count. Also the parameters of liver function test i.e. Glucose, total protein, Albumin Globulin, A/G ratio, Urea nitrogen, SGOT and Alkalin Phophatase decreased significantly. Results showed that blood picture and liver function test became normal on 28 th day after the treatment. These finding are in agreement with Raisinghani *et al.,* (1981), Maqbool et al., (1996),Steinhardt and Thielseher (2000), Knowles et al., (2000), Shelke and Dhami et al.,(2000), omakony *et al.,*(2006), Diaz et al., (2006), Starffor et al., (2007)

The total serum protein (TSP) concentration of the dogs were found decreased before treatment and this is in accordance of the findings of Horak and Clark (1963).Similarly the albumin levels were also found less in all dogs on zero day. Horak and Clark (1963) were of the opinion that the reduction in the plasma protein concentration is mainly due to poor plasma albumin concentration of the blood. However following treatment the total serum protein level increased and was found normal on 28th day post treatment. The serum albumin level increased significantly ($p<0.05$) on 28th day. While A/G ratio also increased significantly ($p< 0.01$) on the same day of observation of post treatment. It is postulated that decreased in infection shows improvement in appetite and lessening of diarrhoea and helped in the increase of plasma protein concentration and albumin globulin ratio. These results are supported by Schalm *et al.,* (1975),

Benjamin (1981), Doormenbal *et al.,* (1988),Gupta *et al.,* (1999), Risvanl *et al.,* (1999),Steinhardt and thielscher (2000) and Knowles *et al.,* (2006).

There was little increase of serum glutamic oxaloctic transaminase (SGOT) level on zero day. Since considerable amount of SGOT activity is found in all the tissues of the mammals, inflammatory changes takes place which rise SGOT level. After treatment level become normal. These results are similar to these of Shelke and Dhami (2001),Kurz and Willett (1991).The serum glutamic pyruvic transaminase (SGPT) levels however did not much alter before and after treatment. The albumin-globulin (A/G) ratio also decreased much on zero day and after treatment become normal as was also repeated by Shelke and Dhami (2001).The alkaline phosphates levels were found to be decline and after treatment become normal. It was noted that alkaline phosphates distributed in high concentrations in intestinal mucosa. It was also noted that serum alkaline phosphatase levels decreased with age whereas total serum protein level increased with age Doormenval *et al.,* (1988).It was also noted that there is an increased in total serum protein and alkaline phosphatase levels in infected dogs as compared to control. In the present study, a decline in SAP level was observed. From the results it was concluded that indigenous and allopathic drugs are effective in the treatment of amoebiasis and an appropriate measures for reducing the risk for amoebiasis are needed and administration of effective chemotherapeutic agents before rainy season is strongly recommended.

In the present study the effect of amoebiasis on serum electrolytes i.e Sodium (Na), Potassium (K), Calcium (Ca) and Magnesium (Mg) was studied. It was noted that there was drop in serum Potassium (K) and Sodium (Na) level *Entamoeba histolytica* infection. After the treatment with allopathic and indigenous drugs all the parameters become normal on 28th day as was reported by Raisinghani *et al.,* (1981). Results of blood picture before and after treatment

with allopathic and indigenous herbal drugs indicated that dogs suffering from amoebiasis exhibited a significant drop in erythrocytic number, haemoglobin contents and neutrophils. With an increase in lymphocytes, monocytes and eosinophils. The blood became normal on 28th day after treatment as was reported by Seegret et al (1930), Raisinghani et al (1981) and Suryanarayana et al (1987).

CHAPTER 6
SUMMARY

An epidemiological study of amoebiasis in dogs and humans, its zoonotic potential, haematology, chemotherapy with different indigenous and allopathic drugs was conducted during December 2008-November 2009. Part 1 deals with month-wise, seasonal, age and sex-wise prevalence of amoebiasis in dogs at Lahore, Pakistan by Tripple fecal test .The highest (30%) prevalence was noted during August followed July whereas the lowest (10%) was during February and May. As regard the season wise prevalence, the highest (21%) prevalence was noted during Summer followed by Autumn and the lowest (12%) during Winter. Prevalence was higher in youngers than adults. As regard sexwise prevalence there is no significant difference between males and females. Results of the prevalence by ELISA indicated that it was the highest (22%) during August whereas the lowest (6%) during November. The highest (15.5%) seasonal prevalence was noted during Summer and the lowest (8%) during Winter. Prevalence was higher in younger dogs than adults. As regard the sex wise prevalence it was noted that it is more in males than females dogs.

Part 2 deals with the month,season,age and sex wise prevalence of amoebiasis in humans by Triple fecal test.The highest (42%) prevalence was noted during August followed by July whereas the lowest (16%) prevalence was noted during January, April and November .Seasonal prevalence indicated, it was the highest(31%) during Summer followed by Autumn and the lowest (17%) during Winter. Prevalence was higher in children than adults.Whereas sex has no bearing effect on the prevalence of disease.Humans of both sex are nearly equally affected.Prevalence by ELISA indicated that it was the highest (34%) during August whereas the lowest (10%) during January.The highest (24%) seasonal prevalence was noted during Summer

whereas the lowest (12.5%) during Winter.Prevalence was higher in children than adults. Whereas sex wise prevalence indicated that it was slightly higher in female than males.

Part 3 deals with the prevalence of *Entamoeba histolytica* cysts in various sources of water i.e tap water and sewerage water. From the results it was noted that 31.66 per cent sewerage and 5.33 per cent tap water samples were harboring *Entamoeba histolytica* cysts.

Parts 4 deals with soil survey regarding the occurrence of *Entamoeba histolytica*.Of the total, 10.83 per cent were found positive for Enamoeba histolytica cysts.

Part 5 deals with meteorological data including the temperature (minimum and maximum), relative humidity and rainfall. It was noted that an optimal temperature ranged from 30c to 35c where significant increase in propagation of infection. Relative humidity 65% to 70% was found to be favorable. Rainfall during summer months helps the cysts for dispersal from one place to another place.

Part 6 deals with the zoonotic potential of the disease. From the study it was noted that in rural areas of city many people use to drink municipal and untreated tap water contaminated with feces (containing *Entamoeba histolytica* cysts) and this water is responsible for transmission of amoebiasis from dogs to humans.

Last part deals with therapeutic trials by using Nigella sativa, Saussurea lappa,Allium cepa and metronidazole. All these drugs were effective at one dose level and highly at 2 dose level. Among indigenous drugs, Nigella sativa at 80 mg/Kg body weight was highly effective than others. This drug is closely effective to metronidazole.This drug is much cheaper than metronidazole and showed no toxic effects.Blood parameters and LFT values were studied in dogs.These values become normal after treatment.

Conclusion

Epidemiological study were under taken in dogs and humans at Lahore revealed that infection rate was the highest during August in both whereas the lowest during May and January,April November respectively.Overall the highest seasonal prevalence was recorded during Summer whereas the lowest during Winter in both dogs and humans . It was recorded that the higher infection rate was recorded in younger (pups and childrens) than adult.Infection was slightly higher in males than females. Severage water is more contaminated than tap water.Oocysts of *Entamoeba histolytica* are also present in soil samples. Meterogical data played very important role in the causation of disease. One hundred and twenty (120) dogs were used in 12 cotrolled experiments to compare the efficacy of Nigella sativa, Saussurea lappa and Allium cepa with metronidazole against amoebiasis.Efficacy was calculated on basis of reduction of oocysts per gram of faeces. The highest efficacy was noted in allopathic drug i.e metronidazole followed by Nigella sativa,Saussurea lappa and Allium cepa.The use of Nigella sativa and Saussurea lappa were recommended for routine use.No side effects were found in any of the indigenous drugs.

It was also concluded thatall serum enzymes, blood parameters and serum electrolytes become normal after treatment.

CHAPTER 7
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