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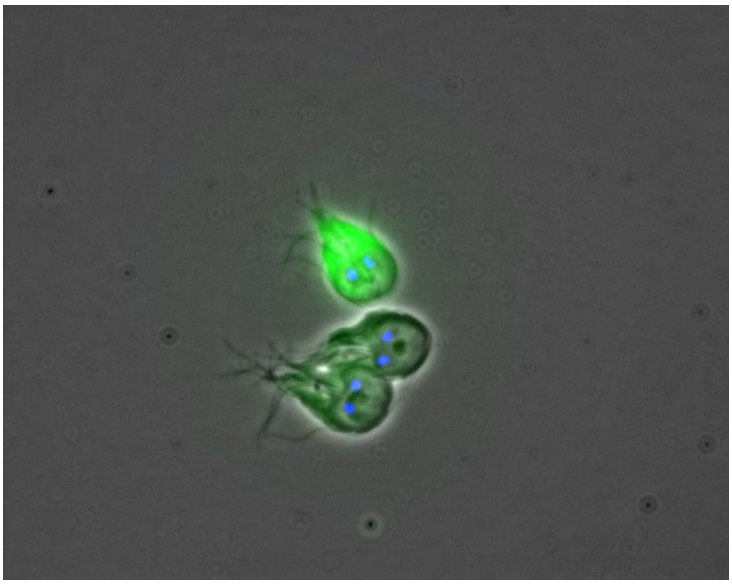
Virulence and oxygen stress genes in *Giardia lamblia*



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1BL426, Degree Project MN3, biology, 30 hp
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Summary

A lot of things are unknown about *Giardia lamblia;* how it causes distress in the host, what it gains from attaching itself to the small intestine, how it protects itself against oxygen, which genes are virulence genes and how does the antibiotic group known as nitroimidazoles actually kill the parasite. In this paper we have taken a look on what genes might be virulence genes as well as oxygen-stress genes. We have also made a suggestion for how the oxygen-stress pathways might work in *Giardia lamblia*. We placed among other proteins thioredoxin and thioredoxin reductase in this map and created transfected trophozoites that over-expressed the proteins with HA tags. We used these transfected parasites to localize the proteins to the cytoplasm in the trophozoite and to find out that the parasite does not survive any better in oxygen with these proteins over-expressed. We also found out that over-expression of thioredoxin and thioredoxin reductase alone does not make the parasites any more sensitive to metronidazole.

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1. Introduction

1.1 Giardia background

Giardia lamblia, also known as *Giardia intestinalis* or *Giardia duodenalis*, is a single-celled eukaryotic parasite. It was first described by van Leeuwenhoek in 1681 when he examined his own stool sample with a home-made microscope(Adam 2001). In 1859 the organism was described in greater detail by Lambl. His thinking was that it should belong to the order *Cercomonas(Adam 2001)*. The name *Giardia lamblia* was not used until later. There is no real consensus on the naming. However, *Giardia lamblia* is the most used term today and also the name used by the medical profession. *Giardia intestinalis* is the name used by European research groups, while Australians use *G.duodenalis* and in the United States they use *G. lamblia*

FIGURE 1

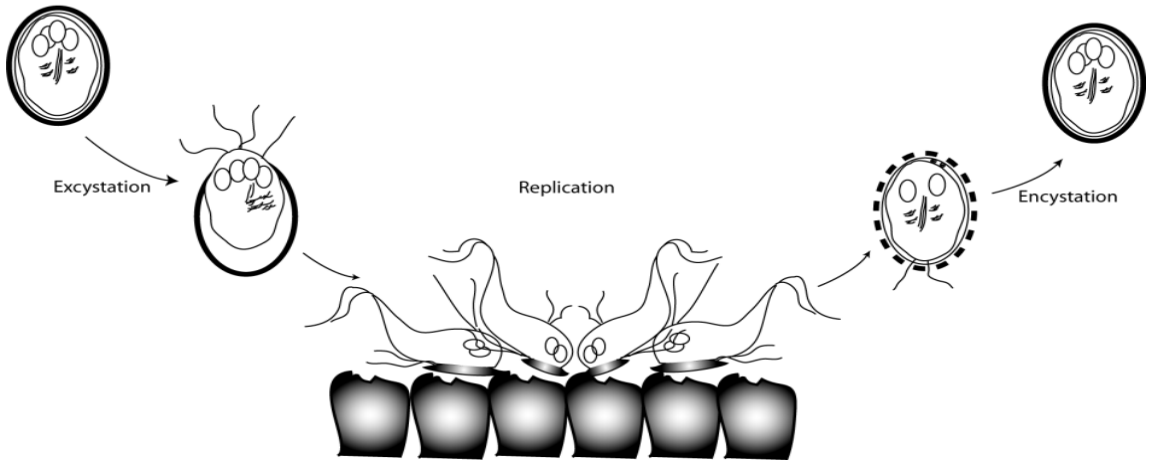

Figure 1. Life cycle of *Giardia lamblia*. The parasite begins its life cycle as an egg-shaped, thick-walled cyst with four nuclei. It is taken up by the host organism via the oral route and the cyst subsequently starts excystation in the stomach. Each cyst forms four pear shaped parasites with two nuclei each, which use a disc on the ventral part of parasite to attach to the intestinal stomach lining. Once attached, the parasite starts to replicate. Encystation generally occurs in the lower part of the intestinal tract and it starts when the parasite has started nuclear replication but the cytokinesis has not yet started. The cyst forms with four nuclei and each nucleus contains two sets of the DNA. Then a hardy cell-wall forms and the cyst exits the host through the fecal route.

The parasite has a two-stage lifecycle (Fig. 1). It first lives as a parasite called trophozoite (Fig. 1) in the small intestine on the epithelial cells. It then starts the encystation process in the lower part of the small intestine were it gains a thicker outer wall, becomes round and cannot move on its own. This encystation is started by a combination of high levels of bile salts, fatty acids and a approximate pH of 7.8 in the large intestine(Ankarklev, Jerlstrom-Hultqvist et al. 2010). The cysts are then expelled from the host with the fecal matter (Fig. 1). The cyst can survive in water or in a humid environment for a long time, until it is taken up by another host whose low pH in the stomach acids starts the excystation process (Fig. 1). The Fecal-Oral route is the primary infectious route. In the third world it is one of the most common parasites, some studies say that there are $2.8 * 10^8$ infected people annually (Lane and Lloyd 2002) and another rapport claims that there are 3000 million people that live in

unsewered conditions and about 30% of them gets sick by *G. lamblia* each year giving a total of infected people closer to 1000 million every year, with 2.5 million deaths annually(WHO press, release 1998). The most common infection route is infection via drinking water, but there have been food epidemics caused by the parasite as well. Due to the lower standard of drinking water in third world countries outbreaks are more common there, but there have been outbreaks in developed countries as well for example Bergen, Norway 2004(Mellingen, Midtun et al. 2010) and United States in 2007(Daly, Roy et al. 2010).

1.2 Giardia infection

The severity of the symptoms and time of *G. lamblia* infection can be very different. The infection can be asymptomatic so that the host does not even know that he has been infected. The parasite can also cause chronic diarrhea, which can result in malabsorption of nutrients. This is severe in children and can cause a "failure to thrive" syndrome which can cause cognitive and growth retardation(Adam 2001).

The infection with *G. lamblia* is generally self-limiting, indicating that the immunesystem recognizes the infection and can combat it, although the exact mechanism is unknown. Nitric oxide (NO) is considered a possible part of the defense against the trophozoite(Eckmann 2003). But the infection can also be chronic and it does not stop unless antibiotics are prescribed. There are several drugs that can fight the parasite, most popular today is Metronidazole, also known commercially as Flagyl. Combined with other antibiotics, are available as over the counter medication in many countries, which leads to certain strains of the parasite developing resistance to the drug.

1.3 Giardia morphology

FIGURE 2

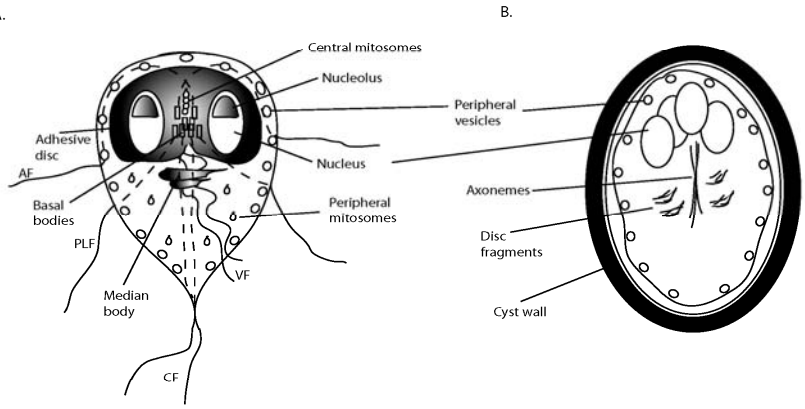

Figure 2. Morphological features of *Giardia lamblia* in trophozoite and cyst form. The trophozoite (A) measures between 12-15 µm in length and 5-9 µm in width. The picture of the trophozoite is shown dorsally. The adhesive disc is located on the ventral side and is used to attach trophozoites to host cells. It is composed of microtubules and is rigid. The basal bodies are the structures were all flagella originate. The dashed lines indicate the interior structures of the flagella. There are four pairs of flagella for a total of eight. Anterior flagella (AF), posterior/lateral flagella (PLF), ventral flagella (VF) and caudal flagella (CF). The median body is a structure of unknown function that is made up of microtubules. There are several peripheral and central mitosomes scattered throughout the trophozoite. The trophozoite contains two nuclei while the cyst contains four. In the tropozoite form the nucleus is diploid. There are lysosome-like vesicles present throughout the peripheral cell structure called peripheral vesicles. The cyst (B) has an oval shape and is measures between 8-12 µm long and 7-10 µm wide with a thick 0.3 – 0.5 µm outer cyst wall. There are flagella and disc fragments that have been disassembled and stored in the cyst. The nuclei are stored in a tetraploid form. The cyst is immobile and is dependent on external factors for spreading itself to new hosts.

Morphologically, *G. lamblia* is pear-shaped and has four pairs of flagella (Fig. 2A). Other major structures include two cell nuclei, a hole on the dorsal side and a disc for attachment to host cells on the ventral side of the parasite in the trophozoite stage. *G. lamblia* has no Golgi-complexes or mitochondria but a mitochondrial relic called the mitosome (Fig. 2A)(Ankarklev, Jerlstrom-Hultqvist et al. 2010). The trophozoite is generally 5-9 µm wide and 12-15 µm long. A cyst contains 4 nuclei (Fig. 2B) and is formed after the trophozite undergoes nuclear replication without the cytokinesis. A cyst is generally 5 µm in lenght and between 7 – 10 µm in diameter with a cyst wall that is 0.3 – 0.5 µm thick and has a generally oval shape (Fig. 2B)(Ankarklev, Jerlstrom-Hultqvist et al. 2010). *Giardia* has variant surface proteins (VSP) covering the whole surface of the trophozoites. These VSP are thought to change to keep the parasite safe from the host immune system as well as allow them to infect different species of hosts(Davids, Reiner et al. 2006).

1.4 Giardia Taxonomy

There are several *Giardia* species that infect different types of animals and they look morphologically different, but there is limited data on how different they are genetically. *G. lamblia* can infect several different mammals, including humans. The exact number of species that can be infected is unclear. The experiments that have been performed to infect one type of mammal with *G. lamblia* cysts from another infected mammal have been inconclusive. This is probably due to these experiments being performed at a time when differentiation of different *G. lamblia* species with molecular method was still lacking(Adam 2001).

G. lamblia is classified as a true eukaryote since it has a cytoskeleton, an endomembrane system and a nucleus. It does, however, lack several of the most common characteristics of a eukaryote. It does not have a smooth endoplasmic reticulum(Adam 2001), a well-defined Golgi apparatus(Adam 2001), peroxisomes(Adam 2001) and most importantly it does not have a true mitochondrium(Adam 2001), a feature that is perhaps considered the most important in determining if a species is an eukaryote or not. *G. lamblia* has some mitochondrial types of genes, which indicates that it once had a mitochondrium or it acquired it from another source(Adam 2001). The metabolism is also atypical of a eukaryote. A standard eukaryote tends to have a phosphorylation pathway, a citric acid cycle and glycolysis. *G. lamblia* has a glycolytic and fermentative metabolism, both under aerobic and anaerobic conditions. The terminal part of the glycolytic pathway is handled by a pyruvate:ferroredoxin oxidoreductase (PFOR) that is a homologue from bacteria that has either replaced the parasite form or the parasite has never evolved a pyruvate dehydrogenase the more common form found in aerobic spices(Adam 2001). All this leads to the conclusion that *Giardia* is an early branching eukaryote and this makes its oxygen protection pathways interesting to study. Its lifecycle leads the parasite's trophozoite form to be exposed to at least some oxygen. However, *G. lamblia* lacks the more common eukaryotic defenses against oxygen, such as Catalase, Peroxidase or Glutathione pathways(Adam 2001). It does have High cysteine membranes proteins (HCMP)(Davids, Reiner et al. 2006), a form of VSPs that are probably expressed in the membrane and one theory is that they might offer some protection against oxygen stress. It also has a flavodiirion protein that transforms oxygen to water(Di Matteo, Scandurra et al. 2008). FixW and PDI4 and PDI6(Brown, Upcroft et al. 1998; Muller, Liebau et al. 2003) proteins which serves as thioredoxin that reduce peroxiredoxin a protein that can reduce Hydrogen peroxide to water.

2. Results

2.1 Bioinformatic analysis of stress genes

Not much is known *about how G. lamblia* cope with stress during an infection or in the environment. Essentially nothing is known about what genes are used or how these genes are regulated. In order to try to study this we analyzed SAGE (Serial Analysis of Gene Expression) data sets, made by Daniel Palm and Dave Reiner at UCSD, California. *Giardia* trophozoites were grown in TYDK medium until the tubes were confluent. The trophozoites were then used to inoculate new cultures to a concentration of 10^5 Cells/ml in 10 ml tubes full with medium and a tight cap to prevent aerobic stress. The tubes were then grown for 21h at 37^0 C. Then incubated with chemicals to encyst and excyst the parasite. mRNA were then harvested at different intervals for SAGE analysis. As a control 10^5 cells/ml were incubated in TYDK.

The raw data has been published at www.mbl.edu/Giardia (now partially removed). The data was assembled by using the software on the site matching the trophozoite against the control sample to try to find a difference in gene expression when the trophozoite is in a more hostile environment considering aerobic and metabolic condition. The data was then analyzed to find how much the gene was upregulated compared to the control. The most significantly changed genes are shown in Table 1.

Table 1. Serial Analysis of Gene Expression (SAGE).

Orf number	Descriprion	Troph 1	Control 2,5 H	Fold Change
8173	Glycerol kinase	0.00263	0.04893	18.60456274
14759	6-phosphogluconate dehydrogenase, decarboxylating	0.01315	0.20089	15.27680608
6289	FixW protein, putative	0.00526	0.06181	11.75095057
137672	High cystine membrane protein, VSP like	0.00526	0.05924	11.26235741
8682	Glucose-6-phosphate 1-dehydrogenase	0.01578	0.17513	11.0982256
8074	Manganes-dependent inorganic pyrophosphatase, putativ	0.00789	0.06954	8.813688213
25296	Protein 21.1	0.01052	0.07984	7.589353612
14521	Peroxiredoxin 1	0.1894	1.26455	6.676610348
137618	Variant surface protein (VSP)	0.10522	0.64902	6.16821897
16587	Kinase	0.01841	0.10817	5.875611081
10971	Hypothetical protein	0.02631	0.13907	5.285822881
8722	Myb 1, like protein	0.08418	0.4095	4.864575909
22677	Nitroreductase Fd-NR2	0.0342	0.1468	4.292397661
91707	High cysteine membrane protein Group 1	0.0342	0.13907	4.066374269
24412	Protein 21.1	0.02631	0.10302	3.915621437
17516	Cathepsin B precursor	0.13679	0.48161	3.520798304
9827	Thioredoxin reductase	0.2499	0.56403	2.257022809

We compared these results against a different experimental setup. An oligo microarray study, made by Emma Ringqvist at Uppsala University(Ringqvist, Avesson et al. 2011), was used to compare the data in SAGE. The experiment was performed in 25 cm^2 bottles containing 10 ml interaction medium. The medium was either DMEM, DMEM and CaCo- 2 cells or TYDK. The TYDK tubes were tightly capped and filled to the limit; the data from the TYDK was used as a normalizing value and is not shown in the table. Parasite load was

equilibrated to an amount 10 times more than the CaCo-2 cells. The trophozoites were washed away and frozen after 1.5 hours of interaction and frozen. The RNA was then extracted and hybridized against an oligonucleotide microarray and analyzed with a GenePix Personel 4100A scanner and GenePix 5.0 software. The columns show how much each gene is upregulated with or without CaCo-2 cells and how many times higher the genes are upregulated when they are attached to CaCo -2 cells. The most significantly changed genes are shown in Table 2.

Tabel 2. Oligo microarray upregulated genes.

Original row	ORF Annotation	DMEM 1.5h	Caco 1.5h	Times Fold change
7786	GL50803_114042 high cysteine membrane protein Group 4	0.15	1.02	6.605229321
1332	GL50803_22385 Protein 21.1	0.91	1.75	1.913687785
4079	GL50803_7715 high cysteine membrane protein Group 1	0.66	1.23	1.877881296
5076	GL50803_16936 high cysteine membrane protein EGF-like	0.79	1.47	1.866828721
6225	GL50803_26140 high cysteine membrane protein EGF-like	0.84	1.24	1.475137176
8039	GL50803_114888 high cysteine protein (HCp)	0.88	1.29	1.465081415
1219	GL50803_22677 Nitroreductase Fd-NR2	0.76	1.06	1.404931806
4623	GL50803_115066 high cysteine membrane protein VSP-like	1.00	1.37	1.370664138
681	GL50803_6289 probable FixW protein	0.89	1.20	1.350147879
9557	GL50803_11540 hexose transporter	1.02	1.24	1.208150224
5506	GL50803_8682 Glucose-6-phosphate 1-dehydrogenase	1.55	1.87	1.207868092
9131	GL50803_8722 Myb 1-like protein (gMyb2)	1.51	1.75	1.160150341
5993	GL50803_91707 high cysteine membrane protein Group 1	1.47	1.66	1.126593795
4043	GL50803_16034 kinase, CAMK CAMKL	1.33	1.44	1.082175608
5574	GL50803_24842 Protein 21.1	1.28	1.08	0.845891706
1185	GL50803_7718 DNA-damage inducible protein DDI1-like	2.30	1.09	0.475117713

There are several interesting genes that are upregulated in both sets. They both have upregulated Glucose-6-phsophate 1-dehydrogenase, Nitroreductase Fd-NR2, High Cysteine Membrane Protein (HCMP) Group 1 (GL50803_91707) and FixW, which is a form of thioredoxin that *Giardia lamblia* has several possible copies of. Both sets have 2 genes of Protein 21.1 that are, however, not the same genes. The SAGE set has only 2 HCMPs upregulated and one Variant Surface Protein (VSP) while the Microarray set have 6 HCMPs and no VSP upregulated. The SAGE set also has several genes upregulated that are involved in oxygen stress: Peroxiredoxin 1, Thioredoxin reductase and Manganese-dependent inorganic pyrophosphates.

One interesting observation that was done during these experiments was that we saw that *Giardia* trophozoites could survive for 24 hours in DMEM in air when they were co-incubated with human CaCo-2 cells. However, in DMEM medium without CaCo-2 cells they died

already after 1.5 to 6 hours (Fig. 3). Thus, the CaCo-2 cells protect the parasites against oxygen stress.

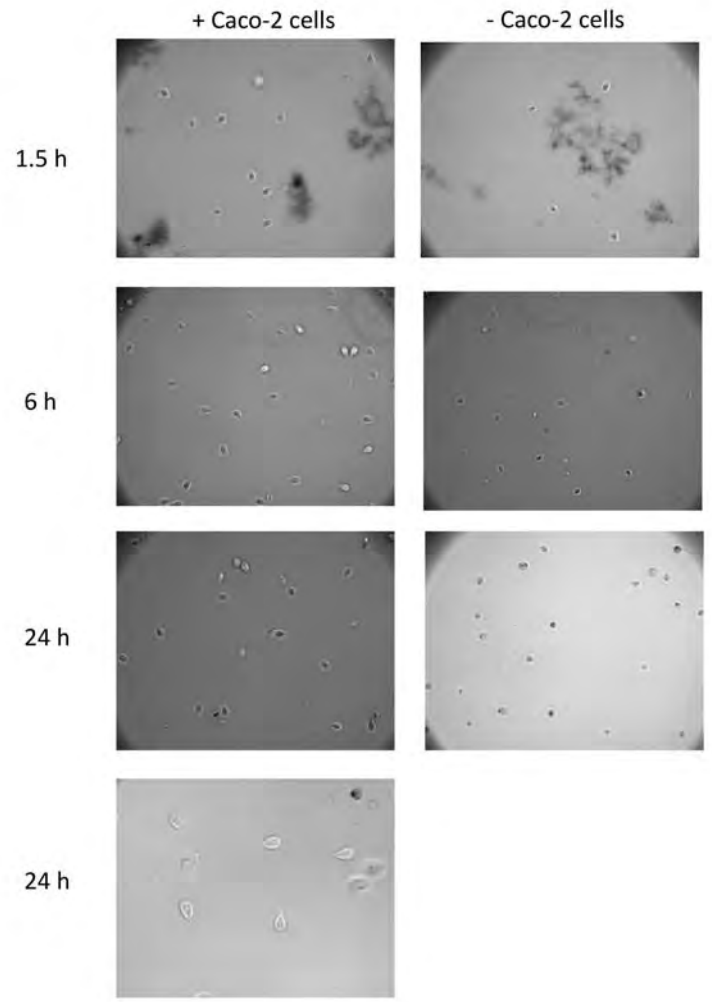

Figure 3. Trophozoite condition with and without CaCo-2 Cells. Confluent CaCo-2 cells whit DMEM medium in open wells were treated with the same amount of trophozoites and observed for 24 hours and photographed with a digital camera through a microscope in even intervals. The trophozoites attached to CaCo-2 cells lived up till the 24 hour mark while the trophozoites without CaCo-2 cells were all dead after 6 hours.

1.2 Proposal for Oxygen-stress protection pathways

By analyzing the data from Table 1 and 2 the following oxygen-stress reaction map in *Giardia lamblia* was extrapolated (Fig. 4)

The oxygen stress can first react witch High Cysteine rich Membrane Proteins (HCMP) that are similar to Variant Surface Proteins (VSP) and that we propose are both to protect the cell by reacting with oxygen and work in a signaling pathway to tell the cell how much oxygen is in the environment. Dioxygen (O_2) can be transformed by NAD(P)H:menadione oxidoreductase (DT-diphorase) that donates one electron to oxygen transforming it to superoxide anion(O_2^-).

Its theorized that $NADP^+$ and NAD^+ might be two of the more important electron donors. Superoxide is then transformed into hydrogen peroxide (H_2O_2), which is another form of oxidative stress coming from outside the cell. What catalyst this reaction uses is unknown. Flavodiiron can take Dioxygen and transform it in to water (H_2O). Hydrogen peroxide causes damage by, among other things, oxidizing proteins and can be reduced to water either by NADH oxidase or Thioredoxin peroxidase/peroxidase. Oxidized proteins and thioredoxin peroxidase are both reduced to a useable form by thioredoxin. There are many proteins that can work as thioredoxin among them several FixW, HCMP and PDI. The disulfide bridge is broken by Thioredoxin reductase, resetting thioredoxin to its reduced form. Thioredoxin reductase needs NADPH as an electron source. NADPH is created in part by NADPH-glutamate dehydrogenase being transformed by Glucose-6-phosphate dehydrogenase.

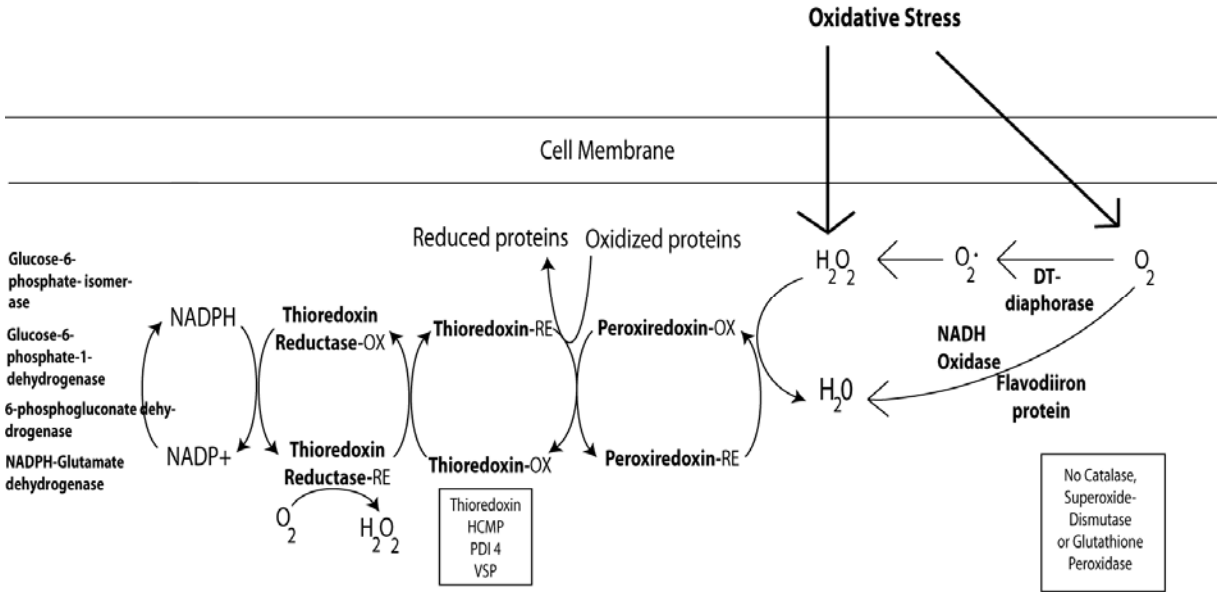

Figure 4. Proposed oxygen-stress protection pathway. This diagram gives an overview of the proposed oxygen protection pathways. ORF numbers and diagrams of the expression data of all named proteins and enzymes in the picture can be found at S1 (Supplemental information 1).

2.3 Cloning and transfection of Thioredoxin reductase and Thioredoxin

Thioredoxin reductase (ORF 9827) and Thioredoxin (among others ORF 3910) are some of the key components in the proposed oxygen-stress protection pathway illustrated in Figure 4. We decided to study these proteins further by cloning them into a newly developed plasmid that contains an HA-tag for epitope tagging of proteins in *G. lamblia* (J.Jerlström-Hultqvist, unpublished). The plasmid (Fig. 5A) contains several points that it can be cleaved by various restriction enzymes and an insert can be put in, in this case BamH1 and Not1 was used. The plasmid also contains three other domains: a PAC domain that stands for puromycin acetyl transferase which is used for selecting for transfectants in *G. lamblia*, a ColE1 domain that is used for replication inheritance in bacteria and an Amp domain that stands for β-lactamase and confers resistance to ampicillin, which is used as a selection tool in bacteria. The total length of the plasmid is 3871 basepairs (Fig. 5A). Primers were constructed to amplify Thioredoxin reductase (GL50803_9827) and thioredoxin (GL50803_3910), one of many possible thioredoxin candidates. The forward primers had to have an area that could be cleaved by BamH1 and the reverse primer was designed with a Not1 cleavage site. The total gene length between the primers is 1074 bp for Thioredoxin reductase and 882 bp for Thioredoxin (Fig. 5B). A gel was run on plasmid heat-shock transferred into *E.coli* to make sure that the inserts were of the right size and active (Fig. 6). The sequencing was made by Uppsala Genome Sequencing Center. Unfortunately the primer design for thioredoxin was not specific enough and the gene sequenced was a homologue to GL_50803_3910, specifically GL50803_104250. The genes are fairly similar though as can be seen by comparing the aminoacid sequences against each other (Fig. 5C).

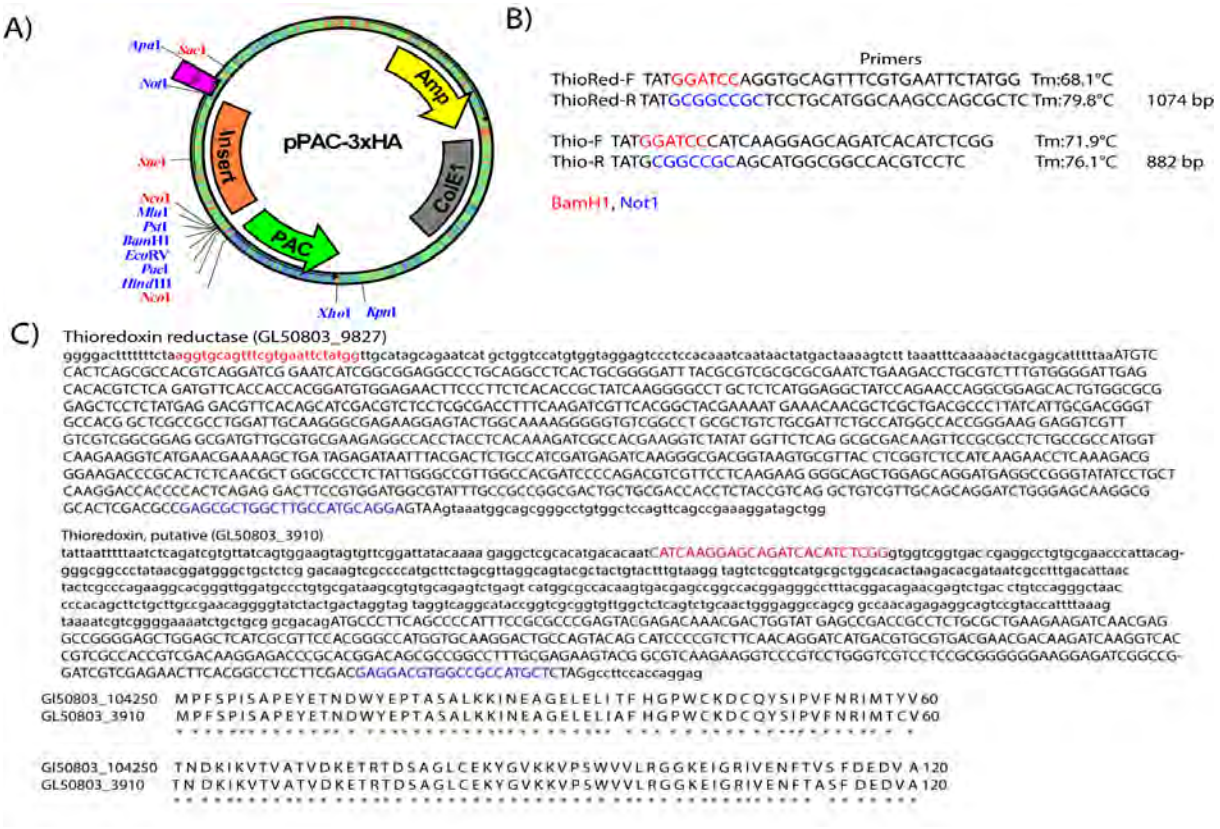

Figure 5. Epitope-tagging plasmid used for HA-tagging of Thioredoxin Reductase and Thioredoxin in *Giardia lamblia*. The red text denotes the forward primer the blue ones reverse primer. The capital letter shows the protein itself and the small promoter or some upstream DNA.

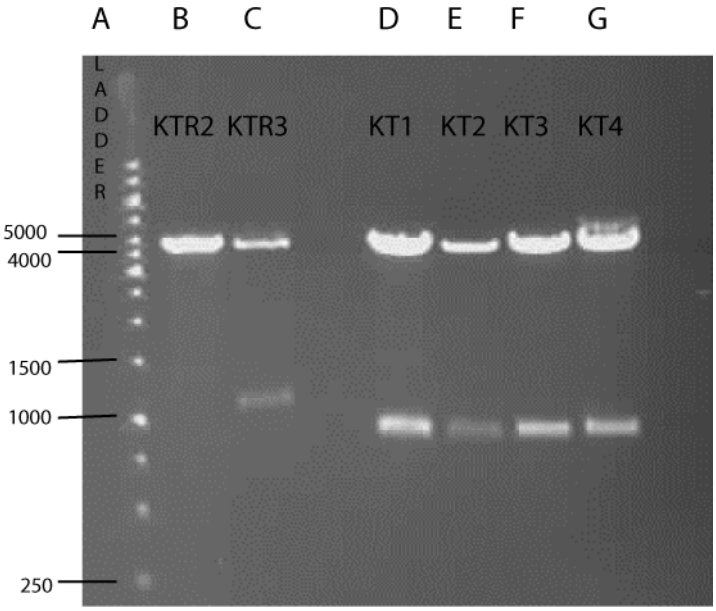

Figure 6. 1% 1xTAE Gel with the following samples: A contains a size ladder for comparison. B –G contains plasmid purified from bacteria and that has then been cut with BamH1 and Not1 to see if the plasmid has a gene insert. A) 1kB GeneRuler from fermentas. B) KTR2 C) KTR3 D)KT1 E)KT2 F) KT3 G) KT4. In the KTR series only KTR3 had an insert that could be thioredoxin reductase. So that sample was automatically qualified for transfection. All samples of the KT series shows a band at the appropriate 900 bp. KT1 was randomly picked for transfection.

2.4 Protein localization using microscopy and survival assay

One plasmid of each gene was transfected into *G. lamblia* and the trophozoites were grown under selection to perform microscopy and analyse where in the trophozoite the proteins are localized. Trophozoites were stained with an anti-HA-A488 antibody against the proteins and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI)(Fig. 7). The first column shows both stains, the second column only the anti-HA-A488, the third column only the DAPI stained, the fourth column is an ordinary black and white photo from the camera. Both proteins seem to be localized in the cytoplasm of the trophozoites.

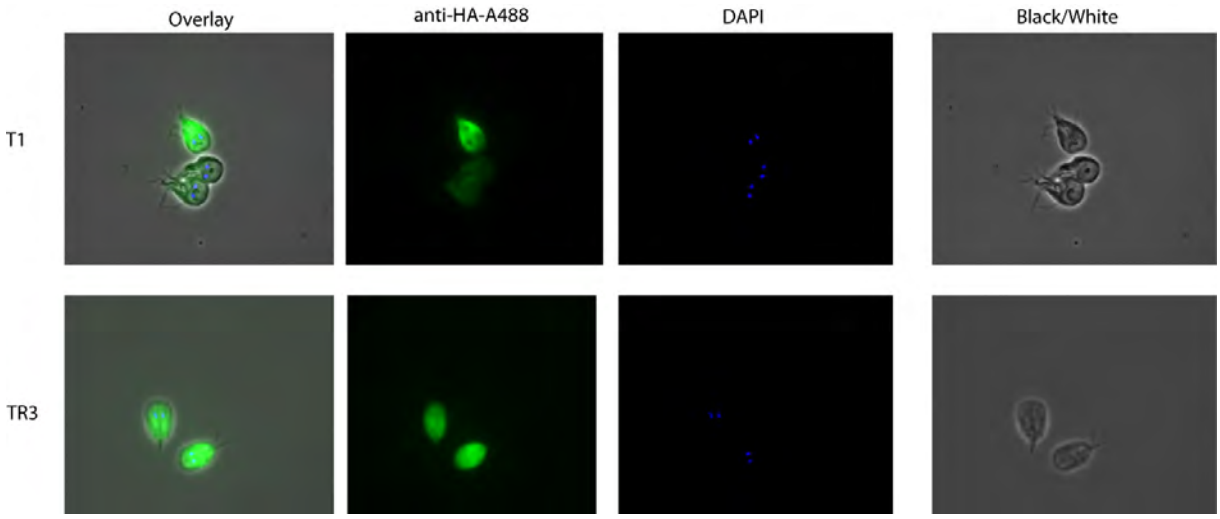

Figure 7. Fixed transfected *Giardia lamblia* with either Thioredoxin 1 (T1) or Thioredoxin Reductase 3 (TR3) proteins over-expressed and containing an HA-tag.

A small experiment was performed to see if the transfectants were more sensitive to nitroimidazole by filling 3 tubes of each type of confluent trophozoite with different concentrations of nitroimidazole (10 µM, 10 µM and 100 nM). The tubes were observed and a comparison was made between the transfected strains and the wildtype strain (data not shown). There was no greater sensitivity to nitroimidazole observed in any of the transfected parasites.

An experiment was performed to see if over-expression of thioredoxin and thioredoxin reductase in the transfected strain actually had an effect on the trophozoites ability to survive in oxygen. Three wells were loaded with 5 ml TYDK and an even number of trophozoites. The trophozoites were followed for 8 hours and pictures were taken with a digital camera through a light microscope (Fig. 8). There was no observable difference between the wildtype and the transfected parasites.

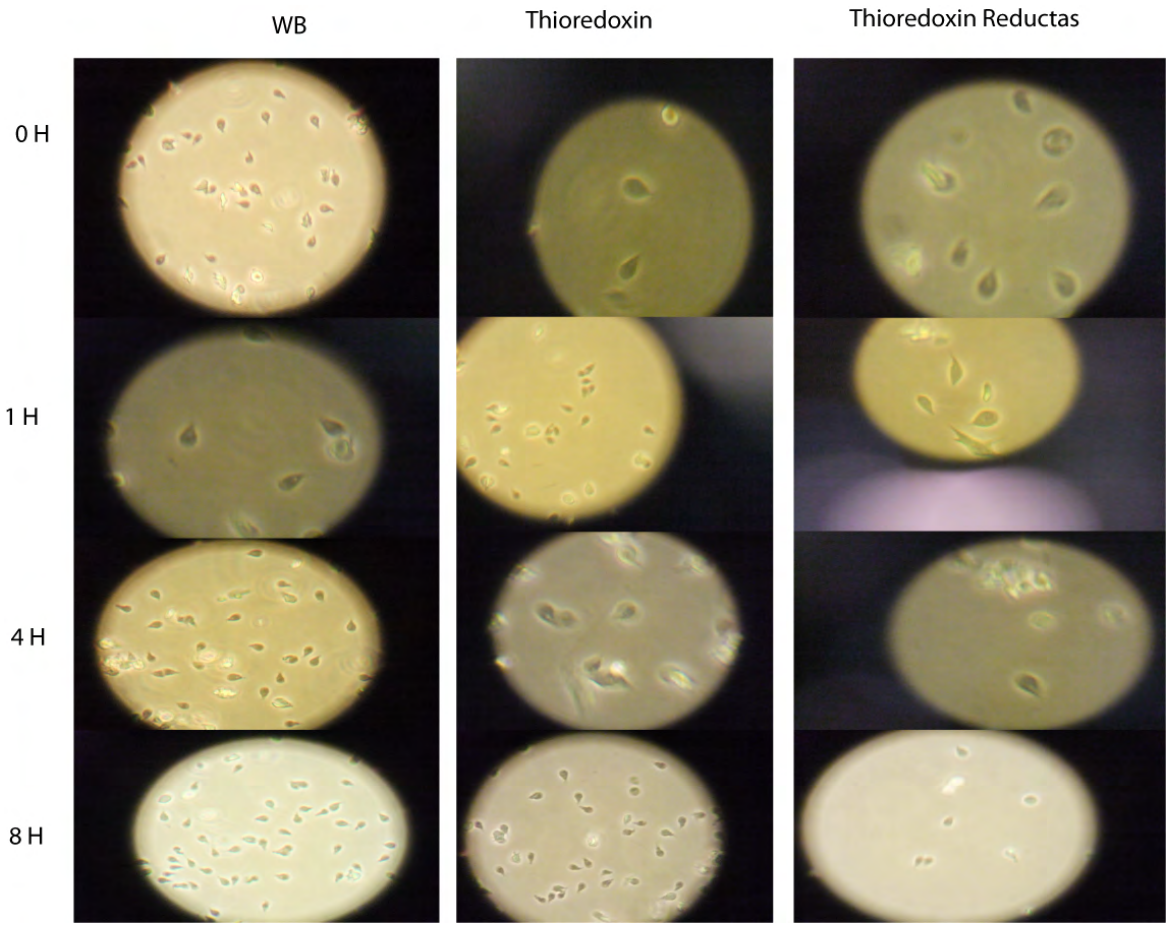

Figure 8. Oxygen stress-test. MULTIWELL™ 6 WELL plate with 3 wells in use at 5 ml TYDK in each well and 7,1 * 10^4 trophozoite in each well for each type WB as control T1 for Thioredoxin and TR3 for Thioredoxin Reductase. The picture were taken at every hour through a microscope with a digital camera for a total of eight hours. No visible difference was observed after eight hours, all three types of trophozoites were looking equally bad.

3. Discussion

In this study we used two different gene expression data sets, generated by microarray and SAGE to identify potential stress genes. There are several genes that are more upregulated in the SAGE data than in the microarray that clearly have to do with oxygen-stress. These are thioredoxin (ORF 6289), thioredoxin reductase (ORF 9827) and peroredoxin 1 (ORF 14521). These are known oxygen stress genes in other organisms(Brown, Upcroft et al. 1998). Thioredoxin reduces proteins that have been oxidized by oxygen or have gone through some other reaction. This includes Flavoproteins and Peroxiredoxin. Thioredoxin itself is oxidized in this reaction and needs to have its new disulfide bond reduced, which is facilitated by thioredoxin reductase. The medium DMEM gives both oxygen- and metabolic stress to trophozoites. However, according to the microarray data DMEM+Caco-2 cells seems to protect the trophozoites from the oxygen-stress to a large degree. This means that in that set there are some oxygen stress, but more metabolic stress genes upregulated for example NEK (ORF 16034 micro or ORF 16587 SAGE)(Pelegrini, Moura et al. 2010), glycerol kinase (ORF 8173 SAGE)(Applebee, Joyce et al. 2011), hexose transporter (ORF 11540 micro)(Slavic, Krishna et al. 2011), and damage inducible protein DDI2 (ORF 7718 micro) all proteins that have a metabolic or damage-control function.

Another gene that might possibly be a stress gene is the putative manganese-dependent inorganic pyrophosphatase (ORF8074) (m-d.i.p.), the exact function of this gene is unknown and it only looks like a manganese-dependent gene found in other organisms. Manganese in general is known to have many different functions among them production of Reactive Oxygen Species (ROS) as part of a detoxification system in bacteria. It is also known to be needed for enzymatic reactions serving as a co-factor or having an inhibiting effect. Several primitive organisms also use manganese ions instead of iron for some functions(Jakubovics and Jenkinson 2001; Wu, Seib et al. 2010). *G. lamblia* has no ROS scavenging systems in the conventional sense and what else manganese does in *G. lamblia* is not that well know. Inorganic pyrophosphates is, however, usually used in other organisms to catalyse the breakdown of pyrophosphates into two phosphate ions. Now normally these phosphates are often used in ATP but *G. lamblia* lacks a normal ATP system and instead only produces ATP by substrate level phosphorylation. This is highly ineffective and as *G. lamblia* is missing one off the key enzymes can only produce two ATPs instead off the normal six, however, *Giardia lamblia* can use a arginine dihydrolase pathway to create 1 ATP as well(Brown, Upcroft et al. 1998). There are also plenty of other possible reactions that need free phosphates there are several in *G. lamblia* important enzymes that are pyrophosphate dependent. The uppregulation of the m.-d.i.p gene in DMEM is interesting. It could mean that several other reactions need to be upregulated because of the stress the parasite is under and as a result more phosphates would be needed.

A gene with a similar reason of upregulation is Glucose-6-phosphate 1-dehydrogenase (ORF 8682) which is present in both data sets and is involved as a key enzyme in the formation of

NADPH. NADPH together with NADH, is one of the major anabolic molecules in *Giardia lamblia*. NADPH is hydrolysed to give electrons to many different reactions among others thioredoxin reductase. This makes the glucose 6-phosphate 1-dehydrogenase gene a major stress gene that needs to help supplying NADPH to many different reactions that help in keeping the trophozoite alive. Interestingly enough if you expose yeast to H_2O_2 this gene gets upregulated showing that it is used in oxygen stress pathways in other organisms as well(http://www.yeastgenome.org/). 6-phosphogluconate dehydrogenase, decarboxylating (ORF14759) is a protein in the same pathway to make NADPH, however, it was only shown to be upregulated in the SAGE data but it is upregulated in some microarray data as well (data not shown). These two genes actually sit together in the genome so it is not implausible that they are regulated by the same factors or that they may be co-dependent in some way (http://www.giardiadb.org/giardiadb). They are both also a big part of the Glucose metabolism normally Glucose is used in two pathways to make ATP and NADH by reducing the Glucose molecule to fructose and then several other steps until all that is left is pyruvate this pathway is complete in *G. lamblia*.

The other branch uses 6-phosphate 1-dehydrogenase, 6-phosphogluconate dehydrogenase and the missing 6-phosphgluconolactase in what is called the Pentose phosphate pathway. This pathway normally creates ATP and NADPH and the end stages are used in purine and pyrimidine scavenging pathways to create amino acids. *Giardia* cannot create ATP with this pentose pathway but it can create NADPH. This is due to the fact that the reaction between the existing enzymes is a spontaneous and the one missing, gluconolactase make the process more beneficial by catalyzing the reaction and creating an ATP molecule. But it is not a critical step and the two existing enzymes. Which is why when the parasite need NADPH to run among other thing the oxygen-stress pathways it need to create more of Glucose-6-phosphate 1-dehydrogenase but when glucose enters this Pentose pathway it leaves less glucose to be reduced to pyruvate leading to less ATP and pyruvate. To offset this it can use the arginine pathway to created ATP but much less than a fully functioning glucose metabolism(Brown, Upcroft et al. 1998). This is why under oxygen-stress the parasite is generally considered to be more arginine dependent. The Pentose pathway is also a suggested drug target in *Trypanosoma cruzi*, and *Plasmodium falciparum* both being parasite with similarities and differences from *Giardia lamblia*(Jortzik, Mailu et al. 2011).

Also interesting are two other genes that are upregulated in both data sets nitroreductase FD-NR2 (ORF 22677) and Myb 1 (ORF 8722). Nitroreductase is a flavoprotein that reduces nitro groups. This protein has earlier been shown to bind to nitazoxanide an antibiotic. It have also been shown to increases the susceptibility of *Giardia lamblia* to nitro-based antibiotics such as metronidazole as well as nitazoxanide(Nillius, Muller et al. 2011). Since there is no drug to reduce in the medium in our datasets, it is interesting that they are upregulated. There are probably some reactions that are stressed by the DMEM medium and need to lose a nitro group. Myb 1 is structurally similar to Myb 2 and is believed to have the same function, namely to help start and control encystation. More specifically most of

the genes controlled by Myb 1 are for cyst wall proteins(Sun, Palm et al. 2002; Huang, Su et al. 2008). The fact that Myb is uppregulated is very interesting, because it shows that the trophozoite senses stress and is trying to form a cyst as a survival mechanism. However, though Myb is upregulated, the parasite does not survive in our setup. Has it lost the ability, if it ever had it, or has the ability to encyst changed so much that it now only can from cysts in certain specific situations? It can also be upregulated because the trophozite needs to activate some metabolic pathways, the formation of a cyst is a major metabolic change perhaps Myb activates some metabolic pathways that helps the trophozoite to survive.

High Cysteine Membrane proteins (HCMP) and Variant surface proteins (VSP) are very similar in form, the HCMP lacks a C-terminal CRGKA sequence in the cytoplasm. There were many upregulated VSP, in the preliminary SAGE data. These were later renamed into HCMP when the data was reanalyzed. They were also not that highly upregulated, putting them below the threshold that is why there are more in HCMP in the microarray data set then the SAGE set. The exact mechanism how HCMP functions is unknown. The functions of HCMP in general is believed to be a protection against oxygen as well as to work as a signal system to let the trophozoite know how much oxygen is in the environment(Davids, Reiner et al. 2006).

Two genes of protein 21.1 are upregulated in both data sets. It is unknown what exactly the protein 21.1 does. The protein has a several ankyin repeat sequence and in other organisms the protein works as a protein-protein interaction domain.

Caco-2 obviously protects the trophozoites from the oxygen stress and possibly the metabolic stress as well this has been known to happen with other organisms as well(Hosogi and Duncan 2005). However it is unknown how and a repetition of the experiment using an O_2 probe to measure the amount of O_2 in the medium would be very interesting. Thioredoxin may not be the primary part of the pathway as we first theorized. Another group Li, Lei et al. published a paper showing that NAD(P)H:menadione oxidoreductase (Dt-diphorase) can transform O_2 to $O_2^{\cdot-}$ and that this then becomes H_2O_2. They also showed that if this gene is downregulated, the trophozoite grows a lot slower. They put forward a theory that this protein drives part of the cell metabolism of the parasite and is responsible for most, if not all, of the major oxygen stress that the parasite experiences(Li and Wang 2006). However, a paper by Adele et al. shows that the Flavodiiron protein has a very high affinity for binding O_2 and a low affinity for NO. This Flavodiiron protein group can in general reduce O_2 to H_2O or NO to N_2O. In this case the *Giardia lamblia* version only protects against O_2(Di Matteo, Scandurra et al. 2008). It is hard to say, how much of the oxygen stress is created internally by the parasite itself. Dt-diphorase must get some oxygen but it is also in combination with Flavodiiron protein and other oxygen dependent metabolic proteins such as for example pyruvate:ferroredoxin oxidoreductase (PFOR). The tropozoite is obviously sensitive to oxygen, but it is doubtful that most of it comes from the internal systems as the parasite seems to survive longer when attached to Caco-2 cells. The most likely explanation of this survival is that the oxygen and oxygen radicals are consumed by the human cells or

that the human cells have some other mechanisms that protect the parasite. This needs to be verified experimentally. It could also be so that the trophozoites take something from the humane cells when they are attached that helps them with the ROS detoxification.

Thioredoxin and thioredoxin reductase seem to be localized in the cytoplasm, meaning they are free floating and not attached to the membrane or one of the proto-organelles. This makes sense as there are many processes and proteins that need to be reduced by thioredoxin. In addition *Giardia lamblia* does not have an extensive transport system within the cell. The overexpression of the proteins thioredoxin and thiredoxin reductase does not seem to infer an ability to handle longer exposure to oxygen(Adam 2001). These overexpression strains need to be studied further.

The exact mechanisms that make nitroimidazoles toxic towards several microbial organisms, is unknown. What is known, is that these compounds are broken down inside the cell and that it is the byproducts of the breakdown not the original compound that cause the cells to die. There was a long held belief that the major, or perhaps the only component, that made this happen was the reduction of Ferredoxin facilitated by PFOR. This was supported by experiments that showed that nitroimdazoles could be broken down by PFOR proteins and reduced levels of PFOR was found in resistant cell lines. However recently Leitsch et al. showed in *Trichomonas vaginalis* that PFOR was not needed at all for the breakdown of nitroimdazoles. They showed that thioredoxin reductase acted as a electron donor to the nitroimdazole(Leitsch, Kolarich et al. 2009). They also showed that if flavin proteins such as thioredoxin were inhibited with diphenyleneiodonium that *Trichomonas vaginalis* was renderd insensitive to metronidazole(Leitsch, Kolarich et al. 2010). Therefore we did a small test to find out if our strains overexpressing thioredoxin and thioredoxin reductase were more sensitive to metronidazol. This proved not to be the case. The same group later extracted thioredoxin protein from trophozoites and added metronidazole and showed that it can be broken down by thioredoxin reductase. This does, at first hand, not make sense. However, this group also had access to several cell lines of resistant *Giardia lamblia* and when they checked them for the expected lower expressed thioredoxin, they found that the levels were the same as in the wildtype. They found that the resistant strains had a lower expression of flavoproteins(Leitsch, Burgess et al. 2011). This indicates that a first step in nitroimdazole toxicity is breakdown by thioredoxin reductase or perhaps PFOR, but there are more steps after this one. For example Nitroreductase, which as mentioned earlier is a flavo protein, could be one of the downstream proteins being affected by metronidazole.

4. Material & Methods

4.1 Cell growth

Giardia intestinalis, strain WB clone 6 (GL_50803) trophozites were cultured in TYI-S-33 (Tryptone-Yeast extract-Iron-Serum) medium in 10 ml Nunclon delta flat side tubes (NUNC, Cat. No 156758) and incubated at 37°C. Transfected trophozoite were selected for by adding 50 µg/ml puromycin. The cells were re-inoculated every 3 to 4 days or upon reaching an approximate confluence of 80-90 %. In order to detach cells from the tube, the cell culture tubes were put on ice for 15-20 minutes and then inverted several times. The trophozoites were subcultured by adding 5 µl of WB, 20 µl of Thioredoxin transfectants and 40 µl of Thioredoxin reductase transfectans to new medium. The different amount is to make all the tubes reach confluence on the same day.

Human colon-derived CaCo-2 cells TC7 clone were seeded in 25 cm^2 cell culture flasks (Sarstedt) at 10^5 cells per flask and maintained in DMEM medium at 37 °C 5% CO_2 in an incubator. The CaCo-2 cells were sub-cultivated two times every week to keep the cultures growing axenically. To make the CaCo-2 cells as similar to small intestine cells as possible, they were allowed to grow between 17 to 21 days with media change two times every week after they reach confluence. The morphological change was then confirmed using light microscopy.

4.2 Medium

TYI-S-33 (TYDK) was consisted of 30 mg/ml Peptone (Becton, Dickinson and Company), 55.6 mM Glucose (Sigma), 34.2 mM NaCl (Merck), 1.14 mM L-ascorbic acid (Sigma), 5.74 mM K_2HPO_4 $3H_2O$ (Fluka), 4.41 mM KH_2PO_4 (Merck), 11.4 mM L-cysteine hydrochloride monohydrate (Sigma) and 0.038 mM ferric ammonium citrate (Sigma), into filtered dH_2O. The pH was set using a PHM210 standard pH meter (MeterLab) and adjusted to 7.0 by adding 5M NaOH. The medium was sterile filtred with a 0.45µm Cellulose Acetate 250/500/1000 ml filter system (Corning). The medium was then supplemented with (0.268mg/ml) bovine bile (Sigma) that had been filtered through a 0.45 µm filter (Sarstedt) and added to the medium to a concentration of 0.5%. Heat-treated Fetal Bovine Serum

(Gibco) was added to the medium to a final concentration of 10%. Dulbecco's Modified Eagle Medium(DMEM) (Gibco) was modified to be used on human cell cultures by adding Fetal Bovine Serum (Gibco) to a final 10% concentration.

4.3 Gels and measurements

All gels were made of 1% 1xTAE and 1 gram/ 100ml Agarose (Sigma) with one to two drops of ethidium bromide (Dutscher Scientific) and run at 100 V for about 30 min. All samples were dyed with 1-5 µl 10x fast Digest Loading Buffer (Fermentas) and 2 µl 1kb GeneRuler (Fermentas) was used as a comparative ladder. All nanodrop measurements were performed on a Nanodrop machine from Savem Werner and analyzed using the program Nanodrop 1000 v3.6.0 from Thermo Fisher Scientific.

4.4 Bioinformatic sources

Serial Analysis of Gene Expression (SAGE) was made by Daniel Palm and Barb Davis at VESPI, San Diego. They did this by growing *Giardia lamblia* in TYDK medium until the tubes were confluent. The trophozoite were then used to inoculate new cultures to the concentration 10^5 cells/ml in 10 ml tubes full with medium and a tight cap to prevent aerobic stress. The cells were then grown for 21h at 37^0 C, and then the cells were then incubated with chemicals to make them encyst and excyst. Afterwards mRNA was harvested at different intervals for SAGE analysis. As a control 10^5 cells/ml were incubated in DMEM and mRNA harvested after 2.5h. Small, around 15 bp snippets called Exprest Sequence Tags (EST) were taken from a cDNA library originally are then hybridized against the mRNA, amplified and quantified. The data was first released in an uncurated raw form at the first Giardia DB site (http://gmod.mbl.edu) with site specific software allowing comparison and expression searches. This site has been discontinud and the data is now only available in a curated form at the new Giardia DB site (http://giardiadb.org). The experimental proceedings are also described in Birkeland et al., 2009.

The Oligo microarray studies during host-cell interaction were made by Emma Ringqvist at Uppsala University. It was performed in 25 cm^2(Sarsdedt) bottles white 10 ml interaction medium. The medium was either DMEM, DMEM and CaCo- 2 cells or TYDK. The TYDK tubes

were tightly capped and filled to the limit; the data from the TYDK was used as a normalizing value and is not shown in the table. The trophozoites were detached by chilling the tubes followed by washing of the trophozoites three time in cold PBS. The amounts of trophozoites were equilibrated to an amount 10 times more than the CaCo-2 cells. The trophozoites interacted with its environment for 1.5h, 6h and 18h. The trophozoites were then harvested by first chilling on ice and then washing them three times in cold PBS to get as many trophozoite cells as possible. The experiment was performed three times. The trophozoites were recollected by cold-centrifugation them and then resuspending them in TRIzol (Life Technologies) and snap-frozen and stored at -80^0C until RNA extraction. Cells were stored frozen in TRIzol solution 1ml per 10^7 cells for maximum of 4 weeks prior to extraction. RNA was extracted from all interaction samples using the Invitrogen TRIzol protocol (Life Technologies). Microarrays were run and analzed as described in Ringqvist et al., 2010. The data from this experiment is published at the Giardia DB site (www.giardiadb.org).

4.5 Oxygen stress test of *Giardia lamblia* with and without CaCo-2 host cells

CaCo-2 cells were grown to confluence as earlier described in small single wells with 3 ml of DMEM and incubated with seven times as many trophozoites as CaCo-2 cells. The controls were *Giardia lamblia* without any CaCo-2 cells. The well was not closed of in any way the lid was resting loosely on the well to allow oxygen to be exchanged. The wells were observed for 24 hours with a light microscope and a digital camera was used to take photos at the different time points through the microscope.

A MULTIWELL^{TM} 6 well (sarstedt) plate with 3 wells in use, one for wildtype control WB, one each for Thioredoxin reductase transfectant and one Thioredoxin transfectant. Each well has 5 ml TYDK and 7.1 * 10^4 trophozoite. The trophozoites were observed for eight hours through a light microscope. A digital camera was used to take pictures through the microscope at 15 minutes after the experiment (zero point) and every hour after the experiment had begun.

4.6 PCR amplification

The Thioredoxin reductase gene GL50803_ 9827 was amplified with forward primer (5´-TATGGATCCAGGTGCAGTTTCGTGAATTCTATGG-3´) with an annealing temperature of 68.1°C and reverse primer (5´-TATGCGGCCGCTCCTGCATGGCAAGCCAGCGCTC-3´) with an annealing temperature of 79.8°C, the Thioredoxin gene GL50803_3910 was amplified with a forward primer (5´-TATGGATCCCATCAAGGAGCAGATCACATCTCGG-3´) with an annealing temperature of 71.9°C and reverse primer (5´-TATGCGGCCGCAGCATGGCGGCCACGTCCTC-3´) with an annealing temperature of 76.1°C ordered from Metabion. All forward primers contained a sequence cleavable by Bam H1 (GGATCC) enzyme and the reverse primers by Not1 (CGGCCGC). The primers were aliquoted down to 20 µm in dH_2O.

PCR was performed on *Giardia lamblia* genome DNA supplied by Jon jerlström-hultqvist. The primers were mixed together pairwise giving a primer mix o 10 µm of each primer in the solution. PCR reaction contained 0.4 µl Phusion Hotstart II Hight Fidelity(HF) Polymerase(Finnzymes), 8 µl 5x Phusion HF buffert(finnzymes), 4 µl dNTPs(Fermentas), 2 µl primer-pair mix, 2 µl *Giardia lamblia* genomic DNA at 20ng/µl and 2.6 µl ddH_2O for a final volume of 40 µl. The PCR program for thioredoxin reductase was 3 min denaturation at 98^0C, the following three steps were repeated 32 times (15 s of denaturation ay 98^0C, 15 s of annealing at 68^0C, 30 s of amplification at 72^0C). The program was finished with a final amplification step at 72^0C for 35 s and run on a PTC-200 DNA Engine (MJ Research) PCR machine. For the thioredoxin amplification was the same, except that the annealing step was at 66^0C. A gel was run to confirm that the correct PCR product was actually obtained.

A PCR purification was run on both PCR products using QIAQuick PCR. The PCR tube was filled with four times as much PB buffer as sample, in this case 200 µl. This was transferred to a SpinCollumn with membrane and centrifuged for 30-60 seconds (s) at 13000 rpm in a table top centrifuge and the supernatant was discarded. The membrane was the washed with 750 µl PE buffer and centrifuged again for 30-60s. The supernatant was discarded and the tube was centrifuged again for 60s and the supernatant is discarded. The membrane part of the collumn was transferred to an Eppendorf tube and filled with 30 µl EB buffert, incubated at 1 min at RT before being centrifuged for 60s. A Nanodrop measurement was

performed to measure the concentration of PCR products in the samples, using EB buffer as a blank.

4.7 Cloning of genes to plasmid

E.coli strain DH5-α containing plasmid pPac-3xHA was inoculated in liquid broth (LB) that contains 50 µg/ml ampicillin and grown over night at 37^0C, and shaking. Then the culture was checked on agar plate with and without ampicillin to make sure that the plasmid was still present. A plasmid purification kit NucleoSpin (Macherey-Nagel) was run on the culture to extract the plasmid from the cell. The culture was centrifuged at 11,000g for 30 seconds, then the supernatant was removed. 250 µl A1 buffer was used to resuspend the pellet, 250 µl A2 buffer was added, the tube was gently inverted a few times and incubated for 5 min at roomtemprature (RT) to lysis the cells. To stop the lyses 300 µl of A3 buffer was added and the tube is gently inverted. The suspension was centrifuged for 5 min 11.000g. Clear supernatant was loaded into a filtered SpinColumn and centrifuged for 1 min at 11,000g. The supernatant was discarded and the filter washed by adding 600 µl A4 buffert and centrifuged for 1 min at 11.000g. The supernatant was then discarded. The tube was centrifuged again for 2 min at 11.000g. The plasmid was eluted from the filter by adding 50 µl AE(elution) buffer incubated for 1 min and then centrifuged for 1 min at 11.000g. A Nanodrop measurement was performed to measure the amount of plasmids in the elution with EB buffer as blank.

Restriction digest of the PCR products was performed with 2 tubes of 200 ng product from each gene mixed with 3ul 10x Fast Digest Buffer, 1 µl of both BamH1 and Not1 (Fermentas) and dH_2O to make a final reaction size of 30 µl, followed by incubation in a heat-block at 37^0C for 2 hours. The tubes from the same gene were collected into one and the QIAquick PCR purification kit was run on both as described above using 240 µl PB buffer and eluted in 30 µl EB fluid. A Nanodrop measurement was then performed to find out the total amount of restriction cleaved material.

The purified plasmid was restriction digested with 1 µg plasmid, 3 µl 10x fast digest buffer, 1 µl each of BamH1 and Not1 and dH_2O to make a final reaction volume of 30 µl followed by incubation in a heat-block at 37^0C for 1 hour. Then 1 µl FASTAP (Fermentas) was added to dephosphorylate the plasmid and stop them from closing again, and then the solution was

incubated for 1 hour. The two reactions containing plasmids were purified using the QIAquick PCR purification kit. It was run as described above using 124 µl PB buffer and eluted in 30 µl EB. A Nanodrop measurement was then performed to measure the total amount of cleaved plasmid.

The ligation was set up in three tubes one for each gene and one blank control containing dH_2O. The cleaved dephosphorylated plasmid was at 50 ng, 2 µl 10x T4 DNA Ligase Buffer, 0,75 µl (Fermentas), 10mM ATP (Fermentas), 0,5 µl T4 DNA Ligase (Fermentas) and 15 µl gene/blank sample, incubated at RT overnight.

4.8 Transformation of *E.coli* strain DH5-α and identification of insert.

100 µl of competent *E.coli* is aliqouated in three tubes. Each tube was given 2,5 µl of its on ligation sample and incubated on ice for 30 min. The tubes were then transferred to a 42^0C heat-block and incubated for 45s. The tubes were put on ice and incubated for 3-5 min. 1 ml of LB was added and the tubes were incubated in 37^0C with shaking for 40 min. The tubes were centrifuged until a pellet could be seen, then most of the supernatant, except 50-100 µl, was discarded. The pellet is resuspended in the liquid that's left and plated out on pre heated Luiquid agar (LA)-Ampicillin (Amp) plates pre-heated to 37^0C. The LA-amp plates were then incubated at 37^0C overnight. The Amp was used for selection so only plasmids that have been closed by the gene fragment and been accepted by the *E.coli* bacteria can form colonies. After overnight incubation, 6 colonies from each LA-amp plate containing a gene were picked and transferred to 3 ml LB and incubated in 37^0C and shaking overnight. The culture went through the SpinColum plamid prep cleaning that has been described above and eluted in 50 µl EB. A Nanodrop measurement was performed to find out the concentration of plasmid. The plasmid was loaded in to a cleaving mix containing 10 µl of plasmid DNA, 2µl FD Buffer, 0.5 µl of both Bam H1 and Not1 and 7 µl dH_2O. The samples were then incubated in a heat-block at 37^0C for 1 hour. The samples were run on a gel to make sure that the plasmid has accepted a gene fragment of the right size. The rest of the bacteria was taken from the tubes with positive plasmids and spread out in a pattern to get single colonies on a LA-amp plate.

Sequencing of recombinants was performed to make sure that the genes were intact and correct. It was performed by Uppsala Sequencing service at Rudbeck laboratories. The

reaction works best with 450 ng of sample in each reaction, two reactions per gene each reaction with its own primer. The primers were PaqSeq Forward 5´-CACCGTGGGCTTGTACTC-3´3 and -20m13 Forward 5´-GTAAAACGACGGCCAGT-3´. The total reaction volume was 20 µl. The sequencing was performed by Uppsala Genome Sequencing Center.

Four tubes were inoculated with WB trophozoite in 10 ml TYDK and allowed to grown to confluence to get enough trophozoite for transfection.

4.9 Cultivation of plasmid for transfection

One colony from each recombinant strain was inoculated in to 50 ml preheated LB and 50µg/ml ampicillin in a 1000 ml E-flask and incubated at 37^0C shaking overnight. The cells were transferred to three 15 ml falcon tubes and centrifuged chilled at 6000 remf. The supernatant was discarded and the SpinColumn plasmid miniprep (Macherey-nagel) was used to extract the plasmid. The pellet was resuspended in 1ml A1 buffer. Then 1 ml of A2 buffer was added to lyse the cells, the Falcon tubes were inverted gently a few times to mix the solutions and this was then incubated for a maximum of 5 min. 1.2 ml of A3 buffer was then added to neutralize the lysis buffer and the Falcon tubes were inverted gently a few times to mix the solution. 1.2 ml of the solution was distributed to eight eppendorf tubes wich were then spun at 13000 rpm for 10-15 min in a microcentrifuge. The supernatant was collected in a falcon tube and placed on ice. SpinColumns were prepared and 700 µl supernatant was deposited in each column and spun for 1 min at 6000 rpm and the flow-through was discarded. This is repeated until all the supernatant in the Falcon tube was used-up. 600 µl AW buffer that have been preheated to 50^0C is added to each SpinColumn to wash the membrane. The SpinColumns were centrifuged at 13000 rpm for 1 min and the flow-through was discarded. The columns were washed with 600 µl of A4 buffer that was centrifuged at 13000 rpm for 2 min and the flow-through was discarded. the columns were centrifuged again for 1 min to make sure that all A4 buffer is gone. The column parts with a filter is moved to an Eppendorftube and placed in a 50^0C heat-block, 100 µl AE buffer that have been preheated to 50^0C, was added to only one SpinColumn and incubated for 1 min. This SpinColumn is then centrifuged at 13000 rpm for 1 min, that elutes the plasmid and the flow through is pipetted to another SpinColumn that have not been eluted. The incubation, centrifugation and pipeting steps were then repeated until the AE buffer have passed

through all four SpinColumns and all the plasmids have been eluted into one sample. A small dilution sample 1:10 is created to be able to make a Nanodrop measurement and gel analyzes with restriction-cleaved plasmid that has been described above, to make sure that the plasmid is still intact.

4.10 Transfection of *Giardia lamblia*

One 10 ml tube of confluent trophozoite was generally calculated to have about $1*10^7$ cells. One tube was used for each plasmid type, they were put on ice to detach the cells for 20 min and then inverted several times. The medium was then transferred to 15 ml Falcon tubes and centrifuged at 850g in 4- 8^0C for 5 min and the old medium was sucked out. The pellet was resuspended in 300 µl ice-cold TYDK medium and this solution was transferred to a 4 mm gap electroporation cuvette (BioRad) and placed on ice. Approximately 40 µg of plasmid DNA was added to the cuvette and mixed by pipeting the solution. The trophozoites were made competent to take up plasmid DNA by electroporation. The pulse was set at 350 V, 960 µF, 800 Ω, afterwards the cuvettes were placed on ice for 15 min. The solution was then transferred to tubes filled with 37^0C TYDK medium supplemented with Gentamicin to a concentration of 100 µg/ml and then incubated at 37^0C, 5% CO_2 overnight. The next day Puromycin was added to concentration of 50 µg/ml to start selecting trophozoites with the plasmid incorporated.

4.11 Microscopy

2% BSA solution 2 grams Bovine Serum Albumin Fraction V mixed with 100 ml PBS and then filtered with a 0,45µm Cellulose Acetat 250 ml filter system.

HBS+Glucose was made by using dH_2O as a base and mixing in chemicals to a concentration of 130 mM NaCl (Merck), 5 mM KCl (Merck), 10mM Glucose (BDH), 1 mM $MgCl_2$ (Merck), 1 mM $CaCl_2$ (Merck), 25mM HEPES (USB). The pH was adjusted to 7,4 using NaOH and measured using a PHM210 standard pH meter. The solution was then filtered using a 0,45µm Cellulose Acetat 250 ml filter system.

Antibody block solution was made by using PBS as a base and adding Bovine serum (adult) (Sigma) until a concentration of 3%. TritonX-100 (Kebo Lab) was added to a final concentration of 0.1%.

1 ml of each strain of the transfected trophozoite was spun down in RT at 600 RPM for 3 min. The supernatant was removed and the cells were washed with 1 ml ice-cold HBS-Glucose and spun down again at the same settings. The trophozoites were resuspended in 50 – 100 µl HBS-Glucose and 20 µl drops were spotted on pre-equilibrated microscope slide (Thermo Scientific) the slide was placed on wetted papper in a petri dish. The dish was placed in a 37^0C incubator and allowed to incubate for 10 min to allow the trophozoite to attach to the slide. The drop was removed using a vacuum suction and the throphozoites are fixated by adding 20 µl of 4% PFA (Paraformaldehyde, Merck) in PBS) and allowed to work for 30 min in the petri dish standing in the incubator at 37^0C. The fixative was removed using vacuum and 20 µl of 0.1M Glycine (Merck) in PBS was used to wash the thropozhoites and the solution was incubated for 10 min at RT. The Glycine was vacuumed away and the cells were permeabilized by adding a drop of 0.2% TritonX in PBS and incubated for 30 min at RT. The permeabilization solution was vacuumed away and a bock solution of 2% BSA was added. This was incubated on the moist paper in the petri dish for 1-2 hours at RT. The block solution was vacuumed away and 10 µl of the antibody Anti-HA-A488:HA.11 clone 16B12 Monoclonal Antibody, Alexa Flour Labeled(A488-101L) (Covance) diluted 1:250 times in antibody block solution. This was incubated at RT for 1-2 hours in moist paper in the petri dish standing somewhere dark. The antibody was vacuumed away and the spot was washed six times using 0.1%TritonX100 in PBS and two times in pure PBS the vacuum was used to suck away the drops. Half of the spots are covered with 3 µl drops of Vectashield+DAPI (VectorLabboritories) and the other half with 3 µl ProlongGo-Gold Antifade radiant with DAPI (Invitrogen), This was to get a layer of fixative with a known refraction index to watch the trophozoites. The Vectashield spots were covered with a coverglass and kept in place by painting the corners with nail polish. The ProlongGold spots were the same except that the nail polish went around the entire glass. Vectashield need to incubate in darkness at RT for 10 min before it have become stabile. ProlongGold needed to incubate at least overnight in darkness before it was stabile. The microscopy was performed using a Zeiss Axioplan II Imaging fluorescence microscope connected to a CCD camera and Axiovision software(Carl Zeiss MicroImagining GmbH).

5. Acknowledgements

I would like to thank the following persons for their help and knowledge, my primary supervisor Staffan Svärd. My primary lab supervisors Jon Jerlström-Hultqvist and Hanna Skarin. I would also like to thank Britta Stadelmann, Elin Einarsson, Emma Ringqvist and Katarina Roxström-Lindquist for their general help and support.

6. References

Adam, R. D. (2001). Biology of Giardia lamblia. Clin Microbiol Rev. **14:** 447-475.

Ankarklev, J., J. Jerlstrom-Hultqvist, et al. (2010). "Behind the smile: cell biology and disease mechanisms of Giardia species." Nat Rev Microbiol **8**(6): 413-422.

Applebee, M. K., A. R. Joyce, et al. (2011). "Functional and Metabolic Effects of Adaptive Glycerol Kinase (GLPK) Mutants in Escherichia coli." J Biol Chem **286**(26): 23150-23159.

Brown, D. M., J. A. Upcroft, et al. (1998). "Anaerobic bacterial metabolism in the ancient eukaryote Giardia duodenalis." Int J Parasitol **28**(1): 149-164.

Daly, E. R., S. J. Roy, et al. (2010). "Outbreak of giardiasis associated with a community drinking-water source." Epidemiol Infect **138**(4): 491-500.

Davids, B. J., D. S. Reiner, et al. (2006). "A new family of giardial cysteine-rich non-VSP protein genes and a novel cyst protein." PLoS One **1**: e44.

Di Matteo, A., F. M. Scandurra, et al. (2008). "The O2-scavenging flavodiiron protein in the human parasite Giardia intestinalis." J Biol Chem **283**(7): 4061-4068.

Eckmann, L. (2003). "Mucosal defences against Giardia." Parasite Immunol **25**(5): 259-270.

Hosogi, Y. and M. J. Duncan (2005). "Gene expression in Porphyromonas gingivalis after contact with human epithelial cells." Infect Immun **73**(4): 2327-2335.

Huang, Y. C., L. H. Su, et al. (2008). "Regulation of cyst wall protein promoters by Myb2 in Giardia lamblia." J Biol Chem **283**(45): 31021-31029.

Jakubovics, N. S. and H. F. Jenkinson (2001). "Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria." Microbiology **147**(Pt 7): 1709-1718.

Jortzik, E., B. M. Mailu, et al. (2011). "Glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase: a unique bifunctional enzyme from Plasmodium falciparum." Biochem J **436**(3): 641-650.

Lane, S. and D. Lloyd (2002). "Current trends in research into the waterborne parasite Giardia." Crit Rev Microbiol **28**(2): 123-147.

Leitsch, D., A. G. Burgess, et al. (2011). "Pyruvate:ferredoxin oxidoreductase and thioredoxin reductase are involved in 5-nitroimidazole activation while flavin metabolism is linked to 5-nitroimidazole resistance in Giardia lamblia." J Antimicrob Chemother.

Leitsch, D., D. Kolarich, et al. (2009). "Trichomonas vaginalis: metronidazole and other nitroimidazole drugs are reduced by the flavin enzyme thioredoxin reductase and disrupt the cellular redox system. Implications for nitroimidazole toxicity and resistance." Mol Microbiol **72**(2): 518-536.

Leitsch, D., D. Kolarich, et al. (2010). "The flavin inhibitor diphenyleneiodonium renders Trichomonas vaginalis resistant to metronidazole, inhibits thioredoxin reductase and flavin reductase, and shuts off hydrogenosomal enzymatic pathways." Mol Biochem Parasitol **171**(1): 17-24.

Li, L. and C. C. Wang (2006). "A likely molecular basis of the susceptibility of Giardia lamblia towards oxygen." Mol Microbiol **59**(1): 202-211.

Mellingen, K. M., A. Midtun, et al. (2010). "Post epidemic giardiasis and gastrointestinal symptoms among preschool children in Bergen, Norway. A cross-sectional study." BMC Public Health **10**: 163.

Muller, S., E. Liebau, et al. (2003). "Thiol-based redox metabolism of protozoan parasites." Trends Parasitol **19**(7): 320-328.

Nillius, D., J. Muller, et al. (2011). "Nitroreductase (GlNR1) increases susceptibility of Giardia lamblia and Escherichia coli to nitro drugs." J Antimicrob Chemother **66**(5): 1029-1035.

Pelegrini, A. L., D. J. Moura, et al. (2010). "Nek1 silencing slows down DNA repair and blocks DNA damage-induced cell cycle arrest." Mutagenesis **25**(5): 447-454.

Ringqvist, E., L. Avesson, et al. (2011). "Transcriptional changes in Giardia during host-parasite interactions." Int J Parasitol **41**(3-4): 277-285.

Slavic, K., S. Krishna, et al. (2011). "Plasmodial sugar transporters as anti-malarial drug targets and comparisons with other protozoa." Malar J **10**(1): 165.
Sun, C. H., D. Palm, et al. (2002). "A novel Myb-related protein involved in transcriptional activation of encystation genes in Giardia lamblia." Mol Microbiol **46**(4): 971-984.
Wu, H. J., K. L. Seib, et al. (2010). "Manganese regulation of virulence factors and oxidative stress resistance in Neisseria gonorrhoeae." J Proteomics **73**(5): 899-916.