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Detection of *Cryptosporidium* oocysts on lettuce and raspberries

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SUMMARY

Cryptosporidium is a parasite which causes gastrointestinal infection and severe diarrhea in humans. The transmissive stage of the parasite is the *Cryptosporidium* oocyst which is excreted in feces and causes infection if it is swallowed by a susceptible host. Protozoan parasites often cause infections in underdeveloped countries with poor hygiene and immunocompromised people, such as people with AIDS, are especially vulnerable. Waterborne outbreaks have also occurred in for example USA, UK and Sweden. There are more waterborne outbreaks documented than foodborne which may be because of lack of appropriate investigative tools for detection in foods and also because foodborne transmission causes more sporadic and widespread outbreaks which are more difficult to document. Today, no international standard method for isolation and detection of *Cryptosporidium* oocysts in food is available. Previous studies have suggested methods for detection of *Cryptosporidium* oocysts on foods. The aim of this study was to compare two previously tested buffers and three extraction methods to find out if any combination was superior for detection of *Cryptosporidium* oocysts on lettuce and raspberries. No significant differences between use of different buffers or extraction methods were found and thus, no combination can be said to be superior to the others. To find any differences, investigations with a higher number of replicates may be required.

INTRODUCTION

Background

Cryptosporidium is a coccidian protozoa and an intracellular parasite which has emerged as an important pathogen for humans in the last 25 years (Smith & Nichols, 2010). *Cryptosporidium* is excreted from the host as a fully sporulated oocyst which is infectious and no time is required for maturation of the oocyst outside the host. The oocysts can survive for weeks or months outside the host especially in a wet environment and at cold temperatures. There are 20 *Cryptosporidium* species and more than 44 genotypes (Smith & Nichols, 2010). In humans, *C. hominis* (formerly known as *C. parvum* type 1) and *C. parvum* are the most commonly detected species. Human infection with *Cryptosporidium* causes gastrointestinal infection and severe diarrhea. Immunocompromised people, such as people with AIDS, are more vulnerable and suffer more severe diarrhea and an infection of long duration which may result in death. Protozoan parasites often cause infections in underdeveloped countries with poor hygiene. *Cryptosporidium* oocysts have also caused waterborne outbreaks in for example USA, UK and Sweden. In Milwaukee, USA, 403 000 people got infected by the parasite through drinking water in the largest outbreak known (Mac Kenzie *et al*., 1994 as cited by Hansen, 2011). There are more waterborne outbreaks documented than foodborne which may be because of lack of appropriate investigative tools for detection in foods and also due to more sporadic and widespread outbreaks which are more difficult to document (Smith *et al*., 2007). Different species of *Cryptosporidium* infects different animals for example livestock, pigs, goats, horses and deer. Animals are primarily infecting each other through a fecal-oral route while humans are infected by contaminated water or foods that are uncooked. The transmission stage of the parasite, the oocyst, is not sensitive to chlorine which is usually used as a disinfectant for bacteria. Freezing or heating of foods including drinking water will inactivate the oocysts.

Transmission of *Cryptosporidium* to humans

The *Cryptosporidium* oocysts is the transmissive stage of the parasite. They are excreted in feces and may cause infection if they are swallowed by a susceptible host. The most common routes of infection are water and food. The infectious dose for humans is low and the oocysts are widely distributed in the environment (Smith *et al*., 2007). *C. parvum* is the major zoonotic species and causes neonatal diarrhoea in livestock (Smith *et al*., 2007). Infected livestock will contaminate their environment with infectious oocysts (Smith *et al*., 2007).

Several ways of food contamination are suggested. Fertilization of food crops with dung from infected animals can result in food contamination. Foods can be contaminated if they are handled or stored in areas where infected animals have contaminated the environment (Robertson & Gjerde, 2001a). Incorrect handling of foods may also increase risk of contamination (Robertson & Gjerde, 2001a).

Contamination of water and foods

Several surveys have detected low levels and widespread occurrence of *Cryptosporidium* and *Giardia* contamination on fruits and vegetables obtained from commercial sources in countries where parasitic infection may be considered endemic (Robertson & Gjerde, 2001a). Low level of *Cryptosporidium* and *Giardia* contamination was found on various vegetable and fruit produce in Norway without there being an outbreak situation (Robertson & Gjerde, 2001a). Several vegetables and fruits were analyzed and 5 (26 %) of the totally 19 positive samples were in lettuce and 14 (74 %) were in mung bean sprouts (Robertson & Gjerde,

2001a). In the same survey, irrigation water was found to contain *Cryptosporidium* and *Giardia*. In this case, lettuce grown in fields irrigated by this water did not appear to contain any parasites but it shows a potential risk of parasitic contamination. An increase in global trading and trends of eating raw or lightly cooked vegetables to preserve taste and nutrient levels, increase the risk of contamination and importance of sensitive standard techniques of detection (Robertson & Gjerde, 2001a, b).

Detection of *Cryptosporidium* oocysts

The method of detection consists of five steps. First the parasites are eluted from the food stuffs into an aqueous suspension. In the second step, the extract is concentrated through centrifugation. Next, the oocysts are isolated from the food stuffs and the suspension through Immunomagnetic Separation (IMS) using commercially available anti-*Cryptosporidium* specific antibodies immobilized on magnetic beads. After that, the oocysts are immobilized on a microscope slide and stained with FITC (fluorescein isothiocyanate) labeled anti-*Cryptosporidium* specific antibodies to allow their visualization. Finally, the samples are screened by fluorescence microscopy.

Optimization of methods

Today, no international standard method for isolation and detection of *Cryptosporidium* oocysts in food is available. It is desirable to develop a detection method that is relatively simple and rapid and the method must also be reproducible and reliable (Cook *et al*., 2006a). Cook *et al*. (2006b) have suggested a method that has been validated by collaborative trials involving eight laboratories in UK and their approach and results are described below.

Concerning the sizes of the food stuffs, the sample should be large enough for detection of low-level contamination (Robertson & Gjerde, 2001b). Using a small sample size will increase recovery efficiency because of less interfering matter from the sample and use of smaller buffer volumes which reduce the losses (Robertson & Gjerde, 2001b). Samples should also be as fresh as possible because it is expected that that more matter will be removed from older food materials (Robertson & Gjerde, 2001b).

Different buffers have been tested to try to increase oocyst recovery. The elution buffer used in an ISO method (ISO 15553, First edition 2006-11-15, Water quality –Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water) for elution of oocysts from filter has been tested for foods by Cook *et al*. (2006a) and also by Robertson & Gjerde (2000) who diluted the buffer 1:4. However, Cook *et al*., (2006a) have reported difficulties with usage of this buffer and they claim that different batches of elution buffer may generate different recovery efficiencies because of the difficulty in dissolving the Laureth 12 detergent during preparation of the buffer. However, this problem has to our knowledge not been described for elution from filter used in analysis of *Cryptosporidium* in water. They also state that all other buffers they tested outperformed elution buffer and that the reason for this should be that the pH of elution buffer is neutral and the molarity too low. Glycine buffer (1 M) performed satisfactorily for both lettuce and raspberries in this study (Cook *et al*., 2006a).

The pH of the glycine buffer was critical for a high *Cryptosporidium* oocyst or *Giardia* cyst recovery from lettuce and two peaks in recovery (pH 3.0 and 5.5) were found for *Cryptosporidium* while one plateau was found between pH 5.0 and 6.0 for *Giardia* (Cook *et al*., 2006, 2007). Thus, a glycine buffer with pH of 5.5 seems optimal for maximizing

recovery of both parasites. The explanation for this pH dependence may be different noncovalent interactions between *G. duodenalis* cysts or the *C. parvum* oocysts and lettuce (Cook *et al*., 2007). It can also be noted that a decrease in pH below pH 4 increased lysis of lettuce cells so that the eluate contained more particles that made it more difficult to extract the oocysts (Cook *et al*., 2006a). The pH of the food materials differ and to increase interaction between antibody and oocyst, pH extremes must be reduced by using suitable buffers (Cook *et al*., 2006a).

Regarding comparison of different extraction methods by the same authors, no significant difference in oocyst recoveries was found when pulsification (rapid beating of the sample in a pulsifier) was compared to stomaching in experiments performed on lettuce and with 1 M glycine buffer pH 5.5 (Cook *et al*., 2006a). In their final method, Cook *et al.* (2006a) chose stomaching rather than pulsification since the former is commonly used in analysis of food which would facilitate comparisons with other laboratories.

Recovery of oocysts

In a study of recovery efficiencies of *Cryptosporidium* oocysts from lettuce, Chinese leaves and strawberries, approximately 42 ±4 % of added oocysts were reisolated (Robertson & Gjerde, 2000). Recovery efficiencies from bean sprouts were usually more variable and lower (Robertson & Gjerde, 2000). In another study, 59.0 ± 12.0 % of *C. parvum* oocysts were recovered from lettuce and 41.0 % ± 13 % from raspberries (Cook *et al*., 2006a).

AIM

Previous studies have suggested methods for detection of *Cryptosporidium* oocysts on foods. The aim of this study was to compare two previously tested buffers and three extraction methods to find out if any combination is superior for detection of *Cryptosporidium* oocysts on lettuce and raspberries.

This study was conducted at the National Food Administration (NFA) in Sweden which is the central supervisory authority for matters concerning food including drinking water. One of the goals for NFA is to work for safe food.

RESULTS

Data analysis

Table 1 shows the results for all experiments. The mean values, standard deviations and 95 % confidence intervals are provided. The yield was calculated assuming that maximum 80 oocysts could be recovered and successfully stained.

Table 1. Data, yield, mean value and statistic parameters. Experimental results from experiments using different food stuffs, extraction methods and buffers.

Food stuffs, Extraction method, Buffer	Results (Number of oocysts)				Recovery efficiency (%)	Mean value	Standard deviation	95 % confidence interval
Lettuce, Stomacher, Elution buffer	1 *	4 *	13	25	13,4	10,8	10,8	17,1
Lettuce, Stomacher, Glycine buffer	22	28	20	6	23,8	19,0	9,3	14,8
Lettuce, Kneading, Elution buffer	8 *	2 *	39	27	23,8	19,0	17,1	27,1
Lettuce, Kneading, Glycine buffer	8	18	30	31	27,2	21,8	10,9	17,3
Lettuce, Shaking, Elution buffer	31	5	17	12	20,3	16,3	11,0	17,5
Lettuce, Shaking, Glycine buffer	28	25	15	7	23,4	18,8	9,6	15,3
Raspberries, Shaking, Elution buffer	9	16	15	3	13,4	10,8	6,0	9,6
Raspberries, Shaking, Glycine buffer	14	14	9	8	14,1	11,3	3,2	5,1

*****These experiments were performed the first day and low values may be due to lack of experience.

Test for comparing samples with different buffers

To find out if there is a significant difference in number of oocysts recovered between the two buffers, a two-sided t-test for two independent samples was used. The test was performed for each of the three extraction methods used, in total three tests for the lettuce samples. The test was also performed once to compare the raspberry samples. T statistics for the tests are provided in Table 2.

Table 2. T statistics from comparison of buffers. Elution buffer and glycine buffer were compared.

Compared methods		T statistic
Lettuce, Stomacher, **Elution buffer**	Lettuce, Stomacher, **Glycine buffer**	-1,2
Lettuce, Kneading, **Elution buffer**	Lettuce, Kneading, **Glycine buffer**	-0,3
Lettuce, Shaking, **Elution buffer**	Lettuce, Shaking, **Glycine buffer**	-0,3
Raspberries, Shaking, **Elution buffer**	Raspberries, Shaking, **Glycine buffer**	-0,1

The null hypothesis is that the expected numbers of oocysts are the same for the two methods and this hypothesis is tested by doing a two-sided t-test. A critical value for the t statistic is chosen so that the hypothesis will be rejected in 5 % of the cases if it is true. The number of degrees of freedom is the sum of the observations in the samples minus two, thus 4+4-2 = 6. This gives a critical value for the t statistic of 2.447.

No significant difference between the elution and the glycine buffer could be observed since the t statistic is below 2,447 and above -2,447 ($\alpha(2) = 0,05$, $f = n_A + n_B - 2 = 6$) for all methods compared.

Test for comparing samples with different extraction methods

To investigate whether there is a significance difference in number of oocysts recovered for the three extraction methods a two-sided t-test for two independent samples was used. The test was performed for each of the two buffers used, in total six times. T statistics for the tests are provided in Table 3.

Table 3. T statistics from comparison of extraction methods. Stomacher, kneading and shaking were compared.

Compared methods		T statistic
Lettuce, **Stomacher**, Elution buffer	Lettuce, **Kneading**, Elution buffer	-0,8
Lettuce, **Kneading**, Elution buffer	Lettuce, **Shaking**, Elution buffer	0,3
Lettuce, **Shaking**, Elution buffer	Lettuce, **Stomacher**, Elution buffer	0,7
Lettuce, **Stomacher**, Glycine buffer	Lettuce, **Kneading**, Glycine buffer	-0,4
Lettuce, **Kneading**, Glycine buffer	Lettuce, **Shaking**, Glycine buffer	0,4
Lettuce, **Shaking**, Glycine buffer	Lettuce, **Stomacher**, Glycine buffer	-0,04

No significant difference between the three extraction methods could be observed since the t statistic is below 2,447 and above -2,447 ($\alpha(2) = 0,05$, $f = n_A + n_B - 2 = 6$) for all methods compared.

DISCUSSION

The buffers investigated were chosen based on previous studies (Cook *et al*., 2006a, Robertson & Gjerde, 2000). The elution buffer is commonly used for detection of oocysts in water and was included based on advice from J Watkins (CREH Analytical, Leeds, UK, oral communication). The glycine buffer has previously been identified as giving the best results in detection from foods in a study by Cook *et al*. (2006a). The chosen extraction methods in this study were stomacher, kneading and shaking. Stomacher is a common method in food analysis for processing foods. Shaking was used based on a method used for extraction of Noroviruses from raspberries and lettuce at National Food Administration in Sweden. Kneading was suggested by J Watkins. Shaking gives a more gentle treatment and it was interesting to test whether this would give higher recovery efficiency since the other more heavy-handed methods may damage the surface of the oocyst. This is important since the antibodies used in the IMS step, and for staining, recognize a carbohydrate structure on a surface protein. A disadvantage with kneading is that this method may be performed differently by different laboratory workers. A standard method should ideally be performed in exactly the same way at different laboratories and by different people for the purpose of comparison and reliability.

Different combinations of extraction methods and buffers were used to evaluate the importance of extraction method and buffer for oocyst recovery. The aim was to find differences in recovery efficiencies for the different combinations used. The results for different replicates with the same extraction method and buffer differ widely. This gives a large standard deviation and wide confidence intervals. No significant differences between use of different buffers or extraction methods could be detected and thus, no combination can be said to be superior to the others. To find any differences, investigations with a higher number of replicates may be required. In further studies to identify the best combination, the time for inoculation could be increased to allow the oocysts to better adhere to the food stuffs. Different buffers or extraction methods could also be investigated.

One drawback in the method is the counting of oocysts on the microscope slides. The reason for this is that counting of the oocysts is a critical step in the experiment which depends on the laborantory worker. When other laboratory workers counted the same slides, a higher number of oocysts were detected (mean value of 23 oocysts detected for the lettuce samples) compared to when I counted the oocysts (mean value 18 oocysts detected for the lettuce samples), which emphasize the importance of experience in this step. In addition, all slides should be scanned and counted several times to minimize the risk that some oocysts are accidently missed or counted more than once due to incorrect movement of the microscope slide. However, the primary aim of this study was to detect a difference in oocyst recovery rather than to determine the exact numbers of oocyst recovered.

Detection could also be performed using molecular methods by exchanging the screening by fluorescence microscopy for PCR. One important difference between detection of oocysts in feces compared to detection in food and water is that while feces may contain up to 10^8 oocysts per gram feces, levels detected in food and water are much lower (Hansen, 2011). This means that if total DNA is extracted from a sample, *Cryptosporidium* DNA would constitute a very small part of the preparation. The IMS will still be required since the *Cryptosporidium* oocysts will constitute a small part of the food stuffs analyzed. Putative problems with molecular methods are that the oocysts are hard to lyse and that substances

inhibiting the DNA polymerases used in PCR are a common problem for several relevant matrices. An advantage of observing the oocysts under the microscope is that both intact and empty oocysts can be detected (Smith & Nichols, 2010).

MATERIALS AND METHODS

Extraction methods

The extraction methods used in this study are summarized in Table 4.

Table 4. Description of extraction methods. Rates and times used in the experiments.

Extraction method	Rate	Time
Stomacher Laboratory blender, Stomacher 400 (Seward)	Normal speed	60 sec
Kneading by hands	-	2 min
Shaking on laboratory shaker	200 rpm	5 min

Extraction buffers

Two different buffers were compared. First an elution buffer from an ISO (The International Organization for Standardization) method for extracting *Cryptosporidium* oocysts and *Giardia* cysts from contaminated water was used (ISO 15553, First edition 2006-11-15, Water quality –Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water). This elution buffer consists of 10 mL 1 M Tris pH 7.4, 2 mL 0.5 M EDTA pH 8.0, 0.1 % Laureth-12 and 0.015 % Antifoam A diluted to 1L.

Secondly, a 1 M glycine buffer pH 5.5 which was found to give the best results in detecting oocysts from foods in a previous study comparing a number of different buffers was used (Cook *et al*., 2006a).

Performed experiments

Cryptosporidium oocysts were released from lettuce samples using three extraction methods (stomacher, kneading and shaking) and two buffers (elution buffer and glycine buffer). *Cryptosporidium* oocysts were also released from raspberries using shaking and two buffers (elution buffer and glycine buffer). Four replicates of each method, i.e. combination of specific extraction method and specific buffer, were performed.

Inoculation of *Cryptosporidium* oocysts

A *Cryptosporidium* suspension (approximately 800 oocysts per mL) was inoculated on 20 g lettuce or raspberries. The *Cryptosporidium* suspension had previously been diluted to approximately 1000 oocysts per mL from a stock solution of 10^7 oocysts/mL (Easy Seed,

TCS biosciences, Buckingham, UK). However, when confirming the actual number of oocysts in eight 50 µL samples of two different aliquots of the spiking suspensions by staining with FITC (fluorescein isothiocyanate) labeled anti-*Cryptosporidium* specific antibodies, it was concluded that the suspension either contained 800 oocysts/mL or that only approximately 80 % of the oocysts were recognized by the antibodies. Therefore, when calculating yields, the number 800 oocysts/mL was used.

The food samples were placed in filter bags (Stomacher® classic 400 filter bags, Seward Limited, Worthing, UK). A total volume of 100 µL of oocyst suspension was added by pipetting 10 µL onto 10 sites of the food sample. The lettuce was cut into small pieces and stored in a refrigerator overnight before inoculation and incubated for 2 h at room temperature with the *Cryptosporidium* suspension on the day of extraction. The raspberry samples were inoculated and incubated for 1 h at room temperature, stored overnight in a refrigerator and were allowed to obtain room temperature before the extraction started.

Extraction and concentration

After addition of 150 mL buffer, i.e. elution buffer or glycine buffer, the sample was processed by one of the extraction methods (stomacher, kneading, or shaking). The sample was distributed in three 50 mL centrifuge tubes with a conical bottom. The plastic bag (if using stomacher or kneading) or the beaker (if using shaking) was rinsed with a small amount of tap water that was poured into the three tubes which were filled to the top. The tubes were centrifuged at 1100×g for 15 minutes at 15-20 °C after which the rotor was allowed to come to a stop without using the brakes. The supernatant was removed until approximately 5 mL remained. The tubes were vortexed and the samples were pooled into one tube. The two remaining tubes were rinsed with deionized water that was added to the sample and the centrifugation repeated. If the size of the pellet exceeded 2.0 mL, two immunomagnetic separation (IMS) reactions were performed. The supernatant was removed until approximately 5 mL remained and the sample was vortexed. To the sample, 4 mL deionized water was added and the sample was vortexed. The sample of approximately 9 mL was transferred to the Leighton tube (Dynal® L10 tubes, Invitrogen Dynal AS, Oslo, Norway) used for the immunoseparation. After that the tube was rinsed with approximately 1 mL water that was transferred to the tube to make a final sample volume of totally 10 mL.

Immunomagnetic separation (IMS) of *Cryptosporidium* oocysts

Immunomagnetic separation (IMS) of *Cryptosporidium* oocysts was performed with the Isolate *Cryptosporidium* kit (TCS Biosciences). To the Leighton tube 1 mL of room tempered buffer A and buffer B was added. The sample of totally 10 mL was transferred in two steps as described above after which 100 µL of crypto magnetic beads was added to the tube. The sample was mixed using a Dynabeads® MX Mixer (Invitrogen Dynal AS) for at least 60 min. After that the tube was inserted into a Dynal MPC™-6 Magnetic Particle Concentrator (Invitrogen Dynal AS) and the magnetic concentrator was rocked back and forth (with an angle of approximately 90 °C) for 2 min. With the tube still held in the magnetic concentrator, the supernatant was poured off and discarded. The tube was removed from the magnetic concentrator to release the beads and 0.8 mL of buffer A diluted 1:10 was added. A long Pasteur pipette was used to transfer the sample to an Eppendorf tube. Thereafter, the tube was rinsed with 0.2 mL of buffer A diluted 1:10 and the liquid was transferred to the same Eppendorf tube.

After mixing, the Eppendorf tube was placed in a DynaMag™-2 Magnetic Particle Concentrator (Invitrogen Dynal AS). The magnetic concentrator was rocked back and forth (now with an angle of approximately 180 °C) for 1 min and then a pipette was used to remove the supernatant. The beads were washed by adding 1 mL of buffer A diluted 1:10 and repeating the rocking procedure and removal of the supernatant. In the next step, 50 µL 0.1 M HCl was added and the Eppendorf tube was vortexed for 15 seconds. Then the tube was incubated 10 min at room temperature after which the sample was vortexed for 15 seconds. Then the tube was placed in the magnetic concentrator which was now held in a horizontal position so that the wall of the tube was placed on top of the magnet and the magnetic concentrator was moved from the left to the right and back. To a well of a Dynal® Spot-On microscope slide (Invitrogen Dynal AS) 5 µL of 1 M NaOH was added. After that the supernatant was transferred to the slide with NaOH.

Staining

Staining was performed using the Easy stain kit from TCS Biosciences but with a slightly modified protocol. In short, the slide was placed on a heating block held at 35 °C and a fan was placed nearby to dry the sample. A positive control for staining was performed using positive control solution with *Cryptosporidium* oocysts provided in the kit. The positive control consisting of *Cryptosporidium* oocysts was dried on the heating block and treated in the same way as the experimental samples. When completely dry, after approximately 30 min, the slide was removed from the heating block but still placed near the fan. Methanol (25 µL) was added to the slide and the sample was allowed to dry completely. Next, 30 µL of a freshly prepared DAPI solution (10 µL of 2 mg/mL DAPI (4´,6´-Diamidino-2-phenylindole dihydrochloride dehydrate) (Sigma-Aldrich, Stockholm, Sweden) in methanol added to 10 mL phosphate buffered saline pH 7.4 (PBS)) was added. The PBS was prepared by dissolving 8.0 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.2 g KCl in 1 L deionized water. The sample was incubated for 2 min at room temperature and then the DAPI stain was removed. Thereafter, 30 µL deionized water was added and the slide was incubated 1 min at room temperature, before the water was removed. To the well, 25 µL staining solution (anti-*Cryptosporidium* antibodies) was added and the slide was incubated in a "humid-chamber" for 15-20 min at 37 °C after which the stain was removed. The "humid chamber" consisted of a glass box with lid and a wet cloth placed at the bottom and was used to prevent drying of the sample.

After removing the staining solution, 200 µL ice cold fixing buffer was added slowly so that it flowed over the edges of the well. The fixation was performed for 2 min at room temperature and then the fixing buffer was removed. The slides were prepared for microscopy using the anti-fade mounting medium included in the kit. Initially, 5 µL mounting medium was used according to the kit instructions but increasing the amount to 15 µL was found to give a better result with less air bubbles trapped under the cover glass. Finally, the cover glass was sealed with clear nail varnish.

Microscopic analysis of *Cryptosporidium* oocysts

The *Cryptosporidium* oocysts were observed under the microscope in the dark under UV at a wavelength of 470 nm and with filters suitable for Fluorescein isothiocyanate (FITC) staining (490 nm excitation, 520 nm emission). Mainly, a magnification of 400× was used. The *Cryptosporidium* oocysts were checked and counted. The oocysts are green fluorescensing and often round with stronger green fluorescence coming from the edges as can be seen in Figure 1. The DAPI staining stained the oocyst DNA which can be seen at 365 nm but due to

a problem with the microscope this was not done in this study. The technique used to count the oocysts is illustrated in Figure 2.

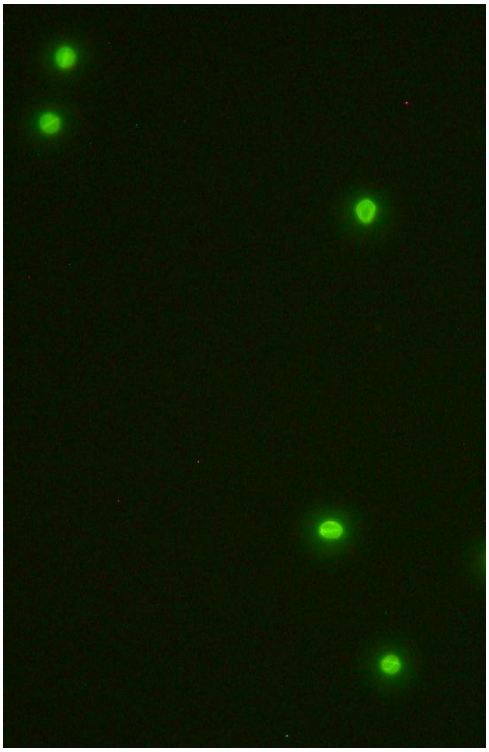

Figure 1. *Cryptosporidium* oocysts. Green fluorescensing oocysts seen under the microscope (photo by Karin Jacobsson, National Food Administration, Uppsala, Sweden).

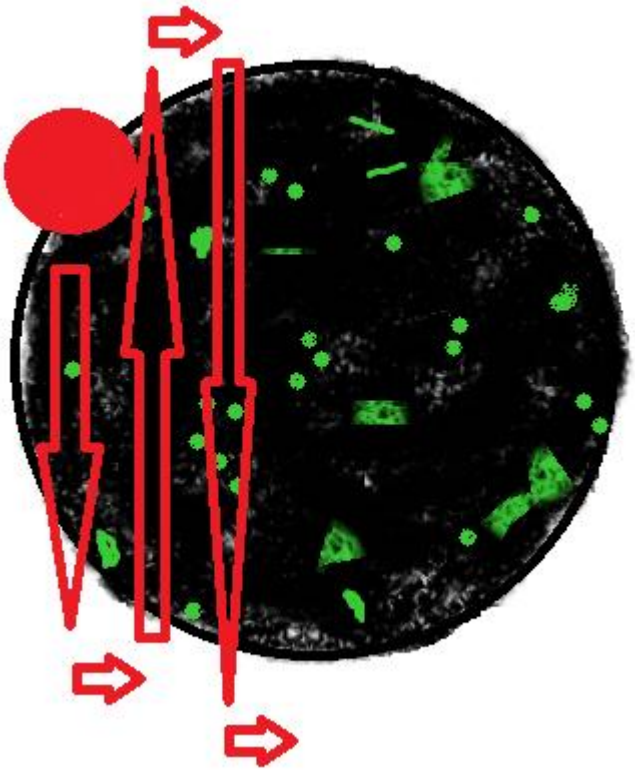

Figure 2. Technique for screening for oocysts by fluorescence microscopy. The well was systematically scanned starting from the upper left corner and moving the slide so that it was scanned downwards to the bottom of the well. Then the slide was moved slightly sideways and scanning continued upwards and so forth as the arrows show. The oocysts were identified among other green fluorescensing materials in the sample and counted (illustration by Cilla Gottfries)**.**

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