Inter and Intra-Assemblage Characterizations of *Giardia intestinalis*: from clinic to genome

JOHAN ANKARKLEV

UPPSALA
UNIVERSITET

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Abstract
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The protozoan parasite *Giardia intestinalis* (syn. *G. lamblia*, *G. duodenalis*) is one of the most common causes of diarrheal disease throughout the world, where an estimated 500 million people are infected annually. Despite efforts in trying to elucidate factors associated with virulence in *G. intestinalis* little is currently known. The disease outcome is highly variable in *Giardia* infected individuals, ranging from asymptomatic carriers to severe disease. The reasons behind the differences in disease outcome are vaguely understood and studies trying to link infectivity to different *Giardia* assemblages or sub-assemblages have rendered conflicting results. Prior to this study, little was known about the prevalence and genetic diversity of different *G. intestinalis* assemblages across the world.

   In this thesis, molecular characterization of clinical *G. intestinalis* samples from Eastern Africa and Central America, has been performed, enabling a better understanding of the prevalence of different *Giardia* genotypes in endemic areas (Papers I and II). A correlation between *Giardia* colonization and the presence of *Helicobacter pylori* in the human host was established. We found that the currently available genotyping tools provide low resolution when used to characterize assemblage A *Giardia*. Also, genotyping of assemblage B isolates at these loci is troublesome due to the polymorphic substitutions frequently found in the sequencing chromatograms. This ambiguity was investigated by using micromanipulation to isolate single assemblage B *Giardia* cells (Paper III). Both cultured trophozoites and cysts from giardiasis patients were analyzed. The data showed that allelic sequence heterozygosity (ASH) does occur at the single cell level, but also that multiple sub-assemblage infections appear to be common in human giardiasis patients.

   Furthermore, genome-wide sequencing followed by comparative genomics was performed in order to better characterize differences between and within different *Giardia* assemblages. The genome of a non-human infecting, assemblage E isolate (Paper IV) was sequenced. The genomes of two freshly isolated human infecting assemblage AII isolates were also sequenced (Paper V). Subsequent, comparative analyses were performed and included the genomes of two human infecting isolates, WB (AI) and GS/M (B). Several important differences were found between assemblages A, B and E, but also within assemblage A; including unique gene repertoires for each isolate, observed differences in the variable gene families and an overall difference in ASH between the different isolates. Also, a new multi-locus genotyping (MLG) strategy for genotyping of assemblage A *Giardia* has been established and evaluated on clinical samples from human giardiasis patients.

Keywords: Giardia, protozoa, parasite infection, diarrhea, genome sequencing, comparative genomics, genotyping, ASH, MLG

Johan Ankarklev, Uppsala University, Department of Cell and Molecular Biology, Box 596, SE-751 24 Uppsala, Sweden.

Till Morsan

Så stolt över att vara din son
Saknar dig som fan…….

List of Papers for This Thesis

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I   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

II   **Ankarklev J**, Hestvik E, Lebbad M, Lindh J, Kaddu- Mulindwa DH, Andersson JO, Tylleskär T, Tumwine JK, Svärd SG. Multi-locus genotyping of *Giardia intestinalis* in Ugandan Children with and without *Helicobacter pylori* colonization. *Submitted manuscript*

III **Ankarklev J**, Svärd SG, Lebbad M. Allelic Sequence Heterozygosity in Single *Giardia* Parasites. *Submitted manuscript*

IV   Jerlström-Hultqvist J, Franzén O, **Ankarklev J**, Xu F, Nohýnková E, Andersson JO, Svärd SG, Andersson B. Genome analysis and comparative genomics of a *Giardia intestinalis* assemblage E isolate. *BMC Genomics*. 2010 Oct 7;11:543

V   **Ankarklev J**, Franzén O, Einarsson E, Lebbad M, Andersson B, Svärd SG. Genomic variation within *Giardia intestinalis* assemblage A isolates. *Manuscript*

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Published Papers Outside the Thesis

I   Wahab T, **Ankarklev J**, Lebbad M, Glavas S, Svärd S, Palm D. Real-time polymerase chain reaction followed by fast sequencing allows rapid genotyping of microbial pathogens. *Scand J Infect Dis*. 2011 43(2):95-9

II   Jerlström-Hultqvist J, **Ankarklev J**, Svärd SG. Is human giardiasis caused by two different Giardia species? *Gut Microbes*. 2010, 1(6):379-82 (Review article)

III   **Ankarklev J**, Jerlström-Hultqvist J, Ringqvist E, Troell K, Svärd SG. Behind the smile: cell biology and disease mechanisms of *Giardia* species. *Nat Rev Microbiol*. 2010 8(6)413-22 (Review article)

IV   Brolin KJ, Ribacke U, Nilsson S, **Ankarklev J**, Moll K, Wahlgren M, Chen Q. Simultaneous transcription of duplicated var2csa gene copies in individual *Plasmodium falciparum* parasites. *Genome biol*. 2009, 10(19):R117

V   Franzén O, Jerlström-Hultqvist J, Castro E, Sherwood E, **Ankarklev J**, Reiner DS, Palm D, Andersson JO, Andersson B, Svärd SG. Draft genome sequencing of *Giardia intestinalis* assemblage B isolate GS: is human giardiasis caused by two different species? *PLoS Pathog*. 2009, 5(8):e1000560

VI   Reiner DS, **Ankarklev J**, Troell K, Palm D, Bernander R, Gillin FD, Andersson JO, Svärd SG. Synchronization of *Giardia lamblia*: identification of cell cycle stage-specific genes and a differentiation restriction point. *Int J Parasitol*. 2008, 38 (8-9):935-44

Contents

Abbreviations

ASH	Allelic sequence heterozygosity
ADI	Arginine deiminase
bg	Beta giardin
CWP	Cyst wall protein
ef 1α	Elongation factor 1-alpha
ESV	Encystation specific vesicle
gdh	Glutamate dehydrogenase
GI	Gastro intestinal
HCMP	High-cysteine membrane protein
IEC	Intestinal epithelial cells
miRNA	microRNA
MLG	Multi-locus genotyping
NO	Nitric oxide
nt	nucleotide
ORF	Open reading frame
PFGE	Pulsed field gel electrophoresis
RNAi	RNA interference
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNA
tpi	Triosephosphate isomerase
UTR	Untranslated region
VSP	Variant-specific surface protein
ZO	Zonula occludens

1 Introduction

My first experience with the beautiful, photogenic protozoan organism, *Giardia intestinalis*, was during a journey in Peru, located on the western coast of the South American continent. After spending days traversing the mountainous deserts of the Peruvian countryside, utilizing different modes of rather questionable transportation, and naively consuming all sorts of amazing foods available, my optimism and negligence finally caught up with me. I eventually found myself huddled up in the dark corners of an auberge in the Urubamba valley at 3500 m altitude above sea level and with a much more humble attitude towards life, and experiencing new cultures (which in this case indubitably refers to the rich microbial "cultures" present in various tropical regions of the world).

Giardia had struck!!

Coincidentally the timing was impeccable, as I was just on the verge of commencing my Master thesis in the Svärd laboratory (*Giardia* Mecca of the North) and had thereby filled my luggage with literature on the topic of *Giardia*. Thus my frequent commutes, between a rather rustic bed and an even more Spartan "bathroom", were pseudo-scientifically monitored and I had the luxury of reading up on what was to come, with regards to the symptom development.

Being a very proud father of an amazing little boy, I recently partook in an event that is referred to as "inskolning" (eng. prep. training) at his daycare. Despite the fact that my above mentioned experience with *Giardia* weren't the most pleasurable days of my life, I still feel fortunate to have experienced the disease first hand, and above all it was the quintessential "inskolning" prior to becoming knighted as a proper Giardian.

1.1 The history of *Giardia*

The first documented observation of *Giardia* occurred 330 years ago to the day, prior to my embarkment of writing this thesis, namely on the 4th of November in 1681 (Dobell, 1920). The keen and enthusiastic Dutch microscopist, Anthony van Leeuwenhoek observed the finding, as he was investigating his own stool for potential villains that were causing him diarrheal illness at the time. His discovery was reported to the Royal Society in London, and reads: *"All these described particles lay in a clear transparent medium, in*

which I have at times seen very prettily moving animalcules, some rather larger, others somewhat smaller than a blood corpuscle, and all of one and the same structure. Their bodies were somewhat longer than broad, and their belly, which was flattened, provided with several feet, with which they made such a movement through the clear medium and the globules that we might fancy we saw a pissabed running up against a wall. But although they made a rapid movement with their feet, yet they made but slow progress." (Dobell, 1920).

In 1859, the first attempt in baptizing the parasite was made, where the Czech scientist Vilem Dusan Lambl suggested the name, *Cercomonas intestinalis*. In 1888, Raphael A. É. Blanchard proposed the name *Lamblia intestinalis* in order to commemorate Dr V. Lambl. It was not until 1915 that the name *Giardia lamblia* was introduced, a name that has partially stuck since then. The name was chosen in order to honor the work of the French Professor, A. Giard, as well as Dr V. Lambl (Ford, 2005). Although the genus name, *Giardia*, has remained since then there are still struggles regarding the species name and as a result, the species complex that in part infects humans is currently referred to as; *G. intestinalis*, *G. lamblia* or *G. duodenalis*, where all three species names refer to the same organism (Table 1). The usage of the different species names is at least partially geographical, where, *G. intestinalis* is mainly used in Europe, *G. lamblia* is commonly used in North America, and *G. duodenalis* seems to be widely preferred in Australia. To date there is still great controversy with regards to the classification of *Giardia intestinalis* and a recent proposal of a revision of the nomenclature of *Giardia* was published in 2009 (Monis et al, 2009) see Table 2. In this thesis however, I will refer to the parasite as *Giardia intestinalis*.

1.2 The global burden of *Giardia intestinalis*

With an estimated 2 billion cases per year, diarrheal disease is one of the leading causes of morbidity and mortality in children across the globe. In low-income countries, diarrhea has been reported as the second leading cause of death in children five years of age or younger, superseded only by pneumonia. In an overall rank of causes of mortality in low-resources countries established in 2004 by the WHO, death by diarrhea comes in third, at 6.9% of all cases (WHO, 2009). Pathogens or opportunists, representing all different kingdoms in the tree of life with the addition of viruses but with some exceptions, may directly cause diarrheal disease in humans. *Giardia*, with an estimated 280 million symptomatic human incidents per year, is regarded as the commonest cause of protozoan diarrheal infection worldwide (WHO, 1996).

Albeit the fact, that areas considered to be endemic *Giardia* regions strictly include low-income countries, the burden of *Giardia* infection is

apparent throughout the world. In Europe, data collected by 23 countries suggested an average notification rate of 58.1 giardiasis incidents per 100,000 individuals, which is higher than the reported cases of both campylobacteriosis and salmonellosis from the same regions (Lujan & Svard, 2011). Data from a surveillance report in the U.S. indicated rates of giardiasis from 1.4 per 100,000 up to 30 per 100,000 individuals in different regions of the country (Yoder & Beach, 2007). It is noteworthy that an estimated 250 symptomatic *Giardia* cases have been expected to occur, per registered case of the disease.

Apart from the broad impact that human giardial infections have on society, *G. intestinalis* is also known to infect a broad range of mammals, other than humans. Here, infection in young farm animals accounts for great economic losses in the agricultural industry (O'Handley et al, 2001). Also the disease is believed to be zoonotic, indicating that transmission between humans and animals possibly occur, see section 1.11.

1.3 *Giardia* phylogeny and host range

Due to current taxonomic uncertainty in the field of *Giardia* I will discuss the taxonomy as it has been progressing during the time of the establishment of this thesis. Based on biochemical, structural and genetic data, *Giardia* is a diplomonad, a group of flagellated, microaerophilic protists that share the uncommon feature of having two nuclei. Phylogenetically *Giardia* belongs to the Kingdom Protista, Phylum Metamonada, Subphylum Trichozoa, Superclass Eopharyngia, Class Trepomonadea, Subclass Diplozoa, Order Diplomonadida and Family Giardiidae (Plutzer et al, 2010). Within the genus *Giardia* there are six different species, based on both morphological and molecular analyses (Adam, 2001).

Table 1. *Giardia* species including the *G. intestinalis* species complex according to the current taxonomy

Giardia **species**	**Host**
G. intestinalis (*G. lamblia*, *G. duodenalis*)	Humans and other mammals
G. agilis	Amphibians
G. muris	Rodents
G. ardaea	Birds (herons)
G. psittaci	Birds (psittacine)
G. microti	Rodents

Five of these species are host specific, where; *G. microti* infects voles and muskrats, *G. muris* infects mice, *G. agilis* are specific to amphibians, whereas *G. ardea* and *G. psittaci* are found in birds. The sixth species, *G. intestinalis*, has been documented to infect a large range of different mammals, including humans (Caccio et al, 2005; McRoberts et al, 1996).

Initially the taxonomy of *Giardia* was based on phenotypic trademarks, such as morphological variations and host specificity (Monis & Andrews, 1998). In the literature a total of 51 species of *Giardia* have been documented, where the basis of characterization has been host occurrence. However, based on morphological and molecular traits only six *Giardia* species have been distinguished. To date, eight different assemblages within the *G. intestinalis* species complex are recognized based on molecular analyses, these include assemblages A-H (Lasek-Nesselquist et al, 2010). Assemblages A and B infect a large array of mammals including humans (Cooper et al, 2007; Lebbad et al, 2008; Monis et al, 1999). Assemblages C through H are thought to be more host specific, with C and D having canines as their specific hosts (Cooper et al, 2007; Souza et al, 2007), E infects ungulates such as cattle and wild ruminants (Lebbad et al, 2010; Sedinova et al, 2003), F infects cats (Souza et al, 2007), G is specific to rats (Lebbad et al, 2010), and the recently discovered assemblage H is proposed to be specific to marine mammals (Lasek-Nesselquist et al, 2009) see Table 2.

Table 2. *G. intestinalis* assemblages, including the recently proposed species names

G.intestinalis Assemblage	Proposed Species	Host
A	*G. duodenalis*	Humans and other mammals
B	*G. enterica*	Humans and other mammals
C/D	*G. canis*	Dogs
E	*G. bovis*	Hoofed animals
F	*G. cati*	Cats
G	*G. simondi*	Rodents

In an attempt to separate the different assemblages of *G. intestinalis* using molecular tools, Monis et al, performed sequence analysis of the *gdh*, *ef*, *tpi* and *ssrDNA* loci. This data was then compared with an allozymic analysis of 23 different genetic loci across the genome. The data generated from the study suggested that the distance found between members within the *G. intestinalis* species complex was indeed greater than those separating certain genera of bacteria (Monis et al, 2009). This data, together with the results

from several genotyping projects as well as the data generated in the recent genome initiatives suggest that there are rather large differences between different *G. intestinalis* assemblages. There is an ongoing debate regarding a revision of the current classification of *Giardia* based on these large genetic differences that have been noted within the *G. intestinalis* species complex, and it has been suggested that the species complex should be broken up into different species, as seen in Table 2.

1.4 The *Giardia* cell

Giardia occurs in two major, metabolic states during its lifecycle; the vegetatively replicating trophozoite and the non-motile, metabolically dormant cyst (Adam, 2001), see Figure 1. Also two intermediate phases have gained recognition and have recently been coined: The excyzoite and the encyzoite stages. The excyzoite is the stage where the parasite rapidly evacuates from its protective chassi, the cyst wall, and transforms into four replicating trophozoites (Bernander et al, 2001). The other one being the encyzoite stage, where the trophozoite slowly rebuilds its protective coating and metamorphosizes back into a state of hibernation (Reiner et al, 2008).

1.4.1 The trophozoite

The *Giardia* trophozoite, the replicating state of the cell, has been described as a pear bisected length-wise with regards to its morphology (Adam, 2001). The trophozoite measures 12-15 μm in length and approximately 5-9 μm in width (de Souza et al, 2004). Movement of the parasite inside the host intestine involves propulsion around its own axis, in a yawing movement, together with the aid of four pairs of flagella. Outstanding features in trophozoite morphology are described below.

As a member of the diplomonads, the giardial trophozoites feature the uncommon trait of harboring two nuclei (Figure 1a), which are bilaterally symmetrical with regards to their positioning and oval shape (Benchimol, 2005). It has been suggested that the two nuclei may differ slightly in function as the two nuclei in a single cell differ in nuclear pore number and distribution (Benchimol, 2005). A very small number of nuclear pores are displayed as the nuclei are dividing, and frequent clustering of nuclear pore complexes in nuclear envelope domains have been seen (Benchimol, 2005).
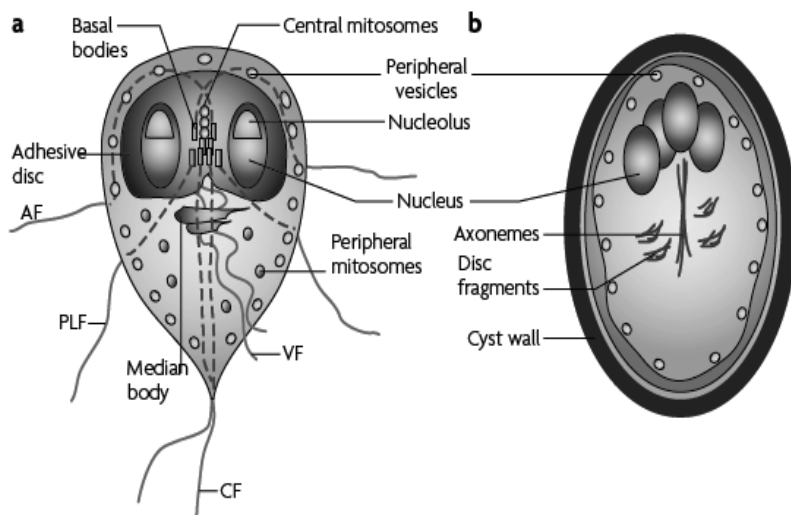
Figure 1. a) The *Giardia* trophozoite the vegetative stage of the life cycle including the two nuclei, the four pairs of flagella, the ventral adhesive disc, and various organelles. b) The dormant cyst, showing the typical feature of four nuclei, the centrally located axonemes, and the scattered ventral disc fragments. *Figure from (Ankarklev et al, 2010).*

Apart from the numerical anomaly, the nuclei share many typical eukaryotic traits such as folding of its genetic content around typical, yet reduced, eukaryotic histones. According to Yee et al., the histone-1, appears to be absent (Yee et al, 2007), suggesting that the *Giardia* nucleosomes are composed solely of the core histone components (H2a, H2b, H3 and H4).

The adhesive disc, present on the ventral side of the trophozoite (Figure 1a), is a *Giardia* specific attribute (Elmendorf et al, 2003). It is considered to be a virulence factor since it is used in attachment to the host epithelium and it is essential for the parasite as a means to avoid elimination due to intestinal peristalsis (Elmendorf et al, 2003; Muller & von Allmen, 2005). Known components of the adhesive disc include members of a *Giardia*-specific protein family of proteins, the giardins, such as; β, γ and δ-giardin, along with α and β-tubulin, SALP-1 and aurora kinase (Davids et al, 2008; Elmendorf et al, 2003). SALP-1 and β-giardin are both immunoreactive when exposed to serum from giardiasis patients as shown by Palm and colleagues (Palm et al, 2003). Electron microscopy imaging of the ventral adhesive disc showed microribbons, extending to the cytoplasm from a wall of microtubuli, which are distributed evenly along the surface of the disc in a spiral layer (Benchimol, 2004; Holberton, 1981; Sant'Anna et al, 2005).

Attachment of the trophozoite to the intestinal lining is documented to result in an imprint in the epithelium, indicating that a rather strong force is involved in parasite attachment (Erlandsen & Chase, 1974; Magne et al, 1991). The attachment is not specific to the host intestine, as *Giardia* tro-

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phozoites are known to attach to the polystyrene surfaces inside *in vitro* culturing vessels. The creation of suction is in part due to the ventrolateral flange that surrounds the ventral disc. Erlandson and colleagues showed that parasitemia was dramatically decreased *in vitro*, when altering the growth substrate from flat to a spiky surface, thus implementing a steric hindrance in parasite attachment (Erlandsen & Rasch, 1994). This indicates that the pressure created under the concave disc is an important factor in parasite attachment. However it has been experimentally shown that attachment is also achieved by surface adhesive lectins, as excyzoite attachment occurs prior to the completion of the ventral adhesive disc (Inge et al, 1988; Katelaris et al, 1995; Weiland et al, 2003).

Like several of its protozoan relatives, *Giardia* is a flagellated organism, with four pairs of flagella that are all rooted to the basal body machinery located between the two nuclei (Figure 1a). Parasite usage of the flagella is multi-faceted, firstly they are involved in trophozoite motility, which occurs in a streamlined and directed manner (Campanati et al, 2002; Ghosh et al, 2001). Secondly, the ventral flagella play an important part in attachment and detachment of the parasite from a surface. Thirdly, it has been implicated that the same flagellar pair used in attachment is also involved in the scavenging of nutrients from the surrounding microenvironment. Structurally, the giardial flagella follow a typical eukaryotic 9+2 arrangement, including a central pair of microtubules surrounded by nine concentric microtubular pairs. Besides the typical eukaryotic flagellar proteins, the *Giardial* flagella contain a kinesin 13 homologue localized at the proximal part of the flagella, α14-giardin localized at both the proximal and distal ends of the flagella, α19-giardin in association with the ventral flagella, and the microtubuli associated protein, EB1 (Kim et al, 2008; Sagolla et al, 2006; Saric et al, 2009; Vahrmann et al, 2008).

As mentioned previously, *Giardia* possesses eight basal bodies, localized between the two nuclei (Dawson et al, 2007; Kim et al, 2008; Saric et al, 2009; Vahrmann et al, 2008) (Figure 1a), which act as points of origin for the four flagellar pairs (Dawson & House, 2010; Sagolla et al, 2006). Basal bodies are highly conserved, cylindrical structures and self sufficient in terms of replication. The giardial basal bodies act as a type of signaling transduction and control centre during the different lifecycle progression stages, i.e. trophozoite cell division (Davids et al., 2008), and differentiation (Abel et al, 2001; Lauwaet et al, 2007; Reiner et al, 2003), as well as trophozoite motility and in particular flagellar assembly (Dawson & House, 2010).

The unique giardial organelle that is named the median body is what gives *Giardia* its characteristic "smile", due to the semi-circular or frown-like shape and its location posterior to the two nuclei, the "eyes" (Figure 1a). Little is currently known about the median body but due to its position, adjacent to the basal bodies and the ventral adhesive disc, several suggestions have been made. Alterations of the median body, which has been docu-

mented during the interphase in trophozoite cell division, indicates that it has a role in mitosis (Bertram et al, 1984). Also Piva et al suggested that it might have one or several of the following functions; a microtubule nucleation site or reservoir, an element used in stabilizing the microtubule during mitosis, or a function in the development of the ventral disc during cytokinesis or excystation (Piva & Benchimol, 2004)

Certain typical eukaryotic organelles are absent in *Giardia*, such as the Golgi. Protein transport is instead conducted via different types of peripheral vacuoles in combination with the complex giardial endoplasmic reticulum (ER) (Hehl & Marti, 2004). The network of ER extends throughout the cell, but is most abundant in the near proximity of the nuclei. Other organelles are reduced in *Giardia*, such as its reduced mitochondrial homologues, the mitosomes. In eukaryotes the mitochondria act as ATP production units, and as such they produce a large fraction of the energy required for the cell. The mitochondria are also involved in lipid metabolism, Fe-S complex synthesis, cell signaling, cell death and differentiation among other things. For long it was thought that *Giardia* completely lacked any form of mitochondria, until the discovery of a chaperonin 60 homologue (Cpn60), which was shown to be expressed in *Giardia* trophozoites (Roger et al, 1998). A few years after this discovery, Tovar and colleagues identified the mitosome; a reduced form of a eukaryotic mitochondrion that lack several typical mitochondrial functions (Tovar et al, 2003). The numbers of these organelles varies from approximately 25 up to 100 per cell and are most abundant in the close proximity of the basal bodies, the central mitosomes. The other type, the peripheral mitosomes are scattered throughout the cytoplasm of the cell (Hehl et al, 2007; Regoes et al, 2005). The only known activity of the giardial mitosome is Fe-S complex synthesis (Ankarklev et al, 2010).

The semi-permeable lipid bilayer that makes up the plasma membrane, is an integral part in the anatomy and physiology of the trophozoite as it allows entry of nutrients into the cell, endocytosis, and exit of waste out from the cell, exocytosis. Apart from that, it is the point from where the organism conducts cell signaling, it is the attachment point for the cytoskeleton, as well as it is the part of the parasite that is involved in interaction with the host environment (Adam, 2001). As such, it acts as a first line of defense against the innate immunity of the host, where a single coat of antigen covers the entire surface of the trophozoite, which is further discussed in section 1.8.4.

1.4.2 The cyst

Outside the host, the *Giardia* parasite is rigorously protected inside a cyst wall (Figure 1b), a hardy capsule consisting of a sugar and protein meshwork at a 3:2 ratio where the major constituent of the carbohydrate moiety is a β(1-3)-*N*-acetyl-d-galactosamine homopolymer. The remaining 40% constitutes cyst wall proteins (CWPs) to which the carbohydrates form a strong

interaction. Three leucine-rich repeat containing cyst wall proteins have been identified, CWP1-3, together with a high-cysteine non-variant cyst protein (HCNHp) (Davids et al, 2006; Sun et al, 2003). The oval shaped cysts are responsible for the transmission of the disease from one host to another (Adam, 1991). The cyst measures up to 12 μm in length and 8-10 μm in width. The filamentous, outer casing of the cyst wall is approximately 0.3-0.5 μm thick with a double membranous lining on the inside (Erlandsen et al, 1996). Cyst wall integrity has been challenged in several *in vitro* studies where it has been documented to sustain stress such as exposure to chemical water disinfection agents and to a certain extent irradiation (Belosevic et al, 2001; Jarroll et al, 1981; Sundermann & Estridge, 2010).

Also, the chemotherapeutic agents involved in treatment of the disease remain inept in affecting encysting cells, after a certain point in the differentiation process, inside the host (Paget et al, 1998). Thus the encystment of the parasite represents an important target for drug development in order to decrease transmission of the disease.

1.5 Differentiation

Differentiation in *Giardia* is one of the most basal eukaryotic developmental processes described and it entails two largely contrasting developmental transitions (Figure 2). These include; the awakening of the dormant cyst (excystation) and the emergence of the excyzoite, leading into the vegetative trophozoite cell cycle, and consecutively the transition back into the latent cyst phase (encystation) as the parasite exits the cell cycle and subsequently gets transmitted into the environment (Bernander et al, 2001; Svard et al, 2003). The entire lifecycle may be carried out *in vitro*, where it has been rigorously assayed, and gene expression analyses have concluded that regulated alterations in expression as a response to changes in the host microenvironment are the driving forces in the differentiation process (Boucher & Gillin, 1990; Gillin et al, 1989; Gillin et al, 1987; Reiner et al, 2008). The *in vitro* establishment of the *Giardia* lifecycle may be utilized as a model to study rudimentary eukaryotic developmental processes as well as a model for other protozoans lacking the complete lifecycle *in vitro*.

1.5.1 Excystation

Outside the host, the *Giardia* parasite remains protected from hypotonic lysis inside the hardy sugar-protein containing capsule that makes up the cyst wall. During the cyst phase, "the dormant phase", metabolism is highly downregulated (Paget et al, 1998). However, upon entering the host, the dormant cyst quickly awakens as it gets exposed to the harsh micro-environmental changes necessary to initiate infection. The low gastric pH in the stomach of the host
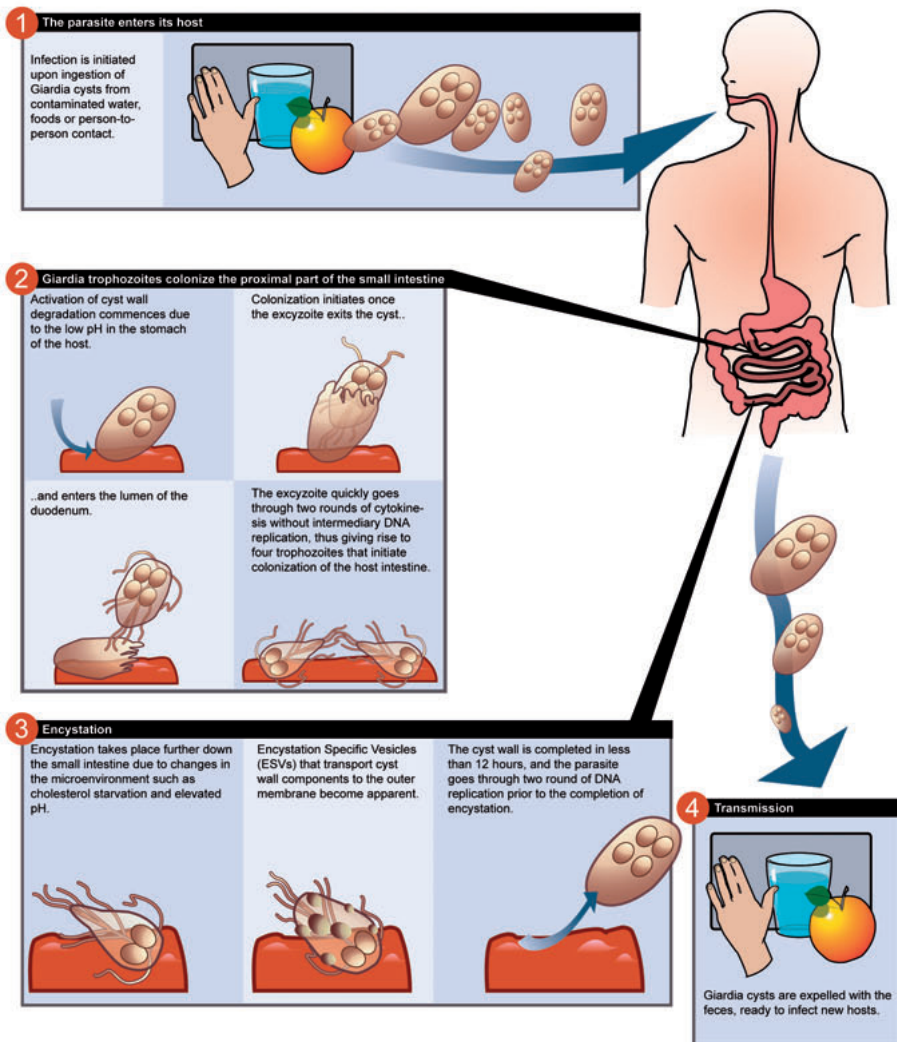
Figure 2. The *Giardia* life cycle, describing the different steps, from infection of the host (1), to activation of excystation and trophozoite colonization of the proximal small intestine (2). Mechanisms behind encystation and cyst development are shown (3), followed by the final excretion of *Giardia* cysts by its host. *(Figure/Text: Pontus Olofsson, Johan Ankarklev)*

works as an initial trigger of excystation. The environmental change leads to a subsequent release of cysteine proteases, which in turn degrade the cyst wall from the inside (Ward et al, 1997) and provides an exit strategy for the cell. It has also been shown that a secreted lysosomal acid phosphatase that acts in dephosphorylation of CWP is an integral part of *Giardia* excystation (Feely et al, 1991; Slavin et al, 2002). Once the activated cyst reaches the duodenum, the excyzoite springs free, a process that takes no more than 15 min post activation (Hetsko et al, 1998). As the excyzoite manages to crack

open the cyst wall at one of the poles, it initially protrudes its flagella followed by the excyzoite body (Buchel et al, 1987). Experimental data has shown that interference of protein kinase A and calmodulin during the excystation process, causes hindrance during the progression of this differentiation step, and thus indicates that they are important players in the process (Abel et al, 2001; Reiner et al, 2003). After the release from the cyst, the excyzoite instantly commences colonization of the small intestine, where two rounds of cytokinesis occur without intermediate DNA replication (Bernander et al, 2001). Thus each ingested cyst ultimately gives rise to 4 trophozoites that subsequently progress into the typical trophozoite cell cycle in the proximal part of the small intestine.

Transcriptional studies of the progression of excystation are scarce, which is largely due to the complexity of this differentiation phase *in vitro*. Excystation of a population of *Giardia* cysts *in vitro* renders a small success rate, where the majority of the population remain as cysts. Thus in order to properly evaluate changes in gene expression at this stage it would be necessary to optimize the method at the population level, or alternatively to select for the part of the population that successfully undergoes excystation. This could potentially be done by isolating single excysting cells from a population, at different stages of the excystation process using a microcapillary based micro-manipulation system like the one utilized in (Brolin et al, 2009) and Paper III of this thesis.

However, as part of a previous, large-scale initiative in the *Giardia* community, serial analysis of gene expression was performed and aimed at looking at changes during various stages of the lifecycle. Data generated from this project give several indications of changes in gene regulation during excystation. These include expression profiles that fit the processes taking place in the cell such as regulation of genes coding for: regulation of protein degradation, proteins involved in mitosis and cell division as well as cytoskeletal proteins (GiardiaDB).

1.5.2 Trophozoite proliferation

Giardia trophozoites complete their cell cycle in approximately 6 to 12 hours *in vitro*, depending on the isolate. The two nuclei replicate synchronously in the S phase and there is a proportionally short G1 phase and a longer G2 phase (Adam, 2000). Upon establishing a reversible *in vitro* synchronization protocol using aphidicolin, which arrests the trophozoites in the G1/S phase of the cell cycle, Reiner and colleagues showed that several *Giardia* orthologues, of known cell cycle dependent genes, change their expression in a cell cycle stage-specific manner (Reiner et al, 2008). Synchronization of *in vitro* cultures has paved way for large-scale expression analysis of the trophozoite cell cycle, which has greatly increased the understanding of the infectious state of this protozoan pathogen.

1.5.3 Encystation

Contrary from excystation, the process of encystation is slow, it is induced further down the alimentary canal (Figure 2) and factors involved in the induction of encystation include; an elevation of the pH and increased levels of bile together with cholesterol starvation (Lauwaet et al, 2007). *In vitro* analyses of aphidicolin-synchronized trophozoite populations have shown that there is a restriction point early in the G2 stage of the cell cycle where the parasite exits the vegetative cycle and commences differentiation (Reiner et al, 2008).

Completion of encystation of the WB isolate *in vitro* takes approximately24-36 h, during this process the encyzoite loses its ability to adhere to the intestinal epithelium due to disassembly of the ventral adhesive disc (Palm et al, 2005) The cell also loses its motility as the flagella becomes internalized, the cell progressively becomes bloated and the formation of encystation specific vesicles (ESVs) become apparent (Ankarklev et al, 2010). These vesicles can easily be viewed using a regular light microscope. The ESVs are Golgi-like organelles where the formation and transport of CWPs occur (Marti & Hehl, 2003).

Approximately a decade ago, Sun et al discovered that a Myb-related protein (Myb2) was responsible for the activation of transcription of encystation specific genes (Sun et al, 2002). From an evolutionary point this is interesting as Myb proteins are responsible for the regulation of differentiation of stem cells in humans (Boheler, 2009). It has also been shown that an AT-rich interaction domain (ARID), together with a GARP-like protein and a WRKY-like protein bind the CWP promoters. These three proteins are known to regulate differentiation and dormancy responses in plant (Ankarklev et al, 2010). Upon exiting the cell cycle in the G2 stage, the parasite has already replicated its genomic content once, but the encyzoite proceeds with a second round of replication (Bernander et al, 2001). Thus, upon completion, fragments of the adhesive disc together with the flagella align in the centre of the cyst, and four nuclei with a 16N ploidy are readily visible, thus allowing the cyst to quickly colonize its next host.

1.6 Transcription

Gene transcription is one of the key fundamental processes in living organisms, where genomic components get copied into complementary RNA. The complementary RNA may in turn result in; transfer or ribosomal RNA (tRNA, rRNA), or alternatively in cases where proteins are encoded the complementary RNA may result a messenger RNA (mRNA) strand, which subsequently is destined for translation into a protein. In congruence with many of its cell biological features, *Giardia* also has a reduced eukaryotic

machinery for transcription. The genome of *Giardia* is very condensed and intergenic regions stretch no more than approximately 0.1-0.4 kbp, on average, between adjacent open reading frames (ORFs) (Franzen et al, 2009; Morrison et al, 2007).

In *Giardia* it has been found that the normally tightly regulated eukaryotic control of transcription is of a looser character. It has been shown that an initiator like AT-rich element just upstream ($\leq$ 60 n.t.) of the transcription start site, seems to be the only activator needed in order to induce transcription in *Giardia* (Elmendorf et al, 2001a). It has been shown that transcription leading to sterile transcripts is commonly occurring in *Giardia* (Elmendorf et al, 2001a) and it is believed that this is caused by loosely regulated transcription due to the nature of the giardial promoters. Additionally, there is a generally adenosine-rich region surrounding the transcription start site. Several transcription related motifs have also been identified in *Giardia*, an AT-rich "box" located 30 nucleotide upstream of the start site may be *Giardia*'s equivalent of the typically eukaryotic CAAT box (Elmendorf et al, 2001b). *Giardia* specific promoter sequences have also been recognized, such as the Myb2 C(T/A)ACAG promoter activation sequence, which in turn upregulates the expression of CWP1-3 and G6PI-B early in the encystation process (Knodler et al, 1999; Sun et al, 2002). Sun et al later went on to identify the promoter regions of two other encystation specific transcription factors, namely the GARP-like proteins (GLP) 1 and 2, with the required (A/G)ATCN sequence for binding (Sun et al, 2006). Several other identified promoter regions in *Giardia* include those of ARID and WRKY, both involved in activation and regulation of CWPs during encystation (Pan et al, 2009; Wang et al, 2007), as are the roles of ran, gdh and α 2tubulin (Elmendorf et al, 2001b; Sun & Tai, 1999; Yee et al, 2000).

In a genomic survey directed at finding transcription related genes in *Giardia*, Best et al found giardial orthologs that comprise one quarter of the 28 polypeptides that make up the RNAPI-III in eukaryotes (Best et al, 2004). One third, of the 12, initiation factors that are generally found in eukaryotes have homologues in *Giardia*. A *Giardia* TATA-binding protein (TBP) was recognized but found to be highly divergent compared to higher eukaryotes and Archaea. Interestingly the giardial TBP was found to be more closely related to Archaea than to the general eukaryotic TBPs (Best et al, 2004). The divergence of the transcription initiation factors found in *Giardia* suggests a considerable difficulty in discovering the potential remainder of factors not yet identified.

Transcribed mRNA sequences in eukaryotes are highly modified prior to translation into proteins. These modifications have also been recognized during transcription in *Giardia*. Hausman et al identified a potential methyl-guanosine cap at the 5' end in *Giardia* transcripts as they showed a block of the transcripts at the 5' ends. They also went on to perform genome mining where they verified the presence of the key enzymes involved in 5' capping

of RNA, which include; RNA triphosphatase, RNA guanyltransferase and RNA methyltransferase (Hausmann et al, 2005). An interesting finding was made in *Giardia*, with regards to the initiation of transcription. In *Giardia* unlike most eukaryotes, the optimal length of the 5'UTR, scanned by the ribosome between the 5'cap and the AUG initiation site, is less than 10 nucleotides in length. This is unusually short compared to the approximate 80 nucleotide-scanning region found in most eukaryotes (Li & Wang, 2004). This is highly likely due to the condensed nature of the genome of *Giardia*.

At the other end, or the 3' end, of mRNA transcripts in eukaryal organisms the presence of a polyadenylated tail is commonly found. In *Giardia* this tail is quite short, somewhat heterogenic and located just downstream of the proposed AGUPuAAPy polyadenylation signal (Que et al, 1996). In the first published genome of *Giardia*, only six out of a total of 23 genes involved in the polyadenylation pathway previously described in yeast were identified (Morrison et al, 2007), which yet again places *Giardia* among some of the earliest branching eukaryotes. In the same study, Morrison et al concluded that only four introns are present in the genome (Morrison et al, 2007). A year later, Chen et al went on to identify giardial orthologues of all small nuclear RNAs (snRNA) involved in intron splicing (U1, U2 and U4-U6) (Chen et al, 2008). This indicates that splicing events possibly occur in *Giardia*, despite the low numbers of introns.

A recent study has also identified a unique giardial gene expression system that entails spliceosomal introns in a split form, or "splintrons". Here it has been suggested that certain genes in *Giardia* are split in the genome, the different gene pieces are transcribed independently and subsequently two of these mRNA fragments form an intermolecular stem structure, which is targeted by the spliceosomes and trans-spliced into a mature mRNA (Kamikawa et al, 2011; Nageshan et al, 2011). This type of trans-splicing of split genes could potentially be commonly occurring in other eukaryotes, especially the ones with genomes almost devoid of introns as is the case in *Giardia*, thus adding to the potential of *Giardia* as an important model system.

Control of gene expression in eukaryotes includes regulation; at chromatin domains, during transcriptional events, of RNA transport, of translation as well as post-transcriptional modifications of mRNA, and the later has been thoroughly investigated in *Giardia*. Post-transcriptional gene silencing or RNA interference (RNAi) has been documented in *Giardia*, where both small interfering RNA and micro RNA (siRNA and miRNA) have been identified (Kolev et al, 2011; Ullu et al, 2004). This machinery is involved in post-transcriptional control of gene regulation through the binding of short antisense RNAs to pre-synthesized mRNAs. Subsequently different pathways within this machinery degrade the mRNA that has been subject to antisense RNA binding. This process was initially described in plants (Napoli et al, 1990; Smith et al, 1990; van der Krol et al, 1990), but has later been

shown to be generally involved in regulation of stress response, cell differentiation and cell cycle control among others (Ullu et al, 2004). Functions of regulation of gene expression will be further described in section 1.8.4.

1.7 Disease characteristics

As previously described, giardiasis is one of the most common causes of diarrheal disease across the world, and among the protozoa, *Giardia* is the number one causative agent of diarrhea both in developing and industrial countries. As of 2004, *Giardia* was included in the World Health Organization's Neglected Disease Initiative, due to the way the parasite seems to flourish in developing countries, together with the general lack of knowledge behind the molecular mechanism of the disease (Savioli et al, 2006).

   Giardiasis may range from asymptomatic to chronic or severe diarrhea, and chronic disorders post-infection have been documented. Asymptomatic hosts may still shed infectious cysts and act as a transmission vehicle for the disease (Hanevik et al, 2009; Ish-Horowicz et al, 1989). The infectious dose has been described to be as low as 10 cysts and was demonstrated by administrating capsules containing *Giardia* cysts to a population of human volunteers (Rendtorff, 1954). However, the size of the infectious dose has shown to influence the clinical outcome, where mice infected with larger doses of cysts have a shorter latency period (Belosevic & Faubert, 1983). The prepatent period of giardiasis, i.e. time after ingestion of the cysts and until the patient starts to shed cysts in the feces, is usually one to two weeks, but may vary as much as a few days up to six weeks (Ortega & Adam, 1997).

   Generally, *Giardia* infection in healthy adults is considered to be self-limiting, and experimental infections suggest that previously infected individuals are less likely to develop symptoms (Nash et al, 1987). Problematic *Giardia* infections, such as refractory infections accompanied with therapy resistance has however been reported in healthy individuals (Hanevik et al, 2007). Individuals suffering from common immune deficiency syndrome (CVID), or acquired immune deficiency syndrome (AIDS) are more likely to develop anorexia or chronic dehydration (Carcamo et al, 2005; Onbasi et al, 2005). Also, infants residing in *Giardia* endemic areas world are more likely to suffer malabsorption, impairment of growth and to develop poor cognitive functions (Farthing, 1997). Irritable bowel syndrome (IBS) is a condition that involves recurring abdominal pain, bloating and may also be accompanied by bouts of diarrhea, but without the presence of a known causative agent. Acute intestinal infections is one of the most common causes of IBS, which in turn is referred to as post infectious IBS (PI-IBS) (Gwee, 2010). Morken et al., have found a potential link between giardiasis and PI-IBS, where the *Giardia* infection is described as a trigger of the IBS symptoms, but the parasite is not necessary for symptoms to persist (Morken et al,

2009a). It has further been shown that *Giardia*-induced PI-IBS patients had reduced IBS symptoms when treated with commensal flora from healthy individuals (Morken et al, 2009b).

Giardia-induced pathophysiological processes that result in symptoms, are not fully understood, however, recent research has generated a clearer indication of what takes place. Firstly, it has been seen that apoptosis of intestinal epithelial cells can be increased due to *Giardia* infection (Troeger et al, 2007). The naturally occurring phenomenon of apoptosis or regulated cell death normally occurs at a rate of 1% in the proximal part of the small intestine. However, in *Giardia* infected patients apoptosis has been shown to be increased by >50%, bringing the total rate of apoptosis from 1% to over 1.5% (Troeger et al, 2007). This increase might be even further elevated in patients with severe giardiasis, as compared to the results from the study, which were based on patients with chronic infections. Giardial activation of the apoptotic proteases, caspase-3 and caspase-9 has been documented to occur *in vitro* (Chin et al, 2002; Panaro et al, 2007). Induction of apoptosis due to *Giardia* infection has also been further indicated based on microarray analysis from an *in vitro* infection assay where genes involved in apoptosis showed expressional activation post infection with *Giardia* trophozoites (Roxstrom-Lindquist et al, 2005). In all the studies described above an increase in intestinal epithelial apoptosis is suggested to lead to a loss of intestinal epithelial barrier and thereby also diarrhea.

Aside from inducing apoptosis in the intestinal epithelium, other pathopysiological aspects have been documented due to *Giardia* infection, such as alterations in the junctional components on the apical sides of the epithelial cells. These components are discussed below, but in short a relocation takes place from the cellular membrane to the cytosol of some of the components responsible in regulating paracellular flow, such as the zonula occludens-1 (ZO-1), as well as α -actinin and F-actin (Scott et al, 2002). Studies in mice have also shown that intestinal permeability increases in mice during a *Giardia* infection, and post infection the membranes restore homeostasis (Scott et al, 2002). Scott and colleagues also discovered that *Giardia* infection in mice leads to the induction of a diffuse shortening of the microvilli that covers the apical side of the intestinal epithelial cell layer (Scott et al, 2004). This in turn leads to malabsorption of nutrients such as sugars but also electrolytes, which are known to minimize the absorption of water (Cevallos et al, 1995; Scott et al, 2004), which in turn could potentially lead to malabsorptive diarrhea. In conclusion, giardiasis like many intestinal diseases, affects the host in a multifactorial fashion where several combined mechanisms are likely to lead to the pathology involved in causing symptoms.

1.8 The site of infection (Host-pathogen interactions)

In its host, *G. intestinalis* colonizes the upper part of the small intestine, the duodenum. As *Giardia* is not invasive, it does not have to actively protrude any membranes for effective parasitism. Instead it found its niche below the mucous layer in the lumen of the small intestine. The epithelial cells in the host intestine are frequently replaced; there is also a continuous luminal flow in the intestine. These two factors make it important for the parasite to be able to efficiently detach itself, swim upstream and re-attach at a proper location (Campanati et al, 2002).

The small intestine is made up of three parts; the duodenum, which is located adjacent to the stomach, followed by the jejunum and lastly the ileum, which connects the small and the large intestine through the ileocecal sphincter (Boron & Boulpaep, 2003). Finger-like projections called villi, cover the linings of the small intestine and the villi are surrounded by glandular structures called the crypts of Lieberkühn. A layer of columnar epithelial cells in turn encases both of these structures. The villi are responsible for nutrient and electrolyte absorption, whereas the crypts major purpose is secretion (Boron & Boulpaep, 2003). Increased secretion of electrolytes and water is a host-response that can wash out parasites from the intestine.

The main turf for *Giardia*, the duodenum, is the location for outlets of secretions from the pancreas and the gall bladder and thus the site where digestive enzymes and bile salts are released for digestion of foods (Marieb & Hoehn, 2007). The chemical microenvironment of the duodenum is constantly balanced with regards to the pH. As food particles enter the small intestine from the stomach there is an immediate decrease in pH, which in turn signals the release of alkaline bile and pancreatic fluid, rendering the microenvironment a slightly alkaline pH (Boron & Boulpaep, 2003). The need of the tough, cysteine rich, exterior membrane of *Giardia* may in part be explained to the harsh environment at its preferred location. Also, upon colonization, the parasite receives a second means of protections as it traverses the mucus layer in order to attach to the epithelial lining.

The mucus layer is formed by a thick coat made up of mucins, which are soluble and membrane-bound glycoproteins, together with lipids and proteins, and with the purpose of protecting the epithelial cells of the host (N'Dow et al, 2004). Goblet cells, interspersed among the intestinal epithelial cells are in charge of producing and secreting the mucus, which apart from acting as a barrier and protecting the intestinal epithelium, also "hides" glycolipid and glycoprotein receptors recognized by invasive pathogens (Navaneethan & Giannella, 2008).

The epithelial cells that make up the intestinal lining are inter-linked through protein interaction by different kinds of junctions, including; desmosome junctions, adherens junctions and tight junctions. The latter are located on the apical, upper half, of the epithelial cells and contain intracellular con-

necting proteins such as claudins, occludins, CAR, and junctional adhesion molecules, which are both involved in cell structure as well as signaling (Hossain & Hirata, 2008). All of these proteins are bound to the actin cytoskeleton, through the ZO membrane adaptors and are thus referred to as ZO proteins. Permeability of the epithelium is largely determined by cellular and paracellular resistance, where the paracellular resistance is considerably lower than that of transcellular resistance, and thereby largely a function of tight-junction structure. The permeability also varies depending on the location in the intestine; an example is the tight junctions in the crypt, where the permeability is greater than in the villus (Boron & Boulpaep, 2003). *Giardia* has been reported to cause a "leaky" intestine (Troeger et al, 2007), where affecting the tight junctions may cause increased symptoms in the host.

1.8.1 Gut ecology

The human or mammalian intestine hosts a rich and abundant flora of commensal organisms and may at the point of a *Giardia* infection, host one or several other pathogens that inhabit the same niche or that in other ways indirectly affect giardial colonization. Although this is a highly unexplored topic in the *Giardia* research field some light has been shed on trying to elucidate the interplay between *Giardia* and its neighboring flora. Extracted exudates from, *Lactobacillus johnsonii*, a commensal that is known to reside in the duodenum of the human host showed an inhibitory effect on trophozoite growth *in vitro* (Perez et al, 2001). Humen et al. went on to assay the affect of per oral (P.O.) administration of *L. johnsonii*, strain La1, to gerbils prior to or during *Giardia* infection, with the consequence of inhibition of colonization or effective elimination of the parasite (Humen et al, 2005). Another interesting study involved the co-infection of *G. intestinalis* together with the nematode *Trichinella spiralis*, which, like most helminthes, induces a strong Th2 immune response in its mammalian hosts. Here co-infection rendered a much stronger *Giardia* infection as compared to that seen in the controls (von Allmen et al, 2006). Studies performed on other entero-dwelling parasites have yielded intriguing results with regards to the effects of their interplay with their neighboring organisms.

Hatching of the eggs of the parasitic nematode *Trichuris muris*, and thus the establishment of infection, have been found to be highly dependent on the microflora in its host where a highly decreased flora renders hatching inept (Hayes et al, 2010). The intestinal protozoan parasites *Entamoeba histolytica* has been found to vary its nuclear DNA content upon changing between xenic and axenic growth conditions *in vitro*, where axenic growth yielded a 10-fold increase in DNA content (Mukherjee et al, 2008). Several recent observations have been made in the interplay between different parasites and between parasites and the commensal flora, which in turn has brought forth a new dynamic to the intestinal niche and a new dimension

regarding current concepts concerning regulation of immunity and intestinal homeostasis in general. Future research aimed at further investigating the giardial interplay with other intestinal organisms likely holds many key factors in store in terms of virulence, as well as potential pro-biotic or prophylactic strategies that may be utilized to combat the disease.

1.8.2 Host immunity

The human immune system is made up of the innate and the adaptive defense strategies, where innate immunity is immediately triggered upon infection and the adaptive system establishes specific response and memory towards a pathogen and is responsible for antibody production. In giardiasis patients it has been documented that the majority of infections do not lead to inflammation in the small intestine and inflammation generally does not seem to correlate with severity outcome of the infection (Oberhuber & Stolte, 1990).

Innate immunity, the first line of defense, acts in many ways upon pathogenic intruders. Along with the pancreatic fluids, antimicrobial peptides such as defensins are secreted from Paneth cells located in the crypts of Lieberkühn (Karam, 1999). Lactoferrin, which has shown a toxic effect on *Giardia* trophozoites *in vitro*, is continuously secreted from the gall bladder (Turchany et al, 1995). Also nitric oxide (NO) together with reactive oxygen species (ROS), both of which have a detrimental effect on giardial trophozoites *in vitro*, are produced and secreted from the intestinal epithelium. Experimental analysis of NO donors such as; S-Nitrosoacetyl-penicillamine, 3-Morphlinosydnonimine and glutathione-S-nitric oxide both inhibit giardial differentiation as well as trophozoite proliferation (Eckmann et al, 2000; Fernandes & Assreuy, 1997).

Innate immunity involved in combating giardiasis, first of all includes the initial contact with the excyzoite/trophozoite and the intestinal epithelium and subsequently dendritic cells (DCs) once the parasite traverses the mucus layer. The DCs, process giardial antigens, which leads to activation of adaptive or humoral immunity through the activation of T cells. Roxström-Lindqvist et al. showed that CXCL1-3, together with CCL2 and CCL20 were upregulated in the human intestinal epithelial cell (IEC) line, Caco2 (Roxstrom-Lindquist et al, 2005). A more recent, clinical study, where *Giardia* infected children were assayed for fecal concentrations of different chemokines, indicated an increase in the level of IL-4, IL-5, IFNγ and CCL2 (Long et al, 2010). Mast cells (MCs) also known as mastocytes are immune cells containing granules that are loaded with inflammatory mediators. Upon stimulation, these granules fuse with the plasma membrane and subsequently become released causing inflammation. One of the major secreted factors is histamine, which is why MCs cause allergic symptoms (Wesolowski & Paumet, 2011). In mouse models MC are strongly correlated to giardiasis, *in*

vivo mouse models, with a defect in MC function or a mutation in c-kit, a factor involved in MC maturation, have demonstrated a lack in the ability to control *Giardia* infection (Erlich et al, 1983; Li et al, 2004). Certain bacterial infections have been documented to down-regulate IgE/allergen stimulated degranulation of MCs (Wesolowski & Paumet, 2011). Upon symptomatic *Giardia* infection on the contrary, patients and particularly allergics, have shown elevated IgE levels in their serum (Di Prisco et al, 1998; Giacometti et al, 2003).

Host adaptive immunity, the second line of defense includes two main cell types, the bursa (bone-marrow) derived B-cells, which are responsible for antibody production and the thymus derived T-cells. The major antibody isotype found in the intestinal lumen is IgA (Langford et al, 2002). The poly-Ig receptor (pIgR) transports IgA through the epithelium of the small intestine and into the lumen. Langford and colleagues reported that the *G. muris* infected, IgA deficient mice could not properly clear the *Giardia* infection (Langford et al, 2002). Also antibodies from *G. muris* infected mice were seen to be toxic in *in vitro* experiments with *Giardia* trophozoites (Belosevic and Faubert, 1987). In humans, individuals suffering hypogammaglobulemia are more prone to prolonged infection as well as elevated symptoms upon becoming infected with *Giardia* (Taylor & Wenman, 1987). Humans have been reported not to be protected against recurrent *Giardia* infection. Infants however, do receive a compelling protection throughout the period that they nurture through breast-feeding, this has been seen in cases where the mothers are previous giardiasis sufferers (Tellez et al, 2003).

The other part of the adaptive immune response, the T-cells, consist of the $CD8^+$ or cytotoxic T-cells (T_c), which bind to and eliminate tumor cells and virally infected cells. $CD4^+$, or helper T-cells (T_h cells) are involved in aiding other lymphocytes in their developmental processes, such as activation of T_c cells and maturation of B lymphocytes (Janeway et al, 2001). T_h cells are likely important in *Giardia* infections as they activate and stimulate antibody production by B-cells. Mice treated with antibodies against T_h cells experienced prolonged infections with *Giardia*, and showed an impaired IgA response compared to immunocompetent mice (Heyworth, 1989).

1.8.3 Virulence

The term virulence is derived from the latin word "virulentus", meaning toxic, or "full of poison" (Casadevall & Pirofski, 2001). The way the term is utilized in infection biology is very broad and essentially entails any means or mechanism that benefits the proliferation of a pathogenic organism inside a susceptible host as well as its mode of transmission. There are several classical definitions of virulence such as, toxicity, or the production of a toxin by

a microorganism causing harm to the host, is one of the most classical definitions of virulence (Casadevall & Pirofski, 2001). Several bacterial species such as *Escherichia spp.* (Fleckenstein et al, 2010), *Bacillus spp.* (Guichard et al, 2011) and *Bacteriodes spp.* (Wick & Sears, 2010) express several toxins that are detrimental to their hosts. To date, no known toxin genes or gene families have been characterized in *Giardia spp*. However a 58-kDa enterotoxin-like molecule was found to activate signal transduction pathways in enterocytes that leads to accumulation of fluids in the intestine and excessive ion secretion (Kaur et al, 2001).

Replication, and transmission are other factors included in the definition of virulence (Casadevall & Pirofski, 2001). Both of these apply to *Giardia*, where rapid colonization is beneficial for the organism in terms of competition. Also, the production and excretion of a large number of viable cysts enhances the capabilities of spreading and establishing infection in new hosts. Adhesion is also described as a mode of virulence. In *Giardia*, attachment by the ventral adhesive disc to the intestinal epithelium is a well documented process (Holberton, 1973; Knaippe, 1990). Factors that aid in trophozoite attachment include, surface lectins (Katelaris et al, 1995; Sousa et al, 2001) and the giardins (Jenkins et al, 2009). Variant-specific surface proteins (VSPs), a gene-family involved in antigenic variation may also have a role in attachment (Bermudez-Cruz et al, 2004). In *Giardia* as well as in many other flagellated organisms, the flagella is considered a virulence factor as well since flagella are known to induce innate immune responses.

Antigenic variation is yet another factor that is considered a virulence mechanism and is discussed in the section below. Although virulence is a microbial entity, it is only upon interaction with a susceptible host that it has a relevant meaning, which makes the general concept very complex. Host immune status along with other host factors indubitably has a significant impact on the disease outcome in terms of virulence or avirulence.

A large number of *Giardia* infected individuals harbor the parasite without showing any symptoms (Adam, 2001). It has also been documented that the disease outcome in individuals from the same household and likely infected with the same *Giardia* strain, varies dramatically (Lebbad et al, 2011). Hence, the concept of virulence includes an array of factors both in the microbe and the host. In the host, immune status, general and induced tolerance to disease as well as the more recently implied host microbiota among other factors all need to be considered, which in turn highly increases the complexity in designing experiments for assaying the host-pathogen interplay in infection biology research.

1.8.4 Antigenic variation

As previously described, adaptive immunity has the potential of clearing a *Giardia* infection. In order to effectively escape this targeted defense applied

by its host, the *Giardia* trophozoite has developed a defense mechanism termed antigenic variation. This implies that the trophozoite masquerades itself with a coat of antigens that it switches over time and thus averts further antibody recognition, making it a difficult target for the host adaptive immune system. Antigenic variation is a process utilized by several human pathogens in order to evade eradication by the host immune responses.

Several pathogenic bacteria including; *Streptococcus pneumonia*, *Campylobacter jejuni*, *Haemophilus influenza* and *Helicobacter pylori* (Moxon et al, 2006) have shown different modes of stochastic switching of genes involved in pathogenic behavior. Also several viruses are known to evade host immunity through high mutational plasticity, examples of important human viral pathogens, that harbor antigenic variation, include; the human immunodeficiency virus (HIV) (Motozono et al, 2010), and the human influenza virus (Chen & Deng, 2009). In eukaryotes, opportunistic fungi, such as *Pneumocystis jiroveci* as well as different *Candida spp.* have been suggested to be able to induce switching in the gene-families encoding the major surface glycoproteins and thus avoid immune recognition (Jain et al, 2008).

Among the human parasitic protozoa, *Plasmodium falciparum* and *Trypanosoma brucei* along with *G. intestinalis* all have relatively well characterized and immensely complex modes of antigenic variation. In *P. falciparum* and *T. brucei* there is evidence of DNA sequence alterations, gene rearrangements and often a requirement of telomere-linked transcription (Lopez-Rubio et al, 2007), all of which have not been found in *Giardia*. The variable gene family that encodes these membrane bound immuno-stimulatory proteins in *Giardia* is referred to as the variant-specific surface proteins (VSPs), see Figure 3. VSPs in *G. intestinalis* have also been shown to be involved in cellular signaling, where modifications of the conserved cytoplasmic tail has been reported to occur. Palmitoylation of the cysteine in the tail aids in regulating the segregation of proteins to lipid rafts in the plasma membrane (Touz et al, 2005), whereas citrullination of the arginine in the tail is involved in regulating the switching mechanism (Touz et al, 2008), see Figure 3b.

The dawn of discovering the occurrence of this antigenic variation in *G. intestinalis* took place in the mid and late 1980's where Nash and Aggarwal detected discrimination in the binding of specific monoclonal antibodies (mAbs) when performing *in vitro* assays on the population level in trophozoites (Nash & Aggarwal, 1986). The same research pair later also confirmed this finding *in vivo* (Aggarwal & Nash, 1988), and the first complete sequence generated of a VSP, namely TSA417, was published a few years later (Gillin et al, 1990). The VSPs vary in size from 20-200 kDa (Prucca & Lujan, 2009), they are highly cysteine rich (10-12%) and the formation of CXXC motifs are involved in establishing disulphide bonds.

The VSPs are composed of three parts; a variable domain, a semi-conserved domain and a highly conserved cytoplasmatic CRGKA-tail

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(Ankarklev et al, 2010). It has been suggested that the entire repertoire of VSP genes, with its proposed 270-300 members, make up approximately 4% of the entire genome content in *G. intestinalis* (Adam et al, 2010). Three major genome initiatives has so far yielded genome sequences of an A (WB), a B (GS) and an E (P15) isolate (Franzen et al, 2009; Jerlstrom-Hultqvist et al, 2010; Morrison et al, 2007) and inter-assemblage comparisons indicated that there are no identical VSPs among the different assemblages of *G. intestinalis*. Expression of VSPs on the trophozoites is done in a mutually exclusive manner except during the events of VSP switching and differentiation when two or several VSPs are simultaneously expressed (Prucca & Lujan, 2009; Svard et al, 1998).
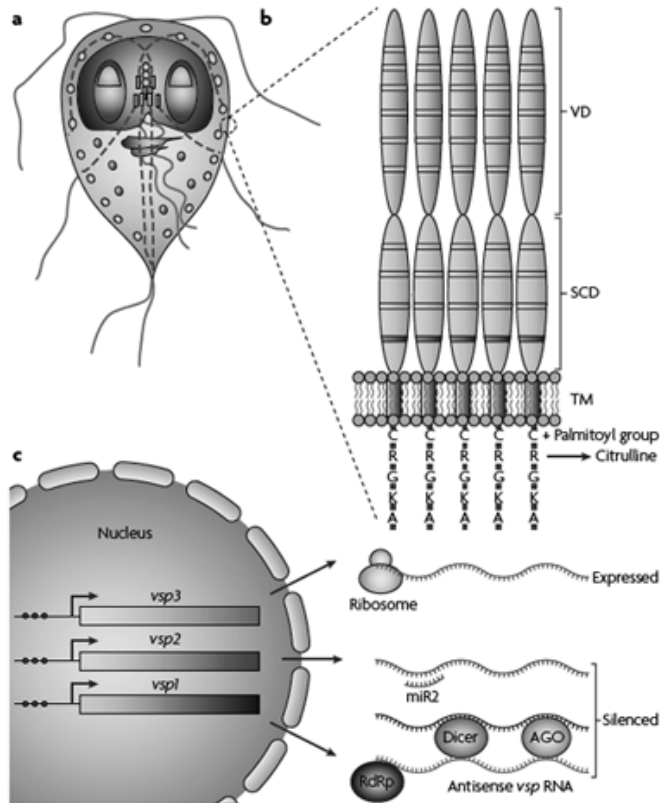
Figure 3. (a) Variant-specific surface proteins (VSPs) and their location at the surface membrane. (b) VSP structural organization, including the variable domain (VD), the semi-conserved domain (SCD) the trans-membrane (TM) region and the conserved, CRGKA-tail including the proposed sites where palmitoylation and citrullination occurs at the CRGKA-tail. (c) The different mechanisms of silencing proposed to occur with regards to antigenic variation in *G. intestinalis*. *Figure from (Ankarklev et al., 2010)*.

The switching of the surface antigen coat occurs spontaneously at approximately every six to 13 generations *in vitro* depending on the isolate and the growth conditions (Nash et al, 1990). The adaptive immunity of the host, indeed triggers switching in the expression of different VSPs. However, a selection of specific VSPs has been seen to occur in *in vivo* models where specific VSPs are selected either for or against in immunodeficient animal models (Singer et al, 2001). Müller et al., also saw a trend in the selection of specific VSPs upon antibiotic stress of the parasite (Muller et al, 2007).

Epigenetic mechanisms involved in regulating the switching of VSPs has been intensively studied and so far three major findings have been reported. Saraiya and Wang found evidence that *Giardia* likely has a snoRNA derived, miRNA mediated transcriptional silencing mechanism, as a miRNA from the GlsR17 snoRNA, miR2, was found to be complementary to the 3' UTRs of at least 22 of the VSPs from the VSP repertoire of the WB isolate (Saraiya & Wang, 2008). It has also been proposed that histone acetylation is involved in regulation of VSP expression in *Giardia* as chromatin-mediated transcriptional silencing can be reversed by histone modifications (Kulakova et al, 2006).

RNA interference, which was previously discussed in section 1.6, has been proposed to be involved in giardial antigenic variation, mechanisms involved in the RNAi pathways block or degrade mRNA that has been antisense-targeted (Ullu et al, 2004), see Figure 3c. A dicer homologue, which is the precursor-cleaving enzyme of RNAi was described in *Giardia* in 2006 (Macrae et al, 2006). Prucca et al found a strong correlation between RNA interference (RNAi) and VSP regulation, as knockdowns of *Giardia* Dicer and RNA dependent RNA polymerase (RdRp) promoted simultaneous transcription of a large number of VSPs, which was verified through nuclear run-on assays (Prucca et al, 2008). In the same experimental set up, the knockdowns of gDicer and gRdRp, were not found to affect trophozoite, growth, function, or ability to encyst. Thereby suggesting that RNAi in *Giardia* is mainly coupled to antigenic variation (Prucca et al, 2008). More recently it has also been shown that the infection of gerbils with trophozoites were a disruption of the VSP regulation has been induced leads to protection against subsequent *Giardia* infections (Rivero et al, 2010). This provides further evidence that VSP switching is essential for immune evasion, but also that disruption of VSP regualtion may provide an important tool in establishing a vaccine against *Giardia*.

1.9 Diagnosis and Treatment

The most commonly used method for diagnosing *Giardia* is through microscopy of fecal samples, directly making wet smears of fresh stool samples or after concentrating the samples using a formol-ethyl acetate protocol. Sam-

ples destined for microscopic analysis may also be fixated using sodium acetate, combined with acetic acid and formalin (SAF), for long-term storage. Microscopy-based diagnosis has been improved due to the development of a direct fluorescent antibody approach (DFA), where a fluorescent molecule is tagged on a CWP1 specific monoclonal antibody (Garcia & Shimizu, 1997).

Enzyme-linked immunosorbent assay (ELISA) is another immunoassay based diagnostic tool that enhances the rate of detection of giardiasis (Boone et al, 1999). PCR-based analysis for the purpose of *Giardia* diagnostics is seldom utilized in routine diagnostic facilities. It is however used for the purpose of genotyping *Giardia* in research laboratories, and sometimes to enable epidemiology upon outbreaks of the disease (Robertson et al, 2006). The recent establishment of real-time based quantitative PCR has however been advancing in *Giardia* diagnostics with an increasing success rate in detecting the disease (Calderaro et al, 2010; Schuurman et al, 2007). Notwithstanding the fact that the more sophisticated molecular or immuno-based technologies for detection of giardiasis yield a higher success rate, facilities necessary for this type of analyses are scarce in *Giardia* endemic areas, where the use of regular light microscopy will likely remain the foremost means of diagnosing giardiasis.

Treatment of *Giardia* infection using chemotherapeutical drugs dates back to the late 1930's where the antimalarial, quinacrine, was shown to be an effective agent in treating giardiasis (Escobedo & Cimerman, 2007). Quinacrine remained the drug of choice until the early 1960s. At this time 5-nitroimidazoles, a group of compounds that were originally discovered due to their efficacy in treating *Trichomonas* infections, was reported to have anti-giardial capabilities (Escobedo & Cimerman, 2007). Out of the 5-nitroimidazoles, metronidazole and tinidazole are the two most commonly used ones in treatment of giardiasis to date. The 5-nitroimidazoles are used in treatment in a variety of anaerobic or micro aerophilic pathogens. The nitroimidazole's mode of action is mutagenesis in the target organism, upon activation through the pyruvate:ferredoxin oxidoreductase (PFOR) pathway it binds transiently to the DNA where it induces strand breaks (Rossignol, 2010).

Chemotherapy against *Giardia* infection also includes the benzimidazole, albendazole that is commonly used in treating helminth infections (Ali & Nozaki, 2007). Nitazoxanide and tizoxanide, its derivative, have recently proven to be up to 50 times more potent on *Giardia* trophozoites than the 5-nitroimidazoles and the mebendazoles in *in vitro* trials, on *Giardia* trophozoites (Cedillo-Rivera et al, 2002). It has also shown to be effective agent in the treatment of *Giarida* infection in HIV patients with metronidazole and albendazole resistant giardiasis, which can possibly be explained due to its different mode of activation compared to the 5-nitroimidazoles and benzimidazoles (Ali & Nozaki, 2007). Additionally, nitazoxanide may have the potential of inhibiting transmission, as it has been reported to damage the outer wall of the cyst.

1.10 *Giardia* genotyping and epidemiology

Currently, molecular genotyping of *G. intestinalis* is based on analyses of one or several of the following genetic loci: small subunit ribosomal DNA, elongation factor 1-α, histone 2b, and histone 4, β-giardin, glutamate dehydrogenase and triosephosphate isomerase (*ssrDNA, ef, h2b, h4, bg, gdh* and *tpi* respectively). The first four (*ssrDNA, ef, h2b, h4*) are considered conserved genetic markers and the latter three (*bg, gdh, tpi*) are considered more variable (Wielinga et al, 2011). Since this thesis mainly focuses on *G. intestinalis* infection in humans I will only describe genotyping of the two human infecting assemblages A and B in this section.

Meta analyses of several molecular typing studies of the human infecting assemblages A and B have shown that assemblage B *Giardia* is more commonly occurring than assemblage A, in humans (Haque et al, 2005; Lebbad et al, 2008; Lebbad et al, 2011). The currently utilized loci and especially the more variable loci, provide ample discrimination of assemblage B isolates, whereas little discrimination is found among assemblage A isolates (Lebbad et al, 2011; Wielinga et al, 2011). The human infecting *G. intestinalis* assemblages (A and B) have been divided into different sub-assemblages based on sequencing results from the *bg*, *gdh* and *tpi* loci. Assemblage A is grouped into AI, AII, and AIII, where AII can be further sub-divided into AII-1 and AII-2 based on two nucleotide substitutions on the *bg* locus. AII is described to typically infect humans, AI has been found in humans, but mainly infects animals and AIII has exclusively been found in animals (Caccio & Ryan, 2008; Lebbad et al, 2010; Lebbad et al, 2011). The routinely used markers for sequence based genotyping of assemblage A provide low resolution between different A isolates, and there is a high necessity to find new molecular markers for genotyping of assemblage A *G. intestinalis*. New tools with higher resolution would highly benefit studies based on elucidating symptom causing vs non-symptom causing *Giardia* genotypes, as well as population based studies aimed at investigating zoonotic potential in assemblage A *Giardia*.

Sequence-based genotyping of assemblage B has been hampered due to a high frequency of mixed base polymorphisms, seen as double peaks at single nucleotide positions in the sequencing chromatograms (Caccio et al, 2008; Lebbad et al, 2011). Although there is a high level of difference in the sequences between different B isolates, there is currently no functional way of categorizing them into different sub-assemblages. This is due to the high frequency of mixed base polymorphisms found in the majority of sequences from clinical samples. Nonetheless, grouping of assemblage B into the BIII and BIV sub-assemblages or sub-assemblage types is still considered commonly occurring. Genotyping and sequenced based epidemiology of *G. intestinalis* will be further discussed in results and discussion.

1.11 Zoonosis

G. intestinalis assemblages A and B are responsible for both human infection as well as infections in a broad range of other mammals (Sprong et al, 2009). Genetic characterizations have been applied to assay the role of animals as transmission vehicles in human giardiasis and vice versa. Nonetheless, the zoonotic potential of *G. intestinalis* is still debated, as there may be variants within the A and B assemblages that have adapted to their respective hosts. Mouse infections with different *Giardia* strains indicate varying results in the level of pathogenesis, where the assemblage B strain, GS/M, successfully colonizes mice but the assemblage A strain, WB, shows none to very limited pathology (Chin et al, 2002; Solaymani-Mohammadi & Singer, 2011). One classic experimental infection of humans has been undertaken, using *G. intestinalis*, assemblage B, cysts from a Gambian giant pouched rat. Purified cysts were given orally to a human volunteer in parallel with oral administration of cysts from the same origin to Mongolian gerbils. Differences were noted between the human and the gerbil subjects, where the latent period was longer in the infected gerbils, the gerbils also continually excreted large amounts of cysts throughout the infection. In the human subject, intermittent excretion of a small amount of cysts was reported and the clinical symptoms in the human volunteer were mild to moderate (Majewska, 1994).

Genotyping data of human and animals infected with *G. intestinalis* suggest that sub-assemblage AI has zoonotic potential, AIII seems to be associated with animal infections. Assemblages AII and B appear to be involved in zoonosis to a lesser extent. Sprong et al., performed a meta-analysis of sequences submitted to the Zoopnet database, including 1440 animal and 978 human sequences. Single locus comparisons indicated zoonotic potential at the sub-assemblage level. However, when they performed a multi-locus genotyping (MLG) approach, including the *bg*, *gdh* and *tpi* loci, only a total of two MLGs showed zoonotic potential of the assemblage A samples and none of the assemblage B samples (Sprong et al, 2009). In order to fully assay the naturally occurring zoonotic potential of *G. intestinalis* it is of high importance to consider epidemiological parameters, such as aiming to collect samples from areas where animals and humans live in a close nit environment. This may include sampling from farm animals and farmers, hunters and wild animals, as well as pets and pet owners. It would also be necessary to use improved molecular markers and an improved MLG strategy to fully assay this important question in the *Giardia* research field.

1.12 The *Giardia* genome

As previously mentioned *Giardia* has two diploid nuclei, that contain equal amounts of DNA, and are both transcriptionally active (Kabnick & Peattie, 1990; Yu et al, 2002). During cytokinesis, or cell division, the nuclei divide in a left to right symmetry so that the daughter cells each receive one copy of each nucleus from the parental trophozoite (Yu et al, 2002). The genome of *Giardia* is similar in size to that of the fungi *Saccharomyces cerevisiae*, at approximately 12 Mbp (Morrison et al, 2007). In the first *Giardia* genome project, Morrison et al., reported the detection of 6470 open reading frames (ORFs), and transcriptional evidence for 4787 of them, and the average intergenic distance was observed to be 372 bp (Morrison et al, 2007). Pulsed-field gel electrophoresis (PFGE) analysis of several different *G. intestinalis* strains indicated the presence of five chromosomes (Adam, 2001). However, a study examining the distribution of the chromosomes in each nucleus suggests that aneuploidy may occur in *Giardia* (Tumova et al, 2007).

Generally, several of the eukaryotic processes seem to be rudimentary in *Giardia*, such as; DNA synthesis, cell division, transcription, RNA processing as well as several cytoskeletal functions, which are simplified processes compared to other eukaryotes such as yeast. *Giardia* also has a limited metabolic repertoire that includes the addition of several bacterial-like enzymes that have been introduced by horizontal gene transfer (Morrison et al, 2007).

Since the first published *Giardia* genome by Morrison et al, all subsequently published *Giardia* genome initiatives have been performed in Sweden by the Svärd group in close collaboration with the Björn Andersson group at the Karolinska Institute and the Jan Andersson group at Uppsala University. The high efficiency of this work is largely attributed to the recent emergence of the current state of the art sequencing platforms that are now readily available. These initiatives have so far generated an assemblage B (GS/M) and an assemblage E genome (Paper IV). Also, two assemblage AII genomes have been sequenced and will be discussed in the next section (Paper V), see Table 3. Comparative genomics of different *Giardia* assemblages have been possible due to the emergence of these recently sequenced assemblage B and E genomes. The assemblage B (GS/M) genome showed 77% nucleotide identity and 78% amino acid identity to orthologous proteins of the WB genome (Franzen et al, 2009). The amount of nucleotide variation observed between these two assemblages would, on the basis of the taxonomy for several other organisms, be enough to categorize assemblages A and B as different *Giardia* species.

Few assemblage specific genes were present in the core genome, which constitutes approximately 90% of the genome. The variable gene families that make up the other approximately 10% of the genome were highly variable between the two assemblages, the VSP repertoires between the two isolates were completely different. *Giardia* has a minimal kinome, where

70% belong to the NEK kinase group (Morrison et al, 2007). Certain kinases were conserved between the two isolates, whereas others were highly diverged or missing. The high cysteine membrane proteins (HCMPs) is another variable gene family that was discovered in the WB genome sequence. The HCMP gene family is similar to the VSP gene family but lacks the conserved C-terminal, CRGKA-domain. These genes have been documented to have a function in encystation (Davids et al, 2006) and during interaction with host cells (Ringqvist et al, 2011). Similar to the NEK kinases, some of the HCMPs were conserved, whereas others were highly diverged or completely missing.

Among the alpha-giardins, a *Giardia* gene family with similarity to annexins, all 21 genes present in the WB genome were highly conserved in GS/M along with their synteny in the genome (Franzen et al, 2009). An interesting finding in the GS genome was the highly elevated level of allelic sequence divergence (0.5%) compared to WB (<0.01%). Since a large number of the allelic sequence variants were non-synonymous it has been suggested that approximately 2,000 new protein variants could be generated. Other interesting findings in the GS/M genome sequence include the absence of a binding site in the promoter region for the encystation-specific transcription factor gMyb. Also a recent introduction of a genomic fragment of bacterial origin was observed in the GS/M genome, this fragment harbored two truncated genes that showed sequence similarity to bacterial genes coding for a signal recognition particle-docking protein (Ftsγ) and carboxynorspermidine decarboxylase. Phylogenetic analysis placed the two genes in the same order as the *Porphyromonas spp* (Franzen et al, 2009), a genus of bacteria that are frequently associated with humans (Kinane et al, 2008). The recently introduced genome sequencing platforms have indeed enabled a fast and efficient way to characterize and detect genetic differences between different *Giardia* assemblages. To date, *Giardia* genome sequences are available for the two human infecting assemblages and assemblage E, that infects hoofed animals. It would be of great interest for the *Giardia* field to establish futher genome sequences of other animal specific *G. intestinalis* assemblages (C, D, F, G or H). It would also be of high importance to sequence the genome of *Giardia muris*, *G. muris* is more commonly used in mouse *in vivo* experiments. However, the absence of a *G. muris* genome sequence hampers the array of experiments possible. Comparative genomics will be further discussed in the results and discussion section of this thesis.

1.13 Sex in *Giardia spp*.

Although sex, or the exchange of genetic material is considered to be fundamental in eukaryotic organisms, several ancestral lineages lack apparent indications of sexual life cycles (Schurko et al, 2009). For a long time it was

assumed that the diplomonads, including *Giardia*, are asexual (Adam, 2001). Recent genomic and population genetic data generated from studies in *Giardia*, however challenges this assumption. The first published *Giardia* genome (WB clone 6, assemblage AI), indicated a surprisingly low amount of allelic sequence heterozygosity (<0.01%) (Morrison et al, 2007). Several genotyping studies of clinical *Giardia* samples, from various global locations, do however show indications that ASH is more common in assemblages B and E (Caccio & Ryan, 2008; Lebbad et al, 2010; Lebbad et al, 2011) and genome sequencing of the assemblage B isolate, GS/M, confirms these findings (Franzen et al, 2009). It has also been suggested that different A sub-assemblages have a higher amount of ASH as compared to the WB strain (Cooper et al, 2007). Andersson recently proposed that ASH found in clinical genotyping studies as well as in genomic data to a large extent may be due to recent out crossings (Andersson, 2011). This model is based on the concept of nuclear fusion, where random chromosome reduction may lead to exchange of genetic material between the chromosomes. The progeny will subsequently reach a tetraploid stage where ASH will be eliminated over time due to gene conversions during asexual proliferation (Andersson, 2011). The model proposed here suggests the occurrence of genetic exchange in *Giardia*, but proposes a low frequency of recombinatory events between different isolates, and also indicates that ASH is accumulated through the common occurrence of an asexual lifestyle. There is no documented evidence for cell fusion in *Giardia,* which is a common trademark of sex in many other eukaryotic organisms, including yeast (Forche et al, 2008). Interestingly however, a process termed diplomixis that entails nuclear fusion, has been observed during *in vitro* encystation of *Giardia* (Poxleitner et al, 2008). It was shown that an episomal plasmid present in only one of the nuclei in the trophozoite could relocate between the nuclei during encystation. Also joint nuclear membranes were observed in encysting cells using electron microscopy, it is not clear however if this observations may have been caused by interrupted nuclear division during encystation.

Genome mining has lead to the identification of giardial homologues for 21 of the 29 genes present in eukaryotes that are known to be involved in meiotic recombination (Melo et al, 2008; Ramesh et al, 2005). Nonetheless, several important genes involved in the meiotic machinery are lacking in *Giardia*, such as the MutL homologue 3 and the MutS homologue 4, the meiotic recombination protein, REC8, along with the sister chromatid cohesion protein, PDS5 (Melo et al, 2008). Also, several genes involved in meiosis are also involved in non-meiotic processes, which leaves little guarantee that the mere presence of meiotic genes is a clear indication of the presence of meiotic recombination.

Although several recent studies suggest that sex indeed occurs in *Giardia*, further evidence is needed to confirm this, and it is necessary to find ways to

experimentally document means by which this organism exchanges genetic material. Atypical sexual events are documented in other eukaryotic microbes; parasexual cycles have been described along with hybrid progeny as a result from rare fusions of divergent cell lineages (Andersson, 2011). An example is the human opportunistic pathogen *Candida albicans* that undergoes a parasexual life cycle. Here, cell fusion is known to account for tetraploid offspring from two diploid cells, the offspring subsequently re-establishes a diploid state through random chromosome losses and where extensive gene conversions have been seen to occur. The final result includes mosaic chromosome pairs with both homo and heterozygous parts (Bennett & Johnson, 2003; Forche et al, 2008). Cell fusions have not been detected to date, and sex in *Giardia* is likely infrequent, furtive or cryptic.

1.14 *In vitro* vs *in vivo* conditions and their implications in infection biology research

The concept of parasitism involves a pathogenic organism and a host on which the organism parasitizes. An parasitic lifestyle of the gastro-intestinal (GI) tract entails several obstacles necessary for the parasite to deal with. These include; the immune system of the host, the commensal flora of the host, biochemical changes in the host microenvironment along with the potential presence of other pathogenic organisms that might directly or indirectly affect parasite survival.

A recent study assaying protein expression in the bacterium *Shigella dysenteriae* serotype 1 strain (SD1), indicated changes in expression of proteins involved in energy metabolism and virulence, based on the *in vitro* or *in vivo* origin of the cells analyzed. A differential protein abundance analysis demonstrated that under *in vivo* conditions (where gnotobiotic piglets were infected) the SD1 cells switched to a state of anaerobic energy metabolism (Kuntumalla et al, 2011). A notable increase of virulence proteins was also seen in *in vivo* derived SD1 cells, where antigens used in the invasion of the colonic epithelium as well as proteins of the Mxi-Spa type III secretion system showed increased expression (Kuntumalla et al, 2011). Studies investigating gene expression between different *in vitro* isolates of *Plasmodium falciparum* have indicated few differences. However, Daily et al recently demonstrated that alternative metabolic states of falciparum malaria do indeed occur inside their human host (Daily et al, 2007). Upon merging the expression data with a library of over 1400 yeast expression patterns three different *in vivo* transcriptional states became apparent, including that of glycolytic metabolism, a starvation response together with an alternative carbon sources metabolism, and lastly an environmental stress response. Interestingly, the data also provides evidence for mitochondrial biogenesis

and thereby a functional mitochondrion in asexually cycling *P. falciparum* parasites (Daily et al, 2007). Different host factors likely affect modifications in parasite biology and essentially, comparisons of *in vivo* strains with strains adapted *in vitro* may provide important clues with regards to factors involved in virulence and transmission, as well as potential targets for disease therapy.

A *Giardia*, *in vitro* infection model has been established, where human *in vitro* adapted intestinal epithelial cell (IEC) lines have been co-cultured with *Giardia* trophozoites (Katelaris et al, 1995; Roxstrom-Lindquist et al, 2005). However, parasite integrity is compromised in these studies due to the differences in *in vitro* growth preferences between *Giardia* trophozoites and human IECs, thus only allowing short-term interactions. Another limiting factor of this model is the *in vitro* adapted nature of the human cells commonly utilized for these experiments. Studies performed to date however utilizing this platform have generated a large amount of interesting data with regards to host expressional changes upon *Giardia* infection (Roxstrom-Lindquist et al, 2005). Here it would be highly interesting to perform similar interaction experiments using primary human IECs and perform comparative transcriptomic analyses using the previously generated dataset as a baseline.

G. intestinalis, unlike *P. falciparum*, may be efficiently studied in animal *in vivo* models, since *G. intestinalis* naturally infects other mammals apart from humans. The most commonly used *in vivo* infection models in *Giardia* research include studies in mice (Li et al, 2004; Roberts-Thomson et al, 1976; Solaymani-Mohammadi & Singer, 2011) and gerbils (Belosevic & Faubert, 1983; Benere et al, 2010; Rivero et al, 2010). Naturally host factors differ between the animal and the human host, in terms of physiology, immunity, as well as the commensal microflora. Nonetheless, this type of *in vivo* system allows experiments conducted over a longer period of time, and it is also possible to alter different host factors in order to verify their direct input on the establishment or clearance of the infection.

Also, experiments involving immune responses to *Giardia* infection as well as microbial factors from the host microflora have been successfully investigated, as previously mentioned. In total, the *in vitro* infection model, combined with the available *G. intestinalis*, *in vivo* models provide an ample platform in order to study host-pathogen interaction in *Giardia* research. It should also be highlighted that with these infection models available, the *Giardia* community should focus more attention on performing assays including both *in vitro* adapted and clinical *Giardia* strains for comparative analyses. More focus should be spent on making clinical isolates available for experimental analysis, it would also be interesting to perform comparative genomic, transcriptomic, and cell culture based phenotype assays on *Giardia* isolates that clinically induce varying symptoms in patients. Stabler et al recently performed comparative genome and phenotype analyses on three different *Clostridium difficile* strains that were clinically termed either

hypervirulent or non-endemic. Here they managed to relate several genetic differences to phenotypic differences that they observed experimentally *in vitro*, with regards to antibiotic resistance, motility, survival and toxicity (Stabler et al, 2009).

Furthermore, it would be of great interest to set up a similar approach as that performed by Daily and colleagues in comparing the expression of parasites, immediately isolated from the host (Daily et al, 2007). The isolation of *G. intestinalis* trophozoites from the human host is more cumbersome as compared to drawing blood from malaria-infected patients, due to the localization of *Giardia* in the body of the host during an infection. However, this could be established by utilizing duodenal gastroscopy. In the *Helicobacter pylori* research field, this methodology has been implemented and followed by experimental analyses (Chung et al, 2010; Maciorkowska et al, 2009). In *Giardia* research, gastroscopy could be utilized in order to isolate parasites straight from the host, as well as host cells from the site of infection. Transcriptome analyses of both the host and parasite and comparison with previously generated data from *in vitro* assays would allow even further depth in these analyses and it would likely provide further insight regarding important factors for pathogenesis, immunity and therapy of *Giardia*.

The experimental analyses suggested in this section are complex, yet manageable. These experimental set ups in combination with the recent developments and advances in the high throughput sequencing platforms will likely bring research in the *Giardia* field further towards approaching the level necessary in order to properly assay the complex networks that host-pathogen interactions entail.

2. Ethical considerations

Written informed consent was obtained from all patients or legal guardians of minor patients from León, Nicaragua, and Kampala, Uganda. Ethical consent for samples collected in León, Nicaragua, was approved from the UNAN, León, Nicaragua, as well as the Karolinska Institute, Stockholm, Sweden. Ethical approval for samples collected of human origin in Kampala was obtained through the Ethics board at the Makerere University, Kampala, Uganda, the ethics board at the University of Bergen, Bergen, Norway, and the ethical committee at the Uppsala University, Uppsala, Sweden. Human fecal samples obtained from the Karolinska Institute were approved through the research ethical committee at the Karolinska Institute.

3. Results and discussion

3.1 Scope of the thesis

The general objective of this thesis was to further the understanding of the genetic differences within the *Giardia intestinalis* species complex. The emphasis has been on the human and animal infecting assemblages, A and B, and the animal infecting assemblage E. Characterization of factors involved in virulence along with host specificity were compared between and within different *G. intestinalis* assemblages along with general comparisons at the genome level. The concept of bridging the gap between the clinic and *in vitro*-based assays in *Giardia* research was applied. Also the efficacy of the current molecular tools used in genotyping and epidemiology were evaluated and the establishment of new and improved molecular markers was anticipated.

3.2 Characterization of *G. intestinalis* in endemic areas (Papers I and II)

At the time of my installment as a PhD student, *Giardia* genotyping was still at a rudimentary stage and little was known with regards to the occurrence of the different human infecting assemblages in endemic regions. It had previously been proposed that *Giardia* infections could cause growth retardation, poor cognition, morbidity and sometimes mortality in young children in endemic *Giardia* regions (Berkman et al, 2002). Also, little was known with regards to the occurrence of *Giardia* in companion animals living in a close proximity to humans in endemic areas. Thus we initiated two genotyping initiatives, one in the New world, where samples were collected in León, Nicaragua, both from humans and dogs, the other one in the Old world, where human samples were collected from asymptomatic children in Kampala, Uganda, with and without *Helicobacter pylori* co-infection.

3.2.1 *G. intestinalis* in León, Nicaragua

Nicaragua is one of the poorest countries in the Americas and it is estimated that almost half the population live below the poverty line, and according to the Food and Agriculture Organization (FAO) of the United Nations, ap-

proximately 20% of the population are undernourished (FAO, 2008). *Giardiasis* has previously been attributed as a major problem in León, Nicaragua (Tellez et al, 1997), an area with a tropical climate and where sanitary conditions are often poor, especially in neighborhoods surrounding the city. In this study we collected 136 *Giardia* positive samples from humans, where 34 out of the 136 had diarrheal symptoms. We were able to perform molecular typing of 119 out of the total *Giardia* samples from human origin. Single or nested *bg* PCR, followed by restriction fragment length polymorphism (RFLP) analysis were performed on all 119 samples. The ratio between different *Giardia* assemblages infecting humans in this region yielded 79% assemblage B and 21% assemblage A. Samples from 100 dogs were also collected; out of the total, eight were diagnosed as *Giardia* positive. Only the canine specific assemblages, C and D, were detected thus not enabling further characterization in order to investigate potential zoonosis.

Of the human infecting isolates, 16 assemblage A and 26 assemblage B isolates, along with the eight dog isolates were additionally analyzed by sequencing at the *bg* and the *gdh* loci. The assemblage A samples from humans were all sub-genotyped as AII, although two single nucleotide polymorphism at the *bg* locus enabled further sub-genotyping, and thus yielding three A2 (AII-1), and 13 A3 (AII-2) sub-assemblages. Sequence analysis of the assemblage B samples showed a high level of mixed base polymorphism, which are seen as double peaks in the sequencing chromatograms. A total of 13 out of the 26 assemblage B samples analyzed, showed mixed bases at the *bg* locus and all sequences except two yielded mixed base patterns at the *gdh* locus. Sequencing of the dog samples also yielded mixed base patterns, but to a lesser extent than what was observed in the assemblage B samples. None of the assemblage A samples yielded mixed base patterns.

At the point in time when this study was performed MLG was a recently introduced concept in the *Giardia* field, and in order to allow for improved comparisons we later decided that a third marker should be added to the MLG strategy (Paper V). In retrospect all PCR positive samples should have been sequenced. Sequencing of the samples allows deeper comparisons as compared to RFLP analysis. When including companion animals in these types of studies it would be advantageous to collect samples from several different kinds of animals. Also, sample collection from a small community over a longer period of time would allow researchers to more closely monitor events of diarrhea and potential giardiasis in companion animals and thus increase the chances to detect potentially zoonotic genotypes.

3.2.2 Concomitant *G. intestinalis* and *H. pylori* infection in Kampala, Uganda

Few initiatives involving molecular characterization of *G. intestinalis* have been undertaken on the African continent, considering the fact that *Giardia*

is considered to be a frequent problem in this region of the world. Several parts of Africa, likely more so than many other parts of the world, are struggling with a poor infrastructure and poor sanitary conditions. According to the FAO, over 30% of children in Uganda are undernourished with stunted growth (FAO, 2010). The common occurrence of people harboring multiple infections is well documented in several parts of the African continent south of the Sahara, such as concurrent infection with worms and protozoa (Imai et al, 2011; Wilson et al, 2010), concurrent infection with different worms (Hotez et al, 2010), virus and protozoa (Moodley et al, 2002) and bacteria and protozoa (Lundqvist et al, 2010). Several cross-sectional studies have brought light on a potential correlation between *G. intestinalis* and *H. pylori* infection (Isaeva & Efimova, 2010; Moreira et al, 2005; Zeyrek et al, 2008).

We performed molecular characterization of *G. intestinalis*, in apparently healthy children with and without concomitant *Helicobacter pylori* infection, in urban Kampala, Uganda. In total 427 fecal samples were collected from children aged 0-12. The prevalence of *H. pylori* was 44.3% in the total study population and *G. intestinalis* was present in 20.1%. Clinical data showed a threefold higher risk of concurrent *G. intestinalis* and *H. pylori* infection, which highlights a correlation between these two important pathogens.

Molecular analysis of approximately half (n=45) of the *Giardia* positive samples was performed, where a sequenced based MLG strategy including three commonly applied molecular markers; *bg, gdh* and *tpi* were used. All samples were also verified using an assemblage specific PCR at the *tpi* locus, a strategy that more readily detects mixed assemblage infections. A total of 34 samples indicated positive results in the PCR, and sequencing of these samples yielded, 73.5% assemblage B, 14.7% assemblage A, and 11.8% yielded mixed assemblage infections. Important to note is that only one sample appeared as a mixed infection prior to analysis with the assemblage-specific primers, and thus highlights the importance of this type of analysis in future studies. MLG analysis of the assemblage A samples concluded that all samples were of the AII-2 sub-assemblage, thus suggesting a very high genetic homogeneity among assemblage A as was the case in León, Nicaragua.

3.2.2.1 Polymicrobial *Giardia* infections

Polymicrobial infections and the concept of synergistic polymicrobial infections, where one microbe creates a favorable environment for another have gained tremendous interest in recent years (Brogden et al, 2005). Synergistic concomitant infections with *H. pylori* have previously been described, where *H. pylori* has been suggested to ameliorate *Salmonella typhimurium* induced colitis. Also, *Schistosoma joponicum* has been shown to alter the antibody response to *H. pylori* (Du et al, 2006; Higgins et al, 2011). Other microbes have also shown to influence the proliferation of *G. intestinalis* both *in vitro* and *in vivo* (Humen et al, 2005; Perez et al, 2001). However, little is known

with regards to how *G. intestinalis* is influenced by or influences other organisms inside the host in general, and in association with *H. pylori* in particular. Thus further studies both *in vitro* and *in vivo* are necessary to validate the mechanisms by which these two important human pathogens influence one another, and which organism ameliorates colonization of the other. This could effectively be investigated as both organisms can be maintained axenically *in vitro* (Keister, 1983; Sainsus et al, 2008) and both successfully infect Mongolian gerbils (Benere et al, 2010; Matsumoto et al, 1997). *In vivo* studies would likely give an indication of which of the two pathogens, *G. intestinalis* or *H. pylori*, that benefits from the other or if an increased colonization rate goes both ways. It would also be of high significance to further investigate the influence of concomitant infections of *G. intestinalis* and other microbial pathogens for potential synergy. Equally interesting would be to investigate the influence of the commensal flora in the host in different locations of the intestine, and potential influences on the *Giardia* cell and life cycle. Differentiation from trophozoite to cyst occurs further down the intestine due to host biochemical changes, but may also likely be due to the changes in the microbiota further down the host intestine.

3.2.2.2 MLG of *G. intestinalis* assemblage A parasites

The low resolution found in the assemblage A samples when performing MLG, with the current molecular tools has greatly hampered the ability to perform phylogenetic analysis and proper epidemiology within assemblage A *Giardia*. We therefore went on to evaluate a recently developed sequencing strategy including two molecular markers, one located on chromosome 3 and one located on chromosome 5. The two genetic loci were previously published in a study assaying clinical *Giardia* samples at a location in Peru (Cooper et al, 2010). The resolution within sub-assemblage AII was slightly higher at these loci, two isolate clusters were formed at the chromosome 5 locus, which differed at four nucleotide positions, and three isolate clusters were formed at the chromosome 3 locus that differed at four positions. One Ugandan *Giardia* isolate had a unique sequence pattern at the chromosome 3 locus, which differed in one nucleotide from the publically available sequences from the study performed in Peru (Cooper et al, 2010). Despite the fact that these new molecular markers provide a slightly elevated frequency of discrimination within assemblage A, it is still of great importance that further efforts are put into generating new tools for genotyping of *Giardia* assemblage A; epidemiology, phylogeny as well as population genetics. A few recent studies have shed light on potential recombination in assemblage A parasites based on population genetics (Cooper et al, 2007; Cooper et al, 2010; Teodorovic et al, 2007). The loci used in these studies, in congruence with the three previously mentioned loci used in our studies (*bg, gdh, tpi*) all lack the necessary resolution to provide conclusive evidence regarding genetic variation and events of recombination between assemblage A isolates.

3.2.2.3 MLG of *G. intestinalis* assemblage B parasites

Sequencing of the assemblage B samples, in congruence with the findings in León, Nicaragua, yielded a high degree of sequence polymorphisms. Only nine out of 26 sequences at the *bg* locus were non-heterogeneous, the rest had one to six heterogeneous mixed base substitutions over a total of 19 positions. Only three sequences each, at the *gdh* and *tpi* loci were non-heterogeneous, the rest had two to 14 heterogeneous mixed base substitutions over 37 positions and 44 positions, respectively. At the *tpi* locus three non-synonymous amino acid substitutions had occurred, two of these resulted in a stop codon, and one indicated a change from a Tyr to a His. Also several positions with heterogeneous mixed bases implied potential non-synonymous amino acid substitutions (n=16). Genotyping of assemblage B, *Giardia* poses a major problem in the *Giardia* community. It has been suggested by us as well as others that the substitution patterns seen are due to mixed assemblage B, sub-assemblage infection, allelic sequence heterozygosity (ASH), or potentially a mixture of the two (Caccio et al, 2008; Lebbad et al, 2011; Wielinga et al, 2011). The number of heterogeneous positions appears to be more frequent when performing analysis on samples from endemic areas (Caccio et al, 2008; Lebbad et al, 2011), which is likely due to an increased rate of infection, and supports the theory of mixed sub-assemblage infections. However the recent genome project of an assemblage B, *Giardia* isolate (GS/M) showed indications of the presence of ASH in a clonal cell line (Franzen et al, 2009), and thus supports the hypothesis of ASH. To properly elucidate this question, we went on to establish a series of methods in order to analyze ASH, as well as mixed infections in clinical *Giardia* samples at the single cell level.

3.3 Allelic sequence heterozygosity at the single cell level in assemblage B *Giardia* (Paper III)

Genetic heterogeneity has become a significant problem in genotyping and molecular epidemiology of assemblage B *Giardia*, as previously described. We validated different techniques and established a pipeline in order to elucidate the origin of the heterogeneous mixed base patterns, at the single cell level. This topic was of tremendous importance for the understanding of *Giardia* biology, and essential for the interpretation of results generated in molecular sequencing projects of assemblage B *Giardia*.

We chose to utilize micromanipulation in order to isolate single trophozoites of the GS/M strain, and cysts from fecal samples from two patients infected with assemblage B, *Giardia*, and one patient that harbored a mixed assemblage A and B infection. We verified different methods for optimal extraction of DNA from single cysts and trophozoites, as it is imperative that all DNA is properly extracted in order to verify ASH at the single cell level.

A protocol based on DNA-releasy, a commercial reagent indicated the proficiency necessary when applied to *Giardia* trophozoites. ASH was indeed present, both in the single trophozoites analyzed as well as in single cysts from the clinical samples, and thus presents a highly interesting phenomenon in *Giardia* biology, as well as it suggests a revision of the currently used approaches in molecular epidemiology and genotyping of assemblage B, *Giardia*.

Development of a new strategy to analyze the data produced with the current standard markers, and thereby include sequences harboring ASH in the analysis may be an option to bypass the current problems. However, this would not rule out any uncertainty regarding the origin of the polymorphism in a given nucleotide position, despite the fact that certain polymorphic positions appear to be conserved between several isolates (Wielinga & Thompson, 2007). Another strategy could be to establish new molecular markers exclusively for assemblage B, *Giardia*, covering regions that are polymorphic between different assemblage B isolates but low in ASH. This strategy would however necessitate further genome sequencing of several different assemblage B, *Giardia* isolates. In conclusion, it is of great importance for the epidemiology and genotyping of *Giardia* to resolve this issue and to find a new strategy for molecular typing of assemblage B.

3.4 Genome sequencing of a non-human infecting *G. intestinalis* assemblage E isolate (Paper IV)

The establishment of the WB and the GS/M genome sequences allowed comparative analysis of the two human infecting assemblages A and B. However, in order to further characterize members of the *G. intestinalis* species complex and to enable detection of genetic factors that differ in non-human infecting assemblages we went on to sequence the genome of the assemblage E, P15 isolate (Table 3). Shotgun sequencing using the 454 Titanium platform (Roche) yielded an average of 47X genome coverage, and allowed an improved gene calling of genes in the variable *Giardia* gene families. A three way comparative genomic analysis, including the WB, GS/M and P15 genomes, indicated that the core genome of all three isolates is highly similar and consists of the approximated 4,557 genes presented in the initial WB genome analysis (Fig 4). An analysis of these orthologs showed that the nucleotide identity, between assemblages A and E, was 87% as compared to 77% between A and B. Thus the hoofed animal-specific isolate is more similar to one of the human isolates (assemblage A). This strengthens our hypothesis that humans are infected by two different *Giardia* species.

The G+C content was compared in conserved syntenic genes among the isolates and indicated the G+C content to be 46% (P15), 47% (GS/M) and

49% (WB). The frequency of ASH was estimated at a similar level as that found in the WB genome, which contradicts findings from genotyping studies, where a higher degree of ASH is commonly observed (Lebbad et al, 2010). Three way comparisons further revealed the presence of assemblage specific genes, where 38 genes were unique to P15, 31 genes were unique to GS/M and 5 genes were unique to WB. These genes, which are not part of the large *Giardia* gene families, do not share sequence similarity to known sequences in the public databases and their function therefore cannot be hypothesized. However one seemingly recently acquired gene, specific to the P15 isolate encodes an acetyl transferase with high sequence similarity to members of the gram-positive bacterial phylum, Firmicutes. Several members of the Firmicutes, such as *Lactobacillus, Clostridium, Anerotruncus* and *Enterococcus* commonly inhabit the gastrointestinal tract of mammals, and thus support the hypothesis of horizontal gene transfer. This discovery in congruence with that found in the GS/M genome, indicates a recent acquisition in *Giardia* of genes of bacterial origin, and brings forth the question whether *Giardia* may also be able to acquire and express genes encoding virulence, such as toxin genes, from bacterial or other origin.
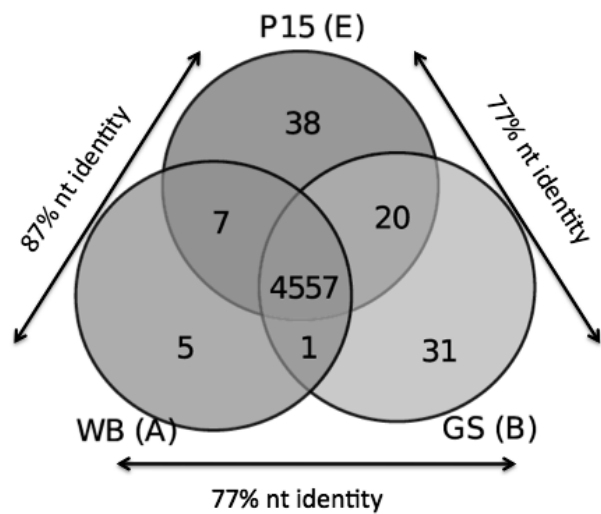
Figure 4. Venn diagram, representing the shared and non-shared gene content of the two human isolates, WB and GS/M, and the hoofed animal-specific isolate, P15. Numbers inside the diagram represent shared or unique genes. Arrows indicate shared nucleotide identity. *Figure from (Jerlström-Hultqvist et al., 2010).*

Among the variable gene families 112 complete VSPs were detected in the genome assembly in the P15 genome. However a large number of VSP sequences are present in small contigs, rendering them inept for comparative analysis. In total it was estimated that P15 has a smaller VSP repertoire than

both WB and GS/M. It was also apparent that the VSPs seldom are located in syntenic regions between the genomes, indicating a high plasticity of this gene family. In common with WB and GS/M the VSPs were found to be scattered throughout the P15 genome. Furthermore, a large number of chromosomal rearrangements were detected between WB and P15, 113 synteny breaks, as compared to 28 between WB and GS/M. VSPs were commonly found in such positions in WB and P15. Other parasites that harbor antigenic variation have shown indications that genome rearrangements are crucial components of this machinery. In *Giardia* it is likely that this plasticity is involved in promoting diversity among the VSP genes through duplications and recombination. The landscape along the *Giardia* chromosomes were found to contain long conserved stretches of gene rich regions, interrupted by regions harboring atypical codon usage and a high abundance of C+G content. VSPs and HCMPs contribute in creating such chromosomal islands. As mentioned in the introduction, the recent genome initiatives have greatly aided in the understanding of *Giardia's* biology and characteristic differences between different assemblages. Differences observed within an assemblage, namely assemblage A is discussed below.

3.5 Genomic and phenotypic comparisons within assemblage A *Giardia intestinalis* (Paper V)

3.5.1 *In vitro* isolation of *G. intestinalis* strains from human patients

Due to the lack of knowledge with regards to virulence and factors contributing to the symptoms of giardiasis, an initiative was set up aiming at establishing clinical *G. intestinalis* strains, *in vitro*, from human patients with documented *Giardia* symptoms. Samples from human patients were collected in an ongoing genotyping study between the Karolinska Hospital and the Swedish Institute for Communicable Disease Control. The study included patients whom had actively sought medical attention due to symptomatic *Giardiasis*, but also included samples from asymptomatic family members that were routinely diagnosed for the presence of giardiasis. Importantly, although medical attention was sought in Sweden, clinical data closely document recent international travels of all patients, thus indicating the origin of infection (Lebbad et al, 2011). Also, a complete clinical analysis was conducted, which minimizes potential misdiagnosis of the symptom origin recorded in the patients. This provides an advantage as compared to sampling *Giardia* from symptomatic patients in endemic areas where concomitant infections are commonly occurring, and potential contributors to the symptomatology may be difficult to diagnose.

Giardia samples were selected due to the abundance of cysts in the samples, where only high cyst loads were prepared for *in vitro* isolation. In addition, a protocol where DAPI was utilized to verify the quality of the DNA in the cyst nuclei of the clinical samples was applied. Thereby only samples with a high level of cysts with apparently undamaged nuclei were included in the isolation trials. Purification of cysts was performed according to the published sucrose gradient protocol. However, we managed to further purify the cysts by filtration through two cellulose acetate membranes. The first filtrations step was through a pore size of 12µm to further eliminate fibers and other larger debris. The second filtrations step was through a pore size of 2µm, in order to eliminate as much of the bacteria from the samples as possible. The filtration greatly decreased subsequent bacterial overgrowth in the axenization step.

The purified cyst samples were excysted using a published excystation protocol (Boucher & Gillin, 1990), but with minor modifications. Briefly, the incubation times were shortened by 10 min in both the induction and excystation steps, also incubation at 37C° was performed on a shaking heating block. All modifications had an apparent increase in the excystation of cysts from the clinical samples. Axenization of excysted parasites involved modification of the growth medium, here the TYI-S-33 standard culturing medium was utilized but with a slightly decreased pH (6.75 as compared to 7.1), and the medium was supplemented with blood serum from human patients as supposed to serum from bovine origin. These modifications yielded a general increase in proliferation of the clinical *Giardia* strains upon axenization, and were also subsequently verified to yield an increase in growth rate in the clinical strains.

Table 3. *Giardia* isolates utilized in the genome sequencing projects of this thesis

Isolate	Assemblage	Level of *in vitro* adaptation	Host details	Country of origin
WB	AI	*In vitro* adapted	Human	Afghanistan
GS/M	B	*In vitro* adapted	Human	U.S.A.
P15	E	Freshly isolated	Pig	Czech Republic
AS98	AII-2	Freshly isolated	Human	India
AS175	AII-1	Freshly isolated	Human	Sweden

In total, four assemblage AII isolates were successfully isolated and maintained *in vitro*. Some differences were observed in growth, which could be depending on the source of the blood serum. This may be linked to the fact that assemblage AII isolates preferentially infect humans, whereas assemblage AI (WB) seems to prefer non-human hosts (Caccio & Ryan, 2008; Sprong et al, 2009).

The aim of *in vitro* isolation of clinical *G. intestinalis* isolates, known to induce symptoms in humans, was to lay grounds for future comparative analysis between different clinical isolates and between clinical isolates and previously *in vitro* adapted *Giardia* cell lines.

3.5.2 Assessment of biological and phenotypic variations of different assemblage A isolates

G. intestinalis, assemblage A, sub-assemblage AII has previously been correlated with symptoms in humans (Haque et al, 2005; Sahagun et al, 2008). Also, the AII sub-assemblage has been found to be dominantly occurring in human patients infected with assemblage A, *G. intestinalis* (Caccio et al, 2008; Lebbad et al, 2011), whereas the AI and AIII sub-assemblages appear to be more or exclusively occurring in animals (Caccio & Ryan, 2008). Thus we decided to perform comparative biological and phenotypic analysis of two of our clinical assemblage AII isolates AS98 (AII-2) and AS175 (AII-1). The *in vitro* adapted WB (AI) isolate was also included in the analyses for comparison. The AS98 isolate was derived from a patient infected in India, and the AS175 isolate from a patient infected in Sweden (Table 3) and both were isolated in our laboratory as described above.

The set up was inspired by the work of Stabler et al, where phenotypic observations in *C. difficile* strains, that exhibited different levels of virulence in humans, were linked to genomic observations performed on the same bacterial strains. In our study, firstly, the growth rate at standard *in vitro* conditions was estimated, where WB and AS98 showed similar trends, but AS175 showed a lower efficiency in *in vitro* growth. This may be attributed to the lack of adaptation to the *in vitro* conditions by AS175. However AS175 showed clinical signs of therapy resistance in the patient, which may be indicative of modifications in this strain that could potentially lead to a slower growth rate. The AS175 strain did subsequently indeed show tolerance to the commonly used *Giardia* deterrent, metronidazole, and showed proliferation at concentrations up to 25μM of the drug *in vitro*. As previously described, the clinical AII strains showed a much higher rate of growth in growth media including human serum, this difference was not detected in WB, which grew equally well in both types of media.

Furthermore we assayed chromosomal differences of the three assemblage A isolates, using pulsed field gel electophoresis (PFGE). Comparisons of the two small chromosomes (chromosomes 1 and 2), which are known to be equal in size in the WB isolate (1.55 Mbp), varied significantly in the two assemblage AII isolates. The sizes of both small chromosomes in AS98 were 1.70 Mbp, whereas the chromosome sizes in AS175 indicated one at 1.55 Mbp and the other at 1.70 Mbp. Such differences have earlier been suggested to be due to recombination between rDNA in telomeric regions

(Prabhu et al., 2007). The size differences in the chromosomes were later analyzed in the genomic sequence data produced. The sequence data showed indications of potential gene duplications due to higher than average amount of reads; it would be highly interesting to further evaluate these findings, which could preferentially be done using quantitative real-time PCR. Also, it would be interesting to do a follow up analysis further verifying potential gene-family duplications in the AII isolates. Another approach would be to use optical mapping of these chromosomes and compare the results with the well-characterized chromosomes of WB in order to further verify the differences in size of the chromosomes detected in the PFGE analysis.

3.5.3 Comparative genomics of three assemblage A isolates

In order to characterize the two clinical assemblage AII isolates (AS98, AII-2 and AS175, AII-1), genome sequencing was performed using the 454 Titanium platform. The genomes of the assemblage AII isolates were compared to the previously published genome of the assemblage AI isolate, WB (Morrison et al, 2007). The divergence between AI and AII was observed to be approximately 1%, represented by approximately 100,000 SNPs. The core genome content was highly similar to that of WB, however; several protein-coding genes were found in the AII genomes that are absent in the WB reference genome (Figure 5). Here several genes shared sequence similarity with several unique genes of the GS/M, assemblage B isolate, also a REP2 viral-like replication protein and a DNA polymerase like protein were discovered. One intriguing hypothesis regarding the genes shared among the AII and the B isolate is that they may have some implications in host specificity, since both assemblage AII along with assemblage B have been closer linked to human infections compared to assemblage AI.
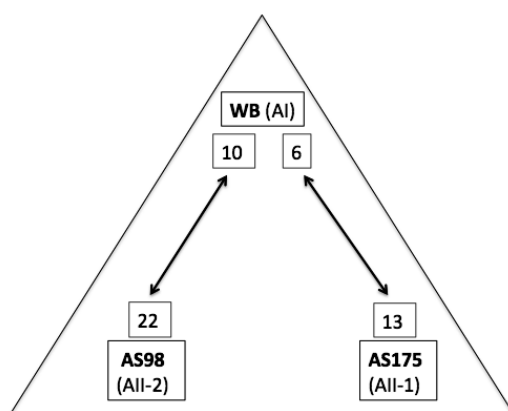
Figure 5. Diagram of the assemblage A isolates included in Paper 5, showing the repertoire of unique protein coding sequences between the two AII isolates (AS98 and AS175) and the reference AI isolate (WB).

Furthermore, the level of ASH in the two AII isolates was interestingly found to be 25 to 30 times higher than in WB (0.25% - 0.3%). Approximately 70% of these polymorphic substitutions were found in either non-coding sequence, or in the variable gene families such as the VSPs or HCMPs. Nevertheless, 30% was found in the core genome, which suggests a potential addition of different protein isoforms.

Previous genome sequencing analyses have lacked full-scale comparative analyses of the variable gene families due to the fact that theses gene families are too divergent on inter-assemblage level. Here the closer relatedness of the isolates analyzed allowed a much higher degree of comparative analyses of the variable gene families. In order to verify differences among the VSP and HCMP variable gene families between the three assemblage A isolates, the relative amount of VSP-like sequence reads were quantified using a profile hidden Markov model. This resulted in division of the VSPs into three main groups, with relatively conserved VSPs found in all three genomes. However, outgroups indicated independent evolution of VSPs in each of the three isolates. A similar trend was observed in the HCMP gene family. These results suggest that the expansion in these two gene families is due to isolate-specific duplications of certain VSPs and HCMPs followed by the divergence of the duplicated genes.

As before mentioned in this thesis, the current tools used for genotyping and molecular epidemiology of assemblage A, *Giardia* lack the resolution necessary in order to properly assay genetic variations within assemblage A. The genome sequences of the three assemblage A genomes provided us with the tools necessary to search for and evaluate new molecular markers for genotyping assemblage A, *Giardia*. A preliminary trial that included regions with high SNP variation between the WB AI-genome and the AS175 AII-genome was set up. The list included six gene fragments of genes known to be immunodominant in *Giardia*. Primers were designed for these regions and DNA from a panel of seven clinical AII *Giardia* isolates of human origin and WB were sequenced using Sanger sequencing. The polymorphisms observed between the AS175 and the WB genome were consistent, however no polymorphisms were present between the different assemblage AII isolates. This prompted us to refine the search for potential candidates, and a search was set up between the two assemblage AII genomes where the criteria was to find regions with ≥20 SNPs, within a 1 kbp region. The search yielded 107 candidates where the chromosomal locations were manually evaluated in the genomes. A final set of six genes was selected, and a nested PCR approach was designed where the primers were located in regions devoid of SNPs among all three isolates (WB, AS98 and AS175). The primers were further designed to have a high and consistent annealing temperature for all target regions. The targets included: Caffeine-induced death protein-1 like protein (CDP, Gene ID: GL50803_16863), DNA repair and recombination protein Rhp26p (DNArr, Gene ID: GL50803_87205), High Cysteine

membrane protein Group 2 (HCMP2, Gene ID: GL50803_22547), High Cysteine Protein (HCP, Gene ID: GL50803_6372), Mitotic Control Protein Dis3 (MCtP, Gene ID: GL50803_112718), NEK Kinase (NEK, Gene ID: GL50803_15411). These six gene targets were interspersed among all five chromosomes with the exception of chromosome 4. On chromosome 4, several potential regions showed nucleotide polymorphisms, but did not however meet all the criteria utilized in choosing the final candidates.

Sanger sequencing of the same seven clinical isolates and WB as in the previous trial yielded a large amount of SNP polymorphisms between the different isolates, including WB. Interestingly, although the sequences generated included over 20 SNPs depending on the target gene, isolate clusters were formed where several different isolates showed homologous substitution patterns either with AS98, AS175 or unique patterns. The WB isolate conclusively showed unique SNP patterns, but at certain loci the WB sequence had higher similarity with certain assemblage AII SNP clusters than what was found between different assemblage AII SNP patterns. Furthermore, the MLG patterns including all six loci indicate variability among the different isolates, which is possibly due to events of recombination between different assemblage A strains. This new MLG approach looks highly promising as a tool to be implemented for molecular epidemiology and population genetics for assemblage A *Giardia*. However, it clearly needs to be further verified on a larger panel of clinical *G. intestinalis* samples.

4. Concluding remarks and future perspectives

The questions raised and the work performed in this thesis has tremendously added to the knowledge regarding inter and intra-assemblage variations in *Giardia intestinalis*. Since the start in 2007, the same year as the first published *Giardia* genome, we have added; four new genomes to the *Giardia* community, improved knowledge regarding *Giardia* genotypes and molecular epidemiology and established new molecular tools for assemblage A *Giardia*. The ability to better characterize *Giardia*, and especially the human infecting assemblages is now at a much more advanced stage. Also a strong emphasis has been put on bridging the gap between *Giardia* in the clinic and experimental research of this important human pathogen. A brief summary of the major findings of this thesis is included below:

• *G. intestinalis*, assemblage B was observed to be dominant in both endemic regions investigated in this thesis project, including León, Nicaragua and Kampala, Uganda. High frequency of mixed bases polymorphisms was detected in assemblage B *Giardia* both in Nicaragua and Uganda, but sequence based analyses of assemblage A, indicated that there is a low resolution in the current genotyping tools

• A significant correlation was found between *G. intestinalis* and *H. pylori* in human patients in Kampala, Uganda

• ASH at the single cell level in assemblage B *Giardia* in concurrence with mixed sub-assemblage infection was found in clinical samples and suggests a revision of the current genotyping strategy for assemblage B *Giardia*

• Genome sequencing of an assemblage E isolate gave clues on differences between the human and non-human infecting *G. intestinalis* assemblages, it suggested a recent acquisition of a gene fragment of bacterial origin, and increased the understanding regarding the gene family involved in antigenic variation in *G. intestinalis*. It also suggests that the two human-infecting assemblages A and B are actually two different *Giardia* species

• *In vitro* isolation of fresh, clinical assemblage AII isolates enabled several biological and phenotype based comparative analyses between clinical AII isolates and the *in vitro* adapted assemblage AI WB isolate

• Genome sequencing of two of the axenized clinical assemblage AII isolates were genome sequenced and comparative genomics were performed involving the assemblage AI WB isolate. Here a comparative analysis could be made of the VSP and HCMP variable gene families, also the level of ASH was found to be higher compared to the WB isolate. Furthermore, comparative genome analysis allowed the establishment of potential new molecular markers for molecular epidemiology and population genetics in assemblage A *Giardia*

The research involved in this thesis has generated a better understanding of *Giardia* biology in general, and strategies involved in clinical *Giardia* research in particular. A large part of the thesis was dedicated to answer fundamental questions in the *Giardia* field and to provide large-scale tools to enable further research. The complex nature of certain of these projects (such as paper V) rendered them highly time consuming. However, now that several clinical isolates are axenized and cryogenically stored, along with the complete genome sequences of two of these assemblage AII isolates, future research possibilities are numerous. Several thoughts regarding future research initiatives have already been suggested throughout the thesis. Thus, I will proceed by suggesting a few projects that I find to be of great importance to the *Giardia* research field today, based on the recent discoveries described in this thesis. The projects described below have already been initiated by me and have already generated a sufficient amount of data, and thus enables certain conclusions to be drawn at this preliminary stage.

Previously our lab has set up a model for *in vitro* infection of *Giardia* on human intestinal epithelial cells (IECs). It has been utilized to assay changes in expression over time on both the human side as well as on the parasite side upon infecting the human Caco2 cell line with the *in vitro* adapted assemblage AI, *G. intestinalis*, WB isolate. With the ability to utilize these data sets for comparative transcriptomic analysis, the same infection model was evaluated for and optimized to suit the clinical AS175 assemblage AII isolate. Interaction between AS175 trophozoites, as well as WB trophozoites, and human Caco2 cells were performed at three different time points. RNA was extracted from both human cells and *Giardia* trophozoites and the Illumina platform was utilized to perform RNA sequencing. Currently, the samples have been sequenced to a sufficient sequence depth; the reads from each time point have been mapped to the respective genome and bioinformatic analyses are currently ongoing.

It will be highly interesting to investigate potential differences between the clinical and the *in vitro* adapted *Giardia* isolates and their regulation of gene expression upon human interactions. This may lead to the identification of new potential drug targets as well a better overall understanding of virulence in *Giardia*. Also, potential differences in expression of human IECs upon infection with different *Giardia* isolates will hopefully further the un-

derstanding of the host responses upon infection with *Giardia*. This interaction assay should preferentially also be followed up by using the same two *Giardia* isolates and RNA sequencing strategy, and applying it to the *in vivo* Mongolian gerbil infection models.

Furthermore, the MLG strategy, which was designed and evaluated in Paper V has been implemented in characterizing a large panel of clinical samples from a previous genotyping study (Lebbad et al, 2011). Here, 40 clinical assemblage A samples have been selected, along with five assemblage A samples from Paper II. Sequencing of the *bg*, *gdh* and *tpi* loci has previouly been performed on all isolates included (Lebbad et al, 2011). The six molecular markers proposed in Paper V, with the addition of the two molecular markers proposed by Cooper et al., 2010, have been evaluated on DNA from this large panel of clinical samples. The addition of the two loci proposed by Cooper et al., in this analysis was done in order to address the importance of consistency when performing genotyping studies, and thus to enable subsequent meta-analyses with the dataset previously produced in the study performed by Cooper et al., 2010.

The results to date are consistent with our preliminary findings in Paper V with regards to clustering of assemblage AII sub-groups, each containing a SNP rich pattern conserved within each assemblage AII cluster. Preliminary analysis of the MLG patterns among the 40 isolates with complete sequences covering all of the eight loci (6 loci, Paper V and 2 loci, Cooper et al., 2010), suggest that there might be a rather common occurrence of recombination within assemblage A *Giardia*. Also, the results produced here challenge the current classification of *G. intestinalis*, assemblage A. In terms of molecular epidemiology, these new molecular markers have so far proven to provide a much necessary new tool that may successfully be utilized in terms of source tracing during *Giardia* outbreaks. Also it will enable further analysis with regards to recombination in *Giardia* at the population level and will hopefully be useful in phylogenetic analyses of assemblage A *Giardia*.

In light of the previously described study a follow up initiative has been made where a panel of assemblage A *Giardia* from animals has been selected (Lebbad et al, 2010). Currently this study is at a very preliminary stage and as of yet it is not possible to draw any conclusions. However, the aim is to utilize the before mentioned MLG strategy in order to investigate the variation among assemblage A in animals, and in particular to further investigate the zoonotic potential in assemblage A, *Giardia*.

5. Populärvetenskaplig sammanfattning på svenska (Summary in Swedish)

Giardia intestinalis (även kallad *G. lamblia*, *G. duodenalis*), är en encellig organism som parasiterar på både människor och på djur. Det uppskattas att ca en halv miljard människor över hela världen årligen infekteras av denna parasit. Giardiainfektion, eller giardiasis, orsakar stora problem i utvecklingsländerna men parasiten förekommer även i Sverige där 1500-2000 fall dokumenteras varje år. Flera av dessa fall är dock länkade till utlandssmitta. Stora ekonomiska förluster inom jordbruket beräknas också vara en direkt konsekvens av *Giardia*, där framförallt unga kalvar drabbas hårt. *Giardias* biologi är väldigt intressant, då den encelliga organismen har två stycken cellkärnor och dubbla uppsättningar av genomet i vardera cellkärnan. Ett annat intressant fenomen hos *Giardia* är att den i likhet med vissa andra parasiter har en metod för att lura immunförsvaret genom att ofta byta ut proteinet i sitt yttre membran och på så sätt maskera sig för värdens immunförsvar. Detta fenomen kallas antigenisk variation. *Giardia* är vidare en organism av urtida påbrå och evolutionära studier har liknat *Giardia* vid "urdjuret" som en gång gav upphov till dagens alla växter och djur.

Definitionen av parasitism innefattar förhållandet där en organism utnyttjar resurser på en annan organisms direkta bekostnad. Ordet "parasit", har inom medicin och biologi kommit att delvis syfta på en grupp encelliga organismer som utgör de så kallade urdjuren (protozoerna), däribland *Giardia*, och inkluderar de protozoer som parasiterar på människor och djur. Andra organismer såsom flera olika typer av sjukdomsorsakande maskar och vissa insekter faller också under kategorin parasiter, till skillnad från sjukdomsalstrande bakterier och virus.

Två olika stadier utgör *Giardias* livscykel, ett där den aktivt förökar sig i övre delen av tunntarmen i sin värd, och ett annat stadium där parasiten kapslar in sig i ett skyddande hölje bestående av socker och protein detta vilostadium kallas för cyststadiet. Cystan är väl skyddad mot sin omgivning och det är i detta stadium som den förflyttar sig från en värd till en annan, tex genom dricksvatten, förorenade livsmedel eller direkt från en värd till en annan.

Giardiasis leder till symtom såsom kraftig, fettrik diarré, kräkningar, aptitförlust, undernäring och allmänt illamående. Magkrämpor och irriterad eller inflammerad tarm kan i vissa fall vara ihållande symtom en tid efter en

giardiainfektion. Det har även dokumenterats att *Giardia* kan leda till mycket svåra symptom hos barn i utvecklingsländer men även hos individer med nedsatt immunförsvar. Det har dock visat sig att giardiainfektion kan leda till svåra symtom eller inga symtom alls, men individer som inte uppvisar symtom kan fortfarande sprida sjukdomen vidare.

Giardia intestinalis består av åtta olika grupper eller genotyper vilka är namngivna från A till H. Vissa av dessa orsakar infektion hos specifika värddjur såsom hundar (C/D), klövdjur (E), katter (F), gnagare (G), eller marina däggdjur (H). Två grupper (A och B) orsakar infektion hos flera olika typer av däggdjur och däribland människan. I nuläget finns det inte mycket dokumenterad information kring varför vissa individer får starka symptom medan andra inte får några symptom alls vid giardiainfektion. Man vet även lite om vad det är som gör att vissa typer av *Giardia* bara infekterar specifika värdar medan andra har en bredare repertoar samt om det är vanligt förekommande att infektion sprids från djur till människa och vise versa. Dessa kunskapsluckor ledde till de grundläggande frågeställningarna inför min avhandling. Vid den tidpunkten fanns det dåligt med tillgänglig data för att kunna utföra jämförelsestudier och på så sätt dra slutsatser kring skillnader i arvsmassa mellan olika genotyper av *Giardia* men även hos isolat från samma genotyp. Den här avhandlingen behandlar frågor kring en del kliniska aspekter så som molekylär epidemiologi och genotypning av *Giardia*, men även storskaliga kartläggningsstudier av *Giardias* arvsmassa där komparativa studier visar tydliga skillnader mellan olika typer av den sjukdomsalstrande parasiten *G. intestinalis*.

För att undersöka förekomsten av olika genotyper av *Giardia* samt öka förståelsen om kliniska aspekter kring sjukdomen giardiasis initierades två studier i endemiska områden, det vill säga geografiska områden där förekomsten av *Giardia* är vanlig (Projekt I och II). Vi valde att undersöka patienter i León i Nicaragua, samt unga barn i Kampala, Uganda. Man visste sedan tidigare att risken för giardiainfektion är hög i dessa områden, men man viste innan våra studier väldigt lite om spridningen av olika genotyper i dessa områden. Vi valde också att undersöka hundar som potentiella smittkällor för *Giardia* i León, samt *Giardia* i relation till den smittsamma bakterien *Helicobacter pylori*. *H. pylori* är vanligt förekommande över hela världen, men den är framförallt vanlig i utvecklingsländerna och har tidigare blivit länkad till magsår, förändringar i mage och tarm samt i vissa fall cancer. Gällande förekomsten av olika genotyper i dessa endemiska områden så upptäckte vi att genotyp B är mest vanligt förekommande hos människan, och hos de hundar vi undersökte i León bar samtliga på genotyper som endast infekterar hunddjur. Hos de individer vi undersökte i Kampala, fann vi en stark korrelation mellan giardiainfektion och förekomsten av *H. pylori*. Vidare så konkluderade vi att de verktyg som generellt används för genotypning av *G. Intestinalis* inte möter den nödvändiga standard som krävs för att kunna utföra betydande genotypning och molekylär epidemiologi. Vidare så fann vi att samma molekylära verktyg inte hade den upplösning som krävs

för att man på ett bra sätt ska kunna särskilja olika varianter inom genotyp A. Dessa upptäckter ledde delvis till de kommande tre studierna i min avhandling.

Förekomsten av de polymorfier som tidigare beskrevs i genotyp B (Paper I och II) har spekulerats kunna vara beroende på en väldigt hög grad blandinfektioner av olika typer av genotyp B, eller beroende på skillnader i arvsmassa i en och samma cell vilket är möjligt då varje *Giardia* har flera uppsättningar av sin arvsmassa. Vi initierade en studie där jag använde en metod som kallas mikromanipulering för att isolera enskilda parasitceller (Paper III). Dessa undersökte vi sedan på molekylärnivå och upptäckte att förekomsten av blandinfektioner inom genotyp B är hög hos en och samma patient men också att en hög grad skillnader inom arvsmassan även förekommer inom en cell. Dessa resultat har hög relevans gällande epidemiologi för *Giardia*, och våra resultat visar att de nuvarande verktygen som finns tillgängliga måste förbättras markant. Utöver det påvisar vår data ett spännande biologiskt fenomen, nämligen att *Giardia* vanligen har flera olika varianter av samma gen på cellnivå. Detta kan innebära att de olika genvarianterna kan koda för olika proteiner, vilket i sin tur innebär att *Giardias* proteinrepertoar är större än man tidigare trott. Belägg för detta har också hittats i ett nyligen publicerat genomprojekt från vår forskargrupp.

För att vidare karaktärisera och undersöka skillnaden mellan olika genotyper inledde vår grupp flertalet initiativ för att kartlägga arvsmassan (genomet) på flera olika genotyper av *G. Intestinalis*. Metoden för att göra detta kalas sekvensering. Då jag inledde mina doktorandstudier fanns ett publicerat *Giardia*genom, detta tillhörande ett genotyp A isolat. Sedan dess har vår grupp helgenomsekvenserat ett genotyp B isolat, ett genotyp E isolat (Paper IV) och två genotyp A isolat (Paper V). Genomsekvensering av genotyp E isolatet ledde till att vi kunde utföra komparativa genomikstudier mellan de två humaninfektiösa genotyperna A och B med ett isolat som man vet inte infekterar människan. I denna studie lyckades vi identifiera gener som är unika för de olika genotyperna och vilka därmed är mycket intressanta att studera vidare med hänsyn till patogenes för de olika genotyperna. Här kan man till exempel tänka sig att unika gener som finns hos de olika genotyperna kan vara kopplade till värdspecificitet. Vidare fann vi att arvsmassan mellan de två humaninfektiösa genotyperna A och B skiljer sig i högre grad än vad genotyp A skiljer sig från den klövdjursspecifika E genotypen. De gener som är sammankopplade med antigenisk variation skiljde sig i hög grad mellan de tre olika genotyperna vilket med största sannolikhet är ett tecken på att dessa ändras relativt snabbt över tid baserat på ett högt selektionstryck från värdens immunförsvar.

I den andra komparativa genomstudien (Paper V) ville vi karaktärisera skillnader hos *Giardia*isolat inom den humaninfektiösa A genotypen. I detta projekt finns flera högintressanta variabler att räkna in. Det första publicerade A genomet inom *Giardia*forskning tillhör subgruppen AI, vilket isolerades från en människa. Det finns dock flera genotypningsstudier och där-

ibland våra från Nicaragua och Uganda som har länkat subgenotyp AII som mest vanligt förekommande i infektioner hos människor, medan AI verkar vara länkat till infektioner hos djur i en mycket högre utsträckning. Genom ett samarbete med Karolinska sjukhuset och smittskyddsinstitutet i Stockholm samlade vi patientprover från patienter som aktivt sökt vård för giardiasjukdom. Jag lyckades isolera och kultivera flera av dessa så kallade kliniska prover i labbmiljö och två prover som båda var av subgenotypen AII genomsekvenserades därefter. Komparativa studier med dessa två AII isolat utfördes mot AI genomet. Här fann vi att dessa genom skilde sig med närmare en procent vilket är en större skillnad än man tidigare spekulerat om. Genfamiljen som är involverad i antigenisk variation kunde vi här jämföra på ett sätt som inte tidigare var möjligt då vi jämfört olika genotyper. Vissa av dessa gener var till en hög grad konserverade medan andra skilde sig markant, och kan ses som ett tecken på att olika grupper av dessa gener har olika funktioner inom *Giardias* biologi. Vissa av dessa gener är troligtvis fundamentala för parasitens överlevnad i värdens tarmmiljö eller har särskilda, mer fundamentala funktioner för den generella biologin, medan andra troligtvis i högre grad är länkade till att lura värdens immunförsvar. Flera gener var unika för AII isolaten jämfört mot AI och vissa av dessa var lika de unika gener som tidigare upptäckts i genotyp B genomet vilket kommer att bli mycket spännande att undersöka i större detalj. Vidare så ledde genomsekvenseringen till att vi kunde identifiera regioner mellan de tre olika genotyp A genomen som hade en hög grad av variation. Dessa regioner har visat sig vara bra kandidater för att användas inom genotypning och molekylär epidemiologi av *Giardia*. Vi har i nuläget analyserat ett stort antal kliniska genotyp A isolat från patienter och skillnaden är drastisk i jämförelse med de tidigare använda genotypningsverktygen för *Giardia*.

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NADA BAR, The no1 Bar in Stockholm, Sweden and the World!!
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