Chapter 2

Review of literature
2. Review of literature

The microaerophilic *Giardia lamblia*, a unicellular, gastrointestinal flagellated protozoan causes one of the most frequent parasitic infections worldwide (Adam R. D., 2001). It lacks conventional mitochondria, Golgi body and peroxisomes. An estimate of 280 million symptomatic human infections has been reported every year (Lane S. & Lloyd D., 2002). The symptoms of giardiasis are watery diarrhea, abdominal pain, irritable bowel syndrome, nausea, vomiting, weight loss and it appears 6-15 days after infection (Farthing M. J., 1997). The disease symptoms have been observed to be more profound in malnourished children and in immunodeficient individual. Metronidazole or other nitroimidazoles comprises the common treatment options. *Giardia* species cannot invade the gut and it secretes no well-known toxin but recent data put forward that *Giardia* increases intestinal permeability by augmenting apoptosis of the inner cell lining of intestine (Scott, K. G. *et al.*, 2002) (Singer, S. M., & Nash T. E., 2000). Due to its potential as a zoonotic pathogen, farm animals get infected hampering the economic yield (O’Handley *et al*., 2001). Even though this infection is chronic nearly half of it is asymptomatic and subsides down by itself (Farthing M J, 1997). It has been put forward that certain gastrointestinal disorders like irritable bowel syndrome can be related to a previous *Giardia* infection (Hanevik *et al*., 2009). Therefore, the symptomatology differs from person to person. The protist genus *Giardia* is a member of the diplomonads, which is a group of binucleated flagellates that are now classified as part of the super group Excavata (Simpson A. G, 2003). Being one of the most divergent eukaryotes examined till date it presents us with unique opportunities to have a better understanding into the key pathways that characterize eukaryotic cells as well as new molecular mechanism within them. *Giardia lamblia* acts as a good model system due to its unique and unusual ultra structure, simple *in vitro* differentiation, sequenced genome and a metabolism resembling that of a bacterium (Lauwaet T. *et al*., 2007). Reductive processes in *Giardia* combined with its parasitic lifestyle play a vital role in its survivability and at the same time highlighting its evolution. *Giardia* is of widespread biological interest because of its early evolutionary position and also because of its striking adaptation to two very different and hostile environments. The flagellated trophozoite form specifically colonizes the small intestinal lumen of human, and the dormant cyst form survives in cold fresh water (Gillin F. D. *et al*., 1996). *G. lamblia* is a micro-aerophilic organism, which does not usually tolerate elevated oxygen pressure. In the upper intestinal cell lining, where this organism generally resides, the oxygen (O₂) concentration there has been measured at 60µM (Raj D. *et al*., 2014). In addition
with this, the amitochondriate parasite lacks some of the conventional enzymes of detoxifying reactive oxygen species (ROS), such as superoxide dismutase (SOD), catalase, peroxidase, glutathione reductase. A recent finding suggest that *Giardia* which is highly susceptible to oxygen tension favor to reside in the proximal small intestine than that to colon as the later has a higher redox buffering capacity (Mastronicola *et al*., 2011). The detail mechanism is unknown until today by which the parasite could aid in the detoxification of ROS produced during an oxidative stress. Cysteine is neither synthesized de novo nor from cystine and is thought to be imported inside the cell by passive diffusion, although active transport has also been accounted for some of the attainment of cysteine (Lujan *et al*., 1994). The toxicity of thiol-blocking agents unable to penetrate intact cells demonstrated the significance of free thiol groups on the surfaces of trophozoites (Gillin *et al*., 1984). *Giardia* is a micro-aerophilic parasite infecting all vertebrates that lacks mitochondria but contains mitosomes (apparent relic mitochondria). This organism is a eukaryote and possesses many typical characteristics such as a distinct nucleus and nuclear membrane, endomembrane system and cytoskeleton; however, other aspects of the cell such as SSU rRNA and some key metabolic enzymes are prokaryotic-like (Svard *et al*., 2003). Although controversial, majority of investigators thinks *Giardia* which has diverged during transition of mitochondrial acquisition has made it an important organism to understand the evolution in eukaryotic cells (Thompson, 2004). The *Giardia* genome project has highly enriched our knowledge about this intriguing organism.

### 2.1. History of the discovery of *Giardia*

*Giardia lamblia*, the intestinal protozoan with a long and venerable history of about 300 years, remains even today, a biological enigma. The great Dutch microscopist Aton Van Leeuwenhoek, who recovered it from his own stool in 1681, is credited for its discovery (Dobell, 1920). The first reports on the morphology of *Giardia* are those of Lambl (1859 and 1860) and Cunningham (1881). A lack of agreement regarding nomenclature in this group has existed from the time this organism was first described and continues to result in confusion. The nomenclature problems involve both genus and species. Two generic names, *Giardia* and *lamblia* are presently used to describe these organisms. The genus name *Giardia* was established by Kunstler (1882). However, Blanchard (1888) suggested that the genus be named *lamblia* to commemorate Lambl (1859). It was Stiles (1915) who named this parasite as *Giardia lamblia*.  

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2.2. Systemic position of Giardia

Conventionally, all living organisms have been classified as prokaryotes or eukaryotes, and some still argue for retaining both the characters (Mayr, 1998). However, the most widely accepted classification now utilizes three major divisions, Archaea (archaebacteria), Bacteria (eubacteria), and Eukarya (eukaryotes) (Woese et al., 1990), which can then be divided into kingdoms. With either classification system, G. lamblia is clearly a eukaryotic organism and has been considered a member of the protozoa, the more "animal-like" of the unicellular eukaryotes. These protozoan organisms have traditionally been classified by their morphology into flagellates, ciliates, amoebae (rhizopods), and sporozoans. Thus, G. lamblia was classified with the flagellated protozoans, including the kinetoplastids (e.g., Leishmania spp. and Trypanosoma spp.), parabasalids (e.g., Trichomonas vaginalis), and Dientamoeba (e.g., Dientamoeba fragilis) (Levine et al., 1980). Giardia has been placed in the order Diplomonadida (two karyomastigonts, each with four flagella, two nuclei, no mitochondria, and no Golgi complex; cysts are present, and it can be free-living or parasitic) and the family Hexamitidae (six or eight flagella, two nuclei, bilaterally symmetrical, and sometimes axostyles and median or parabasal bodies) (Fig. 2.1).

Interestingly, a phylogenetic tree of one of the genes thought to be of mitochondrial origin (cpn60) also suggests that Giardia is an early-branching eukaryote (Fig. 2.2) (Germot et al., 1996; Roger et al., 1998). Iron-dependent hydrogenases of Giardia lamblia give evidence for lateral gene transfer (Nixon et al., 2002, 2003). Recently the triose phosphate isomerase gene has been found to be a very useful genetic marker for the population genetic structure of G. lamblia (Li et al., 2000; Lu et al., 2002). Thus, most of the data suggest that G. lamblia and the other diplomonads are among the most basal of the extant eukaryotes.

<table>
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<th>Taxonomic classification:</th>
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<tr>
<td>Kingdom: Protista</td>
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<td>Subkingdom: Protozoa</td>
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<td>Phylum: sarcomastigophora</td>
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<td>Order: Diplomonadida</td>
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<td>Family: Hexamitidae</td>
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<tr>
<td>Genus: Giardia</td>
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<td>Species: lamblia</td>
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**Figure 2.1:** Classification of Giardia
Over a decade ago, population genetic studies of *Giardia* in endemic communities where the frequency of transmission is very high, found evidence of occasional bouts of genetic exchange in the parasite (Meloni et al., 1995). These authors demonstrated multiple banding patterns in a number of isolates of *Giardia* by allozyme electrophoresis which if a true reflection of the underlying genotypes of the isolates would seem to indicate that *G. lamblia* is functionally diploid, and that recombination or sexual reproduction must have occurred at some stage to produce the apparent heterozygotes (Meloni et al., 1995). These observations have been supported by more recent population genetic studies (Cooper et al., 2007) and molecular analyses which further challenge the idea that *G. lamblia* is a strictly clonal.

**Figure 2.2**: Multikingdom tree inferred from 16S-like rRNA
asexual organism by providing evidence of recombination between homologous chromosomes within and between assemblages (Lasek-Nesselquist et al., 2009). It has also been demonstrated that *Giardia* has maintained some meiotic machinery, the ability of chromosomes to cross-over and some recombination sites (Ramesh et al., 2005; Poxleitner et al., 2008).

It has been suggested that the identification of recombination between assemblages suggests a shared gene pool and calls into question whether it is appropriate to divide the genetically distinct assemblages of *G. lamblia* into a species complex. However, sexual reproduction has never been observed in *Giardia* which could be explained by extremely infrequent sexual reproduction (Ankarlev et al., 2010), as suggested by the population genetic data (Meloni et al., 1995; Cooper et al., 2007). The evolutionary advantage that genetic exchange gives to *Giardia* is the capacity to respond to adversity, for example selection pressures imposed by regular exposure to anti-*Giardia* drugs or competition with co-habiting “strains” in circumstances where the likelihood of mixed infections is common (Hopkins et al., 1999). Thus it may well be a relatively rare event and further population genetic studies are required in foci of infection where the frequency of infection is high. The fact that available data indicates that the genetic assemblages of *Giardia* are conserved in terms of geographic location and host occurrence suggests that any recombination is not reflected at the assemblage and species level.

Recent studies have demonstrated genetic recombination between different isolates of *G. lamblia* (Caccio et al., 2005), which will confuse the interpretation of results based on a single locus. This means that future studies of the phylogeny of *G. lamblia* will continue to require the use of multiple loci. Genotyping studies for epidemiology will also likely require the use of more than one locus to ensure correct identification.

### 2.3. Genotypes of *G. lamblia*

Molecular classification tools have been of great importance in understanding the pathogenesis and host range *Giardia* isolates obtained from humans and a variety of other mammals (Table 2.1). The first study of the molecular differences of *G. lamblia* isolates (Bertram, 1983) was a zymodeme analysis of five axenized isolates, three from humans, one from a guinea pig, and one from a cat, using six metabolic enzymes. In 1985, restriction fragment length polymorphism analysis of 15 isolates was performed using random probes (Nash, 1985). These studies resulted in the description of three groups; group-III was so
different from groups I and II that the suggestion of a separate species designation was made. Subsequently, a number of other molecular classification studies have been performed. Pulsed-field gel electrophoresis (PFGE) chromosome patterns have also been studied (Campbell et al., 1990; Korman et. al., 1992; Isaac-Renton, 1993) but are of limited value for classification because of the frequent occurrence of chromosome rearrangements (Adam 1991; Le Blancq et al., 1991). Likewise, classification by surface antigens (Nash et al., 1985) is limited by antigenic variation of the variant-specific proteins (VSPs) (Nash et. al.,1990, Adam, 2001). These studies have been very useful, but the conclusions that can be drawn from these types of data are limited by the semi-quantitative nature of the data. To allow a more quantitative comparison of Giardia isolates, sequence comparisons of the small-subunit rRNA, triosephosphate isomerase (tim), and glutamate dehydrogenase (GDH) genes have been utilized in a number of subsequent studies (Baruch et. al., 1996; Ey et. al., 1997; Lu et al., 1998; Monis et al., 1996, 1998, 1999, 2003, 2009).

It has characterized and compared the polyadenylated transcriptomes of assemblages A, B and E (Franzen et al., 2013). Analyses of genome content are not dependent on closed genomes or even megabase-sized contigs. If sequencing libraries are random, greater than 95% of the genome is sampled at 3–4x coverage. The genome properties of two Assemblage B strains, GS and 12BC14, and one Assemblage E isolate, P15, have been found. These three genomes were generated using 454 pyrosequencing to approximately 16X coverage. The major findings reported by Franzen et al. indicated that WB and GS share ~78% amino acid identity in protein coding regions and contain very few genes unique to one or the other genome. Although P15 is a non-human isolate, it too contains a very similar gene complement and the same large gene families. The common multigene families are ankyrin-repeat protein 21.1, NEK kinases, VSPs and other high-cysteine proteins, coiled-coil proteins, spindle pole proteins, and endonucleases. Sampling appears to be robust for each of the three isolates, since the components of metabolic pathways are similar and highly conserved groups such as the tRNA synthetases and ribosomal structural proteins are present in expected numbers. However, their antigenic repertoires are very different, which likely explains the differences in epidemiology.
Table 2.1: *Giardia* Genotypes; Adapted from “*Giardia: A Model Organism*” by Lujan & Svard’ 2011

2.4. Cell biology in different stages of life cycle

Life cycle of *G. lamblia* has two stages, the vegetative trophozoite stage and the infective cyst stage. Infection normally follows ingestion of cysts in fecally contaminated water or less commonly via foods (Fig. 2.3) (Adam, 1991). Excystation is activated by the low pH (Bingham et al., 1979) of the gastric acid in the stomach and stimulated by the slightly alkaline pH and proteolytic activity of the fluid in the duodenum (Rice et al. 1981). This key
step in infection is dependent upon a cysteine protease stored in peripheral vesicles of the trophozoite and released into the space between trophozoite and cell wall during excystation (Ward et al., 1997). After excystation, the emerging parasite quickly divides into two equivalent binucleate trophozoites that attaches and specifically colonize the human small intestine. They swim (Ghosh et al., 2001) in the lumenal fluid with four pairs of flagella and also adhere to mucus strand in vivo and in vitro (Adam, 1991). They also penetrate the mucus layer to attach to intestinal epithelial cells via their unique ventral adhesive disc (Nemanic, 1979). In this position they feed on the content of the host gut not by phagocytosing large particles such as bacteria but through micropinocytotic vesicles. Here they multiply by binary fission. When the trophozoites are carried downstream by the flow of the intestinal fluid they encyst, as they could not survive outside the host. The elevated pH and high bile concentration of the intestinal lumen induces the encystation and cyst antigen synthesis (Gillin et al., 1988; Reiner et al., 1989). The most visible overall change during encystation is that trophozoite gradually round up and detaches, lose mobility and become retractile (Fig. 2.4).

![Life cycle](http://www.cdc.gov/dpdx/)

**Figure 2.3:** Morphology and Life cycle of *Giardia sp.*
2.4.1. Trophozoite Structure

The *G. lamblia* trophozoites are pear-shaped and are approximately 12 to 15 µm long and 5 to 9 µm wide. The cytoskeleton includes a median body, four pairs of flagella (anterior, posterior, caudal, and ventral), and a ventral disk (Fig. 2.5). Trophozoites have two nuclei without nucleoli that are located anteriorly and are symmetric with respect to the long axis. Lysosomal vacuoles, as well as ribosomal and glycogen granules, are found in the cytoplasm. Golgi complexes become visible in encysting trophozoites but have not been confirmed to be present in vegetative trophozoites (Gillin *et al*., 1996). However, stacked membranes suggestive of Golgi complexes have been demonstrated (Soltys *et al*., 1996; Lanfredi-Rangel *et al*., 1999).

2.4.2. Cytoskeleton and Ventral Disc

Trophozoites colonize the small intestine of their host, predominating in the mid-jejunum. They attach by their concave ventral surfaces (ventral adhesive disk) to the intestinal wall, where they obtain the necessary nutrients and avoid transport beyond the jejunum. The ventral disk mediates a mechanical attachment not only to the intestinal wall but also to the surface of the container used for axenic growth. Neither cellular invasion nor receptor-mediated

**Figure 2.4:** Giardial cysts are exposed to gastric acid during their passage through the host's stomach, triggering excystation.
attachment has been documented for *Giardia* spp. Therefore, the cytoskeleton and especially the ventral disk play a key role in the survival of the organism in the intestine of the host (Elmendorf *et al*., 2003). The disk contains the contractile proteins actinin, -actinin, myosin, and tropomyosin (Feely *et al*., 1982) as the biochemical basis for the contraction of the disk involved in adherence. Attachment depends on active metabolism and is inhibited by temperatures below 37°C, increased oxygen levels, or reduced cysteine concentrations (Gillin *et al*., 1981, 1982).

### 2.4. 3. Flagella

The trophozoite has four pairs of flagella that begin at two sets of basal bodies that are near the midline and anteroventral to the nucleus. They emerge from the anterior, posterior, caudal, and ventral regions of the trophozoite. Paraflagellar rods extend along one side of the two ventral flagella (Feely *et al*., 1990; Holberton *et al*., 1973). Nine pairs of microtubules encircle two microtubules to form the flagella. The flagella appear to be important for motility but not for attachment. In addition, their early emergence through the cyst wall during the process of excystation suggests their importance in excystation (Buchel *et al*., 1987). Annexin XXI has shown to be specifically localized in the flagella. (Szkodowska *et al*., 2002).

### 2.4. 4. Median Body

The median body is a component of the cytoskeleton that is located in the midline and dorsal to the caudal flagella and consists of a group of microtubules in a tight bundle. It is unique to *Giardia* spp., and its morphology helps define the morphologic characteristics of the different *Giardia* species. *G. lamblia* trophozoites typically have two median bodies that are shaped like claw hammers. Very recent studies have suggested that *Giardia lamblia* has specialized membranes with electron transport and membrane-potential-generating functions (Lloyd *et al*., 2002) supporting the growing evidence that *Giardia* may not be primitive, but is derived from an aerobic, mitochondria-containing flagellate.
Figure 2.5: Cross sectional transmission electron micrograph of *G. lamblia* trophozoite. LC: lateral crest, VLF: ventrolateral flange, VD: ventral disk, N: nucleus, F: flagella, V: vacuole. In the lower panel a higher magnification shows the microtubules (mt) and microribbons (mr).
2.4. 5. Nuclear Structure and Replication

*G. lamblia* trophozoites have two nuclei that are nearly identical in appearance. They replicate at approximately the same time (Wiesehahn *et al.*, 1984) and are both transcriptionally active as determined by uridine incorporation into nuclear RNA. Both the nuclei are partitioned equationally at cytokinesis (Yu *et al.*, 2002) and have approximately equal numbers of rDNA genes as determined by *in situ* hybridization using the rDNA probe. Both have approximately equal amounts of DNA as determined by the intensity of nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) (Kabnick *et al.*, 1990) or propidium iodide (Bernander *et al.*, 2001), although the possession of equal amounts of DNA by the two nuclei has been questioned. It is generally assumed that the two nuclei have the same complement of genes and chromosomes, and results using fluorescence *in situ* hybridization with single-copy genes support this assumption.

2.4. 6. Mitosome

*Giardia* is a member of the diplomonads, often described as an ancient protist group whose primitive nature is suggested by the lack of typical eukaryotic organelles (for example, mitochondria, peroxisomes), the presence of a poorly developed endomembrane system and by their early branching in a number of gene phylogenies (Williams *et al.*, 2002; Riordan *et al.*, 2003). The discovery of nuclear genes of putative mitochondrial ancestry in *Giardia* (Roger *et al.*, 2002) and the recent identification of mitochondrial remnant organelles in amitochondrial protists such as *Entamoeba histolytica* (Koonin, 2003; Gray, 2005) and *Trachipleistophora hominis* (Gribaldo *et al.*, 2002) suggest that the eukaryotic amitochondrial state is not a primitive condition but is rather the result of reductive evolution. Using an *in vitro* protein reconstitution assay and specific antibodies against IscS and IscU—two mitochondrial marker proteins involved in iron–sulphur cluster biosynthesis—have been demonstrated that *Giardia* contains mitochondrial remnant organelles (mitosomes) bounded by double membranes that function in iron–sulphur protein maturation (*Fig. 2.6*). The results indicate that *Giardia* is not primitively amitochondrial and that it has retained a functional organelle derived from the original mitochondrial endosymbiont (Tovar *et al.*, 2003).
2.4. 7. Cyst Structure

Cysts are approximately 5 by 7 to 10 µm in diameter and are covered by a wall that is 0.3 to 0.5 µm thick and composed of an outer filamentous layer and an inner membranous layer with two membranes. The cyst wall of *Giardia* contains proteins and a novel N-acetylgalactosamine (GalNAc) polysaccharide, which is its major constituent. GalNAc is not present in growing trophozoites, but is synthesized during encystment via an inducible pathway of enzymes that produce UDP-GalNAc from fructose 6-phosphate. All of the genes responsible for UDP-GalNAc synthesis during encystment are induced at the transcription level. (Lopez *et al.*, 2003).

2.4. 8. Cell cycle

On the basis of the conventional flow cytometric analysis of the DNA content of encysting cells, early and late cysts, and cells after excystment, a model illustrating the relationship between the *Giardia* cell cycle and life cycle has been proposed (Bernander *et al.*, 2001). During its life cycle, a *Giardia* cell passes through two periods of genome replication that alternate with two phases of nuclear division. In a binucleate flagellated trophozoite, DNA replication (S phase of the cell cycle) precedes mitotic division and cytokinesis (M phase), and both phases are segregated by gap periods (G1 and G2). The trophozoite thus undergoes a canonical cell cycle that allows for the multiplication of a pathogenic stage of the parasite in the intestine. Differentiation into a cyst occurs in cells that have already replicated their DNA and are in G2 phase, the longest period of *Giardia* cell cycle (Bernander *et al.*, 2001; Hofstetrova *et al.*, 2010). A restriction point for initiating encystation is located in G2, though what determines the readiness of the G2 cell to encyst is unknown (Reiner *et al.*, 2008). During encystation, the two nuclei divide, without intervening cytokinesis, forming four cyst nuclei (a possible variant of endomitosis), and DNA content is then duplicated (Bernander *et al.*).
Mature *Giardia* cysts resemble a quadrinucleate syncytium with a replicated genome (Erlandsen and Rasch, 1994). In accordance with other *Giardia* species, except for *G. microti*, with daughter individuals fully developed and separated inside the mature cyst (Feely, 1988), cytokinesis proceeds as late as after excystment. Thus, the gap period between DNA replication and cytokinesis can be as long as several weeks or even more, depending on when the cyst has a chance to excyst. Meanwhile, the cyst must be able to survive in different external conditions, including conditions that could damage the genome, e.g., UV light. The ability of cysts to repair UV-damaged DNA has been reported (Li *et al.*, 2008) and might be a reason why putative meiosis genes involved in DNA doublestrand breaks (DMC1A, SPO11 and HOP11) are expressed in cyst nuclei, although a role for these genes in the mitotic recombination of chromosomes in fused *Giardia* cyst nuclei during diplomyxis has been suggested (Poxleitner *et al.*, 2008). What happens with a quadri nucleate cell during and after excystment is also unclear. One hypothesis, based on a combination of FACS and microscopy data, is that two rapid, consecutive cell divisions produce four trophozoites with a basic level of genome ploidy from a single cyst (Bernander *et al.*, 2001).

### 2.4.8.1 Cell Division of *Giardia* Trophozoites

![Figure 2.7: Schematic representation of cell division in *Giardia*.](image)

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Giardia has a haploid genome size of 12 Mb, with five chromosomes of sizes from 1.4 to 3.4 Mb (Adam, 2000). Each nucleus is thought to be diploid, thus the Giardia trophozoite is essentially a “double” diploid (Bernander et al., 2001). A Giardia trophozoite divides asexually by binary fission; this has been well known for more than 50 years (Filice, 1952). Although different models regarding the plane of cytokinesis have been suggested in the past (Yu et al., 2002; Benchimol, 2004), it is only recently that a combination of findings regarding the course of mitosis (Kulda & Nohynkova, 1995; Tumova et al., 2007) flagellar transformation (Nohynkova et al., 2006) and cytokinesis (Tumova et al., 2007) enabled the true mechanism to become clear. These studies involved monitoring division in live cells and imaging using immunofluorescence, transmission, and scanning electron microscopy. During division, a parent cell splits in the anterior-posterior direction such that the two daughter cells separate ventral-to-ventral in the plane of the daughter adhesive discs. In this way, the left-right asymmetry of the Giardia cell is maintained (Kulda Nohynkova, 1995; Sagolla et al., 2006; Tumova et al., 2007), as exhibited by the counter clockwise (viewing the cell from the ventral side) spiral layer of the disc microtubules. There is also evidence of differences in ploidy between the two nuclei within one cell, as well as between strains in different assemblages (Tumova et al., 2007). When dividing, Giardia alternates between attached and free-swimming phases according to the particular phase of division and the adhesive competence of the parental or newly assembled daughter discs. The whole process can be divided into three phases (Tumova et al., 2007): (1) the initial attachment phase, which proceeds in the adherent parent cell, (2) the free-swimming phase and (3) the terminal attachment phase, which occurs in adherent progeny. During the initial phase, the cell completes the main processes dealing with the duplication of cell structures, namely reorganization of the parent flagella, assembly of new ones, mitosis, and disassembly of the parent ventral disc; the cell also starts to assemble new daughter discs. Cytokinesis is initiated at this phase with the selection of the site of cell division and the start of furrow ingression, which continues in the free-swimming phase. In the terminal attachment phase, abscission occurs and leads to the physical separation of the two cells. Whereas the initial and the free-swimming phases are very short, about 3 and 1 min, respectively, the terminal phase takes up to 50 min and represents the longest phase of the division process (Tumova et al., 2007). Overall, the description of the basic cellular architecture of the specific phases of Giardia cell division is almost complete. However, the molecular basis for these processes and the mechanisms behind their regulation and control mostly remain to be uncovered.
**Mitosis**

Though mitosis and cell division in *Giardia* has recently been quite a controversial topic, it has been investigated at some level for over 50 years (Felice, 1952). The recent description of mitotic stages in *Giardia* using cytological markers, however, confirms that the major cytologic events of mitosis proceeds in the same manner as described in many organisms. *Giardial* mitosis begins with condensation of chromatin (prophase), followed by congression of chromosomes to the spindle midzone (metaphase), the movement of chromosomes to the spindle poles (anaphase A), the separation of spindle poles (anaphase B), and the eventual duplication of flagella and the ventral disc prior to cytokinesis (see details below). Both spindles are bipolar arrays of microtubules with attachments to chromosomes at kinetochores.

**Mechanism of Chromosome Segregation and Mitosis**

Mitotic spindles in protists have been classified using several cytologic features: the continuity of the nuclear envelope during mitosis (open, semi-open, and closed); the position of the spindle relative to the nuclear envelope (pleuromitosis vs. orthomitosis); and the position of chromosomes relative to the spindle axis (Raikov, 1994). In most plants and animals, a complete breakdown of the nuclear envelope occurs during prophase, allowing microtubules direct access to chromatin. As with some fungi, most protists have a closed mitosis, retaining an intact nuclear envelope with an intranuclear spindle (Schuster, 1975; Winey et al., 1995; Ogbadoyi et al., 2000; O’Toole et al., 2003). *Giardia*, in contrast, has a “semi-open” mitosis, a variant of closed mitosis in which kinetochore microtubules from the two extra-nuclear spindles penetrates the nucleus through polar openings in the nuclear envelope.

**Prophase:**

Chromosome condensation, nuclear repositioning, spindle nucleation, and assembly. Extensive chromosome condensation, spindle nucleation/assembly, and nuclear repositioning define the prophase stage of mitosis in *Giardia* trophozoites (Cerva & Nohynkova, 1992; Sagolla et al., 2006). One of the earlier aspects of prophase in *Giardia* is chromosome condensation. Individual Giardial chromosomes can be resolved following chromatin condensation in mitotic prophase. The assembly of the kinetochore on centromeric chromatin and the attachment of the kinetochore to the spindle microtubules are both required for accurate chromosome segregation during mitosis. In other eukaryotes, the centromeric
foci are required to build the mitotic kinetochore by recruiting motors, checkpoint proteins, and additional structural elements (Van Hooser et al., 2001). Presumably this also happens in Giardial prophase, as the centromeres are clearly visible on condensed chromosomes (Sagolla et al., 2006). Spindle nucleation and assembly in *Giardia* occur shortly after chromosome condensation. During Giardial prophase, the nucleation of the two spindles is made apparent by the appearance of MTs between the two nuclei near the flagellar basal bodies. The MTs of the mitotic spindles then extend around each nucleus. Spindle microtubules further elongate during nuclear migration, until each spindle encompasses each nucleus by the end of prophase. Spindle nucleation is mediated by microtubule organizing centres or MTOCs (Fig. 2.7).

**Metaphase:**
Upon completion of prophase nuclear migration, the microtubules surrounding each nucleus form two complete and independent bipolar spindles, one dorsal and one ventral (Fig. 2.7). The opposing poles of each spindle are oriented along the left-right axis of the cell with the chromatin clustered tightly in the centre of each spindle axis. It remains unclear as to whether there is a canonical metaphase alignment of centromeres along a metaphase plate (although this may be present as a short transitory stage).

**Anaphase A and B:**
Chromosome segregation in both nuclei occurs in two stages: chromatid segregation in anaphase A, followed by spindle elongation along the left-right axis of the cell in anaphase B (Fig. 2.7). In *Giardia*, (anaphase-A) initiates first in one nucleus; however, anaphase B occurs simultaneously in the two nuclei. It is unclear which nucleus initiates mitosis or whether initiation always occurs in the same nucleus. During anaphase A, the centromeres localize to the leading edge (near the spindle pole) of the segregating DNA, a behaviour that implies kinetochore attachment to microtubules, rather than some novel mechanism of chromosome segregation. Sister chromatids from each nucleus are segregated to opposite sides (L–R) of the cell, as a result of both nuclear migration and lateral chromosome segregation. Spindle elongation is characteristic of anaphase B, and in *Giardia* this is visualized by the elongation of the nuclei to the extreme L–R sides of the cell, with the nuclear envelopes remaining intact. Centromeric foci remain tightly clustered together at the spindle poles during anaphase B. Centrin foci remain at the spindle poles throughout anaphase A and B (Sagolla et al., 2006). Recently, it has been found that a single mitotic
cyclin in *Giardia* is essential for progression into mitosis. This is the first example of mitosis not regulated by the APC and may reflect an evolutionary ancient form of cell cycle regulation (Gourguechon *et al.*, 2013).

**Telophase:**
Despite differences among diverse eukaryotes in terms of the timing and mechanism of mitosis, the cell division plane is essentially always perpendicular to the axis of chromosome segregation as defined by the mitotic spindle (Balasubramanian *et al.*, 2004). *Giardia* cytokinesis—occurring in the longitudinal plane perpendicular to the spindle axis— is consistent with patterns of cytokinesis in other eukaryotes.

A hallmark of telophase is the presence of a microtubule bundle with unfocused ends that extends between the nuclei, replacing the bipolar spindle arrays (Fig. 2.7). This structure could represent the remaining spindle microtubules following the loss of focused spindle poles, or could arise from *de novo* microtubule polymerization. Centromeres remained clustered in telophase nuclei, based on observations using cenH3::GFP localization in the two daughter nuclei. The two centrin foci on each side of the nuclei move from their anaphase position near the cell periphery to their position between each pair of nuclei as seen in interphase. By the onset of cytokinesis, the DNA decondenses and centromeric foci are visible throughout each nucleus. After karyokinesis, nuclei migrate to their interphase positions in the two daughter cells, prior to cytokinesis. The studies imply that: (1) there is no nuclear fusion during *Giardia* mitosis; and (2) daughters inherit one copy of each parental nucleus in each generation.

**Implications of Mode of Mitosis on Nuclear Inheritance and Heterozygosity**
Beyond establishing the plane of cytokinesis, the mode of *Giardia* cell division has important implications that bear on the maintenance of a unique genetic identity for each nucleus. The genetic content of the two nuclei is presumed to be identical (Kabnick & Peattie, 1990; Yu *et al.*, 2002), and *Giardia intestinalis* assemblage A has been shown to possess a low level (<0.1%) of genetic heterozygosity (Baruch *et al.*, 1996; Lu *et al.*, 1998). Other assemblages including assemblage B have higher (Franzen *et al.*, 2009) or similar levels of heterozygosity (Jerlstrom-Hultqvist *et al.*, 2010) as compared to assemblage A. Inheritance of both copies of the left or right nucleus by one daughter could explain low levels of heterozygosity by eliminating sequence differences in each generation (Yu *et al.*, 2002), but still does not explain ploidy differences (Tumova *et al.*, 2007a). In contrast, inheritance of one copy of both left and right nuclei would preserve any genetic differences between the two nuclei.
among the daughter cells. Canonical meiosis, including the presence of characteristic cytologic evidence such as a synaptonemal complex, has not been directly observed in *Giardia* trophozoites. Alternatively, the inheritance of genetic material from both nuclei may somehow be important for cell survival.

**Division of Microtubule Cytoskeleton**

Just as the nuclei undergo mitotic division in order to maintain cell identity in the progeny, the cytoskeleton, comprising eight flagella, the funis and the adhesive disc, must also be duplicated. Similar to nuclear division, the division of the complex cytoskeleton remained unclear until recently. Recent studies (Nohynkova *et al*., 2006; Tumova *et al*., 2007a, b) have shown that, in a rapid sequence of events that takes less than three minutes, the interphase flagellar apparatus undergoes extensive reorganization, resulting in the transformation of all but the caudal parent flagella to different flagella in the progeny.

**Division of the Flagellar Apparatus**

The basic features of the complex flagellar apparatus of diplomonad *Giardia* are well known and have been reviewed recently (Dawson & House, 2010). It comprises four flagella types: anterior, posteriolateral, ventral, and caudal. As seen in diplomonad cell architecture, each type is represented by two bilaterally symmetric flagella and defined by its location, arrangement, associated structures, and likely functions. Pairs of flagellar basal bodies form two symmetric clusters (tetrads) localized side by side in between the anterior poles of two nuclei. Together with the pertinent flagella, two microtubular root fibers and some other fibrillar appendages, the tetrad forms a structural unit called a mastigont (Kulda & Nohynkova, 1995). When *Giardia* divides, eight parent flagella persist. The parental flagella are inherited by daughter cells in a semi-conservative manner, such that each progeny receives four flagella from the parent cell that are supplemented by four newly arisen flagella to form a complete daughter set. Until recently, it was generally accepted that each daughter *Giardia* inherits the same parent flagella set (one flagellum from each parent pair, i.e., one mastigont). This idea regarding uniform distribution was based on observations showing that the plane of cytokinesis cleaves the dividing cell between segregated mastigonts (Filice, 1952; Cerva & Nohynkova, 1992; Kulda & Nohynkova, 1995). Other models of cytokinesis did not follow the partitioning of flagella or intuitively expected symmetric segregation.
Parent Flagella Distribution

By using monoclonal antibodies for acetylated and polyglycylated tubulins in combination with scanning and transmission electron microscopy, recent work has shown that, before cytokinesis, two parent mastigons exchange half of their flagella components (Nohynkova et al., 2006). This means that in contrast to the general assumption, each daughter receives half of each of the two mastigons, not one of the two mastigons. As a consequence of the arrangement of basal body pairs in tetrads, each of two daughters inherits a different set of four parent flagella. This work has also demonstrated that *Giardia* flagella undergo a maturation process.

Transformation of Parent Flagella during Division

During *Giardia* division, flagella of the parent anterior and caudal pairs are segregated equally between progeny; these flagella are distributed one-to-one per daughter (Fig. 10.2). The flagella of the parent ventral and posteriolateral pairs, on the other hand, are segregated unequally; one daughter receives both ventral flagella, and the other receives both posteriolateral flagella. This is why each daughter inherits a different parent flagella set. Except for the caudal ones, all parent flagella transform into different flagella types in the progeny. The flagella undergo extensive mitotic reorganization, mediated by the reorientation, migration, and segregation of their paired basal bodies, whereby the respective flagella change positions. The parent anterior flagellum transforms into a daughter caudal flagellum in each progeny. Transformation is achieved through exchanging the position of the flagella basal bodies. The basal bodies reorient and it migrate left-right towards opposite cell sides; the left basal body moves toward the right side, while the right basal body moves toward the left side of the cell. This basal body migration results in the gradual pulling of the intracytoplasmic portions of the pertinent axonemes inside the cell, leading to a disjunction of anterior crossing of the axonemes and, finally, to the exchange of the flagella exit positions with respect to interphase. The transformed flagellum joins a segregated parent caudal flagellum to form a daughter caudal flagella pair.

De Novo Assembly of Daughter Flagella

Daughter ventral (V) and posteriolateral (PL) flagella pairs are always formed *de novo* from basal bodies newly assembled during mitosis. Each parent basal body induces the formation of a new one. Due to the configuration of the *Giardia* basal body pairs, each basal body pair in the progeny and, consequently the interphase cell consists of one old (parent) and one new
basal body. Duplication of *Giardia* basal bodies seems to follow the conservative process of centriole/basal body duplication seen in other cells (Loncarek & Khodjakov, 2009). First, each new basal body arises in proximity to a parent basal body, indicating that it is formed by the duplication of an existing structure (Nohynkova, unpublished data). Second, although pairs of *Giardia* basal bodies are not orthogonally arranged in interphase, the new basal body is likely to be formed at right angles with the parent organelle following stringent orientation, which is characteristic of centriole duplication (Tsou & Stearns, 2006; Azimzadeh & Bornens, 2007). This canonical mechanism strictly limits the number of newly assembled basal bodies to only one per parent basal body and cell cycle via an, as yet, not fully understood restriction process (Loncarek & Khodjakov, 2009). Similar control apparently also exists in *Giardia*.

**Maturation of Flagella**

As a consequence of parent flagella transformation and *de novo* flagella assembly, the flagella in each *Giardia* progeny and, therefore, in each interphase cell, have different chronologic ages. Ventral and posteriolateral flagella are the youngest because they are newly formed during mitosis. Anterior flagella are one cell cycle old; they come from the transformation of either a ventral or posteriolateral flagella pair. The right caudal flagellum is old; it is formed through the transformation of a previous anterior flagellum. The left caudal flagellum, the oldest flagellum within the cell, is three or more cell cycle old. This flagellum represents the final stage in the development of a *Giardia* flagellum corresponding to a mature flagellum.

**Developmental Asymmetry of Microtubular Roots of Caudal Flagella**

The caudal pair composed of unequally aged flagella (the oldest and second oldest) is another consequence of the flagellar maturation process. This generational asymmetry, which is exclusive to the caudal basal bodies/flagella, seems to be essential for the maintenance of the left-right cell asymmetry that is phenotypically manifested by a ventral disc. This single-copy component of the *Giardia* cytoskeleton is derived from a microtubular basal body root. Of the two root fibers carried by each caudal basal body in diplomonads, the anterior root is asymmetrically developed in *Giardia* (Brugerolle, 1991).
**Ventral Disc**
Whereas persistent parent basal bodies represent templates for new basal bodies formed in a conservative fashion, new daughter discs are assembled independently of the parent structure, which disintegrates simultaneously with their formation. As a modified microtubular root, the new disc is always formed *de novo*.

**Cytokinesis**
Cytokinesis is the final event of cell cycle that divides one cell into two daughter cells (Fig. 2.7). Although details concerning how the process proceeds differ between eukaryotes (e.g., the position of the division site is determined by the mitotic spindle in animals, the bud-neck in budding yeast and by positioning of the nucleus in fission yeast), the plane of cytokinesis is always perpendicular to the axis of the segregated chromosomes. In animals and yeast, partitioning of mother cell into two daughters is achieved via constriction by the actomyosin ring (Barr & Gruneberg, 2007).

In *Giardia*, the plane of cytokinesis has been a point of controversy for decades. Incongruous models have been proposed (Kabnick & Peattie, 1990; Ghosh *et al.*, 2001; Solari *et al.*, 2003), either based on single observations (Solari *et al.*, 2003; Benchimol, 2004) or derived theoretically from other observations, e.g., single nucleus labeling of episomal DNA by FISH in interphase cells (Yu *et al.*, 2002). Most of these models could not explain the duplication of other cell systems, namely the nuclei and flagellar apparatus.

Coordination is achieved by coupling the initiation of cytokinesis with the inactivation of mitotic cyclin-dependent kinases. During the free-swimming phase of *Giardia* division, the cleavage furrow progresses in the anterior posterior direction, between the caudal axonemes of daughter cells, are splitting the detached parent cell along the longitudinal body axis. For this phase, membrane biosynthesis is crucial. Inhibition of the synthesis of sphingolipids, essential components of eukaryotic membranes, blocks the progression of furrowing (Sonda *et al.*, 2008).

Mechanistically, the two daughter cells separate ventral-to-ventral (“face-to-face”) with two-fold rotational symmetry (biradial, similar to the ying-jiang symbol), which is most obvious from the counterclockwise winding of the ventral discs in both daughters (Tumova *et al.*, 2007). The most unusual event in *Giardia* cytokinesis occurs when, after swimming, progeny that are connected tail-to-tail adhere to the substratum via their newly assembled adhesive discs and crawl apart (moving away from one another), while still connected by a narrow intercellular cytoplasmic bridge (Fig. 10.6). The bridge gradually elongates and
finally breaks (cracks) in the middle (Tumova et al., 2007). Physical forces generated from the opposite movement of the daughter cells may contribute to abscission. How the locomotion is driven and whether there are any specific severing proteins at the site of abscission remain to be identified.

It is evident that, although much progress has been made in recent years toward an understanding of the division of cells, nuclei, and the cytoskeletal apparatus of *Giardia*, much remains to be learned about the molecular background of these processes and their control mechanisms.

### 2.5. Genetics and Molecular Biology

#### 2.5.1. Genome Structure

The genome of *G. lamblia* has the features expected of eukaryotic cells, including linear chromosomes flanked by telomeres that are similar in sequence to those of other eukaryotes (TAGGG) (Adam et al., 1991). Chromosomal DNA of eukaryotes forms chromatin by associating with four core histones (H2a, H2b, H3, and H4) and a linker histone (H1) to form nucleosomes. *G. lamblia* has all four of the core histones, and these histones are very similar to those of other eukaryotes and have no particular similarity to the histone-like proteins of the Archaea (Wu et al., 2000).

Bands that stained more faintly with ethidium bromide were shown to be size variants of chromosome 1, first by using several chromosome-specific probes (Adam et al., 1988) and then by more detailed mapping of the various size variants. These studies showed that chromosome 1 varied in size from 1.1 to 1.9 Mb and that the size variation occurred in both subtelomeric regions (Adam 1992, Hou et al., 1995). Previous studies had shown that tandem repeats of the rDNA (ribosomal DNA) unit were adjacent to the telomeric repeat region (TAGGG) (Adam et al., 1991, Le Blancq et al., 1991, 1992) and were frequently involved in subtelomeric rearrangements. The rDNA repeat is found on different chromosomes among isolates of the same genotype (Adam, 1992). The rDNA repeats are found on one end of chromosome 1 of the ISR isolate, and about 30% of the size difference is accounted for by the difference in rDNA copy number (Adam, 1992). The remainder of the size difference appears to be due to differences in repetitive DNA near the other telomere. The sum of the sizes of the chromosomes (3.8, 3.0, 2.3, 1.6, and 1.6 Mb) (Adam et al. 1988) yields an estimated haploid genome size of 12.3 Mb, similar to the size estimate of 10.6 to 11.9 Mb obtained by densitometric analysis of the NotI fragments of *G. lamblia* DNA (Fan et al., 1991).
The *Giardia lamblia* (WB strain, clone C6) is 12 Mb, organized in 5 distinct chromosomes (chromosome polymorphism has been identified in different strains). Chromosome sizes range from 0.7 to over 3 Mb. The sequencing strategy is shotgun sequencing. These values of approximately 12 Mb are compatible with the estimates obtained by analysis of the *G. lamblia* genome data (www.mbl.edu/Giardia).

For any organism with a ploidy greater than one, a certain degree of allelic heterozygosity is expected. The degree of heterozygosity is generally low in sexual organisms because of meiotic recombination but can become very high in asexual organisms, as has been demonstrated for the rotifers (Welch *et al.*, 2000). Since *Giardia* spp. are assumed to be ancient asexual organisms, one might expect a very high degree of allelic heterozygosity. However, the degree of allelic heterozygosity in *G. lamblia* is actually quite low. The reason for this low degree of heterozygosity has not been determined. Potential reasons could be unrecognized sex in *Giardia* or intermittent loss of a nucleus.

### 2.5. 2. Transfection

The development of transient and stable-transfection systems has contributed substantially to our understanding of the genetics of *G. lamblia*. The initial description of a transfection system consisted of transient expression of luciferase flanked at the 5' end by a short region of the GDH gene and at the 3' end by the putative polyadenylation signal. In this and in subsequent transfection systems, DNA was introduced by electroporation. Subsequently, stable episomal transfection was performed using the puromycin-resistance *pac* gene or the neomycin resistance *neo* gene as selectable markers. Apparently, the bacterial plasmids used as the framework for the transfection vectors contained sequences that functioned as origins of replication in *G. lamblia*.

### 2.5. 3. Transcription and Translation

Transcription in *G. lamblia* is distinctly eukaryotic in nature; nonetheless, it has a number of features that are more characteristic of prokaryotes such as amanitin-resistant transcription using RNA polymerase II (Seshadri *et al.*, 2003). As in all eukaryotes, the transcript is produced in the nucleus and transported to the cytoplasm for translation. The polyadenylation of transcripts is typical for eukaryotes, but the short 5' UTRs and general lack of introns are more characteristic of prokaryotes (although introns may be relatively infrequent in the unicellular eukaryotes). Also, in contrast to other eukaryotes, most *G. lamblia* transcripts do not appear to be capped at their 5' ends.
The short 5' UTRs suggested the possibility that *G. lamblia* promoters might be located near the beginning of the open reading frames. This possibility was supported by the observation of a set of highly conserved motifs in the 5'-flanking regions of a number of cytoskeletal genes. Bi-directional promoter for divergent transcription of a PHD-zinc finger protein gene and a ran gene has also been reported in *Giardia* (Ong *et al.*, 2002). The consensus sequence AATTAAAAA was identified at the site of transcription initiation with the actual initiation site located at the TA (or CA). A second region 20 to 35 nucleotides upstream from the transcription initiation site, CAAAAAA(A/T)(T/C)AGA(G/T)TC(C/T)GAA was proposed as a promoter region, and a third consensus sequence, CAATTT, was found at 40 to 70. When the upstream region was tested in a transfection assay, a 44-bp sequence of the GDH gene provided maximal transcriptional activity, confirming that the GDH promoter was short and located near the coding region. A deletion and mutational analysis of the GDH promoter region confirmed the importance of the AT-rich region at the initiation site as well as a CAAAT region 34 bp upstream (Yee *et al.*, 2000). Specific binding of the immediate 51-bp upstream region to a 68 kDa nuclear extract protein was demonstrated by band shift analysis and by UV cross-linking (Yee *et al.*, 2000). A deletion and mutational analysis of the ran promoter demonstrated that maximal promoter activity was present in the region from -51 to -2 and that regions further upstream did not contribute to promoter function. The most important component for promoter activity was the -51 to -20 region; smaller portions of that region gave reduced promoter activity.

Transcripts have been analyzed to determine whether they have 7-methylguanosine or other caps at the 5' ends. Total polyadenylated RNA was analyzed to determine if the 5' ends were susceptible to T4 RNA ligase (Yu *et al.*, 1998). They were resistant to 5' phosphorylation unless pretreated with calf intestinal alkaline phosphatase to remove the 5' phosphates. These results suggested that the RNA was phosphorylated but not capped. Treatment with the decapping enzyme tobacco acid pyrophosphatase did not increase phosphorylation, also indicating a lack of 5' capping. These results suggest that most polyadenylated RNA does not have a 7' methylguanosine cap. However, since the studies were done using total rather than transcript-specific mRNA, it is still possible that individual transcripts could be capped. In fact, for the differentially processed transcript of the glucosamine-6-phosphate isomerase B gene, the constitutive transcript with a short 5' UTR is not capped while the transcript with the longer (146-nucleotide) 5' UTR expressed during encystation does have a cap, as demonstrated by RNA ligation after treatment with tobacco.
acid pyrophosphatase (Knodler et al., 1999). Whether this was a 7-methylguanosine or another type of cap was not determined.

Small nuclear RNA molecules are involved in the splicing of nuclear pre-mRNA (snRNA U1, U2, U4, U5, and U6) and pre-rRNA (sno-RNA U3, U8, U14, snR10, and snR30) to produce the mature RNA (Niu et al., 1994). Many of these molecules from other eukaryotes have a trimethylguanosine cap at the 5' end. A number of candidate snRNAs from *G. lamblia* were immunoprecipitated by anti-trimethylguanosine antiserum; caps were confirmed by the susceptibility of the antibody reactivity to the decapping effect of tobacco acid pyrophosphatase. The exact roles and identities of the candidate snRNAs have not yet been determined. It will be of especial interest to determine whether splicing of mRNA occurs since introns have not been identified in the *G. lamblia* genes, although, very recently Nixon *et al.* has proposed a spliceosomal intron in *Giardia lamblia* (Nixon *et al*., 2002).

The initial characterization of the rRNA and rDNA genes revealed that the rRNA molecules were much smaller than the typical eukaryotic 18S (SS rRNA) or 28S or large-subunit rRNA (LS rRNA) molecules and in fact were even slightly smaller than the 16S and 23S *E. coli* rRNA molecules. The rDNA repeats were correspondingly small, but had an organization and sequence consistent with that of eukaryotes. In addition, other components of the translation apparatus, including elongation factor 1, elongation factor 2, eRF1, eRF3, RPB1, and RNA polymerase III, are distinctly eukaryotic.

### 2.5. 4. Transposons

Mobile genetic elements, by virtue of their ability to move to new chromosomal locations, are considered important in shaping the evolutionary course of the genome. They are widespread in the biological kingdom. Among the protozoan parasites several types of transposable elements are encountered. The largest variety is seen in the trypanosomatids. Three families of transposons elements were detected in the WB isolate of *G. lamblia* currently being used for the genome sequencing project. Two of these are subtelomeric in location while one is chromosome-internal and dead (Arkhipova *et al*., 2001). Nucleotide sequence analysis of all the elements shows that they are all retrotransposons, and all of them are non-long-terminal-repeat retrotransposons (Bhattacharya *et al*., 2002). These elements are clearly distinct from all other previously described non-LTR lineages. Phylogenetic analysis indicates that these Genie elements (for *Giardia* early non-LTR insertion element) are among the oldest known lineages of non-LTR elements consistent with strict vertical descent (Burke *et al*., 2002).
Although most copies have accumulated mutations, they can potentially encode reverse transcriptase, endonuclease and nucleic-acid-binding activities (Bhattacharya et al., 2002).

2.6. Protein Transport and Degradation

2.6.1. Endomembrane protein transport system

Although structures consistent with endoplasmic reticulum (ER) had previously been identified by electron microscopy (EM), there was some doubt until recently about the existence of ER in G. lamblia. However, the cloning and characterization of SR, as well the identification of an extensive membrane system labeled with antibody to BiP (Soltys et al., 1996), has clearly demonstrated the existence of the ER.

Golgi complexes have not been detected in trophozoites by standard microscopic techniques but have been demonstrated in encysting organisms (Reiner et al., 1990). More recently, transmission and freeze fracture EM of nitrobenzoxadiazole (NBD) ceramide-labeled log phase trophozoites demonstrated heavy staining in the perinuclear region in a pattern similar to that of the Golgi complex from other organisms (Lanfredi-Rangel et al., 1999). However, further investigation is required to reconfirm the presence of a Golgi complex in trophozoites and to determine its characteristics (Lujan et al., 2003). Specific importation of proteins into the nuclei has also been documented by using the simian virus 40 nuclear localization signal to direct green fluorescent protein (GFP) to the nuclei (Elmendorf et al., 2000). The cellular metabolism of Calcium ion and its channeling is regulated by the concerted operation of several transporters present in the plasma membrane, endoplasmic reticulum and acidocalcisomes (Moreno et al., 2003).

2.6.2. Endosome-lysosome vacuoles

Most eukaryotes have a system of endosomes and lysosomes that degrade and recycle endogenous proteins or those acquired by endocytosis or phagocytosis from the extracellular space. Early endosomes internalize endocytosed proteins to allow for their subsequent return to the cell membrane or transport to late endosomes (or, alternatively, maturation of early into late endosomes) followed by transport to and degradation by the lysosomes. Both are acidic, with an endosome pH of <6 and a lysosome pH of 5. Trophozoites have numerous vacuoles encompassing the periphery of the cell, which fulfill at least some criteria of endosomes and lysosomes. These vacuoles are acidic, as shown by their uptake of acridine orange (Feely et al., 1991; Kattenbach et al., 1991). They concentrate exogenous ferritin and lucifer yellow,
suggesting their potential role in endocytosis (Lanfredi-Rangel et al., 1998). *G. lamblia* virus particles appear to be concentrated into the vacuoles by an endocytic mechanism (Tai et al., 1993). Pulse-chase labeling with horseradish peroxidase showed early and persistent labeling of vacuoles, suggesting that there is no distinction between early and late endocytic vesicles as is found in higher eukaryotes. Labeling of a smaller portion of the vacuoles with chemicals that label the ER, such as glucose-6-phosphatase and zinc iodide-osmium tetroxide, and three-dimensional reconstructions (Lanfredi-Rangel et al., 1998), as well as EM using anti-BiP antibody (Soltys et al., 1996), have suggested a continuity of these vacuoles with the ER. The vacuoles also contain a variety of hydrolase activities, such as acid phosphatase, proteinase, and RNase, indicating their lysosomal characteristics. Thus, the vacuoles appear to function as early and late endosomes and lysosomes and may be functionally associated with the ER (Lanfredi-Rangel et al., 1998).

### 2.7. Cell biochemistry

*G. lamblia* is an aerotolerant anaerobe, lack mitochondria but contains lysosome like organelles. The knowledge of biochemistry and metabolism of *Giardia* was very limited till 1980. This situation has changed markedly after the successful axenisation of *G. lamblia* trophozoite *in vitro*. With this breakthrough several papers dealing with the biochemical and metabolic nature of this organism have begun to appear.

#### 2.7.1. Carbohydrate Metabolism

Most eukaryotic organisms depend primarily on aerobic metabolism for their energy production. However, certain eukaryotes, including *Trichomonas* spp., *Entamoeba* spp., and *Giardia* spp., are characterized by their lack of mitochondria and cytochrome-mediated oxidative phosphorylation. They rely on fermentative metabolism (even when oxygen is present) for energy conservation. Glycolysis and its brief extensions generate ATP, with generation dependent only on substrate level phosphorylation. Glucose is not completely oxidized to CO₂ and H₂O as in aerobic metabolism but is incompletely catabolized to acetate, ethanol, alanine, and CO₂. The balance of end product formation is sensitive to the O₂ tension and glucose concentration in the medium.

The metabolism of trophozoites is markedly affected by small changes in oxygen concentration. Under strictly anaerobic conditions, alanine is the major product of carbohydrate metabolism (Edwards et al., 1989; Paget et al., 1990, 1993). Even with the addition of minimal amounts of O₂ (i.e., concentrations of <0.25 µM), ethanol production is
stimulated and alanine production is inhibited (Paget et al., 1993). With further increases in O₂ concentration, ethanol and alanine production are inhibited. At O₂ concentrations of >46 µM, alanine production is completely inhibited and acetate and CO₂ are the predominant products of energy metabolism. These oxygen concentrations are likely to be relevant to the intestinal milieu in which the trophozoites replicate since the oxygen concentration in this environment is estimated to vary between 0 and 60 µM. Thus, the pathway of metabolism of pyruvate appears to be altered for differing anaerobic or microaerophilic environments.

In *Giardia*, there is no metabolic compartmentalization; rather, all the reactions occur in the cytosol or on the cytosolic surfaces of membranes for PFOR-mediated reactions (Lindmark, 1980).

Glucose supplies the major source of energy derived from carbohydrates (Jarroll et al., 1989). Glucose is converted to pyruvate by the Embden-Meyerhof-Parnas and hexose monophosphate shunt pathways (Fig. 2.8). For most eukaryotic and prokaryotic organisms, the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate is an irreversible and regulated step that is catalyzed by an ATP-dependent phosphofructokinase. However, for *Giardia*, as well as for *T. vaginalis* and *E. histolytica*, this reaction is catalyzed by a pyrophosphate-dependent phosphofructokinase. In contrast to the ATP-dependent enzyme, this enzyme catalyzes a reversible reaction and is not a regulated enzyme (Mertens, 1993). The pyrophosphate-dependent phosphofructokinase from *Giardia* has been cloned and characterized (Phillips et al., 1995; Rozario et al., 1995).

Two enzymes that convert phosphoenolpyruvate into pyruvate, ATP-dependent pyruvate kinase (Park et al., 1997), and pyrophosphate-dependent pyruvate phosphate dikinase (PPDK) have been identified (Bruderer et al., 1996; Hiltpold et al., 1999). There is a potential energy advantage in the reaction mediated by PPDK, since two molecules of ATP can be generated by a coordinated reaction involving PPDK and adenylate kinase. Adenylate kinase (Rozario et al., 1995) converts two ADP molecules into ATP + ADP by a reaction that is essentially energy neutral, and PPDK converts phosphoenolpyruvate plus AMP into pyruvate + ATP, resulting in the net generation of two ATP molecules during the conversion of phosphoenolpyruvate to pyruvate, rather than the one ATP produced by the pyruvate kinase reaction. Their relative roles in glycolysis have not yet been determined, but the higher specific activity of pyruvate kinase suggests that it may play a major role in glycolysis (Park et al., 1997).
The conversion of pyruvate to acetyl coenzyme A (acetyl-CoA) is catalyzed by PFOR, which utilizes ferredoxin rather than NAD as the electron acceptor (Lindmark, 1980; Townson et al., 1994, 1996), in place of the pyruvate dehydrogenase complex found in aerobic eubacteria and eukaryotes. PFOR is also found in *E. histolytica*.

Acetyl-CoA can be converted directly to acetate by ADP-forming acetyl-CoA synthetase (Sánchez et al., 2000), resulting in the production of ATP from ADP as acetyl-CoA is converted to acetate. Alternatively, acetyl-CoA is converted to ethanol, using acetaldehyde as an intermediate, by the bifunctional enzyme alcohol dehydrogenase E (Dan et al., 2000; Sánchez, 1998, 1999). Alcohol dehydrogenase E has an acetaldehyde dehydrogenase activity in the amino terminus that catalyzes the conversion of acetyl-CoA to acetaldehyde and an alcohol dehydrogenase activity in the carboxy terminus that converts the acetaldehyde to ethanol.

![Diagram of glucose metabolism](image)

**Figure 2.8: Metabolism of glucose to phosphoenolpyruvate.** The enzymes are labeled as follows: 1 - hexokinase; 2 - glucose phosphate isomerase (proposed); 3 - pyrophosphate-dependent phosphofructokinase; 4 - fructose bisphosphate aldolase; 5 - triosephosphate isomerase; 6 - glyceraldehyde-3-phosphate dehydrogenase; 7 - phosphoglycerate kinase (proposed); 8 - phosphoglyceromutase (proposed); 9 - enolase (proposed)
Figure 2.9: Arginine dihydrolase pathway. The enzymes are labeled as follows: 1 - arginine deiminase; 2 – ornithine transcarbamoylase; 3 - carbamate kinase

Recently, Giardia’s core sugar metabolism has been identified using standard bioinformatic approaches. By comparing Giardia proteomes with known enzymes from other species, enzymes in the glycolysis pathway, as well as some enzymes involved in the TCA cycle and oxidative phosphorylation have been identified. However, the majority of enzymes from the latter two pathways were not identifiable, indicating the likely absence of these functionalities. Enzymes from the Giardia glycolysis pathway appear more similar to those from bacteria (Han et al., 2012). A number of sugar related metabolic pathways for Giardia lamblia have been reconstructed and notable enzyme absences from these pathways has been highlighted. The glycolytic enzymes from Giardia bear a stronger similarity with bacterial enzymes, rather than with eukaryotic or archaeal enzymes (except for phosphoglucomutase and phosphoglycerate kinase which are more similar to those found in eukaryotes). Only a few enzymes were identified from the TCA cycle and oxidative phosphorylation, indicating the likely absence of these pathways.

This approach of analysing metabolic pathways could, in theory, be applied to any organism with genome information but limited annotation. The advantage of using this approach is that it is reasonably quick to give an indication of which pathways are likely to be present and which ones are not. There are however some limitations: for a few proteins, KEGG can allocate wrong EC numbers which will result in false positives if users are not familiar with the pathways. False positives can also occur if one EC group is very similar to another EC group (such as in the case of succinyl-CoA synthetase and acetyl-CoA synthetase). KEGG is a database that is still growing and as yet does not have the enzymes from all species. It is expected that enzyme candidates may not be recovered if they are from
a species extremely different from the known enzymes and species. The overall picture of *Giardia* indicates that glucose is absorbed from the host and metabolised into pyruvate through glycolysis and after that, in order to regenerate the oxidised form of coenzyme NAD+, pyruvate is reduced to ethanol, alanine, or acetate depending on the availability of oxygen. Under aerobic conditions, pyruvate is converted to alanine by a transamination reaction or to acetate by acetyl-CoA synthetase. Also under anaerobic conditions, pyruvate is metabolised to acetyl-CoA by PFOR, and subsequently into acetaldehyde and ethanol. The TCA cycle and oxidative phosphorylation do not appear to occur. These latter results were not completely unexpected since we already know that *Giardia* has an anaerobic life style and has undergone genome reduction (i.e., a smaller genome with fewer unnecessary enzymes will give the parasite advantage when replicating). *Giardia* shares many metabolic attributes of bacteria, including its fermentative energy metabolism which relies heavily on pyrophosphate rather than adenosine triphosphate.

Morrison et al., 2007, looked into *Giardia*’s metabolic repertoire briefly when the *Giardia* genome project was completed. Their results indicated that *Giardia*’s sugar metabolic pathways contained a mixture of eukaryote-like (enzymes that appeared more similar in sequence to those enzymes found in eukaryotes) and bacteria-like enzymes. Morrison et al., (2007) indicated that about half of glycolytic enzymes are eukaryote-like, but they did not distinguish between typical eukaryotic enzymes (i.e., those well studied in mammals, yeasts, and plants) and enzymes from eukaryotic protists. The study has considered protists separately from other eukaryotes, because frequently these eukaryotic protists have prokaryote-like enzymes rather than those from typically studied eukaryotes. Some reasons for *Giardia* having a sizable number of bacteria-like enzymes include the possibilities that mitochondria genes migrated to the nucleus with the loss of this organelle lateral gene transfer of bacterial genes or that the eukaryotic set of enzymes arose after their divergence from the ancestral eukaryote.

There are still many evolutionary questions surrounding *Giardia* and it is expected that the clarification of its somewhat “atypical” metabolism will aid this research. The glycolysis pathway occurs, in nearly all organisms with minor variations. So we ask very briefly if the enzymes in the glycolytic pathway are also conserved in all organisms. *Giardia* annotated proteins (4889 in total) have been compared against 28 bacterial, 12 archaeal species, and 17 other eukaryotic species, and identified four groups of proteins according to the conservation of the proteins in the three domains: Group A contains 37 *Giardia* proteins that are conserved in all three domains of life; Group B contains 849 *Giardia* proteins that are
found in all eukaryotes; Group C contains 274 eukaryotic signature proteins (Hartman et al., 2002; Kurland et al., 2006) which are proteins conserved in all eukaryotes, but not found in any archaea or bacteria; and finally Group D contains 278 Escherichia coli proteins conserved in all bacteria species. The candidates of glycolytic enzymes (20 in total) were compared with the above four groups of proteins. None of the glycolytic enzymes matched were matched to Group A (conserved in all three domains), Group C (eukaryotic signature proteins), or Group D (conserved in all bacterial species). However, there were six candidates matched to Group B.

These results could be explained because Giardia contains a mixture of eukaryote-like and prokaryotic-like enzymes in glycolysis and that glycolysis in bacteria occurs in diverse forms. This means that none of the Giardia’s bacteria-like glycolytic enzymes are likely to be universal to all bacteria and thus less likely to be found matched to those in Group A or Group D. The eukaryotic glycolytic enzymes are more conserved across eukaryotes, and thus some of Giardia’s eukaryote-like glycolytic enzymes were found to be conserved in all eukaryotes. However, homologues of these enzymes conserved in all eukaryotes are also found in some branches of bacteria, hence they did not show up in Group C (eukaryotic signature proteins). This result is due to the large variety of glycolytic enzymes present in bacteria. This above work is merely indicative at this stage and will be the basis for future study.

More pathways, such as those involved in amino acid metabolism, and the RNA degradation pathway can be analysed using this method, adding more pieces to the puzzle of Giardia’s metabolism. This study also identified Giardia candidates for enzymes that had not been recognized before. They bear high similarity to known enzymes of their classes, and although the actual functions of these enzymes have not been confirmed, the above work gives direction to future experimental confirmation with activity assays. Typically a drug target is a key molecule for the infectivity or survival of a microbial pathogen. Selective toxicity would be best achieved if the parasite has a key enzyme that humans do not have or which is remarkably different from the host. For example, PFOR is found in Giardia, but the host (human or mammal) uses the pyruvate dehydrogenase complex to perform the same reaction, and thus drugs targeting PFOR such as metronidazole have been designed. From the glycolytic pathway, we have identified enzymes which are significantly different in Giardia from those in the host, including glucokinase and phosphofructokinase. Glucokinase has been investigated as a drug target for type-2 diabetes, and its potential to be a target for parasite infection is as yet uncertain. Phosphofructokinase has been suggested as a drug target for
Entamoeba histolytica by Byington et al., 1997 and they designed a competitive inhibitor of phosphofructokinase, with the drug inhibiting the growth of the parasite in vitro. These enzymes, and especially those that can be compensated in the host by alternative pathways, hold the possibility of new targets for drugs effective against Giardia. An even better understanding of this parasite’s metabolism will surely provide more ammunition against this worldwide parasitic problem.

2.7.2. Amino Acid Metabolism

Amino acids are becoming increasingly recognized as important components of the energy metabolism of G. lamblia. The uptake of aspartate, alanine, and arginine from the extracellular medium, as well as the documentation of glucose-independent metabolism, suggests the potential importance of amino acid metabolism for energy production in Giardia (Mendis et al., 1992, Schofield et al., 1990, 1991) (Fig. 2.6).

The arginine dihydrolase pathway is one potential source of energy (Edwards et al., 1992, Schofield et al., 1990, 1992). This pathway is present in a number of prokaryotic organisms, but among eukaryotes it has been documented only in T. vaginalis (Lindmark et al., 1982) and G. lamblia. In the arginine dihydrolase pathway, arginine is converted to ornithine and ammonia with the generation of ATP from ADP by substrate-level phosphorylation (Fig. 2.9). Ornithine is subsequently exported in exchange for extracellular arginine by a transporter mechanism (Schofield et al., 1995).

Aspartate is another potential source of energy. It is converted to oxaloacetate by aspartate transaminase, entering the intermediary pathway, where it is converted to pyruvate via a malate intermediate (Mendis et al., 1992).

Alanine also appears to play an important role in allowing trophozoites to adapt to hypoosmotic challenge. With an isoosmolar extracellular environment, the intracellular alanine concentration is 50 mM (Knodler et al., 1994, Park et al., 1997), and with a hypoosmolar challenge, the concentration of alanine rapidly decreases by an active transport mechanism. (In addition to alanine, potassium appears to play a role in osmoregulation of trophozoites (Maroulis et al., 2000). The secretion of alanine occurs via an alanine transporter that also transports L-serine, glycine and L-threonine, L-glutamine, and L-asparagine (Edwards et al., 1993). This transporter acts as an antiport, exchanging intracellular alanine for these other amino acids from the extracellular environment (Schofield et al., 1995).

Aside from the synthesis of alanine as a by-product of energy metabolism, the only other amino acid for which de novo synthesis has been documented is valine. Thus, Giardia
lacks synthesis of most amino acids and depends on scavenging them from the intestinal milieu in which the trophozoite replicates.

One of the notable requirements for axenic growth of *G. lamblia* trophozoites is the absolute requirement for a relatively high concentration of cysteine (16 mM). Cysteine also provides a partial protection from the toxicity of oxygen that is not seen with other reducing agents, including cystine, and therefore appears to be a specific effect of cysteine (Gillin *et al.*, 1981, 1982). Cysteine is not synthesized de novo and is not synthesized from cystine (Lujan *et al.*, 1994). It appears to be imported into the cell by passive diffusion, although active transport may account for some of the acquisition of cysteine (Lujan, H. D *et al.*, 1994). The importance of free thiol groups on the surfaces of trophozoites was demonstrated by the toxicity of thiol-blocking agents that are unable to penetrate intact cells (Gillin *et al.*, 1984). This toxicity suggests that these agents are reacting with thiol groups on the trophozoite surfaces, killing the trophozoites. Cysteine appears to be the major thiol group present (Brown *et al.*, 1993). *G. lamblia* also contains cysteine as main low-molecular mass thiol and GSH has been reported to be absent. The genome, however, encodes putative genes for the GSH biosynthetic enzymes c-glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2) (Morrison *et al.*, 2007). Recently, *G. lamblia* has been shown to possess a monothiol glutaredoxin (1-C-Grx) (Rada *et al.*, 2009). These small redox proteins are characterized by a CGFS active site motif. They can dimerize by coordinating a [2Fe2S] cluster via the active site cysteines of each monomer together with the cysteines of two bound GSH molecules. 1-C-Grxs are involved in (probably the final step of) mitochondrial iron–sulfur cluster biosynthesis. The *G. lamblia* 1-C-Grx is localized in the mitosomes, highly reduced mitochondriatype organelles that harbor iron–sulfur cluster biosynthesis as only known function. The recombinant parasite 1-C-Grx has been shown to form a homodimeric iron–sulfur cluster complex that can be stabilized by GSH (Rada *et al.*, 2009). It is therefore conceivable that *G. lamblia* contains at least low levels of GSH that may specifically be used in iron–sulfur cluster biogenesis. When trophozoites are metabolically labeled with radiolabeled cysteine, most of the label is incorporated into the VSPs (Adam *et al.*, 1988; Aggarwal *et al.*, 1989), suggesting that these surface proteins may play a role in protection of the trophozoite from oxygen toxicity.

### 2.7. 3. Lipid Metabolism

The growth of trophozoites predominantly in the duodenum and jejunum initially suggested the possible importance of bile in the growth of *Giardia* trophozoites. Short-term axenic
growth in the absence of serum can be supported by bile. The biliary lipids cholesterol and phosphatidylcholine and the bile salts glycocholate and glycodeoxycholate will also support this growth (Gillin et al., 1986). Serum is required for longer-term axenic growth, but it has been shown that the Cohn IV-1 fraction of bovine serum (enriched in alpha globulins, lipoproteins, and growth factors) can substitute for whole serum (Lujan et al., 1994). In fact, insulin-like growth factor II, which is present in fraction IV-1, stimulates trophozoite growth and cysteine uptake. The same fraction from a number of other mammals was also effective in supporting growth, although in some cases antibody depletion was required.

G. lamblia trophozoites do not have the capacity of de novo synthesis of fatty acids, with the possible exception of certain minor fatty acids (Ellis et al., 1996). However, free fatty acids are toxic to trophozoites. The trophozoites appear to satisfy their lipid requirements by obtaining cholesterol and phosphatidylcholine from the external environment (Lujan, H. D et al., 1996). The cholesterol and phospholipids are supplied by lipoproteins, cyclodextrins, and bile salts, with transfer of lipids to the parasite surface being facilitated by bile salts. It has also been suggested that a low level of endocytosis of lipids occurs. Exogenous phospholipids have been shown to undergo fatty acid remodeling (by deacylation/reacylation reactions), which allows to alter lipids, bypassing the synthesis of entirely new phospholipid molecules (Das et al., 2002).

Conjugated bile acids appear to be taken up by a carrier-mediated mechanism that includes different carriers for cholytaurine and cholyglycine (Das et al., 1997). The major fatty acids found in axenically grown trophozoites are palmitic acid, stearic acid, and oleic acid. Fatty acid desaturase activity, including desaturation of oleate to linoleate and linolenate, has been documented (Ellis et al., 1996). Arachidonic acid is incorporated into neutral lipids, phospholipids, and a wide variety of cellular lipids (Gibson et al., 1999), while palmitic acid, myristic acid, and oleic acid are transesterified primarily into phospholipids including cellular phospholipids, (e.g., phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol) (Mohareb et al., 1991, Stevens et al., 1997). Interesterification also occurs with incorporation of conjugated fatty acids into phosphatidylglycerol. The toxicity of certain analogs of phosphatidylglycerol for trophozoites has been documented, although the mechanism of this toxicity has not been determined (Gibson et al., 1999).

Isoprenoids are lipids derived from mevalonate that are commonly found in eukaryotic cells. The most notable end product is cholesterol, but isoprenoids are also incorporated into proteins such as the GTP-binding proteins by posttranslational modification.
Isoprenylation of proteins has been demonstrated by the incorporation of radiolabeled mevalonate into trophozoite proteins (Lujan et al., 1995). Incorporation of mevalonate and cell growth was inhibited in a reversible manner by competitive inhibitors of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. Inhibitors of later steps of isoprenylation permanently inhibited cell growth.

2.7. 4. Purine and Pyrimidine Salvage

Many of the pathogenic protozoa, including G. lamblia (Wang, C. C. et al., 1983), depend on salvage pathways for obtaining purine nucleosides. In addition, G. lamblia (Aldritt, S. M et al. 1985), as well as T. vaginalis (Wang, C. C et al., 1984) and Tritrichomonas foetus (Wang, C. C et al., 1983), lack pyrimidine synthetic pathways and depends on salvage pathways for obtaining both purine and pyrimidine nucleosides.

Studies of purine metabolism using radiolabeled precursors have demonstrated the incorporation of adenine, adenosine, guanine, and guanosine into nucleotides but no incorporation of components of the de novo synthetic pathway, such as formate, glycine, hypoxanthine, inosine, or xanthine. The likely scenario is that the purine nucleosides (adenosine and guanosine) are imported by a transporter with broad specificity for nucleosides as well as deoxyribonucleosides (Baum, K. F. et al. 1993; Davey, R. A. et al. 1992). The purine nucleosides are then broken down to the bases by their respective hydrolases. Phosphoribosyl 1-pyrophosphate is synthesized by phosphoribosyl 1-pyrophosphate synthetase (Kyradji, S. et al., 1998) and reacts with the salvaged purine bases to produce the nucleoside-5'-monophosphate in a reaction catalyzed by the respective monophosphate phosphoribosyltransferase (PRTase) (Fig. 2.10). The GPRTases from most eukaryotes utilize hypoxanthine or xanthine as substrate, but the G. lamblia GPRTase is highly specific for guanine (Page, J. P. et al., 1999; Sommer, J. M. et al. 1996), indicating that guanine is the only source for guanine nucleotides. Metabolite analyses showed specific depletion of the guanine ribonucleotide pools in parallel with slower cell growth which revealed that it plays an essential role in supplying guanine nucleotides required for growth and multiplication of Giardia (Munagala et al., 2002). The amino acid sequence of the GPTase enzyme shows less than 20% identity to the human enzyme (Sommer, J. M. et al., 1996). The crystallographic structure of the G. lamblia GPTase has suggested possible reasons for the unique substrate of the G. lamblia enzyme, such as an aspartate substitution for leucine at position 181 (Shi, W et al., 2000). Purine phosphoribosyltransferases catalyze the Mg$^{2+}$ dependent reaction that transforms a purine base into its corresponding nucleotide.
G. lamblia adenine phosphoribosyltransferase (APRTase) shows nucleophilic displacement by electrophile migration (Shi et al., 2002). G. lamblia trophozoites also depend on the salvage of exogenous thymidine, cytidine, and uridine for the synthesis of the pyrimidine nucleotides (Fig. 2.11).

**Figure 2.10:** Purine ribonucleosides salvage pathways. The enzymes are labeled as follows: 1 - adenosine hydrolase; 2 - adenine phosphoribosyltransferase (APRTase); 3 - guanosine hydrolase; 4 - guanine phosphoribosyltransferase (GPRTase).

**Figure 2.11:** Pyrimidine ribonucleoside salvage pathway in Giardia. The enzymes are labeled as follows: 1 - uracil phosphoribosyltransferase (UPRTase); 2 - uridine/thymine phosphorylase; 3 - uridine phosphotransferase (kinase); 4 - UMP kinase; 5 - UDP kinase; 6 - CTP synthetase; 7 - CDP kinase; 8 - cytosine phosphoribosyltransferase (CPRTase); 9 - cytidine hydrolase; 10, cytidine deaminase.
2.8. Immunology

Since the last decade considerable light has been shed on the role-played by the immune system of the host in determining the outcome of *Giardia* infection. The demonstration of spontaneous resolution of infection in man and experimental animals strongly suggests the existence of a protective immune response to *Giardia*. Increased prevalence of *Giardiasis* in hypogammaglobunemic patients emphasizes the potential importance of antibody in the immunity to this parasite. Several *Giardiasis* outbreaks show that individuals repeatedly exposed to *G. lamblia* have a lower incidence of infection suggesting that prior exposure imparts partial resistance to reinfection. In addition a high prevalence of *Giardia* infection in homosexuals (Brown *et al.*, 1973; Hurwitz & Owen, 1978) suggests the role of cellular immunity in *Giardia* infection since many of the homosexuals have acquired T- cells defect. It has also been found that elimination of *Giardia muris* is impaired in hypothalamic mice, and in mice deficient in B-cells and mast cells. Macrophages have also been implicated for immunity to this parasite. More than one immunological mechanism seems to be involved in protection against *Giardia*.

2.8.1. Humoral response

*Giardia* infection induces a local as well as systemic response. Serum antibodies to *G. lamblia* were first demonstrated by Ridley (Ridley & Ridley, 1976) in patients of *Giardiasis* with malabsorption using indirect immunoflourescence antibody test. These antibodies were IgG, IgA and IgM and were found to be directed against surface antigens. The antibody mediated killing may or may not require complements (Deguchi *et al.*, 1987; Nash & Aggarwal, 1986).

The presence of antibodies in high titres in some but not all patients with recurrent infection suggests that antibodies alone are not protective. The cell mediated response may also be necessary for the protective immune response (Eckmann *et al.*, 2003). Thus a combination of immunological events ultimately clears the infection and memory develops in the majority of affected human hosts. But how the secretory antibody or cellular components react with *Giardia* is completely unknown. A few workers have investigated the possibility that specific immunoglobulins may play a role in resistance to *Giardiasis* and have postulated that a deficiency of these immunoglobulins may permit growth of the organism. Thus in intestinal secretion, IgA concentration is found to be lower in patients with *Giardiasis* than those in the control groups (Zinneman & Kaplen, 1972).
On the other hand Char et al. reported the elevation of total serum IgG, IgA and IgM concentrations in children with persistent Giardiasis as compared with controls (Char et al., 1993). Determination of the concentration of Giardia specific antibodies revealed that only IgM was raised while those of IgA and IgG were similar to the controls. The association of high concentration of Giardia specific IgM, low concentration of Giardia specific IgA and IgG and inability to clear the infection suggest that the switch from IgM to IgG or IgA response is inefficient.

2.8. 2. Cell mediated response
Cell mediated immune response in Giardiasis is poorly understood although a role for T-lymphocytes has been well established. It was evidenced that during Giardiasis the total number of T lymphocytes in Peyer patches become double and nude mice (athymic) develop a chronic Giardiasis. Several reports demonstrated that in vitro phagocytosis of Giardia trophozoites by macrophages obtained from both infected and noninfected hosts (Owen et al., 1981, 1979) using scanning microscope, demonstrated that trophozoites finding their way into mucosal breaches are engulfed by macrophages which extend pseudopodea through epithelial basement membrane. The available evidence suggests that macrophages do not spontaneously eliminate Giardia species but it may act as an antigen presenting cell for CD-4 lymphocytes and/or participate in antibody mediated killing of the trophozoites. Theoretically a protective effect of antitrophozoite antibodies might result from inhibition of trophozoite attachment to intestinal epithelial cells or opsonisation of trophozoites for killing or ingestion by phagocytes. Of these possibilities there is evidence that trophozoite attachment to intestinal epithelium is inhibited by antibodies. The nonimmune protection system includes the intestinal mucosal layer, intestinal motility and human breast milk for infants (Adam, 1991, Tellez et al., 2003). A T-cell dependant mechanism is also involved for controlling acute Giardia infection and this mechanism is independent of antibody and B cells (Singer & Nash, 2000). IL-6 has been shown to play a major role in control of acute Giardiasis (Bienz et al., 2003; Zhou et al., 2003).

2.8. 3. Antigenic Variation
2.8. 3. 1. Occurrence of Antigenic Variation
Cysteine-rich surface antigens of G. lamblia trophozoites undergo antigenic variation of a family of immunodominant in vitro and in vivo (Adam et al., 1988; Nash et al., 1988). The
initial studies of the surface antigens of *G. lamblia* showed differences among strains by crossed immunoelectrophoresis and enzyme-linked immunosorbent assay and marked differences in the molecular masses of "excretory-secretory products" from different surface-iodinated *G. lamblia* isolates. These surface antigens varied in number and in size from approximately 50 to 200 kDa in a study of 19 isolates (Nash *et al.*, 1985). A monoclonal antibody (MAb 6E7) for a 170-kDa surface antigen (initially called CRP170 but now called VSPA6) from the WB isolate was cytotoxic for WB trophozoites but not for isolates expressing other surface antigens (Nash *et al.*, 1986). Subsequent data have confirmed that individual organisms express only one VSP at a time (Nash *et al.*, 1990); the detection of multiple surface-labeled bands in some of the studies resulted from subpopulations of trophozoites expressing several different VSP types. In axenic culture, variation occurs approximately once every 6 to 12 generations for a frequency of $10^3$ to $10^4$ (Nash *et al.*, 1990). Variable surface proteins, alpha-giardins, arginine deiminase, ornithine carbamoyl transferase, and fructose-1,6-bisphosphate aldolase have been identified as immunoreactive during acute *Giardia*sis and they are very much important in the development of new diagnostic tools and vaccines (Palm *et al.*, 2003).

### 2.8. 3. 2. Other Antigens

Taglin is a surface antigen that migrates as a 28- and 30-kDa doublet on Western blots (Ward *et al.*, 1987) and has protease-induced lectin activity (Lev *et al.*, 1986). After trypsin treatment it binds to mannose-6-phosphate. This antigen is nonvariable, and expression is constant throughout encystation and excystation (Svärd *et al.*, 1998). Alpha-1 giardin is a highly immunoreactive GAG-binding protein, which may play a key role in the parasite-host interaction (Weiland *et al.*, 2003). *G. lamblia* trophozoites also have a 49-kDa antigen (GP49) that is attached to the membrane surface by a glycosylphosphatidylinositol (GPI) anchor (Das *et al.*, 1991). During synthesis of the GPI anchor, exogenous phosphatidylinositol is incorporated, but *myo*-inositol is converted to phosphatidylinositol prior to incorporation (Subramanian *et al.*, 2000). The GPI anchor is not subject to cleavage by phospholipase C, in contrast to a variety of other GPI-anchored surface molecules, including the trypanosome variant surface glycoprotein. GP49 is constant among different isolates and does not demonstrate variation within single isolates (Das *et al.*, 1991).
2.9. Pathology and pathogenesis

Till now it is not clear how *Giardia* causes the disease. There is mounting evidence that the characteristics of both the parasite and the host may determine the outcome of infection. In symptomatic cases of *Giardia*, trophozoites attach to the microvillus border (mvb) by virtue of their ventral disc and produce a barrier effect or damage to the brush border. This may result in decreased brush border enzymes, which ultimately leads to malabsorption of fat, Vit B-12, lactose and protein. The pathological changes are more marked in hypogammaglobulinaemic patients and may account for increased frequency of overt *Giardiasis* in these cases (Lebwohl *et al*., 2003).

Two antigenically distinct isolates were used to infect gerbils and human volunteers and showed a marked difference in pathogenicity between these two isolates. In subsequent studies it has been found that the same isolate expressed antigen differentially in different hosts. In one group of human volunteers it produced a 72 KDa surface antigen and caused successful infection, whereas in the other group it expressed 200 KDa surface antigens and remained avirulent. Though the data available was not conclusive, yet this attribute of the parasite may be important (Nash *et al*., 1990).

A number of predisposing factors in the host may also take part in causing the disease. Some of these host factors are age, nutritional status of the host, genetic predisposition as suggested by frequency of infection in blood group A and various states of immunodeficiency. *Giardia lamblia* infection has been found to be associated with adult reactive arthritis. Finally it can be said that variable attributes of the infecting strains resulting in varying degree of virulence in conjunction with the host factors determine the susceptibility of *Giardia* infection. Differences in both may be responsible for the wide spectrum of clinical presentations seen in *Giardiasis* (Morimoto *et al*., 2003).

2.10. Epidemiology

*Giardiasis* is a major contributor to the enormous burden of waterborne diarrhoeal diseases that are second only to respiratory infections as causes of mortality and morbidity worldwide (Wolfe, 1992). Prevalence rate of this infection is high in the tropics and subtropics, especially among the socio-economically underprivileged (Nimri, 1994; Mukhtar, 1994; Rajeshwari *et al*., 1994; Overbosch & Ledeboer, 1995; Yassin *et al*., 1999, Diaz *et al*., 2003). Poor hygienic conditions favour its spread; particularly where there is scarcity of clean drinking water and effective sewage disposal systems, resulting in fecal contamination of the
sewage. Though the disease is endemic in the developing countries, it is also responsible for many localized outbreaks in the United States caused by contaminated drinking water. In India, intestinal parasites is a major health problem (Jindal et al., 1995; Saha et al., 1996). *Giardia lamblia* is found to be the most common cause of infection both in adults and children (Singh et al., 1993; Sethi et al., 1999). Direct fecal-oral transmission is the other major means by which *G. lamblia* is spread. Food borne transmission is less common but well documented (Osterholm et al., 1981; Peterson et al., 1988; White et al., 1989). These outbreaks have most probably occurred through contamination of freshly prepared food by infected food handlers.

Globally, the number of immunosuppressed people increases each year, not only due to the continuous spread of the human immunodeficiency virus (HIV) pandemic, but also due to malnutrition, chemotherapy for malignancy, and immunosuppressive therapy. All individuals affected by immunosuppression are at risk of infection by opportunistic parasites (reviewed by Stark et al., 2009). Giardiasis is not considered a major cause of enteritis in HIV-infected patients, and it is not listed among the opportunistic parasitic infections because it does not cause prolonged symptoms and therapy is independent from the patient’s immune status. The observed prevalence varies between 1.5% and 17.7% in the few reports published (reviewed by Stark et al., 2009). The symptoms of Giardiasis in HIV-infected individuals appear to be similar to, and no more severe than, those of Giardiasis in HIV negative individuals, with asymptomatic infection occurring commonly in the presence of HIV (reviewed by Faubert, 2000). However, when CD4+ counts are reduced and cause progressive immunosuppression, the risk of symptomatic Giardia infections increases, with a tendency towards chronic diarrhoea (Dwivedi et al., 2007).

Traditionally, species within the Giardia genus have been considered as eukaryotic organisms that show an absence of sexual reproduction in their simple life cycles (Adam, 2001). This view has been challenged by a number of recent studies (reviewed by Caccio & Sprong, 2010). Besides the interest in evolutionary biology (Logsdon, 2008), there are important implications of recombination for the population genetics, taxonomy, and epidemiology of *Giardia*. Accumulating evidences support the occurrence of genetic exchanges within assemblages (at the individual level or between individual of the same assemblage) and even between assemblages. This has been observed among human field isolates of assemblage A, subgroup AII (Cooper et al., 2007), not only among axenic isolates of assemblages A, subgroups AI and B (Teodorovic et al., 2007), but also among other *G. lamblia* assemblages by comparative and phylogenetic analyses of Genbank sequences.
(Lasek-Nesselquist et al., 2009). It is rather obvious that the distinction between mixed infections and true recombinants is crucial. This will require analysis of single cells (i.e., single cysts) to be formally undisputable. Since genotyping of single cysts is technically feasible (Miller & Sterling, 2007), and assemblage-specific PCR-based assays are available (Geurden et al., 2009; Almeida et al., 2010), research in this direction will be of paramount importance. A prerequisite for inter- and intra-assemblage recombination is that mixed Giardia infections occur in individual host. This seems to be the case, especially in humans and dogs (Sprong et al., 2009), where in MLG analysis ~20% and ~30% of the isolates were inter-assemblage mixtures. The presence of more than one Giardia genotype has important implications for the etiology of Giardiasis: it is unclear how or when humans and animals become infected with two or more genotypes. Either infection with different Giardia genotypes occurs simultaneously, because of environmental mixing, for example in water, or, alternatively, subjects are asymptptomatically infected with one Giardia assemblage, but become ill and symptomatic from a second infection with another Giardia assemblage. The latter hypothesis is supported by the finding of asymptomatic subjects. The occurrence of mixed infections is important for molecular typing of Giardia. Using only one marker for the assignment of isolates to specific (sub)-assemblages is not always reliable, as different markers can give different results. For example, isolates can be typed as ‘‘potentially zoonotic’’ with one marker, but as ‘‘host-adapted’’ with another. More reliable results are obtained when multiple markers are used for typing.

2.11. Clinical features
The clinical manifestation of Giardiasis varies from asymptomatic infection to chronic diarrhoea with malabsorption. Patients with symptomatic Giardiasis have diarrhoea with loose foul smelling stool that is greasy, frothy, or bulky. Other common gastrointestinal symptoms include abdominal cramps and bloating, nausea and decreased appetite. Malaise and weight loss is seen in majority of patients and fever is occasionally present, especially in the early part of the infection (Moore et al., 1969). On the other hand about half of the infected people are asymptomatic, and the infection frequently resolves spontaneously (Adam, 1991).
2.12. Diagnosis

Diagnosis of *G. lamblia* can be done in several ways (Kapoor *et al.*, 2001).

- Microscopic stool examination
- Examination of duodenal contents
- Small bowel biopsy
- Gastrointestinal radiology
- Immunodiagnosis
- Using DNA probe

2.12. 1. Microscopic stool examination

Microscopic examination of stool for cysts and trophozoites is usually the first diagnostic test performed in patients with suspected *Giardiasis*. *Giardia* cysts are found in stool of most patients with *Giardiasis* whereas trophozoites are found less commonly, but correlates with symptomatic infection (Goka *et al.*, 1990). Stool specimens are examined by light microscopy either fresh or fixed with polyvinyl alcohol or formalin and then stained with trichrome or iron hematoxylin (Kim *et al.*, 2003). The sensitivity of a routine examination of a single stool specimen is approximately 50-70% (Goka *et al.*, 1990). Two or three specimens collected on different days should be analyzed as symptoms of illness begins approximately 1 to 7 days before excretion of cyst starts (Hiatt *et al.*, 1995). Sedimentation and immunomagnetic separation of *Giardia lamblia* cysts followed by microscopic examination has shown better sensitivity and specificity (Massanet-Nicolau J, 2003).

2.12. 2. Examination of intestinal fluid

In patients where repeated stool examinations are negative, diagnosis can be done by examination of duodenal contents. The duodenal fluid can be obtained with a duodenal tube or with the enterotest (HEDECO Company, Mountain View, Calif-94043). In enterotest the patient swallows a gelatin capsule on a string. After several hours the capsule is removed from the intestine and examined microscopically. Examination of duodenal content is more sensitive than examination of stool samples (Hopper *et al.*, 2003). The inclusion of routine
duodenal biopsies as part of upper endoscopy in pediatric patients yield additional pathologic findings that otherwise could have been missed (Kori et. al., 2003).

2.12. 3. Small bowl biopsy test
Small intestinal biopsies may yield better diagnosis of Giardia infection where other methods fail. Such specimens may be examined after sectioning and Giemsa staining. The parasite is usually found to attach to the microvillus border, particularly in the crypts. Though both sensitivity and specificity are high for small bowel biopsy and duodenal fluid test but they have little use in diagnosis as these are invasive (Bown et al., 1996).

2.12. 4. Gastrointestinal Radiology
Giardiasis may result in nonspecific intestinal changes that may support disease diagnosis in about 20% of the Giardiasis patients. These abnormalities have been detected by barium examination.

2.12. 5. Immunodiagnosis
Immunooassay procedures offer both increased sensitivity and specificity compared to conventional staining methods. These reagents are also helpful when screening a large number of patients, particularly in an outbreak situation or when screening a large number of patients particularly in an outbreak situation or when screening patients with minimal symptoms. This is based on i) detection of specific antibody in patient’s serum, ii) detection of Giardia antigen in stool and duodenal fluid. Different immunoassay kits based on direct immunofluorescent-antibody assay and ELISA are available in the market (Garcia et al., 2003). Comparative study of these kits wit microscopic and other direct methods (Aziz et al., 2001) have shown higher specificity for these techniques (Rashid et al., 2002). The sensitivity of these kits varies from 94%- 99% while specificity is 100%.

2.12. 6. DNA probe
From the last few years, scientists have been devoting themselves in preparing more sensitive, sophisticated diagnostic systems based on nucleic acid detection. In 1989 Butcher et al. reported a DNA probe for diagnosis of Giardia. In 1990 Lewis et al., reported total genomic DNA probe to detect G.lamblia. It can detect as much as 10µg of G.lamblia DNA, 10^4 trophozoites and 10^4 cysts. But it shows cross reactivity with high amount (5µg) of T. cruzi DNA. Different DNA probes have been designed for detection of Giardia from stool (Shah et al., 2003). The nontranscribed intergenic spacer region of the rRNA gene of Giardia
lamblia has been successfully used to differentiate between Giardia lamblia and other enteric pathogen.

In 1991 Ronald M. Atlas reported the use of polymerase chain reaction in detection of G. lamblia. In this detection system giardin gene has been targeted for amplification. Since then a lot of PCR based detection system has come up (Ghosh et al., 2000). Real time PCR (Verweij et al., 2003) and colourimetric detection of PCR product (Lee et. al., 2003) has also been reported. PCR detection has the advantage over other detection system, both in sensitivity and specificity.

Molecular Diagnosis – Nucleic Acid Detection Methods
PCR-based methods are more sensitive than conventional and immunological assays for detecting G. lamblia in faeces, but the sensitivity of published methods can vary. Molecular techniques are often restricted to specialist laboratories but are necessary to determine G. lamblia assemblages and sub-assemblages. Identifying G. lamblia assemblages infecting humans (A and B) is necessary as it helps determine the epidemiology of disease and likely transmission routes. As DNA sequence based Giardia surveys have also found assemblages A and B in nonhuman hosts, the zoonotic potential of some assemblage A and B isolates must be borne in mind (Caccio & Ryan, 2008; Ortega-Pierres et al., 2009). Molecular sub-typing methods are less developed for G. lamblia than for other protozoan pathogens, but loci on the following genes have been targeted (small subunit ribosomal RNA (ssu-rRNA), β giardin (bg), glutamate dehydrogenase (gdh), elongation factor 1-alpha (ef-1), triose phosphate isomerase (tpi), GLORF-C4 (C4) and the inter-genomic rRNA spacer region (IGS) (Caccio & Ryan, 2008). Caccio & Ryan (2008) highlight potential typing and sub-typing complications caused by (i) intra-isolate sequence heterogeneity (the presence of mixed templates that influence the identification of subtypes within each assemblage) and (ii) the unreliable assignment of isolates to G. lamblia assemblages generated by different genetic markers, especially when single genetic markers are used.

2.13. Treatment of Giardiasis
During the last decade significant achievements have been obtained in treatment of Giardiasis (Petri, 2003). A number of attempts have been made to determine the susceptibility of G. lamblia to different drugs in vitro. Till date drugs viz. Quinacrine, nitroimidazole,
metronidazole, tinidazole and furazolidone are used for treatment of Giardiasis (Minenoa et al., 2003).

2.13.1. Susceptibility of *G.lamblia* to aminoglycosides inhibitor

The aminoglycosides are a large and diverse group of antibiotics a number of which are known to target the SS RNA 3’ end. The 3’ end of SS rRNA plays a critical role in protein synthesis (binding of mRNA and tRNA) and has been characterized as the site of action of several aminoglycoside antibiotics.

Kasugamycin, the first antibiotic for which an rRNA target has been implicated acts on the two methyl groups added to A-1518 and A-1519 in bacteria. *G.lamblia* rRNAs like rRNAs of all organisms includes the AA dinucleotide, although there are no indications of methylation status. *Giardia lamblia* has been found to be partially susceptible to this drug (Edlind, 1989).

Hygromycin acts on the U-1495 and also interacts with G-1494 in. These nucleotides are also present in *G.lamblia* within the highly conserved 1492–1506 sequences. *G.lamblia* WB strain is highly susceptible to this drug at an ID$_{50}$ of 50 µg/ml. But this is of no clinical utility because of the lack of selective toxicity displayed by this aminoglycoside (Edlind, 1989).

Paramomycin interacts at two sites that are separated with respect to sequence but are at the same location with respect to secondary structure. Apparently the 1409-1491 base pair, and not a specific sequence confer susceptibility in *Tetrahymena*. This base pair is present in *G.lamblia* and the parasite has been found to be highly susceptible to this drug (Edlind, 1989). But the drug is rarely used for treatment for its high ID$_{50}$. However since paramomycin, like aminoglycosides is poorly absorbed from the gut, reducing the risk of systemic toxicity, this drug has been recommended in pregnancy.

Kanamycin and apramycin interacts with A-1408 in bacteria. In *G. lamblia* this nucleotide is substituted by G, and so this drug has no effect on this parasite (Edlind, 1989).

Gentamycin interacts at G-1405 of bacteria SS rRNA. Though this is present in *G.lamblia* the parasite is highly resistant to this aminoglycoside (Edlind, 1989). So probably there are other requirements for gentamicin activity.

Other aminoglycosides like neomycin, sisomicin, ribostamycin, butirosin and tobramycin are ineffective against this parasite (Edlind, 1989).
2.13. 2. Susceptibility to Nitroheterocyclic Drugs

*Giardia* infection is presently very effectively controlled by drugs of the 5’nitroimidazole family, metronidazole and tinidazole in particular. They have a broad spectrum of activity against anaerobic bacteria and protozoans.

Metronidazole is widely used for treatment of Giardiasis and is more than effective when given for a 5 day course (Adam, 1991). The mechanism of action of this drug is the reduction of the nitro group to toxic nitro anion radical metabolites via reduced ferredoxin (Edwards, 1993, Townson *et al*., 1994) and is brought about by the enzyme pyruvate ferredoxin oxidoreductase (PFOR) (Townson *et al*, 1996). The toxic radicals have been proposed to bind to DNA disabling the whole cell (Edwards, 1993), but this has not been proved in a nucleated organisms like *Giardia*. There are also indications that the radicals may have toxic effects on the enzymes of the respiratory chain of the parasite (Adam, 1991). The decrease in PFOR activity results in drug resistance of the parasite. Mechanisms that allow *Giardia lamblia* to tolerate oxidative stress may lead to resistance against both oxygen and nitroheterocyclics, with implications for clinical control (Ansell BR *et al*., 2015). The drug is mutagenic in bacteria and in high doses for prolonged period is carcinogenic in mice. In humans, nausea and general malaise are common during therapy and a disulfirum like interaction with ethanol are seen but serious side effects are rare.

Tinidazole is effective when given as a single dose and is also very well tolerated. For this reason it is the drug of choice; although there are evidences of genetic damage induced by Tinidazolein, in human lymphocytes (Lopez Nigro *et al*., 2001).

Furazolidone another member of the nitroheterocyclic drug is also reduced to cytotoxic products in a similar manner to the 5’-nitroimidazoles but not via ferredoxin (Brown *et al*., 1996). In *Giardia* the enzyme NADH oxidase activates furazolidone (Brown *et al*., 1996) but does not appear to be involved in mechanisms of furazolidone resistance. It appears that furazolidone resistance involves membrane changes that prevent access of furazolidone to its site of activation within the parasite.

2.13. 3. Susceptibility to Benzimidazoles

Mebendazole, a benzimidazole is a broad-spectrum antihelmenthic agent that shows activity against *Giardia* through its interaction with β-tubulin.
Albendazole another benzimidazole has *in vitro* activity against *G. lamblia* (Meloni *et al*., 1990). Two different methods - Meloni’s method, based on the loss of adherence of parasites to surfaces, and the Hill method, based on the loss of parasite division capacity, when compared for measuring the susceptibility of *Giardia lamblia* trophozoites to metronidazole and albendazole showed that the adherence method is more sensitive than the multiplication method for low and moderate inhibitory concentrations of albendazole. Conversely for metronidazole the multiplication method seems to be more sensitive for high inhibitory concentrations of the drug. For screening the IC$_{50}$, both methods seem to be effective, however, the inhibition of adherence method have even better performance for the benzimidazole like drugs (Cruz *et al*., 2003). Metronidazole and albendazole resistant *Giardia* can be successfully treated with nitrazoxanide in acquired immunodeficiency syndrome patients (Abboud *et al*., 2001). Albendazole (Abz) and Mebendazole (Mbz) analogues are as active as antiprotozoal agents as Metronidazole (Martinez *et al*., 2001) against *G. lamblia* (Navarrete-Vazquez *et al*., 2003). Albendazole induces oxidative stress and DNA damage in the parasitic protozoan *Giardia lamblia* (Martinez *et al*., 2015).

Secnidazole - another Benzimidazole derivative can be used for treatment of *Giardiasis* to effectively eliminate chronic *Giardia* infection (Escobedo *et al*., 2003).

Fenbendazole is a very effective treatment for *Giardiasis* eliminating *Giardia* trophozoites from the small intestine (O’Handley *et al*., 2001, Keith *et al*., 2003).

Quinacrine is a flavoantagonist and probably suppresses the oxidation of NADPH in *G. lamblia*. Though it is highly effective, gastrointestinal side effects are however common (Upcroft *et al*., 1996).

### 2.13. 4. Other agents

Other agents with *in vitro* activity include chloroquine, pyrimethamine, mefloquine, rifampin, azithromycin, and doxycycline. Ciprofloxacin also has cytotoxic effects in *Giardia lamblia* trophozoites and could be used as an alternative drug in Giardiasis treatment, particularly in infections that are resistant to other antibiotics (Sousa *et al*., 2001). Patients, who fail to respond to treatment, usually respond to second course of treatment with the original or another agent. Combined treatment with quinacrine and metronidazole has been found very effective in treatment of Giardiasis.
2.14. Cultivation of parasite

*G. lamblia* trophozoites obtained from a rabbit, chinchilla, and cat were first grown axenically (in the absence of exogenous cells) in 1970 (Meyer, 1970). HSP-1 medium, a subsequent modification reported in 1976, contained phytone peptone, glucose, L-cysteine HCl, Hanks solution, and human serum (Meyer, 1976). This study reported the first human *G. lamblia* isolate, Portland-1 (P-1). P-1 and WB, an isolate obtained from a symptomatic human who probably acquired his infection in Afghanistan (Smith et al., 1982), belong to the same genotype and have been used for many of the studies of *G. lamblia*. The growth medium has subsequently been modified, and currently the most commonly used medium is modified TYI-S-33 (Keister, 1983). Among the notable requirements for axenic growth are the absolute requirements for a low O₂ concentration, a high cysteine concentration, and the requirement for exogenous lipids, which are obtained from the serum component. When kept at 37°C, the trophozoites adhere to the glass wall of the tube in which they are grown. This adherence is dependent on glycolysis and on contraction of the proteins of the ventral disk (Feely et al., 1982). To date, the other species of *Giardia* (e.g., *G. muris* and *G. agilis*) have not been grown axenically.

2.15. Oxidative Stress:

- An increase in oxidant generation.
- A decrease in antioxidant protection.
- Failure to repair oxidative balance.

Oxidative stress is described as an imbalance between anti-oxidant and pro-oxidant species or as the deregulation of prooxidants and anti-oxidants. Reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), the hydroxyl radical (OH⁻), superoxide anion (O₂⁻), and the RNS, peroxynitrite (ONOO⁻) (Griendling et al. 2000b; Griendling et al. 2000c; Cai et al. 2003a; Taniyama et al. 2003) contribute to the pro-oxidant/anti-oxidant imbalance (Fig. 2.12)
The utilisation of oxygen as a terminal electron acceptor results in the production of the toxic O$_2^-$ radical (1). Subsequent dismutation by superoxide dismutase (2) produces H$_2$O$_2$. Accumulation of O$_2^-$ and H$_2$O$_2$ in the presence of haem-iron results in the regeneration of O$_2$ as well as in the production of the highly toxic hydroxyl radical (OH). The hydroperoxidases, a collective term denoting catalase (3) and peroxidase (4), and the GSH-dependent glutathione peroxidase (5), Glutathione reductase (6) rapidly remove H$_2$O$_2$ thus preventing its accumulation.

2.15.2. Free radicals:

Cell damage is induced by ROS. ROS are either free radical, reactive anions containing oxygen atoms or peroxide and peroxynitrite. The activation of oxygen can take place by stepwise monovalent reduction of oxygen to form superoxide, hydrogen peroxide, hydroxyl radical and finally water.
2.15.3. Sites of free radicals generations:
The main source of ROS in vivo is aerobic respiration, although ROS can also be produced by peroxysomal β-oxidation of fatty acids, stimulation of phagocytosis by pathogens or lipopolysaccharides, arginine metabolism and tissue specific enzymes.

2.15.4. Biological Reactions of Oxygen Free Radicals:
I. Oxidative damage to lipids:
Lipids bilayer membrane is made of mixture of phospholipids and glycolipids that have a fatty acid chain attached to C₁ and C₂ of glycerol backbone by an ester linkage. The peroxidation of lipid involves the following 3 steps:
1. Initiation: \( \text{OH}^* + \text{RH} \rightarrow \text{R}^* + \text{H}_2\text{O} \)
2. Propagation: \( \text{R}^* + \text{O}_2 \rightarrow \text{ROO}^* \)
   \[ \text{ROO}^* + \text{RH} \rightarrow \text{R}^* + \text{ROOH} \]
3. Termination: \( \text{R}^* + \text{R}^* \rightarrow \text{R-R} \)
   \[ \text{R}^* + \text{ROO}^* \rightarrow \text{ROOR} \]

   The hydroxyl radical in the initiation stage, oxidizes the organic substrate (RH) to form \( \text{H}_2\text{O} \). An organic radical (R) the later product has a single unpaired electron and thus can react with oxygen in triplet ground state. The addition of triplet oxygen to the carbon radical leads to the formation of a peroxyl radical (ROO*) which can readily abstract hydrogen from another organic molecule leading to the formation of a second carbon radical and by this way the reaction can propagate. The peroxidation reactions in the membrane lipids are terminated when the carbon or the peroxyl radical cross links to form conjugated products that are not radicals.

II. Oxidative damage to DNA:
The ROS can induce numerous lesions in DNA that causes deletions, mutations and other lethal genetic effects. Both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage and cross linkage to protein. The principle cause of single strand breaks is the oxidation of sugar moieties by hydroxyl radicals. Superoxides and peroxide alone cannot cause strand breaks under physiological conditions and therefore the toxicity is most likely the result of Fenton’s reaction with a metal catalyst. Cross linking of DNA to protein is often of order less abundant than single strand breaks, they are not as readily repaired, may be lethal if replication or transcription proceeds repair.
III. Oxidative damage of protein:
Oxidative attack on proteins results in site specific amino acid modification, fragmentation of the peptide chain aggregation of cross linked reaction products, altered electrical charge and increased susceptibility to proteolysis. The various forms of activated oxygen differ in their potential reactivity. Sulphur containing amino acids and thiols group specifically are very susceptible sites. Activated oxygen can abstract H –atoms from cysteine residues to form a thiol radical that will crosslink to a second thiol radical to form Disulfide Bridge. Alternatively oxygen can add to a methionine residue to form methionine sulfoxide derivatives. Free radical attack on proteins is not reversible e.g., the oxidation of iron sulphur centres by superoxide destroys the enzymatic functions. Many amino acids undergo specific irreversible modification when a protein is oxidised. Tryptophan is readily cross linked to form bityrosine product histidine, lysine, proline, arginine and serine from carbonyl groups on oxidation. The oxidative degradation of protein is enhanced in the presence of metal cofactors that are capable of redox cycling, such as Fe. The metal then reacts with $\text{H}_2\text{O}_2$ in the Fenton’s reaction to form a hydroxyl radical that rapidly oxidises amino acid residues at or near the cation binding site of protein. This site specific alteration of an amino acid usually inactivates the enzyme by destruction of cation binding sites.

2.16. Role of Oxygen concentration and stress responses of protozoan parasites

![Figure 2.13: Overview of protozoal stress responses.](image)

Protozoan parasites are exposed to a wide variety of different environments during their life cycles (Vonlaufen et al., 2008).
Living aerobic organisms, from prokaryotes to complex eukaryotes, have developed elaborate sequences of adaptive mechanisms to maintain oxygen homeostasis and equilibrium. Any deviation from homeostasis, or physiological change in oxygen pressure, is recognized as an exposure to oxidative stress (Fig. 2.13). In *Giardia*, measurement of O\(_2\) consumption as a function of dissolved O\(_2\) indicates that at low levels (0-50µM), the organism is capable of scavenging O\(_2\) (apparent \(K_m\) for O\(_2\) 6.4µM for the trophozoite). On the contrary, in human gut cell lining, *Giardia* resides in a higher oxygen tension where the O\(_2\) concentration has been measured at 60µM. This fact has become more interesting as the conventional enzymes for detoxifying reactive oxygen species (ROS) (viz. superoxide dismutase (SOD), catalase, peroxidase, glutathione reductase) are not found in *Giardia*. Change in the oxygen environment has played the key role in the evolution of aerobic organisms from anaerobes. In case of *Giardia*, it is also evident that change in oxygen pressure has achieved an important role in its survival strategy as well as the evolution and adaptation of this parasite.

In *G. lamblia*, a temperature increase results in a decrease of normal protein synthesis and the enhanced synthesis of HSPs (Lindley *et al.*, 1988). HSP70s in *G. lamblia* show the deepest divergence among eukaryotes and include the endoplasmic reticulum (ER)-resident, GRP78/BiP (Gupta *et al.*, 1994). *G. lamblia* GRP78/BiP expression increases during differentiation, possibly to keep cyst wall proteins soluble during trafficking, thereby preventing premature polymerization inside the parasite (Lujan *et al.*, 1996). The consumption of oxygen by *Giardia* has been particularly puzzling as the diplomonads lack mitochondria and cytochromes (Weinbach *et al.*, 1980; Paget *et al.*, 1993), and rather utilize carbohydrate and amino acid fermentation to meet energy demands (Biagini *et al.*, 1998). *Giardia* is sensitive to oxygen but appears to be devoid of SOD, catalase, peroxidase and GR/glutathione (Brown *et al.*, 1995). The alternative types of potential antioxidant proteins that have been reported include pyruvate itself, which can react with H\(_2\)O\(_2\), and exerts antioxidant activity in *G. intestinalis* (Biagini *et al.*, 2001).

Cysteine is the major low-molecular-weight thiol in *Giardia* and, as in *E. histolytica*, has been proposed to be a protectant from ROS (Fairlamb, 1989). A major flavin-dependent NADH oxidase (Brown *et al.*, 1996a), a thioredoxin reductase class of disulfide reductase (Brown *et al.*, 1996b), a membrane-bound NADH peroxidase (Brown *et al.*, 1998), an NAD(P)H:menadione oxidoreductase (DT-diaphorase) (Sanchez *et al.*, 2001), and putative thioredoxin peroxidases have been cited as potential mediators to protect these protozoa from ROS damage (Li & Wang, 2006). Interestingly, recent work with the DT-diaphorase has demonstrated it to be the likely cause of oxygen sensitivity in *G. lamblia* (Li & Wang, 2006).
2.17. Mode of cell death

Programmed cell death (PCD) is a well-regulated cellular process that has been extensively characterized in multicellular organisms. This process has also been observed in an increasing number of unicellular eukaryotes including the Trypanosomatids, Dictyostelium, Plasmodium, T. vaginalis, E. histolytica and Blastocystis (Chose et al., 2003; Bruchhaus et al., 2007; Tan & Nasirudeen, 2005). PCD can be initiated by various factors both external and internal and these are coordinated by a complex network of regulators and effectors. The various triggers are factors like cellular stress, serum or growth factor deprivation, chemotherapeutic agents, receptor ligand binding, de-regulation of cell division and development or differentiation (Debrabant et al., 2002). The most common types of PCD known are apoptosis and autophagy. Apoptosis (type I PCD) involves an orchestrated cascade of biochemical events leading to characteristic changes in cell morphology which includes proteolytic cleavage by caspases, cell shrinkage, DNA internucleosomal fragmentation, phosphatidylserine exposure, blebbing of the plasma membrane, formation of apoptotic bodies and loss of mitochondrial membrane potential with cytochrome c release to the cytosol (Menna-Burreto et al., 2009). These changes finally lead to cell death. Apoptosis has been divided into 2 forms: caspase-dependent and caspase-independent pathway. The caspase-independent pathway involves the induction of mitochondrial membrane permeabilization (MMP) and the release of apoptosis-inducing factor (AIF), which is controlled by the Bcl-2 family of proteins. In the caspase-dependent pathway, after MMP induction, cytochrome c redistributes from mitochondria to cytosol to activate caspase-9, in collaboration with ATP and the cytosolic factor Apaf-1 (Lorenzo & Susin, 2004). Autophagy (type II PCD) involves the autophagosomal–lysosomal system. Autophagosomes are double-membrane vesicles which are responsible for the engulfment of cytoplasmic constituents, during turnover of organelles. After autophagosome – lysosome fusion, an autophagolysosome is formed in which cellular residues are digested. Autophagy is crucial to maintain the metabolic balance and the recycling of cellular structures during cell growth and development. The de-regulation of such a balance can initiate cell death as a result of prolonged starvation or oxidative stress. In this it has been suggested that limited self-digestion of cell materials, including organelles, can help individual cells provide energy to facilitate survival for up to several days. However, if conditions do not improve, self-digestion continues and eventually results in autophagic cell death (Bruchhaus et al., 2007). Clearly these processes differ from that of necrosis which is typically associated with extreme cell injury causing dramatic alterations in mitochondrial function, cytoplasmic vacuolization
and, ultimately, the breakdown of the plasma membrane. The recycling process of dead cells in necrosis by comparison is slow and less regulated, due to the absence of specific cell signals, leading to an important inflammatory response (Menna-Burreto et al., 2009). With the increasing evidence of PCD in single celled organisms including bacteria (Koonin & Aravind, 2002) it is important to understand how this/these processes evolved with respect to the pathways and also the rational for such systems in unicellular organisms. Although there are a significant number of publications in this area on organisms such as Dictyostelium, Trypanosoma, Leishmania and Plasmodium, our understanding of the pathways involved in many organisms is variable. Giardia is a micro-aerophilic parasite of humans and animals that lacks mitochondria but contains mitosomes (thought to be relic mitochondria). This organism is a eukaryote and possesses many typical characteristics such as a distinct nucleus and nuclear membrane, cytoskeleton, and endomembrane system; however, other aspects of the cell such as SSU rRNA and some key metabolic enzymes are prokaryotic-like (Svard et al., 2003). Although controversial, the majority of workers think of Giardia as a eukaryote that has diverged at or just after mitochondrial acquisition and this has made Giardia an important organism for research into the understanding of evolution in eukaryotic cells (Thompson, 2004).

It has been shown that organisms use different pathways of programmed cell death to actively self destruct. Several studies indicate that Giardia undergoes programmed cell death in response to various stresses and that there are 2 distinct non-necrotic forms, apoptosis-like (Type I) PCD and autophagy-like (Type II) PCD. Our data support studies by other investigators (Pérez-Arriaga et al., 2006; Ghosh et al., 2009; Corrêa et al., 2009; Shemarova, 2010) who have demonstrated through the use of staining that a type of PCD occurs in Giardia. However, in our previous study, we have suggested that apoptotic-like cell death occurs via a mechanism(s) independent of caspases. We were unable to detect caspase activity using a range of approaches including, Western blot, and the use of specific fluorescent substrates and inhibitors. It was demonstrated that Giardia possesses 2 forms of cell death – apoptosis and autophagy (Corrêa et al., 2009). Interestingly, this group proposed the presence of caspases and a caspase-dependent mechanism using CaspaTag and fluorescence microscopy. This method, although frequently used, has been shown by several authors to have questionable specificity (Darzynkiewicz & Pozarowski, 2007). The lack of caspase genes in the genome of unicellular eukaryotes and the possession of an apoptotic pathway independent of caspases has been well documented. In recent years there have been various reports highlighting the involvement of metacaspases in an apoptotic-like PCD in
 unicellular organisms. Metacaspases have shown a well-established functional activity in yeast (Mazzoni and Falcone, 2008), but work in Trypanosoma brucei (Helms et al., 2006) and Plasmodium berghei (Le Chat et al., 2007) has been inconclusive (Atkinson et al., 2009). Caspase independent apoptosis does occur; however, this typically requires the presence of functional mitochondria and therefore this pathway is unlikely in Giardia because this organism lacks mitochondria.

Giardia does, however, have a relic structure termed the mitosome but this organelle is not able to generate energy and no clear metabolic or signalling role has been associated with it (Rosa et al., 2008). Analysis of the Giardia genome for apoptotic-related genes including caspases and metacaspases was unsuccessful in that we were unable to identify any components.

In addition to apoptotic-like PCD, starvation induced death demonstrated evidence of autophagy (type II PCD) in Giardia. This observation was supported by morphological and bioinformatics analysis. It would seem from the genome data presented that the autophagy pathway shows some conservation; however, our survey suggested that the pathway in Giardia is not complete. This is in clear contrast to our ‘apoptotic’-like mechanism(s). However, these data must be looked at carefully as the level of divergence in Giardia proteins may make these types of analysis biased towards those that have significant homology.

The mechanism of programmed cell death (PCD) which takes place in amitochondriate Giardia is still unknown. The pathways involved are not yet clear although we find that the end results are similar to known pathways in other unicellular organisms. Our study supports the claim that PCD originated prior to multicellularity and divergence of the unicellular eukaryotes. These processes would seem to be evolutionarily conserved; however, the mechanisms that underpin these are not common to all eukaryotes.

Finally, it is clear that to delineate such pathways in the protozoans will require a global analysis of transcriptomes, proteomes and metabolomes. Elucidation of these pathways is of great significance not only in terms of evolution but also in terms of identifying novel approaches for the treatment of Giardiasis and for the control of many important protozoan parasites. Efficacious targeting at the molecular level should lead to disease control and produce few undesirable side effects in the host, particularly as the mechanisms involved in both the mammalian and Giardia systems would seem to be significantly different.
2.18. Importance of *Giardia* in Evolution

All organisms that currently occupy the Earth are either prokaryotes or eukaryotes, based on their cellular morphology, but *Giardia* for a long time seemed not to fit well into these categories. More than 2.2 billion years after the time of the increase in oxygen concentration in the Earth’s atmosphere (Hedges *et al.*, 2004), it is hard to imagine that atmospheric oxygen could be toxic; but it was. Back then, the only organisms that could advance towards complex multicellular forms were those that were effectively dealing with the increasing oxygen levels in Earth’s atmosphere.

They did so by giving rise to eukaryotes, organisms having a nucleus and compartments such as mitochondria or plastids that are surrounded by double membranes. How did they advance? According to the serial endosymbiosis theory, they united with small prokaryotic organisms (which were possibly parasitic or symbiotic at that time) that could process oxygen and, over evolutionary time, they became biochemically indispensable for their hosts as a source of cellular energy (Whatley *et al.*, 1979). The energy was generated in the form of ATP derived from the aerobic processing of sugars, a constant supply of which (energy) enables current prokaryotic and eukaryotic life on Earth.

How the endosymbionts became ‘indispensable for their hosts’ is still unclear, because considerable evidence gathered to date indicates that the energy provision might not be the only driving force for establishment and retention of these endosymbionts by the host cell (Moreira & Lopez-Garcia, 1998; Martin & Muller, 1998; Anderson & Kurtland, 1999). It is doubtful that endosymbionts could transport ATP to the host cell at the time of their initial interaction because the ATP translocators are later adaptations in eukaryotic life (Saier, 1999). According to the hydrogen hypothesis, the host cell at the time of the union was an anaerobic archaebacterium, strictly hydrogen dependent and strictly autotrophic, whereas the endosymbiont was a eubacterium that was able to respire and generate molecular hydrogen as a waste product of anaerobic metabolism (Martin & Muller, 1998). Thus, dependence upon the molecular hydrogen produced by the endosymbiont was a driving force for sealing the host–endosymbiont union (Martin & Muller, 1998). Alternatively, according to the metabolic syntrophy (symbiosis) hypothesis, the symbiosis between a methanobacterial-like organism (host) and sulfate-reducing myxobacterium (endosymbiont) was mediated in moderately thermophilic environments, and was based on progressive cellular and genomic cointegration of both types of these prokaryotic partners (Moreira & Lopez-Garcia, 1998). It is worth mentioning that phylogenetic reconstruction analyses of genes coding for proteins involved in
energy metabolism and translation did not support the hydrogen and syntrophy hypotheses but, instead, confirmed the serial endosymbiosis theory (Anderson & Kurtland, 1999).

However, new sequence data challenge the serial endosymbiosis theory, instead raising the possibility that the origin of the mitochondrion coincided with the origin of the nuclear genome of the eukaryotic cell (Lang et al., 1999). In other words, the mitochondrion arose at the same time as the nuclear component of the eukaryotic cell (Gray et al., 1999), rather than in a separate event, as postulated by the serial endosymbiosis theory. Fundamental questions Were the host–endosymbiont union a single event in evolution? This was, indeed, most likely to be the case.

Analyses of genomic organization of mitochondrial genes indicate that these genes derived from an a-proteobacterium-like ancestor (Gray et al., 1999), probably due to a single ancient invasion (Dyall et al., 2004) of an Archea-type host that occurred more than 1.5 billion years ago (Gray et al., 1999). Most experts now agree that mitochondria and mitochondrion-related organelles are monophyletic in origin (Theissen et al., 2003). Is this the only way that ATP can be generated? This is certainly not the case (Gray et al., 1999; Embley et al., 2003). These two questions set up the basis for the debate on Giardia, which has long been considered to be a living fossil, living relic or a primitive early branching eukaryote.

However, Giardia does not really fit into this classification as the most primitive eukaryote or premitochondrial organism (Henze & Martin, 2003; Knigth, 2004; Lloyd, 2004). This is because of the small prokaryotic parasitic or symbiotic organisms that give rise to mitochondria, a characteristic of eukaryotes (Dyall & Johnson, 2000). More and more ultrastructural, biochemical, and molecular phylogenetic details are being revealed that bring into question the lingering textbook definition for Giardia (Henze & Martin, 2003; Knigth, 2004; Lloyd, 2004; Tovar et al., 2003). Giardia is the most widely distributed enteric parasite, and Giardiasis is the most common gastrointestinal disease of protozoan etiology (Wolfe, 1992). This in itself justifies the title of successful protozoan parasite of humans and animals (Wolfe, 1992) yet, surprisingly, phylogeny of this parasite is still intensively discussed. Giardia does not have mitochondria, at least in the sense of the classical eukaryotic ultrastructurally defined organelle. This is also relevant to other microaerophilic protists such as Trichomonas vaginalis, Tritrichomonas foetus and Entamoeba histolytica (Tovar et al., 2003, 2004). Moreover, to alleviate the parasitic bias, many free-living organisms also fall into this microaerophilic protist category (Lloyd, 2004).
A mitochondrion is a double-membraned organelle which makes ATP, requiring oxygen and sugars to do so (Embley et al., 2003). Mitochondria are not uniform across species and represent a variety of extremely diverse organelles. The major function of mitochondria is the electron transport chain mediated oxidation from reduced donors to terminal electron acceptors. *Giardia* also makes ATP; however, it does this in the cytosol, using anaerobic pathways that use Fe–S clusters, cofactors in electron transfers (Tovar et al., 2003). In classical mitochondria, synthesis of Fe–S clusters occurs within the organelle (Henze & Martin, 2003). In *Giardia*, Fe–S clusters are generated in small structures surrounded by two membranes (Tovar et al., 2003). Cell morphologists have described only two types of organelles with double membranes (i.e. plastids and mitochondria), both of which are descendants of prokaryotic endosymbionts. The small membranous structures identified in *Giardia* have no molecular traces of chloroplast biochemical pathways (Henze & Martin, 2003). These organelles (called mitosomes) are ‘equivalent’ to mitochondria, in the sense that they are indirectly involved in production of ATP biosynthesis through the provision of Fe–S clusters (Tovar et al., 2003). *Giardia* mitosomes are characterized by their anaerobic function (Tovar et al., 2003). However, it is unclear whether mitosomes represent a rudimentary mitochondrion as a result of secondary degeneration driven by the adaptation to the microaerophilic habitat (Tovar et al., 2003). It has been recently postulated that a constant supply of Fe–S clusters might have been the selective force for establishment and retention of mitochondrion-derived organelles (Tovar et al., 2003). It is also helpful to remember that the eukaryotes diversified while the ocean was predominantly anoxic (Knoll, 2003) so the anaerobic functions can potentially be seen as biochemical functions of the mitochondrion (Lloyd, 2004).

The mechanism of generating ATP by *Giardia* differs from the mechanisms used by organisms that have typical mitochondria, and this difference has been used in the pharmacological treatment of clinical *Giardiasis* (Henze & Martin, 2003). The F–S cluster mentioned above, in addition to associated hydrogenases, ferredoxins and pyruvate (ferrodoxin oxidoreductase), are essential components for parasite survival in the duodenum, and they represent a successfully used target for pharmacological treatment (Henze & Martin, 2003). To be (aerobic) or not to be the confusion over the energy-generating cellular structures has arisen from the fact that, as aerobic beings, we humans have a basic misunderstanding of the concept of beings that are anaerobic. ‘Anaerobiosis’ is a term frequently used erroneously to characterize various conditions with low, but measurable, oxygen concentrations (as distinct from no oxygen at all). Such environments should be
described as microaerophilic rather than anaerobic. Microaerophilic habitats, for example the intestinal epithelium occupied by *Giardia*, show a measurable concentration of oxygen. In the duodenum, in which *Giardia* lives, the concentration of oxygen might reach 60 mM (Lloyd, 2004). In such places, sulfide can provide reducing power and can serve as an alternative electron acceptor. This is observed in *Giardia*.

Alternatively, in many parasitic, microaerophilic protists, mitochondrial functions become so altered that energygenerating processes and ion-transport functions can be difficult to detect (Lloyd, 2004). Besides, among mitochondria are also hydrogenosomes, which are simply hydrogen-producing forms of mitochondria (Embley et al., 2003). Some microaerophilic protists, for example *T. foetus*, do not have mitochondria but rather hydrogenosomes which produce ATP fermentatively. Hydrogenosomes have double membranes, and phylogenetic analyses have suggested a common ancestry for hydrogenosomes and mitochondria (Anderson & Kurtland, 1999; Dyall & Johnson, 2000). In this context, mitosomes identified in *Giardia* and also in *E. histolytica* (Tovar et al. 2003, 2004) can be regarded as the most recent additions to the mitochondrial family. However, it is still unclear whether mitosomes arose from a common ancestral endosymbiont that gave rise to both the mitochondria and hydrogenosomes.

However, this does not mean that they do not exist. The conglomerate of the lack of understanding of the timescale of evolutionary processes, misunderstanding of basic biological processes and bias of some influential reviews accounted for most of the misconceptions regarding the mitochondrial implications in the tree of life. Terms such as mitosomes, mitochondrial remnants, relic mitochondria and mitochondrion-related relic organelles (Tovar et al., 2003, 2004) are confusing if their evolutionary heritage is not clearly understood. *Giardia* is a highly successful parasite in its own evolutionary, environmental and clinical arena, with an extraordinary physiological plasticity and adaptation to fluctuating external and internal environmental conditions.

*Giardia* is also an ultrastructurally simple organism when compared with other intestinal protozoans. Certainly, *Giardia* is not an ancestor of an organism(s) from before the prokaryotic union. We create definitions to facilitate the understanding of the ambient and evolving world. As further details are revealed, these definitions can be changed. *Giardia* can no longer be regarded as a living relic.
2.18. Study of differential gene expression in *Giardia* using genomic DNA microarray and Real-time PCR technique

Differences in cell types or states are correlated with changes in the mRNA levels of many genes (DeRisi *et al.*, 1997). DNA-microarray technology provides an opportunity to look simultaneously at changes in gene expression in thousands of genes under different physiological conditions (DeRisi *et al.*, 1999).

Oxidative stress triggers a range of physiological, pathological, and adaptive responses in cells either as a result of cellular damage or through specific signaling molecules. These responses ultimately modulate transcriptional outputs to influence cell fate and disease processes. In the past two decades, a number of transcription factors and signaling pathways have been identified and delineated to mediate critical transcriptional responses to oxidative stress. These examples demonstrate the importance as well as the complexity of how alterations in intracellular ROS are converted into discrete and reproducible alterations in gene expression.

However, this is only the beginning of an intensive research period that will perceive *Giardia* spp. being used as model systems to understand genome function and evolution, gene expression, cell biology and intestinal immune responses. The genomes of several *G. lamblia* isolates from different assemblages are being analyzed currently, and now it is possible to develop methods for transfection, gene knockouts, and functional genomics and gene expression studies. The previous decade has contributed substantially to our understanding of protein processing and transport in vegetative trophozoites and encysting organisms. Further investigation promises to aid our understanding not only of the similarities to other eukaryotes but also of the key differences that may provide targets for therapeutic intervention. The next decade will see the application of the current genomic advances to addressing these interesting and important questions. Studies on the transcriptomics, proteomics and metabolomics will allow the identification of the enzymatic components of the various biochemical pathways and will suggest new beam of light on *Giardia* research as well as novel therapeutic strategies.