Molecular Diagnosis and Characterization of Two Intestinal Protozoa:
Entamoeba histolytica & Giardia intestinalis

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I dedicate this thesis to Ruth Svensson,
a pioneer of Swedish protozoology,
who completed her thesis in 1935.

ABSTRACT

Entamoeba histolytica and *Giardia intestinalis* are two of the most important and most widespread diarrhea-related parasitic protozoa in the world. Approximately 1200–1500 cases of *Giardia* and 200–400 cases of *Entamoeba* are reported each year in Sweden, whereas the corresponding numbers are much higher in developing countries like Nicaragua. Traditionally, diagnosis of these parasites depends on microscopic detection of cysts or trophozoites, even though such methodology is neither sensitive nor species specific. Recently, more specific and sensitive molecular techniques have been introduced to identify these infections, but routine use of these tests in diagnostic laboratories is still limited.

Amoebiasis, infection with *E. histolytica,* is a notifiable disease in Sweden. Despite that, diagnosis depends mainly on stool microscopy, which cannot distinguish between the invasive and pathogenic *E. histolytica* and the nonpathogenic *E. dispar*. We used a PCR technique to evaluate the proportions of these two species in patients who had amoebic infections and had initially been diagnosed by microscopy, and we found *E. histolytica* in less than 10% of those cases. Differentiation of *E. histolytica* and *E. dispar* is now a recommended analysis in Sweden, and this approach has greatly decreased the number of patients receiving unnecessary treatment.

Giardia intestinalis consists of seven morphologically identical but genetically distinct genotypes or assemblages, which are designated A to G. Assemblages A and B can infect both humans and other mammals, whereas assemblages C to G are host specific. Sequence-based genotyping of *Giardia* isolates was applied to investigate the relationship between assemblages and symptoms, the zoonotic potential, sequence divergence, and transmission dynamics. *Giardia* samples obtained from humans and dogs living in the same area in León, Nicaragua, as well as from humans and various animals in Sweden were analyzed using molecular methods. The investigation of the human samples indicated the following: assemblage B was the most common genotype in both countries, and this assemblage was largely polymorphic with ambiguous nucleotide positions in many sequences. In contrast, assemblage A isolates were genetically more homogeneous, and multilocus genotypes (MLGs) were easily determined for this assemblage. Most animals were infected with the host-specific assemblages C–G, but assemblages A and B were detected as well. A few animal and human isolates shared the same assemblage A MLGs, which suggests that zoonotic transmission of *Giardia* can occur in Sweden. Determination of MLGs may be a useful tool for source tracing in outbreak situations, although assignment of a specific MLG was hampered in many of the current isolates due to the large sequence polymorphism seen in assemblage B. The only correlation found between assemblages and symptoms concerned flatulence, which was noted to be significantly more common in young children infected with assemblage B.

In conclusion, the combination of microscopy and molecular methods enabled us to differentiate between pathogenic and nonpathogenic *Entamoeba* species and thereby decrease the number of unnecessarily treated patients. Furthermore this methodological approach also gave us basic knowledge about *Giardia* genotypes in humans and animals, both in Nicaragua and Sweden, information that is useful for understanding the transmission and the clinical presentation of *Giardia* infection.
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LIST OF PUBLICATIONS

This thesis is based on the following papers. They will be referred to by their Roman numerals in the text.

I.   Lebbad M, Svärd SG. PCR differentiation of Entamoeba histolytica and Entamoeba dispar from patients with amoeba infection initially diagnosed by microscopy. Scand J Infect Dis. 2005;37(9):680-5.

II. Lebbad M, Ankarklev J, Tellez A, Leiva B, Andersson JO, Svärd S. Dominance of Giardia assemblage B in León, Nicaragua. Acta Trop. 2008;106(1):44-53.

III. Lebbad M, Mattsson JG, Christensson B, Ljungström B, Backhans A, Andersson JO, Svärd SG. From mouse to moose: multilocus genotyping of Giardia isolates from various animal species. Vet Parasitol. 2010 25;168(3-4):231-9.

IV. Lebbad M, Petersson I, Karlsson L, Botero-Kleiven S, Andersson JO, Svenungsson B, Svärd SG. Multilocus sequence based genotyping of Giardia isolates from Swedish patients. In manuscript

CONTENTS

LIST OF ABBREVIATIONS

ALA	Amoebic liver abscess
bp	Base pair
DAPI	4'-6-diamidino-2-phenylindole
DFA	Direct fluorescent antibody test
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
gdh	Glutamate dehydrogenase
GTR	Generalized time-reversible
IUPAC	International Union of Pure and Applied Chemistry
PCR	Polymerase chain reaction
RFLP	Restriction enzyme length polymorphism
SAF	Sodium acetate-acetic acid-formalin
ssrRNA	Small subunit ribosomal RNA
tpi	Triosephosphate isomerase
WHO	World Health Organization

1 INTRODUCTION

The first time I had a chance to become familiar with human intestinal parasites was in Guinea Bissau, West Africa, in 1975. I was fascinated by the appearance of these organisms in the microscope, and I felt that this could be my future. Soon after that I got the opportunity to be trained in parasitology by the legendary "Syster Birgit" at Roslagstull Hospital in Stockholm. In a way, things were easier at that time; a quadrinucleate cyst of the right size and the right morphology was *Entamoeba histolytica*, a Ziehl-Neelsen stained oocyst was *Cryptosporidium parvum*, and different *Giardia* genotypes were unknown to us. Later in life I had another opportunity, this time to learn how to use molecular methods to differentiate species and genotypes that were indistinguishable in the microscope. In this thesis, I have tried to combine my old knowledge in parasite morphology with the new world I am attempting to conquer.

1.1 GENERAL BACKGROUND

Diarrheal diseases
More than 90% of the deaths from infectious diseases worldwide are caused by only a handful of diseases. The World Health Organization (WHO, 2004) ranks diarrheal disease as the second (after acute respiratory infections) most common cause of morbidity and mortality in children in the developing world. The etiological agents of diarrhea include viruses, bacteria, and parasites, and in this context the most important members of the latter group are *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium* spp.

This thesis deals with two of the mentioned species: *E. histolytica* and *G. intestinalis*. Both these parasites have a low infectious dose, are spread through feces-contaminated food and water, have similar clinical presentations, and are commonly found in areas that lack sanitation and clean water. They have comparable, simple life cycles that comprise a resistant, infectious cyst form and a fragile, disease-causing trophozoite, which are also the diagnostic stages. Another common feature is what is called cryptic genetic variation, meaning that it is not possible to distinguish species or genotypes by morphological criteria (Clark, 2000).

2 *ENTAMOEBA HISTOLYTICA*

2.1 HISTORICAL NOTES

In 1875, the Russian physician Friedrich Lösch described the first case of a dysenteric disease in man caused by an amoeba. His vivid description of the motility of the amoebas and the typical nucleus and ingested red blood cells reassures us that he was actually looking at trophozoites of what is now recognized as *Entamoeba histolytica.* Lösch originally chose a simple name for the organism, calling it *Amoeba coli* because it appeared in the colon (Lesh, 1975). In 1903, Fritz Schaudinn changed the name to *E. histolytica* due to the ability of the amoebas to cause tissue lysis (Clark, 1998). In the years that followed, the situation became confusing, since many species names were introduced for amoebas very similar to each other and to *E. histolytica*. In 1919, the famous English parasitologist Clifford Dobell published his now classical monograph "The Amoeba Living in Man" (Dobell, 1919). In this text, he reviewed all published articles that proposed various amoeba species and concluded that all *Entamoeba* that produced cysts with four nuclei were identical to *E. histolytica.*

However, in 1925 the French parasitologist Emile Brumpt suggested the existence of another amoeba with quadrinucleate cysts, which he named *Entamoeba dispar.* Brumpt described this species as being morphologically identical to *E. histolytica* but not associated with disease in its host. He based his assumptions on epidemiological surveys that demonstrated high infection rates of quadrinucleate *Entamoeba* cysts in several European countries where invasive disease was almost unknown. He had also performed a number of experimental infections of kittens, which never led to disease. Despite that, when Brumpt presented his hypothesis at a meeting of the Royal Society of Tropical Medicine and Hygiene in London in 1928, he failed to convince his audience (Brumpt, 1928). First of all, there was evidence that amoebas from asymptomatic carriers were capable of causing disease in other individuals, as had been shown by Walker and Sellard in the Philippines in 1913 (Clark, 1998). Secondly, the suggestion by Brumpt did not suit the clinicians of the time. In the discussion that followed Brumpt's presentation, the prominent English parasitologist Wenyon made this statement (Brumpt, 1928):

> *Professor Brumpt's view if adopted would introduce clinical difficulties. On receiving a laboratory report that quadri-nucleated cysts of the histolytica type were present in the patient's faeces the physician at once proceeded to inject emitine, but with Professor Brumpt's theory put into practice ... the clinician would first have to ask: Is this E. dispar or E. histolytica?*

At that time, the view among protozoologists was that a species always represented certain morphological criteria, which did not agree with the idea that two separate species could have identical morphology (Brumpt, 1928; Clark, 1998). Therefore, Brumpt and *E. dispar* were forgotten for almost 50 years.

In 1978, Sargeaunt and Williams demonstrated that *E. histolytica* strains could be divided into two major groups—invasive and non-invasive—according to zymodemes revealed by isoenzyme electrophoresis performed on cultured parasites (Sargeaunt et al., 1978). Later, about 6,000 separate isolates of *Entamoeba* from all

over the world were investigated using the same technique (Sargeaunt, 1987), and all of them could be assigned to the two groups. The first of these was designated "pathogenic *E. histolytica*" and included isolates from patient with invasive disease and some cyst carriers; the second group, which was called "non-pathogenic *E. histolytica*", consisted mainly of isolates from asymptomatic cyst carriers and contained no isolates from invasive cases.

Biochemical, immunological, and genetic data were accumulated during the years that followed, and by 1993 enough evidence had been gathered to support the existence of two morphologically indistinguishable species (Strachan et al., 1988; Petri et al., 1990; Tannich et al., 1991). Consequently, the invasive and pathogenic parasite was redescribed retaining the name *E. histolytica,* and was set apart from the noninvasive *E. dispar*, in accordance with Brumpt's original hypothesis from 1925 (Diamond and Clark, 1993).

Four years later, in 1997, the WHO accepted this classification into two species and concluded that, in cases involving diagnosis based on light microscopy, the cysts should be reported as *E. histolytica/E. dispar* (WHO, 1997). However, this goal has not yet been reached, as indicated by a PubMed search conducted in June 2010, which revealed that, in 2009, 15 publications still reported the occurrence of *E. histolytica* based solely on microscopy, while 14 described correct finding of *E. histolytica/E. dispar*.

2.2 THE PARASITE

2.2.1 Taxonomy
Entamoebas are anaerobic protozoan parasites that have traditionally been classified in the phylum Sarcodina, members of which are characterized by the presence of pseudopodia that are used for movement and uptake of food. According to more recent classification based on molecular trees, entamoebas belong to the phylum Amoebozoa and the class Archamoebea, and they are closely related to the slime molds (Cavalier-Smith, 2004). A number of species within the genus *Entamoeba* can infect humans, but only *E. histolytica* is of medical importance (WHO, 1997).

2.2.2 Life cycle and morphology
Entamoeba histolytica has a simple life cycle that comprises an infectious cyst form and an amoeboid trophozoite stage. The cysts measure 10–15 μm in diameter and contain one to four nuclei, depending on their level of maturation (Fig 1 A). The cysts have a thick wall made partly of chitin, which makes them resistant to both environmental stress and the gastric acid in the stomach of the host. The trophozoite is 10–50 μm in diameter and contains a single nucleus. The nuclei of both stages have a small central karyosome and thin peripheral chromatin. The trophozoite moves in a characteristic way, which Lösch described in a very expressive manner when he first discovered the parasite in 1875 (Lesh, 1975). *E. histolytica* and *E. dispar* are similar with regard to cyst morphology, and the only morphological criteria that can be used to separate them is the erythrophagocytic trophozoites that are sometimes found in stool samples from patients with *E. histolytica* dysentery (Fig.1B). Humans and occasionally non-human primates (Rivera et al., 2010) are the only natural hosts of *E. histolytica,* thus the zoonotic importance of this parasite is limited. Infection occurs when cysts are transmitted by the fecal-oral route (ingestion of contaminated food or water) or through person-to-person contact. The infectious dose is considered to be low, around 10 cysts, even though the only experimental transmission of *Entamoeba* that has been done used

cysts of *Entamoeba coli* (Rendtorff, 1954a). Excystation occurs in the small intestine, and the trophozoites that are released migrate to the large intestine, where they reproduce by binary fission. Encystation takes place in the colon, thus completing the life cycle by excretion of cysts in the stool. In cases involving diarrhea, trophozoites can be excreted as well, but they can survive for only a short time outside the body of the host. Infections that remain luminal are usually asymptomatic, and clinical amoebiasis occurs only when the trophozoites disrupt the mucosal barrier and penetrate the colon wall, which causes ulcers that lead to amoebic dysentery. Much less frequently trophozoites are spread through the portal vein to the liver, and, very rarely, they even disseminate to other organs such as the lungs and brain.
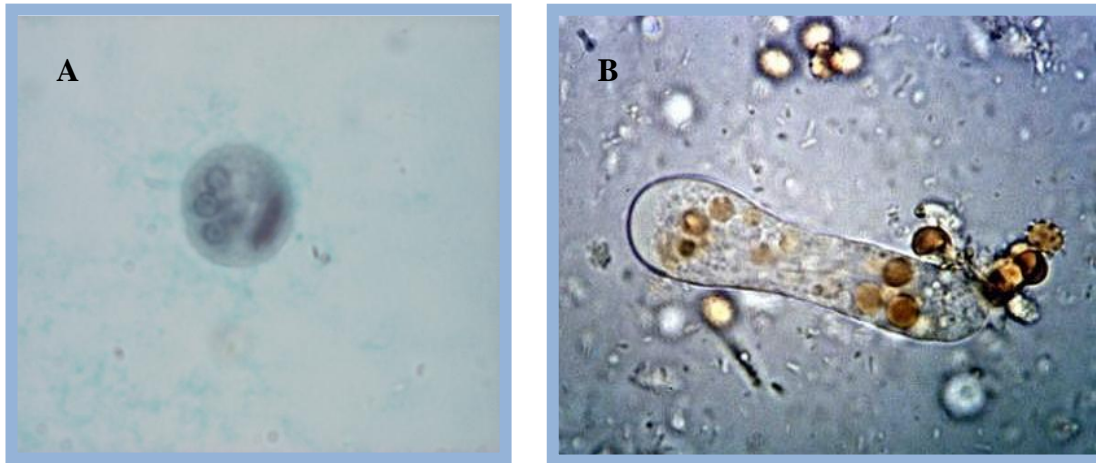
Figure 1. A: Trichrome stained cyst of *E. histolytica* with three visible nuclei and a chromatoid body. B: Trophozoite of *E. histolytica* with ingested erythrocytes (Photo: Birgit Lindberg, Roslagstull Hospital; courtesy of Teodor Capraru).

2.3   CLINICAL PRESENTATION

The term amoebiasis indicates infection with *E. histolytica* regardless of symptoms (WHO, 1997). The symptoms of amoebic colitis/dysentery usually emerge gradually over a period of one to several weeks, and they include abdominal pain and tenderness, painful sudden bowel evacuation (tenesmus), and bloody diarrhea. Less than 40% of the patients exhibit a fever, and some cases involve anorexia and weight loss (Stanley, 2003). *E. histolytica* can also penetrate the colonic mucosa and spread through the bloodstream to the liver, where the trophozoites establish an amoebic liver abscess, which is the most common extraintestinal manifestation. Amoebic liver abscess (ALA) is more common in adult men than in adult women, and the typical patient is a 20–40-year-old male with a 1–2-week history of fever and diffuse abdominal pain in the right upper quadrant (Stanley, 2003).

It is also evident that not all humans infected with *E. histolytica* develop clinical disease. Indeed, studies conducted in countries where *E. histolytica* is endemic have estimated that, at most, only one in four *E. histolytica* infections progresses to disease (Blessmann et al., 2003; Haque et al., 2006; Ali et al., 2008a).

2.4 OTHER *ENTAMOEBA* SPECIES FOUND IN HUMAN STOOL SAMPLES

Although *E. histolytica* is the only intestinal amoeba of clinical importance, it is essential to recognize both the morphological and the genetic variation of other *Entamoeba* species that infect humans, in order to make the correct microscopic identification and to create a foundation for molecular-based diagnostic methods (Santos et al., 2010).

Entamoeba dispar is the most important morphological "twin parasite" of *E. histolytica*. Although they are genetically distinct, these two species are each other's closest relatives in the genus *Entamoeba*. Besides being different with respect to pathogenicity, *E. dispar* parasites are much more difficult to establish in axenic culture (i.e., in the absence of other microorganisms) compared to *E. histolytica* trophozoites (Clark and Diamond, 2002), probably due to disparities related to the uptake of nutrients (Espinosa-Cantellano et al., 1998). Studies involving experimental infections have shown that a number of *E. dispar* strains can form liver abscess in certain animal species (Costa et al., 2006). Furthermore, a recent investigation in Mexico was the first to identify *E. dispar* DNA in human abscess material obtained from patients with bacterial abscesses and from ALA patients, in the latter group in combination with *E. histolytica* DNA (Ximenez et al., 2010). Nevertheless, *E. dispar* is (still) considered to be non-invasive and non-pathogenic in humans, although future studies may reveal a more complex picture.

Entamoeba moshkovskii is a free-living amoeba, which forms quadrinucleate cysts that are morphologically indistinguishable from *E. histolytica/E. dispar* cysts. Even though sporadic human cases had been documented in the past (Clark and Diamond, 1991a), *E. moshkovskii* was previously believed to be an uncommon parasite in humans. However, studies carried out in several countries (Iran, India, Australia, Bangladesh, Tanzania, Tunisia, and Turkey) using the PCR (polymerase chain reaction) technique with specific primers for *E. moshkovskii* have detected this parasite in varying numbers of patients (Ali et al., 2003; Khairnar and Parija, 2007; Tanyuksel et al., 2007; Beck et al., 2008; Fotedar et al., 2008; Nazemalhosseini Mojarad et al., 2010). In one investigation (Fotedar et al., 2008), a correlation was found between *E. moshkovskii* and diarrhea, but the common assumption is that this species is not pathogenic. Interestingly, many of the reported human cases of *E. moshkovskii* have been co-infected with *E. histolytica* or *E. dispar*, but the reason for this is unclear. The occurrence of *E. moshkovskii* in Sweden is not known, but a recent PCR based study of isolates containing quadrinucleate *Entamoeba* cysts from Swedish, Danish, and Dutch patients has suggested that this species is uncommon in our setting (Stensvold et al., 2010b). Notwithstanding, it has been proposed that quadrinucleate cysts should be reported as *E. histolytica/E. dispar/E. moshkovskii* due the similar morphology of these three species (Pritt and Clark, 2008).

Entamoeba coli is found all over the world and is probably the most prevalent of all the intestinal amoebas that occur in humans. Even though a Swedish publication linked this amoeba to diarrheal symptoms (Wahlgren, 1991), it is generally considered to be non-pathogenic. Mature *E. coli* cysts have 8–16 nuclei and pose no diagnostic problems, but immature cysts with four nuclei can be confounded with *E. histolytica/E. dispar* cysts, especially considering that they overlap in size.

Entamoeba hartmanni was originally referred to as the "small race" of *E. histolytica*, but it was redescribed as a distinct species in 1957 (Burrows, 1957). *E. hartmanni* is genetically quite distinct to *E. histolytica/E. dispar*, (Silberman et al., 1999), whereas it differs morphologically, mainly with respect to size. The disparity in size is usually clear (cysts <10 µm), and hence identification is not problematic. However, some isolates can harbor *E. hartmanni* cysts that are >10 µm in diameter, and if these are classified strictly according to the size criterion, they will be incorrectly identified as *E. histolytica/E. dispar* cysts.

The two uninucleated amoebas *Entamoeba polecki* (found in pigs) and *Entamoeba chattoni* (detected in non-human primates), together with two other genetic variants, are now considered to be one species, *E. polecki.* These four variants of uninucleate amoebas have occasionally been observed in human samples and have been further investigated by molecular methods (Verweij et al., 2001; Verweij et al., 2003). Cysts of *E. polecki* might be mistaken for immature *E. histolytica/E. dispar* cysts, because they are equal in size (10–15 µm). *E. polecki* cysts should be suspected when only uninucleate amoeba cysts are seen, especially if the cysts are mature.

Iodamoeba butschlii is another intestinal amoeba whose cysts and trophozoites have a characteristic nucleus that differs markedly from the nuclei of *Entamoeba* species. Despite that, *Iodamoeba* cysts are sometimes confused with immature cysts of *E. histolytica/E. dispar.* Notably, no sequences of *Iodamoeba* have yet been submitted to GenBank, probably due to the difficulties in maintaining this parasite in culture.

2.5 LABORATORY DIAGNOSIS OF AMOEBIASIS

2.5.1 Intestinal infection
Sample collection and preservation
The outcome of any diagnostic procedure depends largely on the methods used for sample collection and preservation, and different approaches are necessary for the two diagnostic stages of *Entamoeba* species, the cyst and the trophozoite. As shown in Figure 2, a formed stool is likely to contain cysts, whereas a watery or dysenteric stool will usually harbor trophozoites. Table 1 presents a comparison of the various fixatives used most often in diagnostic parasitology of intestinal protozoa in Sweden, and it is noteworthy that none of the suggested agents is optimal for all techniques. A fresh stool specimen obtained directly from the patient is seldom available but is essential if the aim is to detect mobile trophozoites. Fecal samples are often forwarded to the diagnostic laboratory by mail, thus it is necessary to use fixatives to preserve the trophozoites.
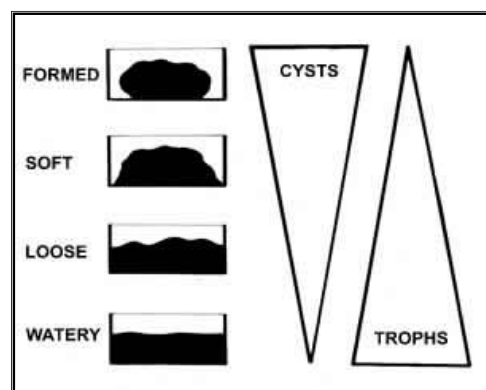

Figure 2. Distribution of cysts and trophozoites in relation to stool consistency (*http://www.dpd.cdc.gov/dpdx/html/DiagnosticProcedures.htm*

Table 1. Comparison of different fixatives used to preserve stool parasites

Analytical method	Unfixed sample	Formalin-fixed	SAF*-fixed	Ethanol-fixed
Direct microscopy of mobile trophozoites	Yes (fresh stool required)	No	No	No
Formalin/Ethyl acetate (ether) concentration	Yes	Yes	Yes	No
Permanent staining of trophozoites	Yes (if fixed immediately)	No	Yes	No
ELISA for *E. histolytica* antigen detection	Yes (less than 48 h)	No	No	No
Monoclonal antibody for *E. histolytica/E. dispar*	Yes	Yes	Yes	Yes
PCR	Yes (cysts)	No	No	Yes (cysts and trophozoites)

*Sodium acetate-acetic acid-formalin

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

Direct wet smear
Direct microscopy of a wet smear requires a minimum of equipment (a slide, a cover slip, and a drop of saline), and it is probably the most widely used method for diagnosing intestinal protozoa throughout the world. It is fast and cheap, but its outcome depends entirely on the skills of the microscopist. A crude fecal sample contains numerous structures such as undigested food particles and human cells, which might be misinterpreted as protozoan cysts or trophozoites. Nonetheless, if interpreted correctly, microscopy is the simplest technique for diagnosing an invasive intestinal amoeba infection, because findings of *Entamoeba* trophozoites with ingested erythrocytes strongly indicate *E. histolytica* (Fig. 1). This is, however, rarely seen in a setting such as Sweden, where most of the *Entamoeba* patients harbor either only cysts or trophozoites without erythrocytes.

Concentration technique
In all Swedish parasitology laboratories, the standard method for detecting fecal parasites is a more or less modified form of the formal/ether concentration technique that was developed by Ritchie in 1948 (Ritchie, 1948). The method has been changed very little since that time, except that ether has been replaced by ethyl acetate (Young et al., 1979), and sodium acetate-acetic acid-formalin (SAF) may be used instead of formalin as the fixative (Yang and Scholten, 1977). With this technique, amoeba cysts are concentrated, whereas trophozoites are found only occasionally.

Permanent staining techniques

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

2.5.2 Extra-intestinal amoebiasis

Few patients with extraintestinal amoebiasis have *E. histolytica* parasites in their stool samples (Fotedar et al., 2007a), thus other methods must be used for diagnosis in such cases. Antibody detection is the preferred laboratory technique in that context, and many different serology methods have been developed. The most widely used is enzyme-linked immunosorbent assay (ELISA), for which a recent study in Bangladesh indicated a sensitivity of 96% for ALA patients but only 46% for amoebic colitis patients (Haque et al., 2010).

When using aspirated abscess material to diagnose *E. histolytica* infection, microscopy offers very low sensitivity, whereas both conventional and real-time PCR have proven to be valuable methods (Zaman et al., 2000; Khan et al., 2006; Othman et al., 2010). However, invasive techniques should be avoided due to the risk of bacterial superinfection or spillage of abscess contents, and they should be used only if absolutely necessary to confirm a diagnosis (Pritt and Clark, 2008). Haque and colleagues (2010) have observed that the combined results of real-time PCR performed on the non-invasive fluids urine and saliva reached a sensitivity of 97% for ALA and 89% for amoebic colitis. This is a promising new approach for diagnosis of ALA, especially in endemic areas where serology is of limited value due to the difficulty of distinguishing between past and present infections.

2.6 DIFFERENTIATION OF *ENTAMOEBA* SPECIES

2.6.1 Zymodemes

The zymodeme technique was the first procedure used to characterize different *Entamoeba* isolates and was long regarded as "the gold standard" for this purpose. However, it requires the use of cultured amoeba trophozoites, and it is tedious and time consuming to perform. Furthermore, the sensitivity is low, because many samples that are positive by microscopy are culture negative. Although the zymodeme method is not currently used for routine species identification, it did play a major role in the early differentiation of *E. histolytica* and *E. dispar* (Sargeaunt et al., 1978).

2.6.2 Antigen detection tests

Specific detection of *E. histolytica* can be achieved by employing commercially available antigen detection kits, such as *Entamoeba* CELISA PATH and TechLab *E. histolytica* II, both of which use monoclonal antibodies against the same target: the Gal/GalNAc-specific lectin (adhesin molecule) of *E. histolytica*. Several studies carried out in Bangladesh, an area with high *E. histolytica* transmission, have shown that antigen detection has a high sensitivity, equal to PCR (Haque et al., 1997; Haque et al., 1998), whereas investigations in non-endemic areas have demonstrated a poor sensitivity for antigen detection compared to PCR (Gonin and Trudel, 2003; Stark et al., 2008). This difference in test results between endemic and non-endemic areas is not

well understood, although it might reflect the fact that PCR is 100–1000 times more sensitive in detection of trophozoites than antigen tests (Mirelman et al., 1997; Stark et al., 2008). The conclusion so far is that the antigen testing, which is both rapid and technically simple, is appropriate in areas with high prevalence of *E. histolytica* but is not useful in settings where there are few cases of *E. histolytica* infection (Gonin and Trudel, 2003; Visser et al., 2006; Stark et al., 2008).

2.6.3 Molecular methods

DNA extraction

The first *Entamoeba* PCR assays were performed on cultured trophozoites, thus DNA isolation was a minor challenge compared to the situation today, when DNA usually is extracted directly from stool samples. It is known that feces contain several PCR inhibitors (Abu Al-Soud and Radstrom, 2000), most of which should be removed by an optimal extraction procedure. Commercial spin columns (QIAamp^{TM} DNA mini kit or QIAamp^{TM} DNA stool mini kit) are among the most widely used devices for extraction of *Entamoeba* DNA directly from stool samples (Verweij et al., 2000; Gonin and Trudel, 2003). The extraction procedures often include initial cyst-disrupting steps such as freezing and thawing, boiling, or bead-beater treatment. Nonetheless, manual extraction is time consuming and inconvenient when analyzing a large number of samples. Since molecular biology methods are becoming part of routine diagnostic techniques for detecting intestinal parasites, use of automatic extraction procedures is increasing (Bruijnesteijn van Coppenraet et al., 2009; Calderaro et al., 2010), although there is a lack of studies comparing manual and automatic extraction of stool parasites.

PCR methods

The earliest methods for differentiating *E. histolytica* and *E. dispar* included two single PCRs that target either the small subunit ribosomal RNA (*ssrRNA*) gene (Clark and Diamond, 1991b) or the gene encoding peroxiredoxin (a 30-kDa protein) (Tachibana et al., 1991). According to a very informative review published by Fotedar and colleagues (2007a), these techniques have been the most commonly used worldwide. Several more recent protocols for detection and differentiation of *E. histolytica* and *E. dispar* are now available, which include duplex PCR, multiplex PCR, nested PCR, and real-time PCR. In addition, a number of procedures for detecting *E. moskhovskii* have been described (Ali et al., 2003; Hamzah et al., 2006; Khairnar and Parija, 2007; Nazemalhosseini Mojarad et al., 2010). *Entamoeba* PCR assays target different loci, but the *ssrRNA* gene has been used most often for all *Entamoeba* species and, due to its multi-copy nature, the analytical sensitivity is usually high.

2.7   MIXED INFECTIONS

Sargeaunt and colleagues (Sargeaunt, 1987) observed very few mixed infections while performing isoenzyme electrophoresis on cultured trophozoites from thousands of *Entamoeba* isolates from many different settings. A plausible reason for this is that, in culture, one species may easily outgrow the other, as has been demonstrated in a model system using *in vitro* co-cultures and PCR detection (Pysova et al., 2009), in which *E. dispar* trophozoites outgrew *E. histolytica* within 48 hours in all assays. It remains to be determined whether the same is true for natural co-infections. PCR performed on DNA extracted directly from stool samples has indicated that mixed infections seem to occur mainly in endemic areas (Ramos et al., 2000; Nunez et al.,

2001) and are rarely reported in European settings, although most *E. histolytica* or *E. dispar* infections that are diagnosed in Europe are acquired in endemic areas.

2.8   GENETIC DIVERSITY OF *E. HISTOLYTICA*

Investigations of genetic variation in coding and non-coding regions of different *E. histolytica* isolates might explain why some infections remain asymptomatic whereas others do not. In a study performed in Bangladesh (Ali et al., 2007), different genotypes were found in isolates from various patient groups (asymptomatic or suffering from colitis or ALA). Moreover, genotyping used in the investigation of a sexually transmitted outbreak of severe amoebiasis in Canada suggested that the patients involved were infected with a highly virulent genotype that differed from simultaneously examined strains that caused less severe infections (Salit et al., 2009). It has also been observed that paired isolates from stool samples and liver abscesses from the same patients differed genetically (Ali et al., 2008b), which implies that the patients in that study were either infected with mixed genotypes, or that some kind of DNA reorganization had taken place in the intestine or liver.

2.9   EPIDEMIOLOGY

The recognition of *E. dispar* as a separate non-pathogenic species meant that the results of all previous prevalence studies based on microscopy were not reliable. It was realized that *E. dispar* gave rise to about 90% of the 500 million new amoeba infections originally estimated to occur each year (Ackers and Mirelman, 2006). It also became evident that, at most, only one in four real *E. histolytica* infections progresses to disease (Ali et al., 2008a). All the same, amoebiasis does have a marked impact on global public health and has been estimated to cause around 40,000–100,000 deaths annually, making it one of the leading causes of mortality from a parasitic disease (Stanley, 2003).

Many prevalence studies have been performed since the introduction of PCR methods and antigen tests that can distinguish between *E. histolytica* and *E. dispar*. The most widespread occurrence of *E. histolytica* has been reported from certain countries in Latin America, Asia, and Africa, although local prevalence is highly variable (Ali et al., 2008a). However, even in countries such as South Africa and Mexico with a high prevalence of *E. histolytica*, *E. dispar* is responsible for a considerable portion of the infections (Ramos et al., 2005; Samie et al., 2006).

Entamoeba in Sweden
The first data on prevalence of *Entamoeba* infections in Sweden appeared in 1935 in Ruth Svensson's groundbreaking thesis "Studies on Human Intestinal Protozoa" (Svensson, 1935). The investigations she performed were based on microscopy of fresh unstained or stained stool preparations or culture (concentration techniques were not used in her thesis), and she found an *E. histolytica* prevalence of 2.3% in rural areas and 1.3% in the Stockholm area. It is impossible to know whether the parasites that she observed were *E. histolytica* or *E. dispar*, because only patients without intestinal symptoms were included in her research (and obviously no trophozoites with intracellular erythrocytes were detected).

In two more recent studies conducted in Sweden (Svanteson et al., 1988; Svenungsson et al., 2000), less than 1% *E. histolytica/E. dispar* were detected in both control and diarrhea patients. In another Swedish investigation (Thoren et al., 1990),

both stool microscopy and serology were used to evaluate treatment of *E. histolytica* in asymptomatic homosexual men. According to the results, none of 42 cyst-positive patients had detectable antibodies, which suggests that the majority of infections were due to *E. dispar.* This might also have been the case in one of the few waterborne outbreaks of *E. histolytica* ever published. It occurred at a Swedish winter ski resort and comprised 1,480 persons who became infected with *G. intestinalis* and 106 with what was at the time believed to be *E. histolytica* (Andersson and De Jong, 1989). Unfortunately, no stool samples were saved from any of those patients, so we will probably never know which *Entamoeba* species was actually involved.

In Sweden, infection with *E. histolytica* is a notifiable disease, but in most cases species differentiation is not performed in the laboratory where the initial microscopic diagnosis is made, and samples are infrequently sent for further analyses. Consequently, the data reported are inconclusive, and the true number of *E. histolytica* infections remains unknown. However, accumulated data from the section of Diagnostic Parasitology and Mycology (PDM) at the Swedish Institute for Infectious Disease Control (SMI) indicate that less than 10% of the microscopically diagnosed cases are due to *E. histolytica* (Paper I).

2.10 TREATMENT

The WHO recommends that *E. histolytica/E. dispar* should be differentiated whenever possible and that patients should not be treated based on microscopy findings alone. Yet, regardless of symptoms, all cases identified as *E. histolytica* should be treated due to the risk of invasive disease, whereas cases found to involve only *E. dispar* should not be treated. If a patient with *E. dispar* (or *E. moshkowskii*) has intestinal symptoms, a search should be made for other causes of disease. Asymptomatic *E. histolytica* infection should be treated with a luminal amoebicide (diloxanide furoate or paromomycin), and invasive intestinal or extra-intestinal amoebiasis should be handled by administering a tissue amoebicide (metronidazole) followed by luminal treatment (WHO, 1997).

3 *GIARDIA INTESTINALIS*

3.1 DISCOVERY

Almost 330 years ago, Antony van Leeuwenhoek examined his own diarrheal stool under a single-lens microscope and was the first to detect a human infection with an intestinal protozoan, describing the organisms he observed as "oval animalcules a little bit bigger than blood cells moving around" (Dobell, 1920). In November 1681, van Leeuwenhoek sent a letter to the Royal Society in London to report his findings. In the book "Leeuwenhoek and His Little Animals," Dobell concluded that van Leeuwenhoek's description corresponded well with the vegetative (trophozoite) stage of *Giardia* (Dobell, 1932). But the question is, using a microscope of the time, was it really possible to distinguish all the details that were recounted, such as the "small paws" that the organism used to move around with? The answer is yes. The observations were recreated by the British scientist Brian Ford, who used the same kind of single-lens microscope as van Leeuwenhoek and was able to clearly view the *Giardia* trophozoites with surprisingly good resolution (Fig. 3A-C) (Ford, 2005).
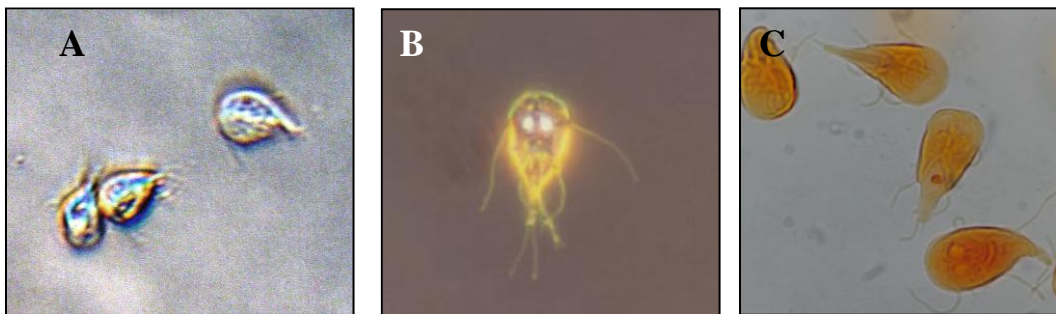

Figure 3. *Giardia* trophozoites as van Leeuwenhoek saw them in 1681 (**A**) and as we see them in 2010 (**B, C**). The picture in **A** was adjusted by use of Photoshop CS to account for the way that the human eye optimizes a microscopic image (photo courtesy Brian Ford). Photo **B** and **C** Anders Magnusson

3.2 THE PARASITE

The name
After Leeuwenhoek's observations, it took about 200 years before the organism was given a name. Lambl rediscovered *Giardia* in 1859 and called it *Cercomonas intestinalis*. In 1882 Kunstler established the generic name *Giardia* for a flagellate (*Giardia agilis*) found in a tadpole. Over the years different species names have been proposed for the parasite infecting humans: *Giardia lamblia*, *Giardia duodenalis*, and *Giardia intestinalis*. These three names are now used in parallel, with some regional and personal preferences (Adam, 2001).

Giardia species
The genus *Giardia* is a member of the diplomonads, a group of binucleated flagellates that belongs to the eukaryotic supergroup Excavata (Adl et al., 2005). *Giardia* is the only genus of diplomonads that infects humans, and its closest relative found in the human intestine is the uninucleate flagellate *Chilomastix mesnili*. The following six species of *Giardia* have been described, based on the morphology of the trophozoites and median bodies and the characteristics revealed by electron microscopy: *G. intestinalis* (humans and mammals), *G. agilis* (amphibians), *G. muris* (rodents),

G. ardae (birds), G. *psittaci* (birds), and G. *microti* (rodents) (Adam, 2001). If not otherwise indicated, in this thesis *Giardia* refers to *G. intestinalis.*

3.2.1 Life cycle and morphology

Like *E. histolytica, Giardia* has a simple life cycle that comprises a resistant infectious cyst stage and a mobile disease-causing trophozoite. The cysts are transmitted via water, food, or person-to-person contact, and it has been estimated that 10–25 cysts are sufficient to achieve infection (Rendtorff, 1954b). A cyst is oval in shape, measures 8–12 μm x 7–10 μm, and has four nuclei. The nuclei are hardly visible in unstained preparations but are distinct after trichrome or DAPI (4'-6-diamidino-2-phenylindole) staining (Fig. 4A, B). Excystation is triggered by exposure to the gastric acid in the host's stomach and continues in the small intestine. This process starts with a short-lived excyzoite, which divides twice and gives rise to four trophozoites that measure 5–9 μm x 12–15 μm and have two nuclei, eight flagella, and a median body with unknown function. The living trophozoite moves in a very characteristic way, like a "falling leaf", and this feature is used as a diagnostic criterion to discriminate *Giardia* trophozoites from other protozoan flagellates in fresh stool samples. The trophozoites are very "photogenic" in Giemsa-stained preparations, appearing as a face in which the two nuclei form the eyes and the median body constitutes the smile (Fig. 4C). The trophozoites are either free or become attached to the intestinal epithelium via an adhesive disc. Encystation into new cysts takes place in the lower part of the small intestine, and the excreted cysts, which are immediately infectious, are also resistant to environmental factors and can survive for a long time under favorable conditions (Ankarklev et al., 2010).
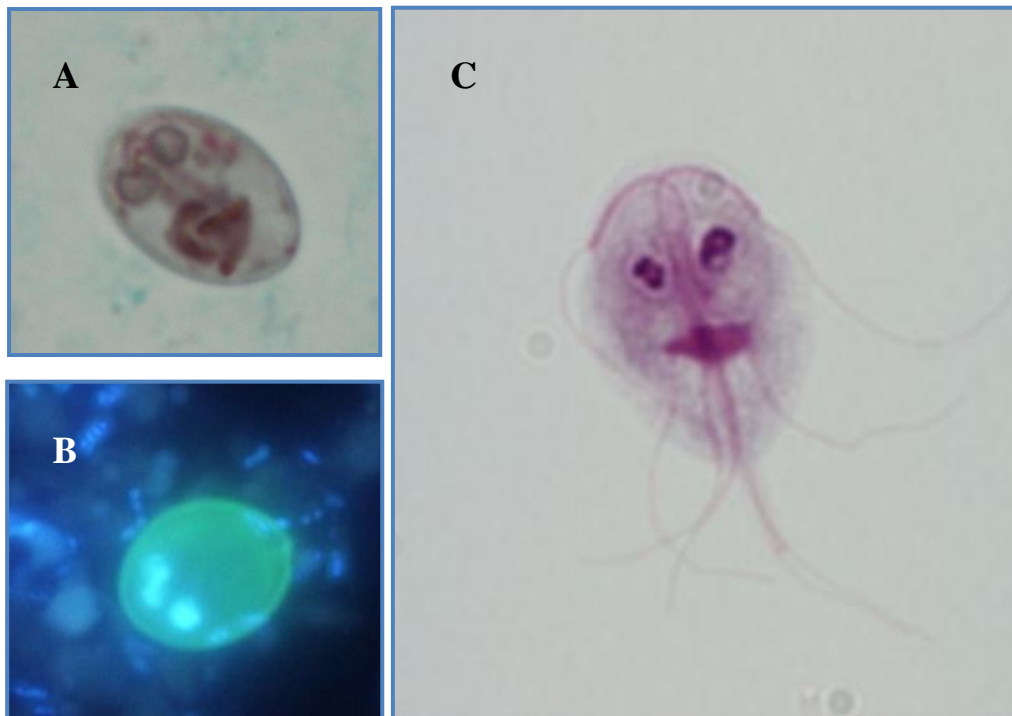

Figure 4. Micrographs of *Giardia* showing a cyst stained with trichrome (**A**), another cyst stained with DFA + DAPI (**B**), and a trophozoite stained with Giemsa (**C**)

3.2.2 Clinical presentation

Although *Giardia* trophozoites were detected in a diarrheic stool more than 300 years ago, the organism was not considered pathogenic until 1978, and this assertion was

based on observations of symptoms such as malabsorption, and the pathology of the upper part of the small intestine in patients with giardiasis (Kulda and Nohýnková, 1978; Faubert, 2000). Koch's postulate was fulfilled in 1987, when all of five volunteers inoculated with the GS/M strain (assemblage B) became infected, and two of them also developed typical symptoms of giardiasis (Nash et al., 1987). The average incubation time from infection to onset of symptoms is one week but can be as long as three weeks (Jokipii and Jokipii, 1977). The typical patient has symptoms for at least one week, including foul-smelling diarrhea, nausea, abdominal cramps, flatulence, and intense fatigue (Petri, 2005). Prolonged diarrhea and malabsorption may also be present, although many of the patients have only mild diarrhea or remain asymptomatic, especially in endemic areas. Unlike *E. histolytica*, *Giardia* is a non-invasive organism, and the pathogenesis is not fully understood. The disease is now considered multifactorial, including apoptosis of enterocytes, loss of epithelial-barrier function, hypersecretion of chloride ions, inhibition of brush-boarder enzymes, and malabsorption of glucose, water, and sodium ions (Ankarklev et al., 2010).

3.3   LABORATORY DIAGNOSIS OF GIARDIASIS

Microscopy
Microscopic detection of *Giardia* cysts in a stool specimen, either directly in a wet smear or after formol-ethyl acetate concentration, is the most frequently used method for diagnosis of giardiasis worldwide. Less often, diagnosis is based on detection of trophozoites in fresh stool samples or SAF-fixed material. Compared to identification of *Entamoeba* spp., microscopy of *Giardia* cysts and trophozoites is more straightforward, and there is little risk of confusion with other parasites. Moreover, only "ghost" cysts with an empty appearance are sometimes not recognized as *Giardia* parasites (Collins et al., 1978). However, the sensitivity of microscopy is quite low due to the intermittent excretion of *Giardia* cysts (Rendtorff, 1954b; Danciger and Lopez, 1975), and thus it is recommended that at least three samples be examined in order to rule out giardiasis.

Antigen detection methods
Quite a few commercial kits are available for detection of *Giardia* antigen. Two techniques that are often used are enzyme-linked immune-sorbent assay (ELISA) that assesses soluble antigens and a direct fluorescent antibody (DFA) test that detects intact organisms. Several studies have shown that these two methods offer greater sensitivity compared to light microscopy (Zimmerman and Needham, 1995; Garcia and Shimizu, 1997), but they are not available in all parasitology laboratories due to the high cost and substantial workload they entail, and also limited access to the required equipment. An alternative technique involves a solid-phase immunochromatographic test card system (ImmunoCardSTAT! Cryptosporidium⁄Giardia rapid assay), which allows concurrent detection of *Cryptosporidium* and is also fast, easy to use, and does not require extra equipment (Johnston et al., 2003). Unfortunately, the sensitivity of such a system is lower than microscopy, and thus this test is not recommended for follow-up of patients with *Giardia* treatment failure (Strand et al., 2008).

PCR
Conventional single or nested PCR analyses are not often performed for diagnosing giardiasis, except at specialized centers. In contrast, there is increasing use of real-time PCR as a diagnostic tool that can detect *Giardia*, often in combination with other enteric protozoa, such as *Cryptosporidium* spp*., E. histolytica*, and *D. fragilis*. This approach has proven to provide higher sensitivity compared to conventional methods,

and it also entails a lower workload (Verweij et al., 2004; Haque et al., 2007; Schuurman et al., 2007; Ten Hove et al., 2007; Bruijnesteijn van Coppenraet et al., 2009; Ten Hove et al., 2009; Calderaro et al., 2010). However, in the parts of the world where giardiasis is highly endemic, microscopy will probably remain the routine procedure for detecting stool parasites, including *Giardia*, for a long time to come.

3.4 EPIDEMIOLOGY

G*iardia* is a parasite found in all parts of the world and in a large number of mammals, including humans, livestock, pets, wildlife, and aquatic animals (Thompson, 2000; Lasek-Nesselquist et al., 2010). Several recent reports have also described *G. intestinalis* in various birds and even fish, although true infections remain to be confirmed in these animals (Lasek-Nesselquist et al., 2008; Yang et al., 2010b). The scope of this thesis is limited mainly to human giardiasis, and thus *Giardia* infection in animals will be discussed in light of the potential zoonotic risk, not from a clinical veterinary perspective.

Giardiasis worldwide
The prevalence of *Giardia* in humans varies in and between countries, and it is higher in areas where environmental hygiene is low. According to estimates from the WHO about 200 million people have symptomatic giardiasis, and around 500,000 new cases occur each year (WHO, 1996). Studies in different European countries have indicated a prevalence of 1–17%, and up to 100% of the population can be infected in certain highly endemic areas (Plutzer et al., 2010).

Giardiasis in Sweden
In 1889, Muller made one of the earliest observations of giardiasis in Sweden, when he described several layers of trophozoites of what he called *Cercomonas intestinalis* (the name used by Lambl in 1859) attached to the mucosa of the small intestine of an executed murderer (Muller, 1889). Ruth Svensson's studies of parasites in various healthy populations in the 1930s in Sweden revealed a *Giardia* prevalence of 4.7% in urban regions and 7.0% in rural areas (Svensson, 1935). In the 1970s, several Swedish reports were published concerning giardiasis in children at day care centers and among people who had traveled to Leningrad (Kettis and Magnius, 1973; Kettis and Thoren, 1974). No prevalence studies have been performed in Sweden since Ruth Svensson's days, but in a survey of adult individuals conducted in 1996–1997, *Giardia* cysts were found in 2% of the diarrhea patients and in none of the 203 healthy controls (Svenungsson et al., 2000).

An investigation of calves in Sweden detected *Giardia* infections in 29% of the animals that were diarrheic and 23% of those that were healthy (Bjorkman et al., 2003). Similar results were obtained when diarrheic and healthy lambs were investigated: 25% and 20%, respectively, were infected with *Giardia* (Ljungström et al., 2001). Another study of dogs in Sweden revealed that *Giardia* was common in puppies (33%) but rare in the adult animals, and none of the dog owners harbored cysts (Castor and Lindqvist, 1990).

3.5 MOLECULAR DIFFERENTIATION

3.5.1 Assemblages and subtypes

Giardia intestinalis consists of seven genetically different assemblages, designated A to G, and an additional genetic variant was recently described in seals (Lasek-Nesselquist et al., 2010). All assemblages have similar morphology, and they cannot be differentiated by microscopy. Based on genetic and host-specific data, it has been proposed that assemblages A to G be re-classified as separate *Giardia* species (Monis et al., 2009), but this issue has not yet been settled (Table 2). Previous research based on enzyme analysis and DNA sequencing of isolates of *Giardia* adapted to growth *in vitro* have shown that each of the two assemblages A and B can be further divided into two main sub-assemblages, which are designated AI and AII, and BIII and BIV (Monis et al., 1996; Monis et al., 1999). It was long assumed that AI was the only zoonotic A sub-assemblage, and that AII occurred only in humans, although it has been shown that the latter group has a wider host range as well (Sprong et al., 2009). An additional sub-assemblage denoted A III has also been described (Lalle et al., 2007), which is considered to be strictly non-zoonotic, because it has been identified solely in animals (mainly wild ungulates). So far, no host preferences have been attributed to sub-assemblages BIII and BIV. Furthermore, it is evident that more "subtypes" exist within assemblages A and B, as well as within the other assemblages. The nomenclature for *Giardia* subtypes has not been strictly standardized, and various researchers have used different designations for the level below sub-assemblage, calling it subtype, sub-genotype, subgroup, or genotype. In this framework and in Paper IV, the term "subtype" is used to refer to the level under sub-assemblage, while the term "sub-genotype" was used in Paper II and III. In some instances, Arabic numerals are also used to denote established subtypes (e.g., subtypes A2 and A3 at the β-giardin locus) (Caccio et al., 2002).

Table 2. *Giardia* assemblages and proposed species names

Assemblage	Sub-assemblage	Host	Proposed species name
A	AI AII AIII	Humans and animals Humans and animals Animals	*Giardia duodenalis*
B	BIII BIV	Humans and animals	*Giardia enterica*
C		Dogs	*Giardia canis*
D		Dogs	*Giardia canis*
E		Ruminants and horses	*Giardia bovis*
F		Cats	*Giardia felis*
G		Rodents	*Giardia simondi*

3.5.2 Molecular typing methods

Compared to other protozoan parasites, such as *Cryptosporidium* spp. and *Plasmodium falciparum*, genotyping techniques for *Giardia* are not particularly advanced. The following loci are most often used as markers: the small subunit ribosomal RNA (*ssrRNA*), β-giardin, glutamate dehydrogenase (*gdh*), and triose phosphate isomerase (*tpi*). Considering differences between these genes, *tpi* and *gdh* are the most variable, followed by β-giardin, and the *ssrRNA* gene is the most conserved. These disparities are mirrored in differences in substitution patterns, since the β-giardin gene shows few

non-synonymous changes, whereas *gdh* and *tpi* seem to tolerate amino acid replacements (Caccio and Ryan, 2008). The *ssrRNA* gene has frequently been used for genotyping of both human and animal isolates. This gene has a multi-copy nature that results in greater sensitivity of PCR compared to the sensitivities achieved when analyzing the other commonly used genes, and its conserved features facilitate design of the primer. However, the discrimination power is low for the *ssrRNA* gene, and differentiation is possible only between assemblages, not between sub-assemblages or subtypes. By comparison, higher resolution can be achieved for the β-giardin, *tpi*, and *gdh* genes, and numerous subtypes within each assemblage have been identified, many of them occurring only in single isolates (Sprong et al., 2009). Recently, the strategy of applying the 5.8S rDNA and intergenomic rRNA spacer regions (ITS1 and ITS2) for differentiation of all assemblages has gained attention as a useful method for both genotyping and identification of species other than *G. intestinalis*, including *G. muris* and *G. microti* (Caccio et al., 2010).

The tool used most often for identifying different assemblages has been conventional PCR in combination with either direct sequencing or analysis of restriction fragment length polymorphism (RFLP). PCR with assemblage-specific primers (Amar et al., 2002; Geurden et al., 2008; Levecke et al., 2009) and real-time PCR with assemblage-specific probes (Guy et al., 2004; Almeida et al., 2010) have also been described.

RFLP
The RFLP technique is frequently used to genotype protozoan parasites such as *Cryptosporidium* spp. and *Leishmania* spp. (Spano et al., 1997; Xiao et al., 2001; Marfurt et al., 2003). RFLP is simple to perform and less resource demanding compared to sequencing, but the information obtained is limited, and point mutations in the restriction sites can give rise to unexpected RFLP patterns. A number of investigators have described the use of PCR-RFLP methods for *Giardia* genotyping at several loci (primarily in human isolates): β-giardin (Caccio et al., 2002; Lalle et al., 2005b), *gdh* (Homan et al., 1998; Read et al., 2004), and *tpi* (Amar et al., 2002; Lalle et al., 2009). However, in the future, RFLP will probably be replaced by sequencing, because the latter method is becoming increasingly affordable and is generally more informative than RFLP.

3.5.3 MLGs, mixed infections, and assemblage swapping
The resolution of genotyping using only one gene is not considered to provide sufficient discriminatory power, and hence a multilocus approach has been suggested by Caccio and colleagues (2008). According to their proposal three markers (the β-giardin, *gdh*, and *tpi* genes) should be used, and the sequences (subtypes) of each locus should be combined into multilocus genotypes (MLGs) which can make it possible to to enable comparison of isolates from different sources. The occurrence of mixed infections has also been noted lately in some investigations using assemblage-specific *tpi* primers, which allows detection of a much larger number of mixed assemblage A and B infections than can be done when using more general primers (Geurden et al., 2008; Levecke et al., 2009). Another feature observed in *Giardia* genotyping studies is the occurrence of "assemblage swapping," which means that different markers can give rise to different results for the same isolate. This phenomenon has been attributed to preferential amplification of one assemblage over the other in "true" mixed infections or to recombination between assemblages (Caccio et al., 2008; Caccio and Sprong, 2009). Thus, it is clear that in-depth studies focusing on several loci and using assemblage-specific primers are needed both to clarify the issue of zoonotic transmission and to discern the correlation between assemblages and disease pattern.

3.5.4 Allelic sequence divergence

Several investigators have observed that double peaks (overlapping nucleotides) often occur in sequences from assemblage B, as well as assemblages C, D, and E, but are rarely seen in assemblage A or F (Caccio and Ryan, 2008). This feature has been attributed to mixed subtype infections or allelic sequence divergence, or a combination of both. The frequent occurrence of double peaks in the assemblage B sequences also complicates assignment of isolates to sub-assemblages and to specific MLGs.

3.6 MOLECULAR EPIDEMIOLOGY

3.6.1 *Giardia* assemblages in humans

Genotyping of a large number of human *Giardia* isolates from different parts of the world has demonstrated that humans are almost exclusively infected with assemblage A or B. Other assemblages have occasionally been found in humans: C and D (Read et al., 2004; Traub et al., 2004), E (Foronda et al., 2008), and F (Gelanew et al., 2007), but these findings require further confirmation. Table 3 presents examples of the results of genotyping of human isolates collected worldwide (outbreak-related investigations are not included). According to this compilation, assemblage B dominates in Asia, Australia, Europe, and Latin and South America, whereas it seems that assemblage A predominates in Africa. It is not clear whether these data reflect the different populations investigated, the different geographical sites, or the various methods used. Table 4 shows the incidence of assemblages in studies using only one marker (data extracted from Table 3). According to analysis based on the *tpi* marker, assemblage B is twice as common as assemblage A, whereas the opposite results are obtained by use of the β-giardin marker (i.e., twice as many assemblage A as assemblage B isolates). A similar trend is seen among the sequences deposited in the ZoopNet database (Sprong et al., 2009). The reason for these discrepancies is unknown, although it has been speculated that primer sequence mismatches might reduce the ability to amplify certain assemblage B isolates at the β-giardin locus (Robertson 2007).

Table 3. Distribution of assemblages A and B in 2471 human cases of giardiasis
An asterix* indicates that correlation between assemblages and symptoms was investigated

Origin	No. of isolates	Loci tested	Ass A	Ass B	Ass A+B	Reference
Argentina	28	*tpi*	0	28	0	(Molina et al., 2007)
Argentina	43	*tpi*	3	40	0	(Minvielle et al., 2008)
Brazil	62	ß-giardin	62	0	0	(Volotao et al., 2007)
Brazil	37	*gdh*	29	8	0	(Souza et al., 2007)
Brazil*	58	*ssrRNA*	9	43	6	(Kohli et al., 2008)
Cuba*	20	ß-giardin, *gdh*	9	11	0	(Pelayo et al., 2003)
Mexico	9	ß-giardin	9	0	0	(Lalle et al., 2005a)
Nicaragua	119	ß-giardin, *gdh*	25	92	2	(Lebbad et al., 2008)
Peru	16	*gdh*	10	6	0	(Perez Cordon et al., 2008)
Peru	25	*tpi*	6	19	0	(Sulaiman et al., 2003)
Canada	15	ß-giardin	3	9	3	(Guy et al., 2004)
Total America	**432**		**165**	**256**	**11**	

Country	N	Gene	Assemblage A	Assemblage B	Mixed	Reference
Australia	8	*gdh, ssrRNA*	2	6	0	(Read et al., 2004)
Australia*	23	*ssrRNA*	7	16	0	(Read et al., 2002)
Australia	124	*gdh, ssrRNA*	31	93	0	(Yang et al., 2010a)
New Zealand	30	ß-giardin	23	7	0	(Winkworth et al., 2008)
Total Australia and New Zealand	**185**		**63**	**122**	**0**	
Bangladesh*	267	*ssrRNA, tpi*	20	231	16	(Haque et al., 2005)
India*	101	*tpi*	7	88	6	(Ajjampur et al., 2009)
India	16	*tpi*	5	8	3	(Traub et al., 2004)
Malaysia	42	*ssrRNA*	1	41	0	(Mahdy et al., 2009)
Nepal	35	*ssrRNA, tpi*	7	26	2	(Singh et al., 2009)
Palestine	8	*gdh*	5	3	0	(Hussein et al., 2009)
The Philippines	133	*tpi*	18	83	32	(Yason and Rivera, 2007)
Thailand	61	ß-giardin, *gdh, tpi*	5	31	25	(Tungtrongchitr et al., 2010)
Thailand	12	*gdh*	5	7	0	(Ratanapo et al., 2008)
Thailand	30	ß-giardin	12	17	1	(Kosuwin et al., 2010)
Total Asia	**705**		**85**	**535**	**85**	
Egypt*	41	*tpi*	31	8	2	(Helmy et al., 2009)
Egypt	17	*tpi*	1	16	0	(Foronda et al., 2008)
Egypt*	87	*tpi*	58	10	19	(Abdel-Moneim and Sultan, 2008)
Ethiopia*	52	ß-giardin	31	13	8	(Gelanew et al., 2007)
Ivory Coast	14	*tpi, gdh*	0	14	0	(Bertrand et al., 2005)
Western Sahara	36	*tpi, gdh*	16	18	2	(Lalle et al., 2009)
Total Africa	**247**		**137**	**79**	**31**	
Albania	22	*ssrRNA*	10	12	0	(Berrilli et al., 2006)
Belgium	72	ß-giardin, *gdh, tpi*	16	30	26	(Geurden et al., 2009b)
France	25	*tpi, gdh*	9	16	0	(Bertrand et al., 2005)
Italy	11	ß-giardin	5	5	1	(Giangaspero et al., 2007)
Italy	30	ß-giardin	24	6	0	(Caccio et al., 2002)
Italy	37	ß-giardin	17	15	5	(Lalle et al., 2005b)
Italy	42	*ssrRNA*	19	13	10	(Giangaspero et al., 2007)
Italy	68	*ssrRNA*, ß-giardin	23	45	0	(Calderaro et al., 2010)
Norway	63	ß-giardin, *gdh,*	4	59	0	(Robertson et al., 2007)
Portugal	7	ß-giardin	2	5	0	(Almeida et al., 2006)
Portugal	25	*tpi*, ß-giardin	25	0	0	(Sousa et al., 2006)
Spain*	108	*tpi*	43	61	4	(Sahagun et al., 2008)
The Netherlands	98	*ssrRNA, gdh*	32	64	2	(van der Giessen et al., 2006)
The Netherlands*	18	*gdh*	9	9	0	(Homan and Mank, 2001)
Turkey*	44	*tpi*	19	25	0	(Aydin et al., 2004)
UK*	199	*ssrRNA, tpi*	48	145	6	(Breathnach et al., 2010)
UK	33	*tpi*	9	21	3	(Amar et al., 2003)
Total Europe	**902**		**314**	**531**	**57**	

Table 4. Assemblage incidence in isolates genotyped at one locus only (data extracted from Table 3)

Locus	No. of isolates	Assemblage		
		A	B	A+B
tpi	676	200	407	69
β-giardin	283	188	77	18
gdh	91	58	33	0
ssrRNA	187	46	125	16
Total	1237	492	642	103

3.6.2 *Giardia* assemblages in animals

Cats and dogs
Quite a few genotyping studies have included *Giardia* isolates from cats. A recent review identified only 11 investigations in which at least one cat isolate was genotyped (Ballweber et al., 2010). The host-specific assemblage F was more common (44 isolates) than the potentially zoonotic assemblage A (30 isolates), and sub-genotyping of a limited number of the assemblage A isolates revealed mainly sub-assemblage AI. We found similar proportions of assemblage F and A in cats in Sweden, although no classical sub-assemblages AI or AII variants were identified (Paper III).

Dogs have been studied more extensively than cats, which has revealed that the host-specific assemblages (C and D) also predominate in canines, although assemblage A and to some extent assemblage B are found as well. Mixed-assemblage infections are frequently identified in dogs (Sprong et al., 2009; Ballweber et al., 2010). Most researchers performing sub-genotyping of assemblage A isolates have detected sub-assemblage AI. Notably, in a study conducted in Belgium, 38 of 119 successfully genotyped canine isolates were identical to subtype A2 or A3 (at the β-giardin gene) but none were identical to sub-assemblage AI (Claerebout et al., 2009). By comparison, dogs investigated in Sweden were found to harbor almost exclusively the host-specific assemblages C and D, and only one of 31 dogs in that study was infected with assemblage A (Paper III).

Smaller pets
Although *Giardia* infections are well recognized in smaller pets like rabbits, guinea pigs, and hamsters, few researchers have included such animals in their genotyping studies. Notably, in one of the few documented foodborne outbreaks of giardiasis, the food preparer's infected pet rabbit was highly suspected, although no genotyping was performed at the time (Porter et al., 1990). In our investigation in Sweden (Paper III), assemblage B was identified in one rabbit and one guinea pig, and as far as we know these animals lack host-specific assemblages, and thus assemblage A or B could be expected. Indeed, this was demonstrated in an Italian study that included two rabbit

isolates, one of which was found to contain assemblage A and the other assemblages A and B (Giangaspero et al., 2007).

Livestock
Genotyping studies worldwide have included a large number of isolates from cattle and sheep, and to a lesser extent also goats, pigs, and horses. In livestock, it seems that assemblage E is most prevalent, followed by assemblage A, although there are some exceptions. Considering investigations of cattle samples, assemblages E and B have been detected in Canada (Coklin et al., 2007), whereas only assemblages A and B (no assemblage E) were found in New Zealand (Winkworth et al., 2008). Mixed assemblage infections were reported in only a few of the cited studies, however when Geurden et al. (2008) used assemblage-specific primers to study calves in Belgium, these researchers found that 31% of the investigated samples contained both assemblages A and E. In Sweden, we found only assemblage E in cattle, whereas several of the sheep samples we investigated contained variants of assemblage A (Paper III).

Non-human primates
Non-human primates carry the same *Giardia* assemblages as humans, namely, A and B. Genotyping of isolates from gorillas and howler monkeys in Uganda and Brazil, respectively, detected only assemblage A (Graczyk et al., 2002; Volotao et al., 2008), whereas a study of various captive non-human primates in Belgium revealed both A and B, and also showed that mixed assemblage infections were common (Levecke et al., 2009).

Wildlife
This group includes various mammals, such as wild ruminants (moose, deer, buffalos, and muskoxen), carnivores (foxes, coyotes), rodents (rats, voles), and aquatic animals (beavers, seals). Rodents are usually infected with the host-specific assemblage G, whereas AI and AIII are the most prevalent sub-assemblages in wild ruminants. Both assemblages A and B have been identified in foxes, beavers, and seals (Hamnes et al., 2007; Lasek-Nesselquist et al., 2008).

3.7   ZOONOTIC ASPECTS

In 1979, the WHO elected to include giardiasis on their list of diseases that might be zoonotic and this decision was based on the occurrence of large-scale waterborne outbreaks of giardiasis in North America, in which wild animals were assumed to play a major role (WHO, 1979). WHO officials also referred to a report recounting experimental infections of humans and animals with *Giardia* cysts obtained from beavers (Davies and Hibler, 1979). Beavers are the animals that are most widely suspected of being sources of giardiasis in the United States and Canada, but the opposite scenario is actually more likely: beavers are probably infected through water contaminated by humans (Xiao and Fayer, 2008). In the first fully described experimental infection of a human volunteer with *Giardia* obtained from a non-human source, the cysts used were isolated from a giant pouched rat carrying assemblage B (Majewska, 1994).

Sub-assemblage AI and, to a lesser extent, also sub-assemblage A II and assemblage B are considered to have zoonotic potential. Many reports have claimed findings of zoonotic genotypes, but few studies have included both human and animal samples from the same area. Nevertheless, at least four investigations have proposed zoonotic

transmission between animals and humans living in the same communities. In the first of these, Traub and colleagues (2004) found that humans and dogs in two households in northern India carried the same A or B genotypes. The second study (Inpankaew et al., 2007) examined samples from humans and dogs at 20 different temples in Bangkok, and a notable observation was that, among the sequenced isolates, assemblage A occurred in one dog and two monks at the same monastery. The third investigation (Marangi et al., 2009) showed that six children and eight dogs from a closed community in Italy harbored sequences exhibiting 99.5% identity to sub-assemblage AI. The fourth study (Winkworth et al., 2008) included samples from 30 humans and 40 calves from the same area in New Zealand, and it was found that isolates from 13 of the humans and 24 of the calves had identical sub-assemblage AI sequences. Furthermore, isolates from four of the humans and four of the calves had identical assemblage B sequences and, interestingly, this particular subtype was also found to be common in human isolates in Belgium (Geurden et al., 2009b), as well as in 18 isolates analyzed in our study of human giardiasis in Sweden (Paper IV).

Of the four investigations discussed above, only the one conducted in India (Traub et al., 2004) used more than one marker (i.e., *ssrRNA* and *tpi*), and the results of sequencing at the two loci gave inconsistent results for samples from both humans and animals (assemblage swapping). The study in Bangkok (Inpankaew et al., 2007), used the *ssrRNA* locus, and the remaining two investigations sequenced the β-giardin gene. Notably the β-giardin gene fragments sequenced in the last two studies differed substantially in length (191 and 511bp, respectively).

Sprong and colleagues investigated the zoonotic potential of *Giardia* by using a large number of sequences submitted to the ZoopNet databank (Sprong et al., 2009). These researchers compared sequences from 1,440 animal and 978 human isolates analyzed at four genetic loci (*ssrRNA*, β-giardin, *gdh*, and *tpi*) and found that both assemblages A and B had an evident zoonotic potential at the level of assemblages and sub-assemblages. However, when a multilocus (β-giardin, *gdh*, and *tpi*) approach was used, a zoonotic potential was apparent for only two MLGs, both from assemblage A. In their article zoonotic potential was defined as an identical *Giardia* genotype isolated both from animal and humans, and they did not consider epidemiological parameters. They also found that several isolates were assigned to different assemblages at different loci, which further complicated the picture.

3.8   ASSEMBLAGE AND DISEASE

Several of the studies included in Table 3 explored intestinal symptoms in relation to assemblages. A number of investigators observed that clinical symptoms were associated with assemblage A (Read et al., 2002; Aydin et al., 2004; Haque et al., 2005; Ajjampur et al., 2009), whereas others found a stronger relationship with assemblage B (Pelayo et al., 2003; Gelanew et al., 2007). Also, two studies demonstrated that assemblage B exhibited more extensive association with persistent symptoms, while assemblage A was found in connection with intermittent diarrhea (Homan and Mank, 2001; Helmy et al., 2009). Sahagun and colleagues (2008) found a correlation between assemblage A and symptoms in patients less than five years of age, whereas other investigators noted that fever was more common in assemblage A patients, but found no additional associations (Breathnach et al., 2010). Moreover, no correlation was detected between assemblages and symptoms in analyses performed in Egypt and Brazil (Abdel-Moneim and Sultan, 2008; Kohli et al., 2008). Most of the mentioned studies used one or two markers, and it is noteworthy that the *tpi* gene was more often

found to be associated with symptomatic assemblage A patients than the *gdh* and β-giardin genes. It is not yet known whether this is merely a coincidence, or if it mirrors the ability of different primers to amplify certain assemblages. The published results also suggest that the less prominent assemblage in an area might be the one that is most prone to produce symptomatic infection, as was seen for assemblage A in two studies conducted in Asia (Haque et al., 2005; Ajjampur et al., 2009).

3.9   MULTI LOCUS GENOTYPING AS A TOOL FOR SOURCE TRACING

In outbreak situations, it is of great interest to trace the source of infection. Waterborne outbreaks of *Giardia* are quite common, but source tracing is seldom successful. This might be due primarily to the relatively long prepatient period of the infection; once the symptoms appear and the diagnosis is established, the source is eliminated. Another plausible reason is that the number of cysts present in contaminated water is usually low, and thus detection requires very sensitive PCR methods. In a waterborne outbreak in Bergen, Norway, there were 1300 laboratory-confirmed cases, and genotyping of stool samples revealed different subtypes of assemblage B; however, molecular analysis of the few *Giardia* cysts found in the drinking water four weeks after the onset of the outbreak was not successful (Robertson et al., 2006). Foodborne outbreaks of giardiasis are rarely described, and no successful genotyping has been reported. Genotyping performed in connection with outbreaks at day care centers revealed assemblage B in all amplified isolates in one study (Amar et al., 2002) and assemblage A in all isolates in another investigation conducted in Sweden (Svenungsson et al., 2007). Regrettably, the methods used in those two studies (i.e., assemblage-specific *tpi* PCR and sequencing of the β-giardin gene, respectively) are not discriminatory enough to allow correlation of cases.

4 AIMS

The general aim of this thesis was to differentiate and characterize intestinal protozoa through the introduction and development of molecular methods.

Specific aims:
- to estimate the proportion of *Entamoeba histolytica* and *Entamoeba dispar* in patients with amoebic infections diagnosed by microscopy (Paper I);

- to investigate *Giardia* assemblages in human and animal isolates from Sweden and Nicaragua (Paper II, III, IV);

- to genotype *Giardia* isolates from humans and animals, and to investigate their zoonotic potential (Paper III, IV);

- to investigate the correlation between *Giardia* assemblages and clinical symptoms (Paper IV);

- to evaluate sequence-based multilocus genotyping as a potential tool to investigate different *Giardia* isolates from humans and animals (Paper III and IV);

Ethical clearance was obtained for Paper I, II, III and IV.

5 *ENTAMOEBA* IN SWEDEN (PAPER I)

5.1 STUDY DESIGN AND STUDY POPULATION

In Sweden, routine stool parasitology is performed at around 30 different laboratories. At the time this study was conducted (2001–2002), none of these laboratories used molecular analyses or antigen tests to differentiate *Entamoeba* species, and thus reporting as *E. histolytica/E. dispar* when quadrinucleate cysts were found, leaving it up to the physician to decide whether or not treatment should be implemented. According to the Swedish Communicable Diseases Act, it was mandatory for clinicians to submit notification of identified *Entamoeba* infections, and those records indicated that around 400 cases were detected each year. At that time, the majority of those cases were probably treated as true *E. histolytica* infections, and thus there was an obvious need for specific species diagnosis. All infectious disease clinics and parasitology laboratories in the country were given information about our study and were invited to participate. The inclusion criterion was the detection of *E. histolytica/E. dispar* parasites by microscopy.

There were a few obstacles to overcome before we could begin the study. The first problem was the fact that formalin or SAF fixative was frequently used as a transport medium for stool samples sent to the parasitology laboratories for examination. Initial assays performed at our laboratory at SMI had shown inconsistent PCR results for DNA extracted from formalin- or SAF-fixed samples. In short, the sensitivity of PCR was hampered when analyzing stool specimens that had been exposed to either of the mentioned fixatives, and this effect appeared to be both dose and time dependent. We realized that it was not suitable to perform PCR on the original stool sample, and thus it was necessary for the parasitology laboratories to inform the doctors in charge that new samples had to be sent to us at SMI in order to achieve species differentiation. The next obstacle to overcome was that *Entamoeba* DNA degrades over time in unfixed stool samples. This was particularly apparent in samples containing mainly trophozoites, as would be the case in patients with *E. histolytica* dysentery. Figure 5 shows how the sensitivity of PCR diminished over time in a sample containing *E. dispar* trophozoites. The sample was kept at room temperature, and DNA was extracted at different time intervals. After 48 hours, no detectable DNA was amplified. Due to limited access to true *E. histolytica* samples, we used a stool specimen containing *E. dispar* parasites, because we assumed it would provide comparable results.

Figure 5. PCR analysis of *Entamoeba dispar* DNA extracted from a stool sample at different times after collection. Results: lane 1, 0 h; lane 2, 2 h; lane 3, 4 h; lane 4, 6 h; lane 5, 8 h; lane 6, 10 h; lane 7, 24 h; lane 8, 48 h; lanes 9 and 10, negative controls; lane 11, *E. dispar* control

We also found that fixation in 70% ethanol preserved the *Entamoeba* DNA quite well, at least on the lab bench, which prompted us to evaluate ethanol as a transport medium for stool samples intended for *Entamoeba* PCR. All participants in the study received a "kit" containing sampling instructions, vials with and without ethanol, request forms, and a stamped envelope addressed to the SMI. DNA extraction and PCR were performed using a commercial kit for DNA extraction (QIAamp[TM] DNA mini kit) and conventional single PCR methods (Clark and Diamond, 1991b; Tachibana et al., 1991). The primers used for *Entamoeba* differentiation are listed in Table 5.

Table 5. Primers used for *Entamoeba* differentiation*

Primer ID	Primer sequence (5´–3´)	Locus	Target	bp	Reference
PspF PspR	GGCCAATTCATTCAATG AATTGAG CTCAGATCTAGAAACAA TGCTTCTC	*ssrRNA*	*E. histolytica*	876	(Clark and Diamond, 1991b)
NPspF NPspR	GGCCAATTTATGTAAGT AAATTGAG CTTGGATTTAGAAACAA TGTTTCTTC	*ssrRNA*	*E. dispar*	878	(Clark and Diamond, 1991b)
P11 P12	GGAGGAGTAGGAAAGTT GAC TTCTTGCAATTCCTGCTT CGA	PRX[a] 30-kDa protein	*E. histolytica*	100	(Tachibana et al., 1991)
EntamI EntamII	GTTGATCCTGCCAGTATT ATATG CACTATTGGAGCTGG AATTAC	*ssrRNA*	*Entamoeba* spp	580[b]	(Verweij et al., 2001)

*The annealing temperature was 55 °C for all primers.
[a]Peroxiredoxin gene
[b]Amplicon size depends on the *Entamoeba* species; 579–580 bp for the *E. polecki* complex.

5.2   RESULTS AND DISCUSSION

Altogether, stool samples from 207 patients were received and analyzed by microscopy and PCR at SMI. Microscopy performed after concentration of the unfixed sample detected the *E. histolytica /E. dispar* complex in 161 patients, all of whom were positive in PCR (10 for *E. histolytica* and 151 for *E. dispar*). In addition, samples from 14 patients who were negative by microscopy were positive for *E. dispar* in PCR analysis. In seven of those cases only the ethanol fixed samples were positive in PCR, indicating that degradation of DNA occurred in unfixed samples during transport.

In all, only 5.7% of the PCR-positive samples contained *E. histolytica* DNA. This low proportion of *E. histolytica* compared to *E. dispar* agrees with data from settings similar to ours, such as Australia, Canada, Greece, Spain, and the Netherlands (Verweij et al., 2000; Evangelopoulos et al., 2001; Gonin and Trudel, 2003; Visser et al., 2006; Fotedar et al., 2007b; Gutierrez-Cisneros et al., 2010). After conclusion of our study, differentiation of *Entamoeba* species became a part of routine analyses at SMI. The results of PCR performed on stool specimens from April 2001 to December 2009 indicate that *E. histolytica* infections have never represented more than 10% of the total number of positive PCR reactions per year (Table 6).

Table 6. PCR differentiation of *E. histolytica* and *E. dispar* in stool samples 2001–2009

	Entamoeba histolytica	*Entamoeba dispar*	Negative in PCR	Total PCR
April 2001–Dec 2002	10 (5.7%)[a]	165	32	207
2003	4 (5.7%)	66	24	94
2004	3 (6.6%)	42	18	63
2005	6 (7.8%)	71	37	114
2006	5 (4.5%)	105	29	139
2007	8 (6.1%)	122	33	163
2008	8 (6.9%)	115	33	156
2009	11 (8.3%)	121	-	-

[a] Total number of *E. histolytica* (% of all positive PCR)

No mixed infections with *E. histolytica* and *E. dispar* were found during the study period, and the same absence of mixed infection has also been observed in other non-endemic areas. This reflects either the true situation or the fact that only DNA from the most dominant species is amplified in PCR. Since 2003, we have used two primer pairs that target different *E. histolytica* loci in all samples investigated by PCR (Table 5). Despite that, mixed infections have continued to be very rare, or, more precisely, has occurred only once, in a child from Somalia.

Samples from 46 patients were negative for *E. histolytica/E. dispar* by microscopy performed at SMI. Fourteen of those specimens contained *E. dispar* DNA, whereas the remaining 32 were negative in both PCRs. All but two of those patients had sent in paired samples (one unfixed, one ethanol fixed), and hence there was probably no DNA degradation. A shortcoming of our study was the lack of access to the original samples and the time delay between initial diagnosis and the second sample collected for *Entamoeba* PCR and thus one or more of the following scenarios explain the negative results of microscopy and PCR:

a) patients were treated after initial diagnosis and before providing the second sample;
b) spontaneous cure could have occurred, especially if there was a long delay between the first and the second sample;
c) intermittent excretion of cysts gave false-negative microscopy and PCR results;
d) the initial diagnosis was mistakenly based on amoeba species other than *E. histolytica/E. dispar*.

Scenarios **a** and **b** were out of our control, whereas **c** was already under partial monitoring during the study period. We received two or three samples (taken on different occasions) from 17 of the study patients, and in only one of those cases did the findings change from negative to positive.

Scenario **d** is a more delicate issue. Thirty-two patients had samples that were negative for *E. histolytica* and/or *E. dispar* in microscopy and PCR performed at SMI, and samples from 22 of those subjects were found to contain cysts of other amoeba species (Table 7). Since we did not have access to the original samples, we can only suspect that the initial diagnosis was incorrect in those cases. Over the years, we have seen the same trend as during the study period, namely, that 15–30% of the samples sent to SMI for species differentiation are negative by PCR (Table 6), and that around half of the PCR-negative samples contain amoeba species other than *E. histolytica*/*E. dispar*. Microscopy of *Entamoeba* cysts is a real challenge, and studies in other countries such as Thailand, Egypt, Ethiopia, and Nicaragua have reported experiences similar to ours (Kebede et al., 2003; Parija and Khairnar, 2005; Hamzah et al., 2006; Leiva et al., 2006). This difficulty to differentiate intestinal protozoa was also noticed in a European multicenter investigation in which only fair or moderate agreement between reference laboratories was found for most *Entamoeba* species (Utzinger et al., 2010).

Table 7. Microscopy of 32 samples that were PCR negative for *E. histolytica* and *E. dispar*

Parasite species detected by microscopy	No of samples
E. hartmanni	6
E. hartmanni + *E. coli*	2
E. hartmanni + *I. butschlii*	1
E. coli	8
E. coli + *I. butschlii*	1
I. butschlii	1
E. hartmanni + *I. butschlii* + *G. intestinalis*	1
E. polecki	2
G. intestinalis	1
E. histolytica/E. dispar	0
No cysts found	9
Total	32

In our laboratory, we have had access to a monoclonal antibody (kindly provided by Hugo Lujan, Catholic University of Córdoba, Argentina) that can distinguish between the *E. histolytica/E. dispar* complex and cysts of other amoeba species commonly found in humans, but unfortunately not between *E. histolytica* and *E. dispar* cysts (Fig. 6).
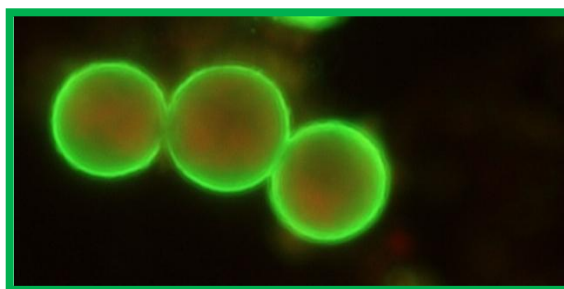

Figure 6. *E. histolytica/E. dispar* cysts stained with a monoclonal antibody

Nevertheless, this antibody has been a very valuable complement to ordinary microscopy in cases involving cysts that were difficult to identify, and it has been especially useful for separating cysts of *E. polecki* type from immature uninucleate *E. histolytica/E. dispar* cysts. Cysts of *E. polecki* occurred during the study period and have appeared intermittently since then. Due to this infrequent incidence and the similarity to immature *E. histolytica/E. dispar* cysts, it is difficult for local laboratories to identify these cysts.

The final diagnosis of *E. polecki* is made by PCR sequencing, either after amplification by universal *Entamoeba* primers (Table 5) or by use of primers specific for uninucleate *Entamoeba* species (Stensvold et al. manuscript in preparation). Interestingly, almost all samples containing uninucleate cysts that have been analyzed in our laboratory since 2001 have had a genetic variant of *E. polecki* hitherto identified only in humans. Of the other three variants of uninucleated cysts that are occasionally discovered in humans, *E. polecki* (pigs), *E. chattoni* (monkeys), and *E. struthionis* (ostriches), only *E struthionis* has been observed in two of our patients. Based on genetic data, these four variants are now considered one species, *E. polecki* that comprises four different subtypes (Stensvold et al. manuscript in preparation).

From the beginning of our study in 2001 up to 2009, 55 patients were diagnosed with *E. histolytica* DNA in their stool samples (Tables 6 and 8), and 22 patients were found to have *E. histolytica* DNA in abscess material. As shown in Table 8, most of the infections involving both intestinal amoebiasis and ALA originated in Asia. Furthermore, two patients with intestinal amoebiasis had almost certainly been infected in Sweden; one of these individuals was probably infected by her partner who was diagnosed with *E. histolytica/E. dispar* cysts after a visit to Thailand (PCR was not performed), whereas no likely source of infection was determined for the other patient. The occurrence of domestic *E. histolytica* infections in other countries in Western Europe is not known, although it appears to be low, since only few reports, (two from Italy and two from the Netherlands) were found in a PubMed search (Gatti et al., 1995; Vreden et al., 2000; Gatti et al., 2002; Edeling et al., 2004).

Table 8. Origin of cases of *E. histolytica* infection detected by PCR analysis of stool samples or liver aspirates 2001–2009

	Sweden	Europe	Asia	Africa	South America	Origin not known	Total
Stool samples	2	3	23	6	8[a]	13	55
Liver aspirates	0	0	14	4	3[a]	1	22

[a]One patient from Brazil was positive for *E. histolytica* in both stool and liver abscess material.

Future work on *Entamoeba* in Sweden will include application of real-time PCR at our laboratory and also implementation of differentiation of *E. histolytica/E. dispar* at different local microbiological laboratories. This work will include a new approach to collection of fecal specimens in which use of the combination of SAF-fixed and unfixed samples should enable us to solve most of the difficulties related to fecal parasitology.

6 *GIARDIA* IN NICARAGUA AND SWEDEN (PAPER II, III AND IV)

6.1 STUDY POPULATIONS

6.1.1 *Giardia* in Nicaragua (Paper II)

In Nicaragua, as in the rest of Latin America, *Giardia* infection is common and contributes to the diarrheal disease burden of children (Tellez et al., 1997). At the time we performed this study (2002–2003) few attempts had yet been made to genotype *Giardia* isolates in Latin America. Our work was done in cooperation with Universidad Nacional Autónoma de Nicaragua (UNAN) in León, Nicaragua, and it was based on human stool samples (n = 136) that had already been collected for other purposes and had been found to contain *Giardia* cysts/trophozoites by light microscopy. Inasmuch as many of the inhabitants in the study area were dog owners, and the dogs were allowed to roam freely in the neighborhoods, we also performed microscopy to screen 100 canine samples for *Giardia* cysts, and those that were found to contain cysts were included in our molecular analyses.

6.1.2 Swedish animals (Paper III)

In Sweden, *Giardia* infection in animals has received little attention, and no *Giardia* isolates from animals had been genotyped prior to our study. Our goal was to identify *Giardia* assemblages in various animal species, including livestock, pets, and wild life, and to determine the host specificity and zoonotic potential of the assemblages that were found. To obtain animal samples, we cooperated with two of the largest laboratories performing veterinary diagnostic parasitology in Sweden: the National Veterinary Institute in Uppsala and VIDILAB in Enköping. These two facilities do not routinely investigate all samples for *Giardia*, and hence it took considerable time to collect isolates from various sources and representing all of the different assemblages (2002–2008). Another impediment was that parasitological examination of animal samples is frequently performed on fecal samples that are pooled (by either the animal keeper or the staff at the diagnostic laboratory). This was a new experience for us, because in human parasitology each specimen represents a single patient. Thus, many of the animal samples had to be rejected from the study, and only pooled samples (two from sheep, four from cats, and 11 from dogs) that contained unique or otherwise interesting genotypes were included.

6.1.3 Swedish patients (Paper IV)

Prior to the study reported in Paper IV, the only human *Giardia* isolates that had been genotyped in Sweden were from an outbreak at a nursery school, and analysis at the ß-giardin locus showed that they were exclusively of subtype A3 (Svenungsson et al., 2007). Our present investigation was conducted between May 2007 and April 2009 in cooperation with a nearby hospital laboratory that perform diagnostic parasitology (the Department of Clinical Microbiology, Karolinska University Hospital, Stockholm) and the Department of Communicable Disease Control and Prevention in Stockholm (Smittskydd Stockholm). Fecal samples that had not been preserved in formalin or SAF fixative and had been diagnosed with *Giardia* parasites were forwarded to SMI for further analyses. A questionnaire concerning symptoms, recent traveling, and possible routes of transmission was sent to all patients by mail. In some cases, the patients were also interviewed by telephone.

6.2 METHODS (PAPER II, III AND IV)

6.2.1 Preservation of fecal samples and DNA extraction

Many studies of intestinal protozoa are carried out in developing countries that have limited laboratory resources, and thus there is often a time delay before DNA extraction can be performed. The results of our investigation of *Entamoeba* spp. had shown that ethanol fixation was useful for preserving *Entamoeba* DNA from human samples, and hence we applied this approach to *Giardia* DNA as well. Some of the human samples collected in Nicaragua (n = 33) were subjected to DNA extraction in Leon, and the remaining human samples (n = 103) were kept in ethanol at 4 °C for 3–10 months until transportation to Sweden was available. This approach was quite successful, as indicated by amplification at one or two loci in 86% (119/136) of the samples. On the other hand, initial attempts to preserve feces from Nicaraguan dogs directly in ethanol before DNA extraction were disappointing, since amplification failed in all samples, despite positive microscopy for *Giardia*. In a subsequent collection of canine fecal samples, the cysts were isolated on a sucrose gradient (on the spot in Leon), fixed in ethanol and thereafter transported to Sweden. This time the results were encouraging, because all microscopy-positive canine samples (n = 8) were successfully genotyped. In later analyses conducted on specimens collected in Sweden, cyst isolation on a sucrose gradient was used for all *Giardia* samples from animals, whereas *Giardia* DNA was extracted directly from human stool samples (usually ethanol fixed). The cysts were disrupted by the use of a Bead-Beater (Biospec Products Inc., Bartlesville, OK, USA) before performing extraction using a QIAamp^{TM} DNA mini kit. In our laboratory, this combination of isolation on a sucrose gradient (mainly for animal samples), mechanical disruption of cysts, and spin column purification has led to successful DNA extraction for *Giardia* PCR, as well as for PCR analysis of other intestinal protozoa (*Entamoeba* spp., *Cryptosporidium* spp., and Microsporidia spp.).

6.2.2 Molecular methods

Primers used for *Giardia* genotyping in the present research are listed in Table 9. AmpiTaqGold (Applied Biosystems) was employed in all PCR reactions. RFLP was performed on amplicons generated from β-giardin, *tpi*, and *gdh* PCR according to published methods (Caccio et al., 2002; Read et al., 2004; Lalle et al., 2005b; Lalle et al., 2009). PCR products came mainly from nested or semi-nested amplification and in some cases also from single PCR, and they were purified using ExoSAP-IT (Fermentas) and sequenced in both directions at SMI using appropriate primers and the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems), or they were sent to AGOWA (Germany) for sequencing. Chromatograms and sequences were examined manually and aligned using the BioEdit sequence analysis program (*http://www.mbio.ncsu.edu/BioEdit/page2.html*). Nucleotide sequences were compared with sequences in the GenBank database by use of the BLAST tool (*http://www.ncbi.nlm.nih.gov/blast/*) and also with locally available sequences by applying the BioEdit sequence analysis program.

6.2.3 Phylogenetic analyses

Phylogenetic trees were constructed to determine the genetic relationships between different *Giardia* isolates. New sequences were combined with reference sequences of representative isolates from different assemblages and sub-assemblages. The trees were generated by maximum likelihood analysis using the generalized time-reversible (GTR) substitution model and among-site rate variation. More detailed information is given in the methods sections of Papers II, III, and IV.

Table 9. Primer sequences used in the studies reported in Papers II, III, and IV

Primer ID	Primer sequence (5´-3´)	Locus	Annealing temp °C	Amplicon size bp	Paper	Reference
Gia2029	AAGTGTGGTGCAGACGGACTC	ssrRNA	55	497	II, III	(Appelbee et al., 2003)
Gia2150c	CTGCTGCCGTCCTTGGATGT					
RH11	CATCCGGTCGATCCTGCC		59	292		(Hopkins et al., 1997)
RH4	AGTCGAACCCTGATTCTCCGCCAGG					
G7	AAGCCCGACGACCTCACCCGCAGTGC	β-giardin	65	753	II, (III, IV)	(Caccio et al., 2002)
759G	GAGGCCGCGCCCTGGATCTTCGAGACGAC					
376	CATAACGACGCCATCGCGGCTCTCAGGAA		55	384	II	(Lalle et al., 2005b)
βFd	GAACGAGATCGAGGTCCG		55	511	II, III, IV	
βRw	CTCGACGAGCTTCGTGTT					
GDHeF	TCAACGGTYAAYCGYGGYTTCCGT	gdh	56	458	II, III, IV	(Read et al., 2004)
GDHiR	GTTRTCCTTGCACATCTCC					
GDHiF	CAGTACAACTCYGCTCTCGG			432		
Ghd1	TTCCGTRTYCAGTACAACTC	gdh	50	755	III, IV	(Caccio et al., 2008)
Gdh2	ACCTCGTTCTGRGTGGCGCA					
Gdh3	ATGACYGAGCTYCAGAGGCACGT		50	530		
Gdh4	GTGGGCGCARGGCATGATGCA					
AL3543	AAATIATGCCTGCTCGTCG	tpi	50	605	III, IV	(Sulaiman et al., 2003)
AL3546	CAAACCTTITCCGCAAACC					
AL3544	CCCTTCATCGGIGGTAACTT		50	530		
AL3545	GTGGCCACCACICCCGTGCC					
AssAf	CGCCGTACACCTGTCA	tpi ass A	64	373	IV	(Geurden et al., 2008)
AssAr	AGCAATGACAACCTCCTTCCGTTGTTGTTG					
AssBF	CTCCCTCCTTT	tpi ass B	62	400	IV	(Levecke et al., 2009)
AssBR	CCGGCTCATAGGCAATTACA					
TPIDF	CCGTTCATAGGTGGCAACTT	tpi ass D	50	530	IV	(Lebbad et al., 2010)
TPIDR	GTAGCCACTACCAGTTCC					

6.3 RESULT AND DISCUSSION

6.3.1 Nicaragua study (Paper II)

In this investigation, we examined the distribution of *Giardia* assemblages in humans and dogs in Leon, Nicaragua. Initially, all 136 human samples were subjected to single PCR and RFLP analysis at the ß-giardin locus (Caccio et al 2002). Since that approach gave information only on assemblage type, we also performed PCR with subsequent sequencing of the ß-giardin and *gdh* genes in a limited number of human samples (n = 42). The concordance between the two loci in those samples was good, and the only discrepancy found was that two isolates, determined as single assemblage A infections at the ß-giardin locus, were identified as mixed assemblage A and B infections at the *gdh* locus. We also noted that assemblage B (79%) was more common than assemblage A (21%). This finding is in contrast with studies performed in Mexico and Brazil that detected only assemblage A (Lalle et al., 2005a; Volotao et al., 2007) but it agrees with more recent investigations conducted in South America, which revealed a predominance of assemblage B (Kohli et al., 2008; Minvielle et al., 2008). We also demonstrated that assemblage B sequences were highly polymorphic compared to sequences from assemblage A, the latter showing only two subtypes (A2 and A3) at the β-giardin locus. The eight canine samples were investigated at the *ssrRNA*, β-giardin, and *gdh* loci, and all of them were found to contain the host-specific assemblages C or D, or a mixture of both.

6.3.2 *Giardia* in animals in Sweden (Paper III)

At the onset of this investigation, essentially nothing was known about the distribution of different *Giardia* assemblages in animals in Sweden. The work was conducted over a long period, and DNA extraction and PCR-RFLP and sequencing of the β-giardin gene were performed continuously on all 114 isolates. Later on, molecular analyses of two other markers, the *gdh* and *tpi* genes, were performed on all samples. Initially, the *ssrRNA* locus was also investigated, but the information was limited to assemblage level, and hence this gene was later used only when no PCR products were obtained with the other markers or to verify unusual results. The *ssrRNA* PCR was useful for identifying *Giardia* species other than *G. intestinalis* (i.e., *G. muris* and *G. microti*), for which the success rate was very low when using the other PCR primers. One reason for that may have been that the samples from rodents contained very few *Giardia* parasites, and thus a PCR directed towards a multi-copy gene, such as the *ssrRNA* locus, was more sensitive. Another plausible explanation is that the β-giardin, *gdh*, and *tpi* primers are designed for *G. intestinalis* and therefore are not optimal for other *Giardia* species. We also found that assemblage D from canine samples was not amplified in the nested PCR using the *tpi* primers described by Sulaiman et al. (2003). Sequencing of the product from the primary *tpi* PCR demonstrated three substitutions at the forward primer site and four at the reverse primer site, and, based on this observation, modified primers for assemblage D were designed (Table 10). This strategy shows that universal primers for *G. intestinalis* designed using information on sequences from only a few isolates might not achieve equal amplification of all assemblages, and it also highlights the importance of having a panel of DNA from all various assemblages before a new PCR is established. However, according to our experience, this goal is not easily achieved, since it took us a considerable amount of time to assemble such a collection.

Assemblage A was detected in a total of 15 animals (nine ruminants, five cats, and one dog) and assemblage B in five animals (three monkeys, one rabbit, and one guinea pig). Three sheep were infected with both assemblages A and E, and the remaining animals harbored the host-specific assemblages C to G or other *Giardia* species. The zoonotic

aspects and other implications of the findings are discussed under the respective headings below.

Table 10. Primers for nested *tpi* PCR (Sulaiman et al., 2003) compared with sequences of assemblages A, B, and D

Assemblage		Acc. no. Genbank	Position from the start of the gene								
			18	24	27	30	528	531	534	537	543
AI	-	L02120	C	C	C	T	C	G	C	G	C
B	-	L02116	C	C	T	T	C	G	T	G	C
D	Swedog105	EU781028	G	A	T	C	A	T	T	A	T
D	Swedog112	-	G	A	T	C	A	T	T	A	T
D	Swedog119	-	G	A	T	C	A	T	T	A	T
Primer F AL3544			C	C	I[a]	T	-	-	-	-	-
Primer R AL3545			-	-	-	-	C	G	I[a]	G	C

[a]I = inosine used at ambiguous positions

6.3.3 *Giardia* in patients in Sweden (Paper IV)

As a complement to the study of *Giardia* assemblages in animals in Sweden, we initiated an investigation of patients diagnosed with giardiasis in this country. Stool samples from a total of 214 persons were forwarded to SMI. The specimen was sent in together with the sediment obtained after formol/ethyl acetate concentration, and thus microscopy using the DFA and DAPI techniques could be performed on all samples. For all isolates, we attempted PCR amplification of the β-giardin, *tpi*, and *gdh* genes, as well as *tpi* PCR with primers specific for assemblages A and B, respectively. RFLP analysis was done on all products of the three PCRs. Lastly, sequencing was performed on all samples positive in β-giardin PCR and on almost all samples that were successfully amplified at the *tpi* and *gdh* loci. The success rate was good at all three loci, as depicted in Figure 7. In all, 73 patients were infected with assemblage A and 128 with assemblage B, and six had mixed assemblage A+B infections. However, in seven isolates, no amplicons were obtained at any locus; six of these samples contained a few *Giardia* cysts that were empty (i.e., lacked nuclei) according to DAPI staining, and the remaining sample had probably been exposed to formalin.
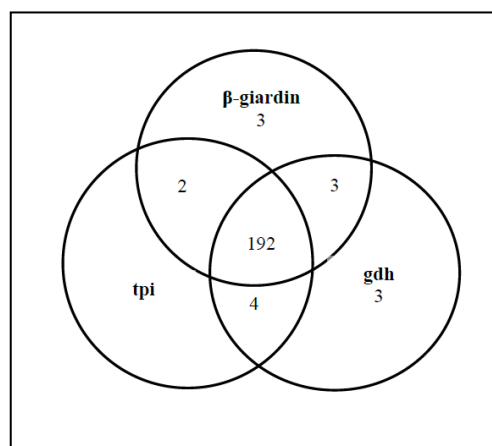
Figure 7. Distribution of 207 *Giardia* isolates successfully amplified at the ß-giardin, tpi, and gdh genes.

6.3.4 Association of assemblages with symptoms (Paper IV)

The patient group consisted of 109 females and 105 males. The distribution of assemblages in relation to gender was remarkable: among the females, 36 had assemblage A and 64 assemblage B; among the males, 37 had assemblage A and 64 assemblage B. For 181 of the 214 patients (85%) that responded to the questionnaire that had been sent to all participants detailed information regarding various symptoms was obtained. From the remaining 33 patients partial data concerning symptoms were extracted from the mandatory notifications submitted by the clinicians. The merged information from these two sources showed that a total of 180 persons had symptoms, and 34 were asymptomatic. No association was seen between assemblages and symptoms when the whole patient group was examined (p-value 0.58). A more detailed analysis was also performed on 145 symptomatic patients (including 51 with assemblage A and 87 with assemblage B) that responded to the questionnaire and that were not co-infected with other diarrhea-related organisms. The questionnaire included items about diarrhea, bowel movements per day, abdominal pain, bloody stools, vomiting, flatulence, fever, and weight loss. For all patients, the most common symptom was diarrhea (97%), followed by flatulence (78%), and weight loss (73%). The only assemblage-symptom correlation found concerned flatulence, which was more common in patients with assemblage B (p-value 0.006). Separate analysis of 12 symptomatic children aged 0–5 years revealed that all six of the children who had assemblage B, but none of the other six with assemblage B, reported flatulence. When children 0–5 years of age were excluded from the analysis, the correlation between assemblage B and flatulence was no longer apparent (p-value 0.16). These findings demonstrate that there is a strong correlation between flatulence and infection with assemblage B parasites in the age group 0–5 years. At present, we have no explanation for why flatulence was not evenly distributed among the children, although it is possible that assemblages A and B parasites differ with regard to metabolism. It has been shown that assemblage A parasites produce hydrogen gas (Lloyd et al., 2002), and, although not yet studied, the level of such gas production might be higher in assemblage B. It is also plausible that members of these two assemblages differ with respect to how they affect the growth of gas-producing bacteria in the host's intestine.

According to the questionnaire responses, 10 patients received intravenous fluids while they were hospitalized for diarrhea. Seven of these patients were infected with assemblage B, one with assemblages A+B, and two with assemblage A. Co-infections with *Shigella* and *Campylobacter* were observed in one of the assemblage B patients and in the patient with mixed assemblage infection. Notably, the two patients with assemblage A that received intravenous fluids were both infected in Sweden with a unique assemblage A MLG that had previously been found only in our study of *Giardia* genotypes in Swedish animals (see under 6.3.7 Zoonotic transmission).

Another observation was that, of 10 patients who reported joint pain, eight were infected with assemblage A and two with assemblage B. None of the patients harbored bacteria associated with reactive joint pain, although one patient with assemblage B was co-infected with *Cryptosporidium*. All patients were adults, but the study design did not permit us to further explore these findings with regard to location or intensity of pain. Reactive joint pain is a well-known symptom following infections with enteric bacteria, and it has infrequently been described in patients with giardiasis, mainly children (Goobar, 1977; Shaw and Stevens, 1987). An investigation of the health sequelae of human cryptosporidiosis revealed that joint pain was more common in

Cryptosporidium patients than in healthy control subjects, and it was correlated with previous *C. hominis* infection (Hunter et al., 2004).

6.3.5 Mixed assemblages (Papers II, III, and IV)

The issue of mixed-assemblage infections has recently received considerable attention, and, to further address this question, we subjected the *Giardia* isolates from Nicaragua that we had already sequenced at the β-giardin and *gdh* loci to additional investigation at the *tpi* locus. This was done using both universal primers and assemblage A- and B-specific primers (Sulaiman et al., 2003; Geurden et al., 2008; Levecke et al., 2009). Table 11 shows that this approach confirmed the two mixed infections that we had previously detected at the *gdh* locus and identified one more patient with assemblage A+B infection, resulting in 7% (3/42) mixed assemblage A+B infections in the Nicaraguan subjects. These observations nearly agree with the findings reported in Paper IV, which were obtained using the same approach and showed that only 3% (6/207) of the Swedish patients were infected with both assemblages. In a study in Great Britain (Breathnach et al., 2010), another *tpi* PCR was performed using the assemblage-specific primers described by Amar (2002), and, similar to our results, mixed infections were found in 3% (6/199) of the patients that were investigated. However, these findings differ from those obtained in a Belgian study that used exactly the same approach as ours and demonstrated that 32% of the patients had double infections (Geurden et al., 2009a). The reason for this discrepancy is not clear, considering that the patient populations in the three studies were quite similar and represented a mixture of domestic and travel-related infections. The assemblage A- and B-specific *tpi* primers were tested in our laboratory using diluted DNA from cultured parasites and a very high sensitivity was found when DNA from assemblage A was diluted in a high concentration of assemblage B DNA and vice versa (data not shown). Thus it seems that the low rate of mixed-assemblage infections in our studies was not related to methodology.

Table 11. Comparison of results of β-giardin, *gdh*, and *tpi* PCR performed on 42 *Giardia* isolates from Nicaragua

No. of isolates	Nested β-giardin	Nested *gdh*	Nested *tpi*	*tpi* A+ B PCR
13	A	A	A	A
1	A	A+B	A	A+B
1	A	A+B	B	A+B
1	A	A	A	A+B
26	B	B	B	B

Lately, considerable attention has also been focused on assemblage swapping (i.e., different assemblages indicated by analysis of different loci in the same isolate) (Caccio et al., 2008; Almeida et al., 2010). We did not observe such exchange in any of the isolates (from humans or animals) that exhibited single-assemblage infections at all three loci. Altogether, we have successfully sequenced all three gene loci in 234 human isolates and 89 animal isolates, and, apart from the mixed-assemblage infection in nine human and eight animal isolates, there was no apparent disagreement between assemblages.

6.3.6 Intra-isolate sequence divergence (Papers II, III, and IV)

Sequencing of all three genes revealed a high degree of polymorphism with frequent double peaks (overlapping nucleotides) at specific positions in the chromatograms. This occurred most often in human assemblage B isolates from both Nicaragua and Sweden, although it was also observed in assemblage C, D, and E sequences from animals. Double peaks were lacking for almost all sequences from assemblage A, except for a few β-giardin sequences at the specific positions distinguishing subtype A2 from subtype A3 (Paper IV). A high degree of polymorphism in assemblage B isolates has also been noticed in other studies (Robertson et al., 2007; Caccio and Ryan, 2008; Lalle et al., 2009) and has been further investigated by cloning (Lasek-Nesselquist et al., 2008; Hussein et al., 2009; Kosuwin et al., 2010). The complete genome sequencing of the assemblage B isolate GS/M (Franzen et al., 2009) showed that the average allelic sequence divergence was much higher in this isolate (0.5%) than in the previously sequenced assemblage A isolate WB-C6 ($< 0.01\%$) (Morrison et al., 2007). This suggests that the greater abundance of mixed positions in assemblage B sequences is due to the higher allelic sequence divergence. In addition, the possibility of infections with multiple assemblage B strains complicates the picture even more. We observed a strong correlation between isolates that generated double chromatogram peaks for all three genes and represented infections acquired outside Europe (p-value < 0.001). This implies that at least some of the double peaks produced by our assemblage B sequences were due to mixed infections, which are presumed to be more common in areas with a high *Giardia* prevalence (Caccio and Ryan, 2008).

Single-cell PCR (unpublished data)

To further investigate the occurrence of intra-isolate sequence divergence, we performed single-cell PCR analyses on trophozoites from the assemblage B isolate GS/M (*in vitro* cultured) and on single cysts from three separate clinical isolates, all with double peaks in the original sequences. Two of the isolates (Sweh197 and Sweh212) were from patients infected with assemblage B and the third (Sweh207) from a patient with mixed assemblage A+B infection. Cysts from the human samples were separated using a sucrose gradient. Single trophozoites and cysts were isolated through micromanipulation (Fig. 8).
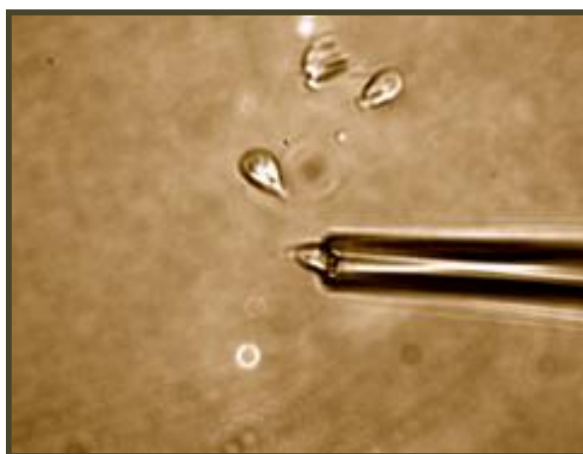
Figure 8. Isolation of a single *Giardia* trophozoite from culture by the micromanipulation technique (Photo Johan Ankarklev)

In short, to ensure that only single cysts were isolated, selected cysts that had been stained *in vitro* with FITC-conjugated *Giardia* antibodies were isolated by a micromanipulation technique and then transferred to multi-well microscope slides and detected visually in a fluorescence microscope. Each well (n = 44) contained only a single cyst. DNA from single cysts and trophozoites was liberated using DNAreleasy (NIPPON Genetics) and amplified by nested PCR, and this was followed by sequencing of the amplicons. The β-giardin and *tpi* loci were investigated. Sequences from nine single GS/M trophozoites showed double peaks in the chromatogram at the same positions as the *tpi* locus in the crude isolate (Table 12). Single cysts from assemblage B isolate Sweh197 generated both *tpi* sequences with double peaks at certain positions and sequences without any double peaks (Table 13). Similar results (i.e., sequences with and without double peaks) were obtained for single cysts isolated from Sweh212 both at the β-giardin and *tpi* loci (Fig. 9). Single cysts from isolate Sweh207 (original isolate with mixed A+B infection) generated 10 assemblage B and two assemblage A sequences (identical to subtype A2) at the *tpi* locus, whereas only assemblage B sequences (n = 15) were identified at the β giardin locus. The assemblage B sequences from both loci showed a high degree of polymorphism. These results indicate that heterogeneous alleles that give rise to polymorphic sequences are present within single parasites. The results also showed that single cysts from a patient sample with mixed assemblages generated either assemblage B or A sequences. This indicates that the sample comprised two distinct isolates and that there was no recombination between assemblage A and B at the loci tested (Ankarklev et al., manuscript in preparation).

Table 12. Nucleotide changes in isolate GS/M at the *tpi* locus. IUPAC nucleotide codes were used indicate multiple nucleotides at polymorphic positions (**R**=**A/G**; **Y**=**C/T**).

Isolate		Sub-assemblage	Position from start of gene		
			39	45	26
		BIII	**G**	**T**	**G**
		BIV	**A**	**T**	**G**
GS/M	Crude isolate	BIV	**R**	**Y**	**R**
GS/M_071	Single trophozoite		**R**	**Y**	**R**
GS/M_072	Single trophozoite		**R**	**Y**	**R**
GS/M_073	Single trophozoite		**R**	**Y**	**R**
GS/M_074	Single trophozoite		**R**	**Y**	**R**
GS/M_076	Single trophozoite		**R**	**Y**	**R**
GS/M_077	Single trophozoite		**R**	**Y**	**R**
GS/M_078	Single trophozoite		**R**	**Y**	**R**
GS/M_079	Single trophozoite		**R**	**Y**	**R**
GS/M_080	Single trophozoite		**R**	**Y**	**R**

Table 13. Nucleotide changes in isolate Sweh197 at the *tpi* locus. IUPAC nucleotide codes were used indicate multiple nucleotides at polymorphic positions (**R**=**A**/**G**; **Y**=**C**/**T**).

Isolate	Material	Sub-assemblage/subtype	Position from start of gene			
			39	114	165	280
		BIII	G	C	C	A
		BIV	A	C	T	A
Sweh197	Crude feces	Mixed	R	Y	Y	R
Sweh197_SC200	Single cyst	Mixed	R	Y	Y	R
Sweh197_SC084	Single cyst	Mixed	R	C	Y	R
Sweh197_SC201	Single cyst	Mixed	R	C	Y	R
Sweh197_SC086	Single cyst	Mixed	R	Y	C	R
Sweh197_SC098	Single cyst	Mixed	R	Y	C	R
Sweh197_SC149	Single cyst	Mixed	R	Y	C	R
Sweh197_SC213	Single cyst	Mixed	R	Y	C	R
Sweh197_SC196	Single cyst	Novel subtype 1	A	C	C	G
Sweh197_SC197	Single cyst	Novel subtype 1	A	C	C	G
Sweh197_SC212	Single cyst	Novel subtype 1	A	C	C	G
Sweh197_SC207	Single cyst	Novel subtype 2	G	T	C	A
Sweh197_SC215	Single cyst	Novel subtype 3	G	C	T	A
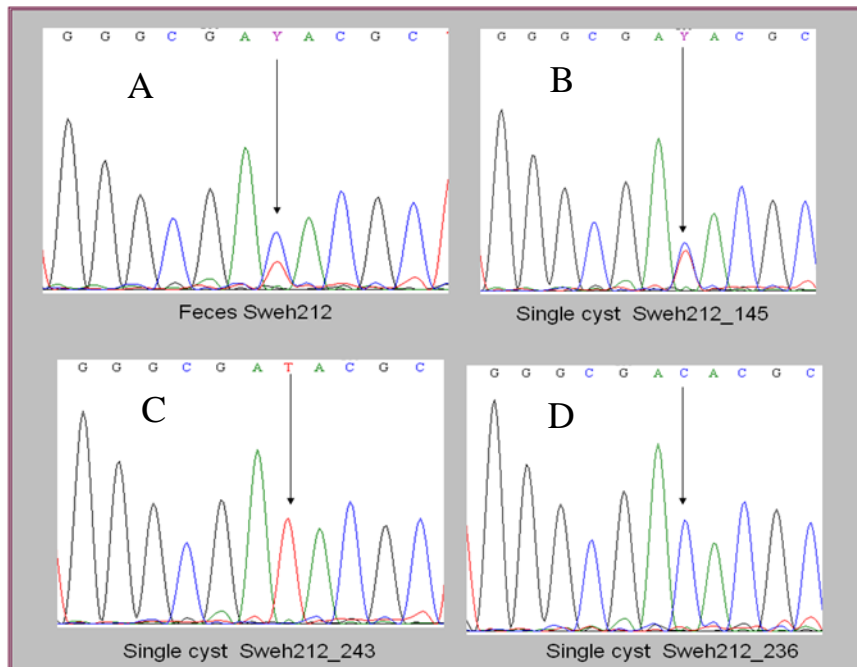
Figure 9. Four chromatograms from isolate Sweh212 representing the same 11 nucleotide region at the β-giardin locus. Sequences from crude isolate (A) and single cysts (B, C, and D).

6.3.7 Zoonotic transmission (Papers III and IV)

Most of the animals we studied in Sweden were infected with the host-specific assemblages C–G, although assemblages A and B were also found. Figure 10 presents possible transmissions routes for *Giardia* in our country, based on our investigations of both animal and human isolates. Among 28 canine samples that were successfully genotyped, only one contained assemblage A, and the remaining harbored the host-specific assemblages C and D. Accordingly, it seems that dogs do not play an important role in zoonotic *Giardia* transmission in Sweden. The opposite has been reported from many other countries, where dogs have been pointed out as potential sources of human infection because they frequently harbor assemblage A (Claerebout et al., 2009; Sprong et al., 2009). The canine samples from Nicaragua that we analyzed also displayed assemblages C and D, but, since only eight of the 100 dogs we investigated were positive for *Giardia*, it is impossible to draw any conclusions. In our samples from Sweden, we found assemblage A mainly in sheep and wildlife (moose and deer), but also in some cats. Phylogenic trees and MLG analysis showed that none of these animals harbored sub-assemblage AII, which was the most common sub-assemblage identified in the human assemblage A isolates (Fig. 11). Unexpectedly, in October 2008, isolates from three patients were observed to exhibit the same novel assemblage A RFLP pattern (unpublished data) at the β-giardin gene that had previously been demonstrated for most animal isolates with assemblage A. Later, we found that these three patients had MLGs identical to those identified in some ruminants and cats. Two of these patients had not been in contact with any particular animal, but the third was a moose and deer hunter who had taken part in a hunting expedition shortly before he became symptomatic. All three patients reported that they had not traveled abroad prior to infection, and, as already mentioned, two of them were hospitalized and received intravenous fluids.

Few of the animals we studied in Sweden were infected with assemblage B, and thus comparison of human and animal isolates was possible in only a few cases. One rabbit (Rabbit176) had diarrhea and its owner had previously been treated for giardiasis, and, considering all three markers this animal had subtypes that had also been detected earlier analyses of human *Giardia* isolates, although the sequence combination (MLG) observed was not exactly the same as that seen in any of our patients infected with assemblage B (Fig. 10). Unfortunately, no parasites from the rabbit owner were available for analysis. Rabbits and guinea pigs are often kept as pets, but remarkably few *Giardia* studies have included these animals (Giangaspero et al., 2007).

The above-mentioned examples certainly pinpoint the difficulties involved in connecting human and animal *Giardia* cases when zoonotic transmission is suspected. Nevertheless, our findings strongly suggest that zoonotic transmission of *Giardia* can occur in Sweden, although such dissemination is not very common.
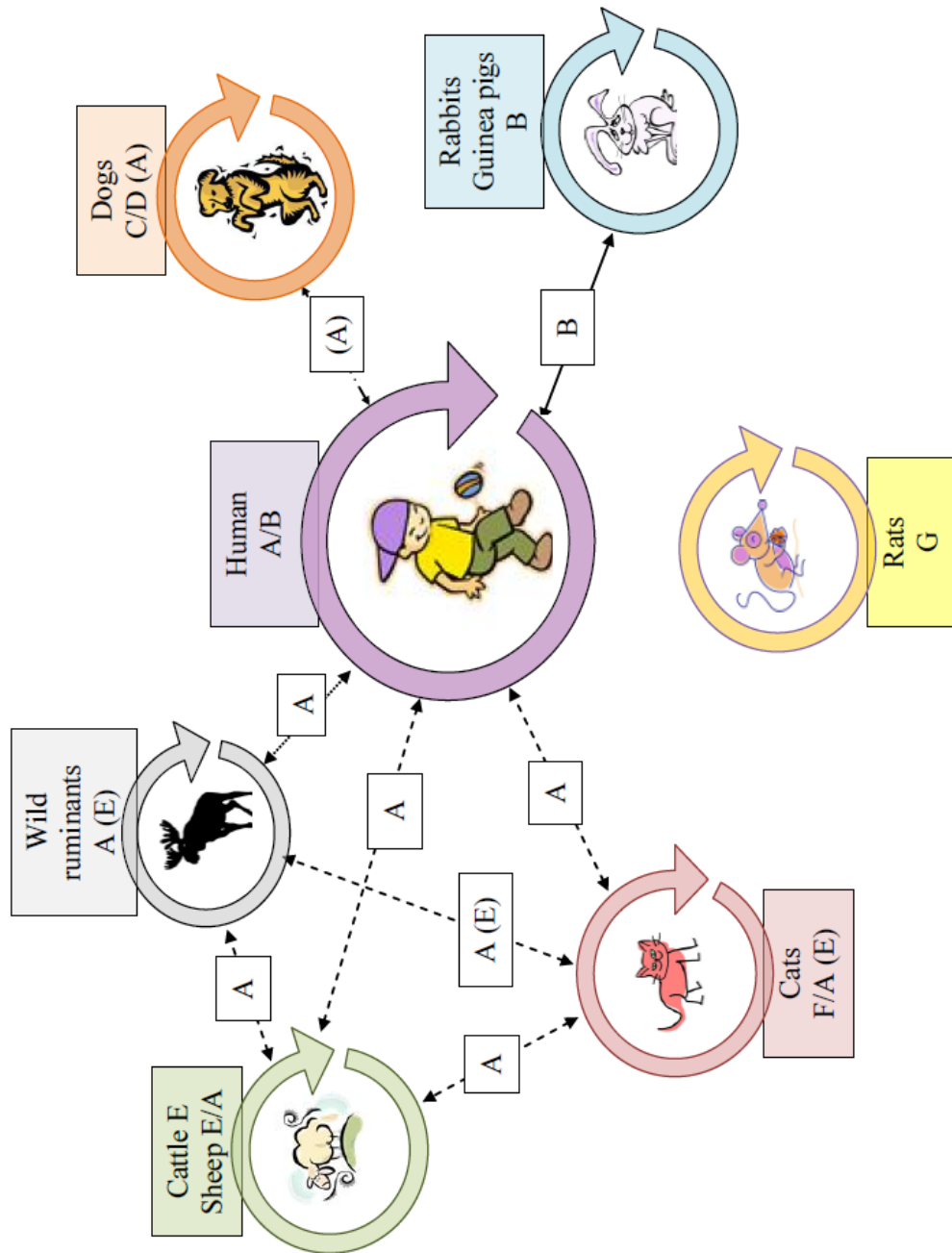
Figure 10. Possible routes of *Giardia* transmission in Sweden.
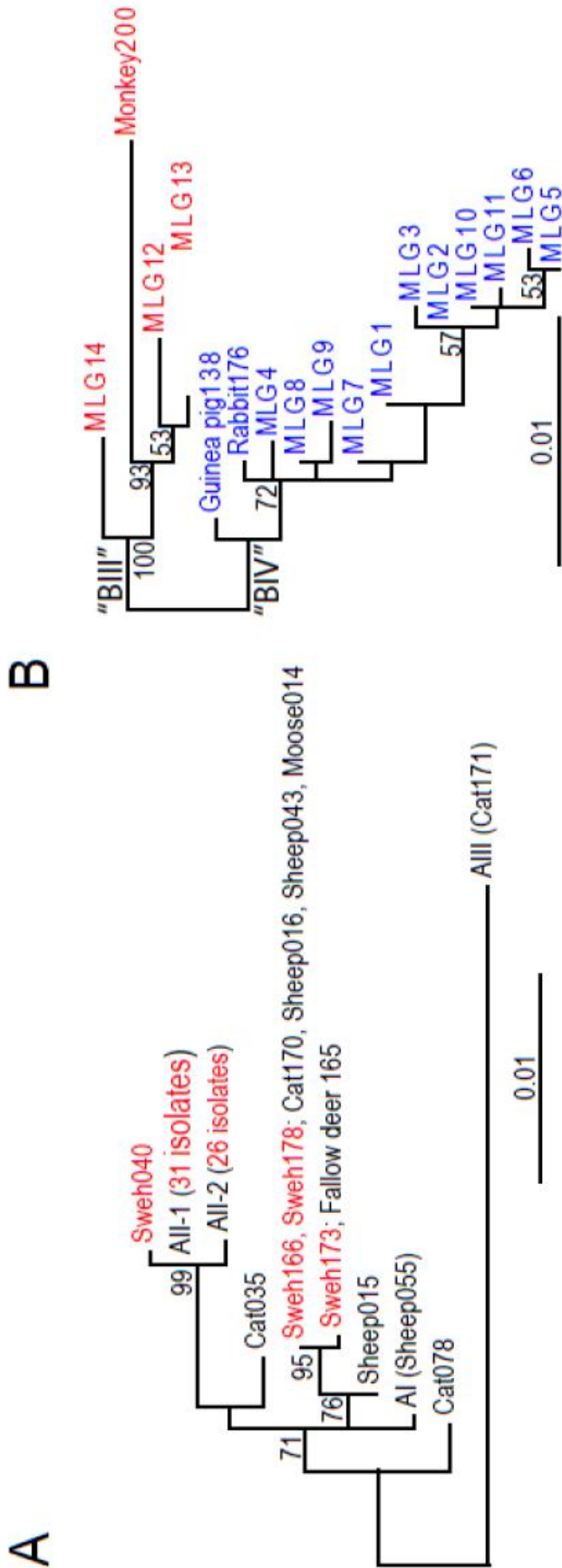
Figure 11

Nucleotide maximum likelihood trees based on concatenated datasets for ß-giardin, gdh, and tpi gene sequences. MLGs with unambiguous sequences identified in Paper IV (Table 3 and 4) are combined with reference isolates and isolates from our MLG study of animals in Sweden (Paper III). (A) Phylogenetic tree based on 1884 aligned positions of assemblage A isolates. Human isolates identified in Paper IV are indicated in red. (B) Phylogenetic tree based on 1358 aligned positions of 17 assemblage B MLGs identified in Paper III and Paper IV. BIII (red) and BIV (blue) isolates are assigned according to their clustering with reference isolates in phylogenetic trees of the individual genes (Fig. S1 Paper IV). Only bootstrap support values >50% are shown.

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6.3.8 Source tracing (Paper IV)

No larger outbreaks of giardiasis occurred in the Stockholm area during the study period, but 18 small family clusters were identified, each of which involved two to five individuals. In two of these clusters, the family members harbored either assemblage A or B, and thus it was quite obvious that they were infected with parasites from different sources. In six other clusters infected exclusively with assemblage A and whose MLGs were successfully determined, the value of genotyping was limited, because these A MLGs were common in our other patients as well. In contrast, multilocus genotyping of assemblage B isolates appeared to be a reliable tool for source tracing in some instances. This was indicated by the observation that in five family clusters the members shared identical MLGs. On the other hand, in several of the clusters in which assemblage B isolates were identified, the frequent occurrence of overlapping nucleotides in the sequences hampered the allocation of the isolates to a particular MLG. Clearly, investigation of sequences obtained from crude feces isolates cannot reveal whether this polymorphism was due to infections with mixed subtypes or allelic sequence divergence, or a combination of both. A similar observation was made during a large waterborne outbreak of giardiasis in Bergen, Norway, where genotyping of assemblage B isolates from the affected patients showed substantial variability (Robertson et al., 2006). So the paradox is that either there is insufficient genetic variation in the loci investigated (assemblage A) or the polymorphism is too extensive (assemblage B) to allow reliable source tracing.

6.3.9 Treatment and treatment failure (Paper IV)

The Swedish Society for Communicable Disease Prevention and Control stipulates that all cases of giardiasis should be treated, regardless of symptoms. Information concerning treatment was obtained for the majority of the patients in our investigaiton, but follow-up after treatment was outside the scope of the present research. Notwithstanding, during the study period we noted that 10 patients had delivered a second stool sample after being treated with metronidazole or tinidazole, and we analyzed those samples by use of the same molecular tools as we had applied to the other samples. The findings showed that assemblage B was more common among those 10 patients and also indicated that several of those individuals were infected in India, although the genotyping results were inconclusive due to the extensive polymorphism in the assemblage B isolates. Reduced sensitivity to the drugs commonly used to treat *Giardia* is recognized in the literature and has been thoroughly assessed after an outbreak situation (Morch et al., 2008). Despite that, little is known about the assemblages involved in sporadic cases, and hence further research is needed in that context (Robertson et al., 2010).

7 PAST, PRESENT AND FUTURE WORK

Prior to the studies underlying this thesis, no investigations had been conducted to examine the distribution of *E. histolytica* and *E. dispar* in Sweden, and thus over-treatment due to incomplete diagnostic procedures was probably the rule. Over the years, it has become increasingly evident that making a correct laboratory diagnosis is highly important, and, ideally, this should be achieved for all samples containing *E. histolytica/E. dispar* parasites. Therefore, it is important to ensure that future work on *Entamoeba* in Sweden focuses on implementation of methods for species differentiation at local parasitology laboratories, so that all patients can receive a correct diagnosis together with the initial report based on microscopic findings.

The present work has also generated a large set of DNA samples from different amoeba parasites, part of which have already been used to study the genetic diversity of Entamoeba (Stensvold et al., manuscript) and the occurrence of *E. moskhowskii* in our part of the world (Stensvold et al., 2010b). Moreover, the investigation of *Entamoeba* in humans has drawn interest in the genetic diversity of amoebas found in animals, a hitherto rather unexplored subject (Stensvold et al., 2010a).

Through our analyses of *Giardia* isolates from humans and animals in Sweden and Nicaragua, we have accumulated basic knowledge concerning assemblages and genotypes in these populations. We have also compiled a unique collection of well-defined *Giardia* DNAs that have been investigated at several loci and represent assemblages A–G, as well as a large number of subtypes. A selection of these DNAs has recently been used to develop a rapid technique for genotyping based on real-time PCR with subsequent sequencing (Wahab et al. accepted manuscript). In addition, more than 100 unique *Giardia* sequences have been submitted to the Genbank database. Together, these efforts have created a framework for further *Giardia* research in Sweden, especially regarding zoonotic transmission, connection between genotype and symptoms, and identification of virulence and drug-resistance genes.

As a spin-off of the research reported in this thesis, we established in vitro cultures of two *G. intestinalis* assemblage AII isolates representing subtypes A2 and A3 in β-giardin. The whole genomes of these two isolates have been sequenced using the 454 technique, and the data thus obtained have revealed differences in chromosomal patterns, sensitivity to drugs, growth rate, and encystation efficiency compared to the standard assemblage AI isolate WB (Ankarklev et al., manuscript in preparation). Hopefully, one outcome of these data will be the identification of a gene locus that is more efficient for source tracing of *Giardia* assemblage A than those that are presently used. Another consequence of the current work has been the single-cell PCR (performed on trophozoites and cysts, and described in part here), which has proved what has long been suspected but not previously seen, namely, that mixed alleles occur in single *Giardia* parasites (Ankarklev et al., manuscript in preparation)

Microscopy has been the basic technique for detection of parasites in this research, and molecular methods have constituted the tool for further identification. The current trend in diagnostic parasitology is heading towards newer strategies, either immunology- or molecular-based. Hopefully, the art of microscopy will not be forgotten, but instead employed in combination with other techniques.

8 ACKNOWLEDGEMENTS

It's been a long and winding trip from my first encounter with a stool parasite to the completion of this thesis. Many people along the path have supported and encouraged me. At times you have had more faith in me than I've had myself! Your friendship, your wisdom and your support have urged me on to this end. I thank you all!

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