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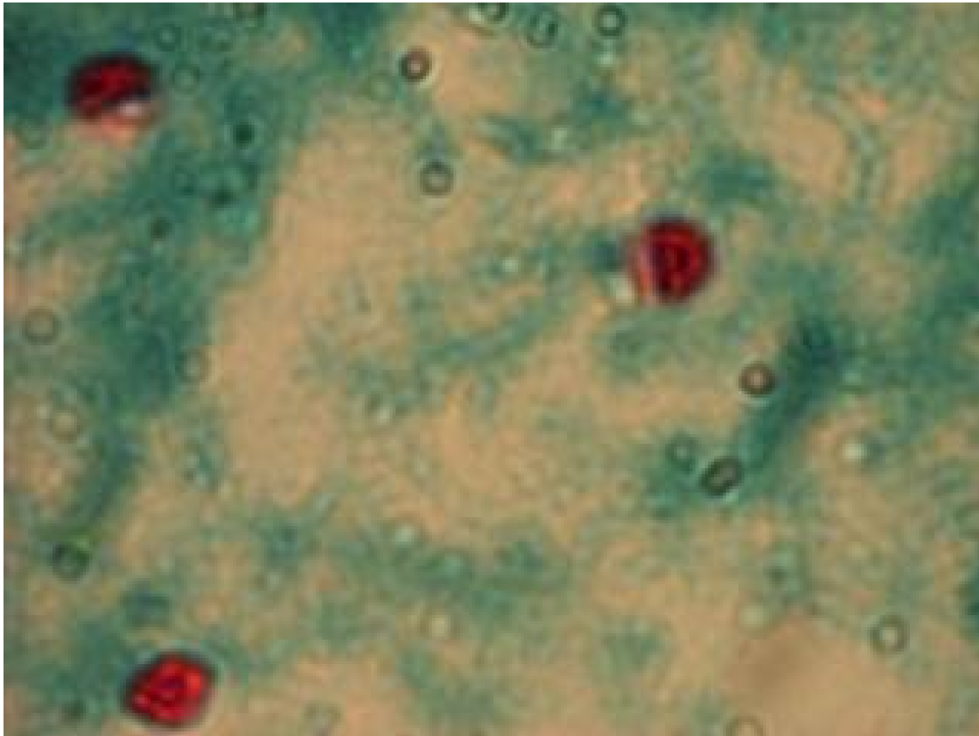
Development of *Cryptosporidium* enrichment methodology to facilitate whole genome sequencing of *Cryptosporidium* from patient samples



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List of abbreviations

BLAST	The Basic Local Alignment Search Tool
CDC	The American Center for Disease Control and prevention
COWP	*Cryptosporidium* oocyst wall protein
FOI	The Swedish Defence Research Agency
g	Relative centrifugal force
gp60	Glycoprotein 60
IFL	Immunofluorecent labeling
IMS	Immunomagnetic separation
MALBAC	Multiple Annealing and Looping Based Amplification Cycles
MSB	Swedish Civil Contingencies Agency
RFLP	Restriction fragment length polymorphism
Rpm	Revolutions per minute
SLV	The National Food Agency
SMI	The Swedish Institute for Communicable Disease Control
SVA	The National Veterinary Institute

ABSTRACT

Cryptosporidium, an oocyst forming protozoan, is one out of four pathogens responsible for the majority of severe diarrheal infections in the world. Children in developing countries and immuno-compromised individuals are especially vulnerable groups where an infection may lead to death.

There are over 20 species of *Cryptosporidium* and even more subtypes. The different species are spread in a variety of different ways: through water or food or by zoonotic or human to human transmission. Today, only two whole genomes of *Cryptosporidium* have been sequenced and the tools available for differentiating between different species and subtypes are insufficient.

The Swedish Institute of Communicable Disease Control, in cooperation with four other agencies, has started a project with the aim of creating a genomic databank for *Cryptosporidium* in order to identify better gene markers to differentiate between species and subtypes for faster detection of the transmission routes.

Cryptosporidium cannot be cultured *in vitro* and to determine the species and subtype, samples must be taken directly from human fecal sample for sequencing. Human fecal sample contains a lot of contamination such as bacteria, fungi and plant and animal cells. Such contamination need to be removed prior to sequencing. Today there is no adequate method for purifying *Cryptosporidium* oocysts from human fecal sample.

In this project we have developed a basis for the purification of oocysts from human fecal sample. The method includes two flotations followed by immunomagnetic separation and whole genome amplification.

Sequencing of the purified material gave results of 28.3% and 77% *Cryptosporidium* DNA in the samples, which covered 0.57 and 0.7 fractions of the reference genome respectively, indicating the method to be a good ground for future work of developing a method for purification of *Cryptosporidium* oocysts before whole genome sequencing.

Introduction

Cryptosporidium

General information

Diarrhea accounts for approximately 10.5% of the world's deaths of children under the age of five (Liu L. *et al*. 2012) and *Cryptosporidium* is one of the four pathogens which is responsible for the majority of diarrheal infections (Kotloff K.L. *et al.* 2013).

Cryptosporidium is one of the most common protozoan parasites in children with enteric diseases in developing countries (Gatei W. *et al*. 2006) and can cause severe disease in immunocompromised individuals, such as those suffering from HIV.

Cryptosporidium is an oocyst forming protozoan from the group of parasitic protists called Apicomplexan protozoan, which also includes parasites such as *Plasmodium*, causing malaria, and *Toxoplasma*, causing toxoplasmosis. Infection with *Cryptosporidium* leads to cryptosporidiosis which became a notifiable disease in Sweden in July 2004.

The species that are most frequently found to infect humans are the zoonotic *Cryptosporidium parvum,* which is also common among calves, and the human specific *C. hominis*. However, other species such as *C. meleagridis, C. felis*, *C. canis, C. ubiquitum, C. cuniculus*, *C. viatorum* and *Cryptosporidium* chipmunk genotype I, have also been shown to infect humans (Pieniazek N.J. *et al* 1999; Xiao L. *et al* 2001; Fayer R. et al 2010; Chalmers R.M. et al. 2011; Lebbad M. *et al*. 2013).

The genome of *Cryptosporidium* is approximately 9 Mb and is composed of eight chromosomes. There is no apicoplast present, as there is in most Apicomplexans, and the mitochondria is degenerated and lack genes. The lack of apicoplastic and mitochondrial DNA are two factors leading to fewer protein-encoding genes in *Cryptosporidium* compared to what is estimated for the Apicomplexan parasites *Plasmodium* and *Toxoplasma*. (Abrahamsen M.S. *et al* 2004; Xu P. *et al* 2004)

Life cycle

Cryptosporidium needs a host to reproduce and is therefore classified as a parasite. *Cryptosporidium* has a sexual and an asexual life cycle (Figure 1). When ingested, oocysts reach the small intestine and release sporozoites. The sporozoites attach to the epithelium cells, invade the cell and develop intracellularly into trophozoites. Trophozoites further develop into Type 1 meronts, each containing 8 daughter cells called Type 1 merezoites. Once the Type 1 merezoites are released they can either lead to autoinfection, infecting nearby epithelial cells, or develop into Type 2 meronts. The Typ 2 meronts contain 4 merozoites which once released, form either microgamonts or macrogamonts, leading to the formation of micro- and macrogametocytes. The gametocytes can fuse, to form zygotes. The zygotes develop into two forms of oocyst: approximately 20% are thin-walled and can reinfect the

host by releasing sporozoites, whilst 80% of the oocysts are thick-walled and excreted into the environment. (Current W.L. and Garcia L.S. 1991)

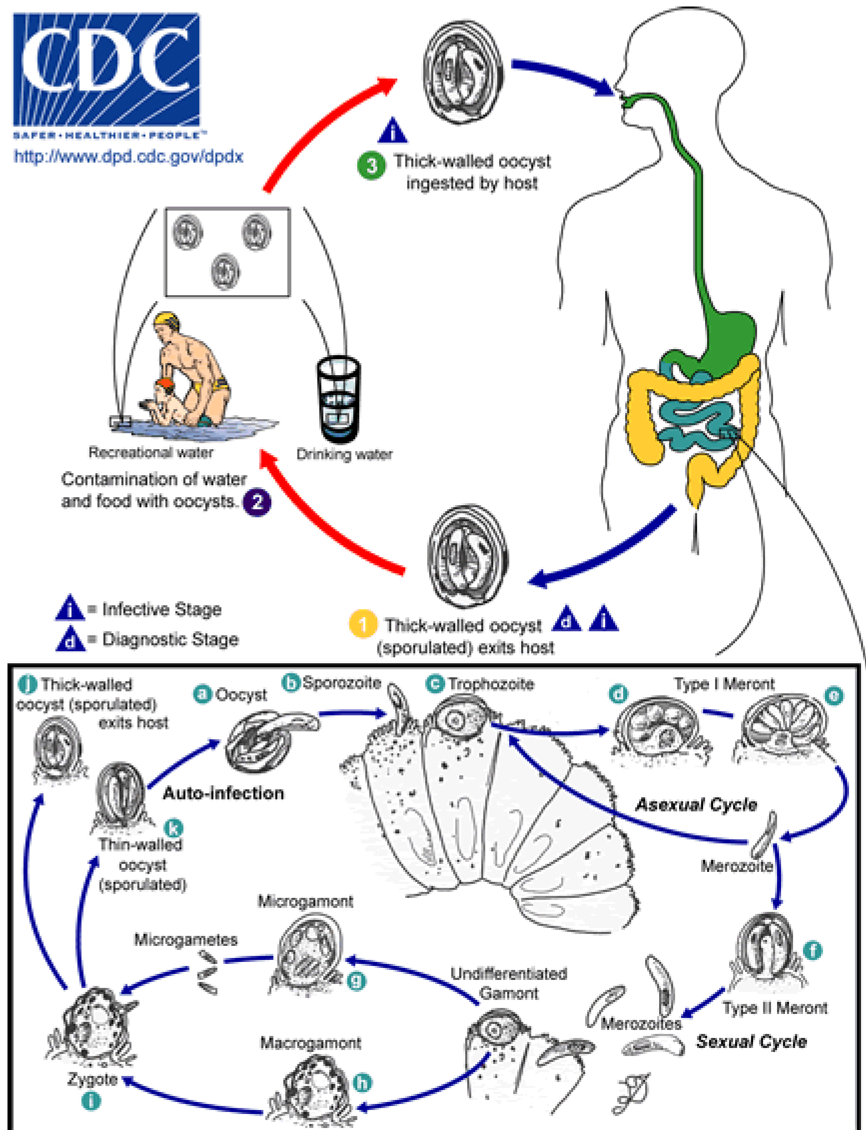

Figure 1, Life cycle of *Cryptosporidium* (www.cdc.gov)

Cryptosporidiosis

Symptoms and treatment

Symptoms vary from none to loss of appetite, nausea, vomiting, abdominal pain and diarrhea. The infectious dose can be low, ranging from 1 to 1042 oocysts ingested (Dillingham R.A *et al*. 2002), varying with the strain virulence and host susceptibility (Okhuysen P.C. et al. 1999). The incubation time ranges from two to twelve days and the symptoms usually last for one to two weeks. However, for immunocompromised individuals the symptoms can become progressively worse with time and may lead to death (Boothe C.C. *et al.* 1980).

Since cryptosporidiosis is commonly self-limiting, the treatment is generally symptomatic, with supplies such as rehydration. However, some drugs such as nitazoxanide, albendazole, metronidazole and paromomycin have been tested for treating *Cryptosporidium* infections (Theodos C.M. *et al.* 1998; Rossignol J.F.A. *et al*. 2001; Masood S. *et al.* 2013).

Nitazoxanide is thought to inhibit the pyruvate:ferredoxin/flavodoxin oxidoreductase enzyme-dependent electron transfer reaction (Hoffman P.S. *et al.* 2007) and has been approved by the US. Food and Drug administration for treatment of individuals with healthy immune systems, but the effectiveness of nitazoxanide for treating cryptosporidiosis in immunosuppressed persons is unclear (www.cdc.gov).

In Sweden no drugs to treat cryptosporidiosis have been accepted on the market but the drugs albendazol, paromomyci, spiramycin and azitromycin are available under special circumstances (www.lakemedelsvarket.se)

Transmission routes

There are more than 20 different species of *Cryptosporidium* and they all share a similar morphology. However, the transmission route differs. The parasite can spread by zoonotic, foodborne, waterborne, as well as person-to-person transmission.

Diagnostics

Today most *Cryptosporidium* cases are diagnosed by microscopic morphology, but over the last decade, molecular tools have been developed to improve the detection of *Cryptosporidium*, as well as to differentiate between different species and subtypes.

Currently, *Cryptosporidium* is typed for species by RFLP and/or sequencing of the 18S rRNA gene. *C. parvum* and *C. hominis* can further be subtyped by sequencing and counting the number of tandem repeats in the gp60 gene, which encodes for a 60 kDa glycoprotein. The name starts with the family designation (Ia, Ib, Ic etc. for *C. hominis* and IIa, IIb, IIc etc. for *C. parvum*) and is followed by the number of TCA, TCG and TCT repeats. The TCA repeats are shortened to the letter A, the TCG to the letter G and the TCT to the letter T. There could also be rare repeats present which are shortened to the letter R. For example, IbA10G2 stands for the *C. hominis* Ib subtype family, with 10 TCA repeats and 2 TCG repeats.

However, the tools currently available are either too broad, detecting only the dominant genotype in cases where the infection is of a mixture of different species or subtypes because of the exponential amplification by PCR, or too narrow detecting only the most common species, *C. parvum* and *C. hominis* and species closely related to them, failing to detect other species (Xiao L. 2010).

Cryptosporidiosis in Sweden

As mentioned earlier, cryptosporidiosis became a notifiable disease in Sweden the 1th of July 2004 and since then around 100 to 200 cases have been reported per year (Table 1) to the Swedish national surveillance system (SmiNet). The exceptions were during 2010 and 2011 when there were two large outbreaks in Östersund and Skellefteå (www.smittskyddsinstitutet.se).

Table 1, Total Cryptosporidium cases reported from the year 2004 to 2013

Year	2013	2012	2011	2010	2009	2008	2007	2006	2005	2004
Total	188	238	379	392	159	148	110	103	69	47

The largest *Cryptosporidium* outbreak in Sweden was in 2010 in Östersund. Approximately 20 000 individuals were infected. The infection spread through the drinking water and the citizens of Östersund had to boil their water to avoid the infection to spread even more. (SMI rapport: *Cryptosporidium i Östersund* 2011)

Shortly after the outbreak in Östersund, another large outbreak appeared in Skellefteå. The source of the infection is thought to have been the water treatment plant Abborren. However, no oocysts could be found in the raw drinking water. Therefore, citizens were informed to boil their water as a precautionary measure, minimizing the likelihood of a similar scale outbreak to that in Östersund. (SMI rapport: *Cryptosporidium i Östersund* 2011)

In both cities *C. hominis*, subtype IbA10G2, often associated with waterborne outbreaks, was the species detected and responsible for the outbreak.

Other minor outbreaks have also taken place in Sweden during the last years. The first ever reported swimming pool associated outbreak in Sweden occurred in 2002 at Lidingö where approximately 1000 individuals were infected. (Insulander M. *et al*. 2005)

In October 2010 a link was established between *Cryptosporidium* cases in Stockholm/Uppsala and *Cryptosporidium* infections at a national conference in Umeå using molecular subtyping. The outbreak was found to be food-borne and the *C. parvum* subtype IIdA24G1, a subtype previously found in lambs and goats (Quílez J. *et al*. 2008), was found at both locations. At the same time, an outbreak at a private party in Örebro another subtype, *C. parvum* IIdA20G1e, also described in a Swedish calf suggesting a zoonotic source, was identified disconnecting this outbreak from those occurring in Stockholm/Uppsala and Umeå. (Gherasim A. *et al.* 2012)

Cryptosporidiosis around the world

The largest outbreak ever documented in the world was in the town of Milwaukee in 1993. The outbreak started from the southern water treatment plant where water from Lake Michigan enters to supply parts of Milwaukee with drinking water. The financial cost of the illness is estimated to be $96.2 million, including medical cost and productivity losses (Corso

P.S. *et al.* 2003) and it was estimated that more than 400 000 people were infected, although the actual figure may have been even higher. (Mac Kenzie W.R. *et al.* 1994)

Water-borne outbreaks have been reported from all over the world. In England and Wales private water supplies and swimming pools contribute significantly to annual *Cryptosporidium* cases (Smith A., *et al*. 2006). In France contamination from waste water backflows was found in the distribution network and at the water collection facilities (Beaudeau P. *et al*. 2008) and in New South Wales, Australia, public swimming pools also contribute to the spread of *Cryptosporidium* (Waldron L.S. *et al.* 2011).

The American Center for Disease Control and prevention (CDC) have classified *Cryptosporidium* as a class B bioterrorism agents due to the parasites resistance to many disinfection processes such as chlorination (Fayer R. 1995), and the low infectious dose necessary for infection.

Separation and purification methodology

Since *Cryptosporidium* cannot be cultured *in vitro* and because the oocysts are small in size, there are difficulties in working with *Cryptosporidium*. To ensure the subtype being infectious to humans, the oocysts must be purified from human fecal samples. Fecal samples contain lots of contamination, from human cells to bacteria, fungi and plant cells. For whole genome sequencing, the sample needs to have a large quantity of DNA and to be free from contamination. For this reasons the enrichment methodology is very complicated and separation and purification becomes crucial.

Sucrose flotation, formalin-ethyl acetate sedimentation, Percoll or Ficoll gradient centrifugation, cesium chloride gradient centrifugation and discontinuous sucrose gradient in combination with Percoll gradient isolation are some methods which have been used for isolation of C*ryptosporidium* from feces, primarily for detection (McNabb S.J. *et al.* 1985; Waldman E. *et al*. 1986; Arrowood M.J. and Sterling C.R. 1987; Kilani R.T. and Sekla L.1987; Weber R. *et al*. 1992; Truong Q. and Ferrari B.C. 2006). These methods have also been used in isolation of other parasites, for instance, sucrose flotation for isolation of *Toxoplasma* from soil (Matsuo J. *et al.* 2004) and formalin-ethyl acetate sedimentation for a variety of parasites, including amoebae, flagellates, cestodes, nematodes, and trematodes (Truant A.L. *et al.* 1981).

Another method, salt flotation, has been used in other studies to purify *Cryptosporidium* oocysts (O'Brien C.N. and Jenkins M.C. 2007; Moriarty E.M. *et al.* 2005) and is based on the fact that substances with lower density will float on top of substances with higher density. The density of a substance is the mass per unit volume (kg/m^3) of the substance. Water in room temperature (20°C) has a density of 0.9982 kg/m^3 while *Cryptosporidium* oocysts has a density of 1.0454 kg/m^3 (Medema, G.J. *et al.*1998) making the oocysts sink in water. When adding salt (NaCl) to the water, until the water is saturated, the density of the water will increase to 1.2 kg/m^3 making the oocysts float in saturated NaCl water. With the use of a

centrifuge the time of separation is reduced. By centrifugation and changing the density of the medium, *Cryptosporidium* is separated from most of the background material in a sample.

Furthermore, immunomagnetic separation, a method to isolate cells out of a fluid, has also been used in other studies (Bukhari Z. *et al.* 1998; Giovanni G.D. *et al.* 1999; Rochelle P.A. *et al.* 1999). Paramagnetic beads coated with antibodies bind to antigens of the cell. With a magnet, the beads in complex with the cells are collected. The cells are further mechanically removed from the beads by vortexing. Hydrochloric acid (HCl) is added to prevent rebinding, whilst NaOH is added to neutralize the sample.

The MSB project

The interest in *Cryptosporidium* in Sweden has greatly increased since the recent large outbreaks. Creating a genome reference data bank by whole genome sequencing to develop better methods of defining the species and subtype is of great importance. With more genomes sequenced, new and better genetic markers could be defined to differentiate between different species and subtypes. This would facilitate the process of identifying the source of an outbreak. Since *Cryptosporidium* cannot be readily cultured *in vitro* a critical step of the project is to purify and concentrate the oocysts from patient samples before sequencing. The small amount of sample usually obtained from patients further complicates the process.

Therefore, in January 2013, The Swedish Institute for Communicable Disease Control (SMI) in cooperation with The National Veterinary Institute (SVA), The Swedish Defence Research Agency (FOI) and The National Food Agency (SLV) started a project, financed by Swedish Civil Contingencies Agency (MSB) with the aim to have 30 genomes sequenced by the end of 2014. Utilizing this database, should allow for the characterization of new genetic markers, as well as the development of more sensitive and specific methods for tracing infection. The intention is that these methods will be implemented via the four authorities by the end of 2015.

The MSB project aims to improve the capability for detection, early warning and analysis of natural and deliberate spread of *Cryptosporidium spp*. and *Giardia intestinalis*. However, this student project was only focused on improving the methodology of *Cryptosporidium* purification, although it is possible that some of the conclusions derived from the current work, could also be applied to *Giardia* purification and sequencing.

Aim

The aim of this project was to separate and purify *Cryptosporidium* oocysts from human fecal samples prior to whole genome sequencing. In order to achieve this, a method including two salt flotations, immunomagnetic separation, DNA extraction and whole genome amplification was developed before sequencing of the genome.

Material and methods

Sample

Experiments were performed from 4 ml of a single patient fecal sample, positive for *Cryptosporidium parvum*, subtype IIaA16G1R1b. The process, starting with two flotations followed by IMS, DNA extraction, whole genome amplification, PCR purification and sequencing of the material, was repeated two times. The processes will be referred to as Experiment 1 and Experiment 2.

Salt flotation

To separate the oocysts from the rest of the feces, two flotations were performed. The protocol for flotation was developed at The National Veterinary Institute (SVA). Unless stated otherwise all procedures were performed at room temperature. Furthermore, all centrifugation steps in the flotations were performed using a Labofuge™ 400R, function line, centrifuge (Heraeus™ instruments)

Flotation 1

Using a 50ml falcon tube, 4ml of the fecal sample (filtrated and preserved in 2% potassium dichromate) was vortexed and Milli-Q water was added to a volume of 50 ml. The tube was centrifuged for 5 min in 1540 x g. The supernatant was removed and the addition of Milli-Q water and centrifugation was repeated two more times.

The pellet was resuspended in Milli-Q water, up to a volume of 4 ml, and transferred to a 15 ml tube, along with 4 ml of saturated NaCl in water. The tube was vortexed for 10 seconds, shaken for 10 seconds and vortexed for another 10 seconds. The tube was centrifuged for 1 min at 1540 x g. Then, the supernatant was transferred to a 50 ml tube containing 42 ml of Milli-Q water, by carefully pipetting the upper layer, where most of the oocysts are thought to be situated, making sure the oocysts did not attach to the tube and by rinsing the pipette in the water. The tube was centrifuged for 10 min at 1540 x g. Supernatant was removed, until there was 5 ml left in the tube. The tube was vortexed and Milli-Q water was added to a volume of 50 ml. The tube was centrifuged for 10 min at 1540 x g. The supernatant was removed, until there was 5 ml left in the tube. The 5 ml sample was transferred to a 10 ml tube and Milli-Q water was added to the tube, via rinsing of the pipette and the 50 ml tube, until the 10 ml tube was filled. The 10 ml tube was centrifuged for 10 min at 1540 x g. The supernatant was removed until there was 1.5 ml left in the tube.

20 µl of sample was smeared onto a glass slide for purification validation with Ziehl-Neelsen staining (see 3.4) and 10 µl was used for estimation of parasite numbers with immunofluorescent labeling (IFL).

Flotation 2

Milli-Q water was added to a final volume of 4 ml to the material from Flotation 1 (approximately 1.5 ml). After the addition of 4 ml of saturated NaCl in water, all further steps of sample purification were repeated as described in Flotation 1. A second 20 µl of sample was smeared onto a glass slide for purification validation with Ziehl-Neelsen staining and 10 µl was used for counting with IFL.

IMS

To further separate the oocysts from the remaining fecal material, immunomagnetic separation was performed.

20 µl of material from Flotation 2 was used for Immunomagnetic separation preformed with the Dynabeads® GC-Combo Kit (Applied Biosystems®) according to the manufactures description. However, only the beads coated with anti-Crypto antibodies was used. In short, the sample was mixed with buffers and beads before placed in a Stuart tube rotator SB3 (Stuart equipment) for mixing, with a speed of 17 rpm for 1 h. The beads, in complex with the oocysts, were then captured using a magnet and washed before the oocysts were mechanically removed from the beads by vortexing. Instead of spreading sample onto glass in the Post IMS process, the sample was transferred to an eppendorf tube and 5 µl of 1N NaOH was added. Approximately 50 µl of sample was obtained.

20 µl of further purified sample was smeared onto a glass slide for purification validation with Ziehl-Neelsen staining and 10 µl was used for counting with IFL.

Immunofluorescent labeling (IFL) and Ziehl-Neelsen staining

Immunofluorescent labeling was used to count the oocysts after each flotation and the IMS. The sample was stained with anti-Cryptosporidium monoclonal antibody (Cellabs Pty Ltd), observed at a magnification of x20 and counted as wet-prep in fluorescent microscope (Nikon Japan Y-FL). The antibody and sample was mixed in a 1:1 ratio and incubated in 37°C for 30 minutes before loading 5 µl of sample onto slides.

Further, background contamination was validated through Ziehl-Neelseen staining. 20 µl of sample was smeared onto a glass slide and allowed to dry for at least 30 min. Samples were fixated in methanol for 5 min and placed in a colour stand. The sample was stained in carbol fuchsin (Merck Millipore, Germany) for 20 min before being washed twice with hydrochloric acid in ethanol (0.37% hydrochloric acid in 95% ethanol) and tap water. The sample was further stained with malachite green (1% in Milli-Q H2O) for 3 min before washing with tap water. The sample was allowed to dry before mounting with Pertex mounting media (Leica biosystems) and observed at a magnitude of x40 in light microscope (Nikon eclipse E400).

DNA extraction

A modified protocol for DNA extraction from fecal matter, derived from the Qiagen-extraction of DNA from tissue protocol and QIAamp DNA Micro Handbook Protocol: Isolation of genomic DNA from tissue (Qiagen), was used to extract DNA form the oocysts, as described below.

Approximately 2 cm of a 1.5 ml conical bottom tube was filled with 0.5 mm Zirconia beads (BioSpec Products). 400 µl pre-warmed ASL buffer was added to the tube. 20 µl of the material from IMS was added. The tube was placed in a Bullet blender™ (Techtum lab) and shaken for 1 min at maximum speed. The sample was then allowed to settle at room temperature for 15 min. 250 µl of the supernatant was transferred to an eppendorf tube and 25 µl of proteinase K was added. The sample mix was incubated in a waterbath (Heto DT Hetotherm, Bergman Beving Lab) for 2 h at 56°C. 250 µl of AL buffer was added and the tube vortexed and put on a heat block (Grant QBD2) at 70°C for 10 min. 250 µl of ethanol (96%) was added and the tube was vortexed and incubated at room temperature for 5 min. The lysate was transferred to a QIAamp mini elute column and centrifuged for 1 min at 6000 x g. The column was transferred to a new collection tube, 500 µl of AW1 buffer was added and the tube was centrifuged for 1 min at 6000 x g. The column was transferred to a new collection tube, 500 µl of AW2 buffer was added and the tube was centrifuged for 1 min at 6000 x g. To remove potentially remaining AW2 buffer, the column was transferred to a new collection tube and centrifuged for 3 min at 20000 x g. The column was transferred to a new collection tube, 20 µl of RNase free water was added and the tube incubated for 5 min at room temperature before DNA was eluted with centrifugation for 1 min at 20000 x g. Centrifugation was performed in an eppendorf centrifuge 5417C (Hettich labinstruments).

DNA amplification

To amplify the genomic DNA following extraction, a single cell whole genome amplification kit (Yikon genomics) was used. Amplification was performed according to manufacturer's instructions. The kit includes a cell lysis step, a MALBAC (Multiple Annealing and Looping Based Amplification Cycles) pre-amplification step and an exponential amplification step. The lysis step extracts the DNA from the oocysts. The MALBAC pre- amplification step performs a close-to-linear pre-amplification of the entire genome and is followed by an exponential amplification by PCR. All reactions are performed in one single tube. Reaction mixes are prepared separately before adding to the tube.

1 µl of material from DNA extraction was used. The PCR machine (GeneAmp® PCR system 9700 PE, Applied biosystems) was set to 17 cycles in the exponential amplification. 2-4 µg DNA is estimated to be the maximal quantify of DNA which can be obtained in the reaction.

For Experiment 1, two reactions were made, one including (A) and one excluding (B) the lysis step of the protocol. For Experiment 2, only one reaction including lysis step was made.

PCR purification

A QIAquick PCR purification Microcentrifuge and Vacuum kit (Qiagen) was used to purify the amplified material from inhibiting substances, according to the products protocol.

All the material from amplification (65 µl) was used. The elution was performed with 30 µl RNase free water and approximately 30 µl purified sample was received. All centrifuge steps were performed in a Heraeus biofuge pico centrifuge (DJB labcare).

DNA concentration

The concentration of unamplified and amplified genomic DNA was measured using Qubit® Flourometer (Life Technologies) using the high sensitive buffers. The samples were measured in triplicates with 1 µl sample in each replicate.

For Experiment 1, the reaction with amplification including lysis step (A) and the reaction with amplification excluding the lysis step (B) were pooled before measurement.

Sequencing

Sequencing was performed at SMI Core facility. A DNA library was prepared in an AB Library Builder™ System (Life technologies). Inserts with around 400 bp were selected and amplified using ION PGM template OT2 400 kit and whole genome sequencing was performed with an Ion PGM™ System for Next-Generation sequencing (Life technologies).

Sequence annotation

Sequence annotation was performed at SMI Core facility. The annotation was done with the software CLC Genomics workbench 6.0.1. The raw data was downloaded in BAM format from Torrent server. A reference genome (*C. parvum*, strain Iowa II) was downloaded from www.ncbi.nlm.nih.gov/. The sequence was mapped to the reference genome.

The report function in CLC genomics workbench 6.0.1 was used to find the coverage and the total number of mapped bases.

A de-novo assembly of all the unassembled reads was done. The three longest reads and the three reads with the highest coverage was BLASTed using blastx to NCBI.

Results

Purification and quantification

All material was monitored during each step of the purification process (after the first and second flotation, and after IMS) using Ziehl-Neelsen staining (Figure 2). Reduced background contamination was observed following each stage of purification.

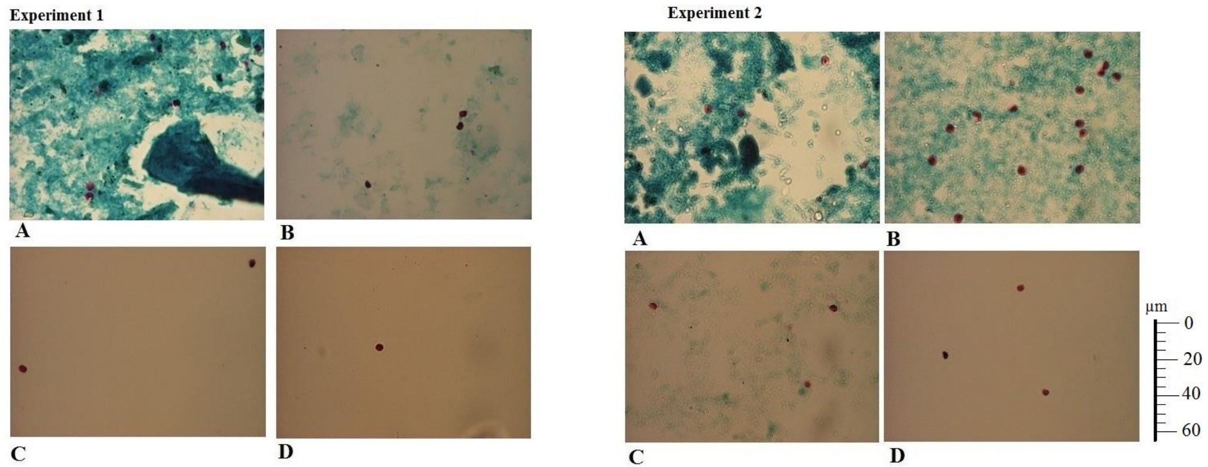

Figure 2, *Cryptosporidium* stained with Ziehl-Neelsen observed under bright field light at a magnification of x40. *Cryptosporidium* are seen in red colour and background in green colour**. A.** Starting material**. B.** After flotation 1**. C.** After flotation 2**. D.** After IMS.

Cryptosporidium oocysts were counted after the first flotation, the second flotation, and after IMS using immunofluorecent labeling (Table 2). A reduction in oocyst quantity was observed.

For Experiment 1, 53.4 % of the oocysts were lost from the first flotation to after the second flotation. Out of the 6 260 ocysts (313 oocysts/µl x 20 µl) from the second flotation going into IMS, 1 150 oocysts remained after the IMS which is a loss of 81.6% oocysts.

For Experiment 2, 17.3% of the oocysts were lost from the first flotation to after the second flotation. Out of the 15 680 oocysts (784 oocysts/µl x 20 µl) from the second flotation going into IMS, 4 950 oocysts are remaining after the IMS which is a loss of 68.4%.

Table 2, Quantification of oocysts using IFL

	Material	**Oocysts / µl**	**Total**
Experiment 1	After flotation 1	671	1 006 500 oocysts in 1.5 ml
	After flotation 2	313	469 500 oocysts in 1.5 ml
	After IMS	23	1 150 oocysts in 50 µl
Experiment 2	After flotation 1	948	1 422 000 oocysts in 1.5 ml
	After flotation 2	784	1 176 000 oocysts in 1.5 ml

	After IMS	99	4 950 oocysts in 50 µl

DNA amplification and concentration

Experiment 1

The samples (A+B) treated with two flotations, IMS, DNA extraction and amplification including and excluding lysis step, were pooled. DNA concentration was measured for the pooled sample and for the unamplified sample treated only with two flotations, IMS and extraction using Qubit® Flourometer.

The pooled sample (A+B) had an average concentration of 2.07 ng/µl and the unamplified sample had less than 0.005 ng/µl.

As part of the control process, the first batch of samples were screened (pre- and post-amplification) using qPCR targeting the 18S rRNA gene. *Cryptosporidium* DNA was present and had been amplified (data not shown).

Experiment 2

The sample, treated with two flotations, IMS, DNA extraction and amplification including lysis step, was measured using Qubit® Flourometer. The sample had an average concentration of 2.5 ng/µl and the unamplified sample treated only with two flotations, IMS and extraction had less than 0.005 ng/µl.

Sequencing and annotation

Experiment 1

608 144 reads with an average length of 227 bp were achieved.

The sequence was mapped to the reference genome (*C. parvum,* strain Iowa II). A total of 138.6 Mbp were sequenced and 39.2 Mbp were mapped. 28.3% of the total DNA in the sample was *Cryptosporidium* DNA. The coverage was 4.25, the standard deviation of coverage was 10.95 and the relative standard deviation of coverage was 2.58. A 0.57 fraction of the reference was covered (Table 3). For whole genome sequencing with Ion Torrent the sample needs to contain at least 10% of the genome of interest.

Table 3, sequencing results Experiment 1

Mbp total	Mbp mapped	Percentage crypto (%)	coverage	coverage std dev	RSD coverage	Fraction of ref covered
138.6	39.2	28.3	4.25	10.95	2.58	0.57

A de-novo assembly of all the unassembled reads was done. The three contigs with the longest reads and the three contigs with the highest coverage was BLASTed using blastx to NCBI. The blast hits with the longest reads were the gram positive bacterium *Propionibacterium acnes* and the blast hits with the highest coverage were human *Homo sapiens* (Table 4).

Table 4, Contaminations in Experiment 1, longest contigs and contigs with the highest coverage

Contamination	Coverage	Length
Propionibacterium acnes	6.8	6004
Unknown, most likely bacterial	12	5728
Propionibacterium acnes	11	4982
Homo sapiens	387	505
Homo sapiens	385	235
Homo sapiens	289	524

Experiment 2

468 327 reads with an average length of 244 bp were achieved.

The sequence was mapped to the reference genome (*C. parvum,* strain Iowa II). A total of 147.4 Mbp were sequenced and 114.2 Mbp were mapped. 77% of the total DNA in the sample was *Cryptosporidium* DNA. The coverage was 12.4, the standard deviation of coverage was 35.5 and the relative standard deviation of coverage was 2.86. A 0.70 fraction of the reference was covered (Table 5)

Table 5, sequencing results Experiment 2

Mbp total	Mbp mapped	Percentage crypto (%)	coverage	coverage std dev	RSD coverage	Fraction of ref covered
147.4	114.2	77	12.4	35.5	2.86	0.70

A de-novo assembly of all the unassembled reads was done. The three contigs with the longest reads and the contigs with the highest coverage was BLASTed using blastx to NCBI. The blast hits with the longest reads were the bacteria *Methylobacterium sp*., the fungi *Melampsora pinitorqua* and the bacteria *Nitrobacter winogradskyi*. The blast hits with the

highest coverage were human *Homo sapiens* and the bacteria *Xanthobacter autotrophicus* (Table 6)

Table 6, Contaminations in Experiment 2, longest contigs and contigs with the highest coverage

Contamination	Coverage	Length
Methylobacterium sp.	41	12868
Melampsora pinitorqua	15	9257
Nitrobacter winogradskyi (low similarity)	36	9224
Homo sapiens	186	560
Xanthobacter autotrophicus (low similarity)	85	4633
Mammalian (most likely *Homo sapiens*)	72	455

Discussion

The results of this study indicate that the process of purifying *Cryptosporidium* oocysts from human samples for whole genome sequencing is achievable.

When comparing material before and after salt flotation the samples had more background contamination before the flotation, although 53.4% and 17.3% of the oocysts were lost. Attempts to further optimize the flotation protocol, by altering the time and speed of centrifugation, as well as the volume of saturated salt water, were tested during the development of this method, without any significant improvement. However, salt flotation is a promising method as a first step of purification.

This is in agreement with another study, made by O'Brien C.N. and Jenkins M.C. in 2007, where salt flotation has been successfully used to purify *Cryptosporidium* oocysts from fecal sample from calves. The volume of sample in their study was much greater, 1-2 L of feces collected each day for 5-7 days, and the process also included another extraction step, to remove lipids commonly found in great amounts in calf feces, and a short sodium hypochlorite treatment step. Their results show minor protein contaminations and no bacterial colonies appeared on Petri dishes with LB agar and blood agar. On the other hand, all contaminations found in feces will most likely not grow on Petri dishes with agar.

Immunomagnetic separation (IMS) has been used successfully for recovery of *Cryptosporidium* oocysts from environmental samples in other studies (Bukhari Z.*et al.* 1998; Giovanni G.D. *et al.* 1999; Rochelle P.A. *et al.* 1999). Using IMS, as a second step of

purification from fecal sample, resulted in even more background contamination lost. However, many oocysts were also lost using IMS, probably due to unsaturated beads or due to the oocysts not detaching from the beads. To overcome this problem, one possible solution would be to use more material in the IMS. In this study only 20 µl of material was used, where the whole volume from flotation (~1.5 ml) could have been used and perhaps the beads in the IMS would thereby saturate.

The step in the IMS where the oocysts are removed from the beads is another critical step. The process is done mechanically by vortexing and most likely not all oocysts are removed from the beads. A way to overcome this problem could be to try to extract the DNA directly from the oocysts when they are still attached to the beads, perhaps using heat as a denaturizing factor.

The concentration of DNA which can be extracted from the sample after IMS, when using 20 µl of material, is too low to be sequenced. Therefore, the whole genome amplification step is crucial. When running a screening qPCR of the material from experiment 1, the copies of the *Cryptosporidium* 18S rRNA gene were shown to have been amplified with the amplification kit from Yikon genomics, between 632 and 1900 times from the original material (data not shown). This indicates the amplification kit had successfully amplified *Cryptosporidium* DNA. The amplification kit also includes a lysis step and it is possible that the DNA extraction before the amplification can be excluded from the purification process since there will be losses of DNA in the extraction.

Sequencing of the purified, and later amplified, material revealed the sample to contain 28.3% *Cryptosporidium* DNA which covered 57% of the reference genome in Experiment 1 and 77% *Cryptosporidium* DNA which covered 70% of the reference genome in Experiment 2. By the use of a larger chip and deeper sequencing the results could be further improved.

The large difference in percentage *Cryptosporidium* DNA in the first and second experiment (28.3% and 77%) could be explained by the difference in number of oocysts going into amplification and sequencing in the different experiments (23 oocysts in Experiment 1 and 99 oocysts in Experiment 2). In addition, differences in the amount of background contamination remaining following the different flotations could further explain the difference. An alternative explanation could be competitive binding of the beads in IMS where Experiment 2 had a larger number of oocysts going in to the IMS, perhaps thereby outcompeting the binding of contaminations. As a result of differences in the pre-amplification process, the efficiency of DNA amplification could vary significantly.

As seen in the blast hits (Table 4 and 6) a large fraction of the contamination is bacterial DNA. By including a chlorination step in the purification process the amount of bacterial DNA would most likely be reduced.

Today only two whole genomes of *Cryptosporidium* have been sequenced, *C. hominis* and *C. parvum* (Abrahamsen M.S. *et al.* 2004; Xu P. *et al.* 2004). A genome sequence for *C. muris* (*Cryptosporidium muris* RN66 2008-10-16) was sent to the *Cryptosporidium* data bank by a group from the U.S.A. but that has yet not been fully characterized.

The *C. hoiminis* genome that was sequenced was the *C. hominis* isolate TU502 which was derived from an infected child and further propagated in gnotobiotic piglets and purified from the feces by salt flotation followed by centrifugation on a Percoll or Nycondenz gradient and bleach treatment (Xu P. *et al.* 2004). The C. parvum genome that was sequenced was the Iowa “type II” isolate of *C. parvum* where the oocysts had been isolated from calves using a discontinuous sucrose gradient and bleach treatment (Abrahamsen M.S. *et al.* 2004).

In both of these studies the oocysts had gone through a passage through hosts other than humans before sequencing. A study made by Akiyoshi D.E. and colleagues (2002) states the *C. hominis* isolate TU502 to be stable passing through humans, piglets and calves. The study used *Cryptosporidium* oocyst wall protein (COWP) PCR-RFLP, microsatellite analysis, sequencing of the SSU rRNA and the β-tubulin genes and genotype-specific PCR to analyze the stability of the isolate.

The results of the sequencing of the *C. hominis* isolate TU502 genome have been of great importance for the research of *Cryptosporidium*. Unfortunately, the risk of other unanalyzed, however important, regions of the genome being affected by the passage through different hosts remains with the use of this enrichment method.

The purification method used before sequencing of the Iowa “type II” isolate of *C. parvum* is stated to yield “highly pure preparations minimally contaminated with bacterial or host DNA” (Abrahamsen M.S. *et al.* 2004). A discontinuous sucrose gradient has been successfully used as an isolation method of oocysts from calf feces in other studies, then in combination with a Percoll or cesium chloride gradient (Arrowood M.J. and Donaldson K. 1996; Arrowood M.J. and Sterling C.R. 1987). Working with calf feces provides a different set of parameters to deal with as compared to human samples (e.g. by the composition and volume of material) but the method might also be applicable for human samples and remains to be investigated.

In comparison to the methods described above for *C. hominis* and *C. parvum*, the method described in this project will not only save time and resources, no ethical issues need to be addressed and more genomes will be able to be sequenced.

By sequencing more genomes, future work in the field of *Cryptosporidium* research (both from a basic research and diagnostic perspective) will be facilitated. For instance, identifying genes responsible for different stages or steps during the life cycle of *Cryptosporidium*, or finding crucial metabolic pathways, might help establishing suitable drugs for treating cryptosporidiosis.

With more genomes sequenced, better gene markers could be defined and more sensitive methods for analysis could be developed. In the event of an outbreak the gene markers could be used to specify the species and subtypes responsible and thereby hasten the process of finding the source of the outbreak.

Conclusion

In this project we demonstrated a purification process of *Cryptosporidium* oocysts from a patient sample involving two gradient flotations, IMS, DNA extraction, whole genome amplification and PCR purification, which will serve as a foundation for future work on the development of a method optimized towards the regular and routine sequencing of the *Cryptosporidium* genome, and may be applied for other parasites as well.

With more genomes sequenced, the field of *Cryptosporidium* would rapidly increase and with better gene markers the work of minimizing the number of infected individuals in the event of an outbreak would be greatly facilitated.

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