Optimization and verification of changes made to US-EPA 1623 Method to analyse for the presence of *Cryptosporidium* and *Giardia* in water

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Dissertation submitted in fulfilment of the requirements for the Degree of M -Tech in the Department of Biosciences,

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March 2010

DECLARATION

This work has not previously been accepted in substance for and degree and is not being concurrently submitted in candidature for any degree.

Signed _____

Date 15 March 2010

ACKNOWLEDGEMENTS

I hereby wish to express my gratitude to the following individuals who enabled this document to be successfully and timeously completed:

- Prof. H du Preez
- M Grundlingh
- A Grundlingh
- C De Wet
- G Booysen
- K Sigudu
- P Stegmann
- R Khoza

Abstract

Methods for detecting the presence of *Cryptosporidium* oocysts and *Giardia* cysts have been developed and continuous improvement is being done to improve the recovery rate of the target protozoa. Rand Water has adopted their method for isolation and detection of *Cryptosporidium* oocysts and *Giardia* cysts in water from United State Environmental Protection Agency (US-EPA) Method 1623, 1999. In 2005 changes were made by US-EPA to the Method 1623.

A study was done to improve the performance of the Rand Water Method 06 (2007) used for isolation and detection of *Cryptosporidium* oocysts and *Giardia* cysts. Three methods namely: Rand Water Method 06 (2007), US-EPA Method 1623 (2005) and Drinking Water Inspectorate standard operating procedures (2003) were compared and key different steps in the methods were identified (wrist action speed, centrifuge speed, immunomagnetic separation procedures and addition of pre-treatment steps). Different experiments were conducted to verify and evaluate the difference between two wrist action shaker speeds, three different centrifuge speeds, two slightly different immunomagnetic separation procedures and when a pre-treatment step was included in the method.

Three different types of water matrices (reagent grade water, drinking water and raw water) were used for the experiments and secondary validation. Data obtained from the experiments and secondary validation was statistically analyzed to determine whether there was a significant difference in the recovery of *Cryptosporidium* oocysts and *Giardia* cysts. Secondary validation of the Rand Water Method 06 (2007) was performed by implementing the study experiments' findings into the method.

The results indicated an increase in the recovery rate of *Cryptosporidium* oocysts and *Giardia* cysts when data was compared with the previous

secondary validation report. The mean recovery of *Cryptosporidium* oocysts in reagent grade water samples increased from 31% to 55%, drinking water samples increased from 28% to 44% and raw water decreased from 42% to 29%. The mean recovery of *Giardia* cysts in reagent grade water samples increased from 31% to 41%, drinking water samples increased from 28% to 46% and raw water decreased from 42% to 32%.

Furthermore, even though the recovery rate of raw water decreased the use of pre-treatment buffer reduced the number of IMS performed per sample by reducing the pellet size. Enumeration of microscope slides was also easier as there was less background interference. The optimization of the Rand Water Method 06 (2007) was successful as the recovery rate of *Cryptosporidium* oocysts and *Giardia* cysts from water increased. All the changes that were verified and that increased the recovery rate were incorporated into the improved Rand Water Method 06.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ANNEXURES

GLOSSARY OF TERMS

Definitions

Cryptosporidium	*Cryptosporidium* is a protozoan parasite potentially found in water and other media. It is determined by brilliant apple green fluorescence under UV light, size (4 to 6 µm), and shape (round to oval), excluding atypical organisms specifically identified as other microbial organisms by FITC and DIC.
Crytosporidiosis	A parasitic disease caused by *Cryptosporidium*, a protozoan parasite in the phylum Apicomplexa. It affects the intestines of mammals and is typically an acute short-term infection. It is spread through the fecal-oral route; the main symptom is self-limiting diarrhoea in people with intact immune systems.
Cysts	A phase or a form of an organism produced either in response to environmental conditions or as a normal part of the life cycle of the organism. It is characterized by a thick and environmentally resistant cell wall.
Eutrophic	A body of water, commonly a lake or pond has high primary productivity due to excessive nutrients and is subject to algal blooms resulting in poor water quality. The bottom waters of such bodies are commonly deficient in oxygen, ranging from hypoxic to anoxic.
FITC	A fluorescent dye that may be conjugated to antibodies for cell surface labelling. FITC emits green light with an emission peak at approximately 515nm

Giardia	*Giardia* is a protozoan parasite potentially found in water and other media. It is determined by brilliant apple green fluorescence under UV light, size (8 to 18 µm long by 5 to 15 µm wide), and shape (oval), excluding atypical organisms specifically identified as other microbial organisms by FITC and DIC
Giardiasis	It is popularly known as beaver fever or backpacker's diarrhoea, it is a disease caused by the flagellate protozoan *Giardia lamblia* (also sometimes called *Giardia intestinalis* and *Giardia duodenalis*). The *Giardia* organism inhabits the digestive tract of a wide variety of domestic and wild animal species, as well as humans.
Hypertrophic	Excessive accumulation, high nutrient levels in water.
Immunomagnetic separation	A purification method that uses microscopic, magnetically responsive particles coated with antibodies targeted to react with a specific pathogen in a fluid stream. Pathogens are selectively removed from other debris using a magnetic field.
Matrix spike	A sample prepared by adding a known quantity of organisms to a specified amount of sample matrix for which an independent estimate of target analyte concentration is available. A matrix spike is used to determine the effect of the matrix on method recovery efficiency.
Mesotrophic	Greater than oligotrophic but less than eutrophic.

Oligotrophic — The environment that offers little to sustain life. The term is commonly utilised to describe bodies of water or soils with very low nutrient levels.

Oocysts — The encysted zygote of some sporozoa: for example *Cryptosporidium.* The oocyst is a phase or form of the organism produced as a normal part of the life cycle of the organism. It is characterized by a thick and environmentally resistant outer wall.

Protozoan parasites — Are human parasitic protozoans affecting the gastrointestinal tract and they usually occur in aquatic environment throughout the word. They are larger in size than either bacteria or viruses and are uniquely designed to survive in water.

Reagent grade water — Water demonstrated to be free from the analytes of interest and potentially interfering substances at the method detection limit for the analyte.

Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ASTM	American Society for Testing and Materials
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential Interference Contrast
DWI	Drinking Water Inspectorate
FA	Immunofluorescence assay microscopy
FITC	Fluorescein isothiocyanate
HIV	Human Immunodeficiency Virus
IMS	Immunomagnetic separation
MPC	Magnetic particle concentrator
NTU	Nephelometric turbidity units
PTFE	Polytetrafluoroethylene
EPA	Environmental Protection Agency
UV	Ultravoilet

Symbols

°C	degrees Celsius
µl	microlitre
%	percent
<	less than
>	greater than

Alphabetical characters

cm	centimeters
g	gram
kg	kilogram
G	acceleration due to gravity
ID	inside diameter
in.	inch

L	litre
m	meter
mg	milligram
mL	millilitre
mM	Millimolar
N	normal; gram molecular weight of solute divided by hydrogen equivalent of solute, per litre of solution

CHAPTER 1

1 INTRODUCTION AND OVERVIEW OF THE STUDY

1.1 The importance of water

Water is essential for all life to exist, as it makes up more than 70% of most living things. While a human can survive more than a week without food, a person will die within a few days without water since every part of our body relies on water. Globally water is the lifeblood that sustains economic growth and enhances human development.

Worldwide, approximately 1.1 billion people lack access to safe drinking water, 2.6 billion people have no access to proper sanitation and more than 5 million people die each year from water related diseases (Rand Water, annual report, 2007). South Africa is located in a predominately semi-arid part of the world and about 7.2 million people do not have access to clean water. However according to the Constitution of the Republic of South Africa, 1996, and the Bill of Rights everyone has the right to have access to an environment that is not harmful to their health or well being.

1.2 Natural water resources

South Africa's drinking water resources are in global terms scarce and extremely limited. Two main sources of water in South Africa are surface water (rainfall and its runoff into rivers or dams) and groundwater (water that is collected in underground stores or aquifers). Research done by Department of Water Affairs and Forestry in 2007 indicated that the total flow of all the rivers in South Africa combined amounts to about 49 200 million cubic meters (m^3) per year and the total amount is less than half of the Zambezi River, which is the closest large river to Southern Africa.

Groundwater is a relatively small component of South Africa's water resources, accounting for approximately fifteen percent of the South Africa's water consumption (Department of Water Affairs and Forestry, 2002). The groundwater plays a vital role in some rural and more arid areas, as it serves as the only source of water available for domestic requirements, stock watering and small-scale irrigation. The main method of abstracting ground water is by means of boreholes although in other rural areas with extensive but generally low yielding aquifers the wind pump is used for abstraction. In South Africa it is estimated that there may be more than 1.1 million water yielding boreholes.

Quality of groundwater abstracted is not always fit for consumption. The water may be contaminated by different factors for example, chemicals produced by mines as waste liquid that runs to groundwater, contamination by faecal pollution, viruses, bacteria, protozoa and helmiths which can cause waterborne diseases. Groundwater in the Southern Africa often has high nitrate values, which can exceed 50 mg/l (exceeding the permissible level of nitrate in drinking water which is 20 mg/l according to South African National Standard 241:2006). Excess nitrate in water when consumed can cause a number of health concerns including spontaneous abortion or stillbirth, gastric and other cancers and hypertension. One disadvantage of using a borehole can be over abstraction that can result in adverse impacts on groundwater dependent ecosystems including estuaries (mouth of large rivers where they reach the sea), wetlands and springs.

1.3 Challenges related to water affecting South Africa

South Africa is affected by some of the global challenges where water is involved which include (1) eutrophication or excessive plant (including algae) growth in dams, (2) access to water services and (3) affordability of water services. According to annual reports of the Department of Water Affairs and Forestry (2007) it is indicated that about fifty percent of dams in South Africa are seriously affected (hypertrophic) while the rest range in quality from good

(oligotrophic) to poor (mesotrophic). Eutrophication is the process of nutrient enrichment of waters that results in the stimulation of an array of symptomatic changes amongst which increased production of algae and aquatic plants. This process can have negative impacts on the environment which include negative ecological impacts, aesthetic, recreational and human health impacts. Contamination of the water bodies in the eutrophication process contributes to blooms of toxic algae.

Another challenge is access to piped water as not all South Africans have access to piped potable water directly to their homes. Boreholes and water tanks provided by government are sometimes the only source of water for some people. Rivers, streams and dams serve as sources of water in some rural communities. Scarcity of water is not the only reason some communities do not have access to potable water, political or economic policies implemented can be the cause as well. Lack of the technical and financial assets to access potable water and competition for scarce water resources are some of the examples that illustrate that poor people are deprived of their right to clean safe water.

The South African government has implemented plans in some areas of the country to meet the communities' demands of having access to potable water, for example by designing programs to educate people on how to save water. Another program by government was to give each household about 6 kilolitres of free water every month, disadvantaging the poorest large households without access to piped water. The use of pre-paid water meters was introduced in different areas like Orange Farm and Soweto. The pre-paid meter system works by granting an initial lifeline of 6 kilolitres water per month free of charge to families before they start paying for water. In order to get more than basic lifeline one has to buy credit on the water meter in advance which is sometimes impossible for poor families.

Furthermore South Africa is facing another challenge of informal settlements, where communities do not have basic day-to-day requirements like potable water, good sanitation conditions and electricity. Under those conditions those communities are at risk of diseases like waterborne diseases. In addition, the rural communities where they rely on untreated water from streams, rivers or dams are also at risk of waterborne related disease outbreaks. Outbreaks of cholera and other waterborne diseases are other problems affecting African countries like South Africa, Mozambique, Democratic Republic of Congo and Zimbabwe to mention a few. In South Africa, Eastern Cape, Kwa-Zulu Natal and Mpumalanga provinces are prone to cholera outbreaks due to poor sanitation and hygiene at household level. Some outbreaks are not reported, recorded or exposed by the media because of the environment people live in.

The South African government has relied on cost recovery policies and privatisation to deliver water and different water policies have been implemented. In some areas where drinking water is supplied by the government, the inadequate monitoring and management of water quality often result in drinking water quality failing to comply with the national requirements (South African National Standard 241:2006).

1.4 Quality of drinking water

Access to clean, safe drinking water is a basic human right and essential to people's health. Safe, clean drinking water is water that is acceptable for humans to drink and use for other domestic purposes such as food preparation and bathing (Department of Water Affairs and Forestry: 2005).

The quality of drinking water may be monitored and controlled through a combination of protection of water sources, control of treatment process and management of the distribution and handling of the water. World Health Organisation guidelines (2006), in support of the framework for safe drinking water, provides a range of supporting information including microbial aspects,

chemical aspects, radiological aspects and acceptability aspects as discussed below.

Microbial aspects: Contaminated drinking water can lead to infectious diseases caused by pathogenic bacteria, viruses and parasites. Infectious diseases are the most common and wide spread health risk associated with drinking water. Any breakdown in water supply safety may lead to large scale contamination and potentially to detectable disease outbreaks.

Chemical aspects: Most chemicals arising in drinking water are of health concern only after extended exposure of years rather than months. The principal exception is nitrate. Several of the inorganic elements for which guidelines values have been recommended are recognized as essential elements in human nutrition. The chemical guideline values are not set at concentrations of substances that cannot reasonably be measured.

Radiological aspects: The guideline does not differentiate between naturally occurring and artificial or human made radionuclide. Radiological hazards may derive from ionizing radiation emitted by a number of radioactive substances (chemicals) in drinking water.

Acceptable aspects: Consumers may have no means of judging the safety of their drinking water themselves, but their attitudes towards their drinking water supply and their drinking water suppliers will be affected to a considerable extent by the aspects of water quality that they are able to perceive with their own senses. The provision of drinking water that is not only safe but also acceptable in appearance, taste and odour is of high priority. Water that is aesthetically unacceptable will undermine the confidence of consumers, leading to complaints and more importantly possibly lead to the use of water from sources that are less safe. The appearance, taste and odour of drinking water should be acceptable to the consumer.

Drinking Water Inspectorate (May 2005) provides guidance on the implementation of the water supply regulations 2000 and 2001, which apply to water companies whose areas of supply are mainly in England and Wales.

There are different regulations for example, regulation on water supply zones; wholesomeness; monitoring of water supplies; monitoring additional provisions; investigations, authorisations of departures; water treatment and records and information. As stated in regulation: thirty-two, a notice is given by the secretary of the state to a water company requiring them to make an application for approval of any process. The notice may also prohibit use of the process for a specified period.

South African National Standard, SANS 241: 2006 defines the quality of acceptable safe, clean drinking water in terms of microbiological, physical, organoleptic and chemical parameters at the point where water is delivered to the customer for consumption. In South Africa, the Department of Water and Forestry ensures that water supplied by Water Services Authorities to the community meet the requirements of South African National Standard 241:2006. The government also has introduced an incentive based regulation by giving the Water Services Authorities Blue and Green drop status, which is designed to allow customers to drink water from the taps with confidence and be secure in the knowledge that wastewater is managed and discharged in a sustainable environmentally acceptable manner. Blue or green flags will indicate good quality of water that is complying to set criteria by the Department of Water Affairs and Forestry requirements, while red or purple flags will be issued to Water Services Authorities, who fail to adhere to the requirements (Department of Water Affairs and Forestry September, 2008).

The quality of water can be measured by performing different types of chemical and microbiological tests to measure certain parameters. The results of tests are compared to set criteria or levels stated in SANS 241:2006 to indicate the quality of water. Microbiological, physical, organoleptic and chemical requirements are mentioned in annexure A. According to South African National Standard 241:2006 the exceeding of the alert levels shown in Table 1 require immediate remedial action and non-

routine follow up sampling to be continued during investigation until non-conforming parameters comply.

Table 1: Operational water quality alert values for Microbiology (South African National Standard 241:2006)

Determinant	Unit of measure	Alert level
Heterotrophic plate count	Count per ml	5000
Total coliform bacteria	Count per 100ml	10
Somatic coliphages	Count per 10ml	1
Cytopathogenic viruses	Count per 100L	1
Protozoan parasites (*Cryptosporidium / Giardia*)	Count per 10L	1

According to South African National Standard 241:2006 the government prefers that all drinking water samples are analysed in laboratories accredited by a recognised accreditation body or the methods used are Department of Water Affairs and Forestry approved methods of analysis, as defined in the Department of Water Affairs and Forestry Drinking Water Quality Laboratory Strategy, September 2005.

1.5 Monitoring of drinking water quality

1.5.1 Purpose of microbiological monitoring and testing of water

The microbiological examination of water determines if the water is potable, based on the isolation and identification of pathogenic microorganisms after the water purification processes. Therefore drinking water that is tested microbiologically is already in the distribution system and by the time the results are released after approximately twenty-four hours the consumer would have already been exposed to the pathogen if present in water. Thus, microbiological tests are based upon detecting microorganisms whose

presence indicates the possibility of the pathogenic microorganisms by using indicator organisms.

1.5.2 Microbiological monitoring methods

Some of the Microbiological analyses performed on drinking water are mentioned in South African National Standard for microbiological analysis of water SANS 5221:2007 and they include:

Heterotrophic plate count method which indicates the water treatment efficiency by estimating the number of total viable heterotrophic bacteria - microbes that use organic compounds as their major carbon source.

Isolation of total coliforms bacteria, which are gram negative, oxidase negative and non-sporing rods capable of growing aerobically on agar medium containing bile salts.

Isolation of faecal bacteria which are bacteria capable of acid, aldehyde and gas production within 24 hours of aerobic growth at 44.5 ± 0.5°C on medium containing lactose as the carbon source or capable of producing typical blue colonies on m-FC medium. The presence of *Escherichia coli* in water indicates potential faecal pollution and provides information on water treatment efficiency.

Determination of somatic coliphages serves as a model for human enteric viruses and provides information on water treatment efficiency.

Determination of viruses which confirms a risk of infection and faecal pollution and provides information on water treatment efficiency.

A specialized test for isolation and detection of *Cryptosporidium* oocysts and *Giardia* cysts confirms a risk of infection and faecal pollution and provides information on water treatment processes efficiency as well.

1.5.3 Indicator organisms

It is practically impossible to test water for each of the wide variety of pathogens that may be present in water therefore microbiological monitoring for indicator organisms is done. Indicator organism is the organism which, when present in water is evidence of pollution of the water by feacal material

(Pelczar, Chan & Krieg 1993). Many indicators have been studied and recommended for water quality assessment (International Organization of Standards 1990. Detection and enumeration of coliform organisms, thermotolerant coliform organisms and presumptive *Eschericia coli* has been done according to Standard Methods for examination of water and wastewater, (Clesceri & Eaton 1995). Evaluation of the reliability of indicators is carried out by comparison of their incidences and survival in water and treatment processes with that of selected pathogens (Regli, Rose, Haas & Gerba 1991).

The indicator organism, *Escherichia coli* is used to indicate the microbiological safety of water, but the detection of *Escherichia coli* and to a lesser extent total coliforms in drinking water has significant consequences for water providers, healthcare professionals and regulators. *Escherichia coli* is unreliable as an indicator for the presence or viability of oocysts and cysts. When bacteria are compared to parasites, the parasites are infectious at much lower doses, 1-10 cysts or oocysts than the infectious bacterial levels required; 1 000 –100 000 (Mackintosh, Ramba, Delport & Genthe 1999).

Investigations done in Norway during the waterborne disease outbreak in 2004 indicated that raw water used as source water had high levels of feacal indicator bacteria and *Escherichia coli* and in treated water samples *Escherichia coli* was not detected. There is no link between *Escherichia coli* present in water and the protozoan parasites for example *Cryptosporidium* and *Giardia* present in water. Chlorination effectively removed the indicator organism, the results indicated that there was no *Escherichia coli* present in water but chlorine resistant pathogens such as protozoan parasites were still present in water (Robertson, Hermansen, Gjerde, Strand, Alvsag & Langeland 2006).

1.6 Data obtained from monitoring water

The monitoring of drinking and source water play a role in managing treatment plants, giving an indication of the quality of water, especially for source water. Decisions can be taken on which chemicals and concentration or methods to be used, based on the results obtained from such tests. When samples are analyzed accurately and accurate results released, results are then compared to the set standards for example South African National Standard 241:2006 is used for potable water. If the results do not comply to the set standards required action is taken as described in South African National Standard 241:2006.

There are many factors that determine the correctness and reliability of the tests performed by a laboratory. As stated in International Organization of Standards 17025:2005 the factors include: human factors, accommodation and environmental conditions, equipment, test and calibration method and method validation, measurement traceability, sampling, handling of test and calibration items and are described in Table 2.

Table 2: Contributing factors to correctness and reliability of test results (International Organization of Standards 17025:2005)

Human factors	Personnel: Competent personnel to operate specific equipment, perform tests, evaluate results and sign test reports.
Accommodation and environmental conditions	Environmental conditions must not invalidate the results or adversely affect the required quality of any measurement. Laboratory facilities must be such as to facilitate correct performance of the test.
Equipment	All equipment used must be capable of achieving the accuracy required and comply with specifications relevant to the tests concerned and equipment must be operated by authorized personnel.

Table 2: continued	
Test and calibration method and method Validation	Only use tests which meet the needs of the customer and which are appropriate for the test it undertakes. Methods can be laboratory-developed methods or non-standard methods and all methods must be validated before use.
Measurement traceability	All equipment used for tests must be calibrated before put into service.
Sampling	Laboratory must have a sampling plan and procedures for sampling of substances.
Handling of test and calibration items	There must be procedures for the transportation, receipt, handling, protection, storage retention and / or disposal of test items, including all provisions necessary to protect the integrity of the test item and to protect the interest of the laboratory and the customer.

1.7 Specialized test

Africa has very few water services that monitor the presence of *Cryptosporidium* oocysts and *Giardia* cysts in water because it requires a specialized method. In South Africa few laboratories can perform the specialized method to detect *Cryptosporidium* oocysts and *Giardia* cysts; they include Rand Water, South African Council for Scientific and Industrial Research and Umgeni Water. The monitoring of protozoan parasites in water provides information on water treatment processes' efficiency as the parasites are resistant to the concentrations of disinfectant used during water purification. The protozoan parasites can only be trapped during certain steps of water purification provided the water purification processes work adequately and quality control checks are done regularly.

The method for detection of protozoan parasites, which are *Cryptosporidium* oocysts and *Giardia* cysts is a complex and time consuming method. Every step in the method is crucial therefore improvements should be done continuously to optimize, improve the recovery rate of cysts and oocysts and reduce the factors that can affect the recovery rate.

The main aim of the project is to optimize and verify the current Rand Water Method 06 (2007) for isolation and detection of protozoan parasites *(Cryptosporidium* oocysts and *Giardia* cysts), to improve the recovery rate of cysts and oocysts and also increase accuracy and reliability of results.

The specific objectives are:

1. Literature review focusing on protozoan parasites, *Cryptosporidium* and *Giardia.*
2. Literature review on the *Cryptosporidium* and *Giardia* detection methods.
3. Compare the methods and identify steps that differ.
4. Perform separate experiments to determine the effect of the different steps on recoveries of oocysts and cysts.
5. Secondary validation of the method by including the different experiments results into the current Rand Water Method 06 (2007).

CHAPTER 2

2 PROTOZOAN PARASITES: *CRYPTOSPORIDIUM* AND *GIARDIA*

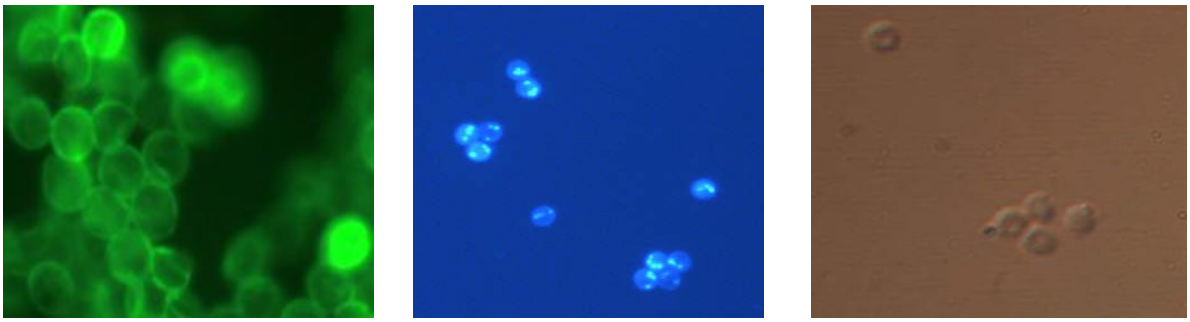
2.1 Protozoan parasites

Protozoan parasites are human parasitic protozoans affecting the gastrointestinal tract and usually occur in aquatic environments throughout the world. Species within the genera cause human cryptosporidiosis and giardiasis, which probably constitute the most common causes of protozoan diarrhoea worldwide, leading to significant mobility and mortality in both developing and developed countries. They are larger in size than either bacteria or viruses and are uniquely designed to survive in water. Three most prevalent gastrointestinal parasites known, according to Betts, Casemore, Fricker, Smith & Watkins (1995:3) are *Giardia duodenalis, Cryptosporidium parvum* and *Entamoeba histolytica* and they are transmitted by the faecal–oral route and inadequate sanitation.

2.2 *Cryptosporidium*

2.2.1 General description

Cryptosporidium is a single–celled, coccidian protozoan parasite that inhabits the human and animal intestines. The protozoan parasite *Cryptosporidium* is transmitted in the oocyst, a stage that has a protective covering and is environmentally resistant (Smith & Thompson 2001). The spherical oocysts are 4-6 µm long, thick walled and each containing four sporozoites shown in Figure 1.



A: FITC B: DAPI fluorescence C: DIC

Figure 1: *Cryptosporidium* oocysts microscopy A: FITC examination; B: DAPI fluorescence examination and C: DIC examination (American Water Works Association, 1995).

Cryptosporidium consists of the following known species, each with a specific host:

Cryptosporidium parvum, host is mammals including humans

Cryptosporidium bailey and *Cryptosporidium meleagridis*, host is birds

Cryptosporidium muris, host is rodents

Cryptosporidium serpentis, host is reptile collection particularly in snake

Cryptosporidium saurophilum, host is lizards

Cryptosporidium nasorum, host is fishes (Mara & Horan 2003).

The oocysts are very persistent in water and extremely resistant to the concentration of disinfectants normally used in drinking water treatment processes and can remain in favorable environments for long periods without loss of infectivity (Korich, Mead, Madore, Sinclair & Sterling1990).

2.2.2 Life cycle of *Cryptosporidium*

Cryptosporidium has a multi-stage life cycle that takes place in the host's intestine involving six major developmental stages:

(1) excystation,

(2) merogony,

(3) gametogeny,

(4) fertilization,

(5) oocysts wall formation

(6) sporogony, as shown in Figure 2.

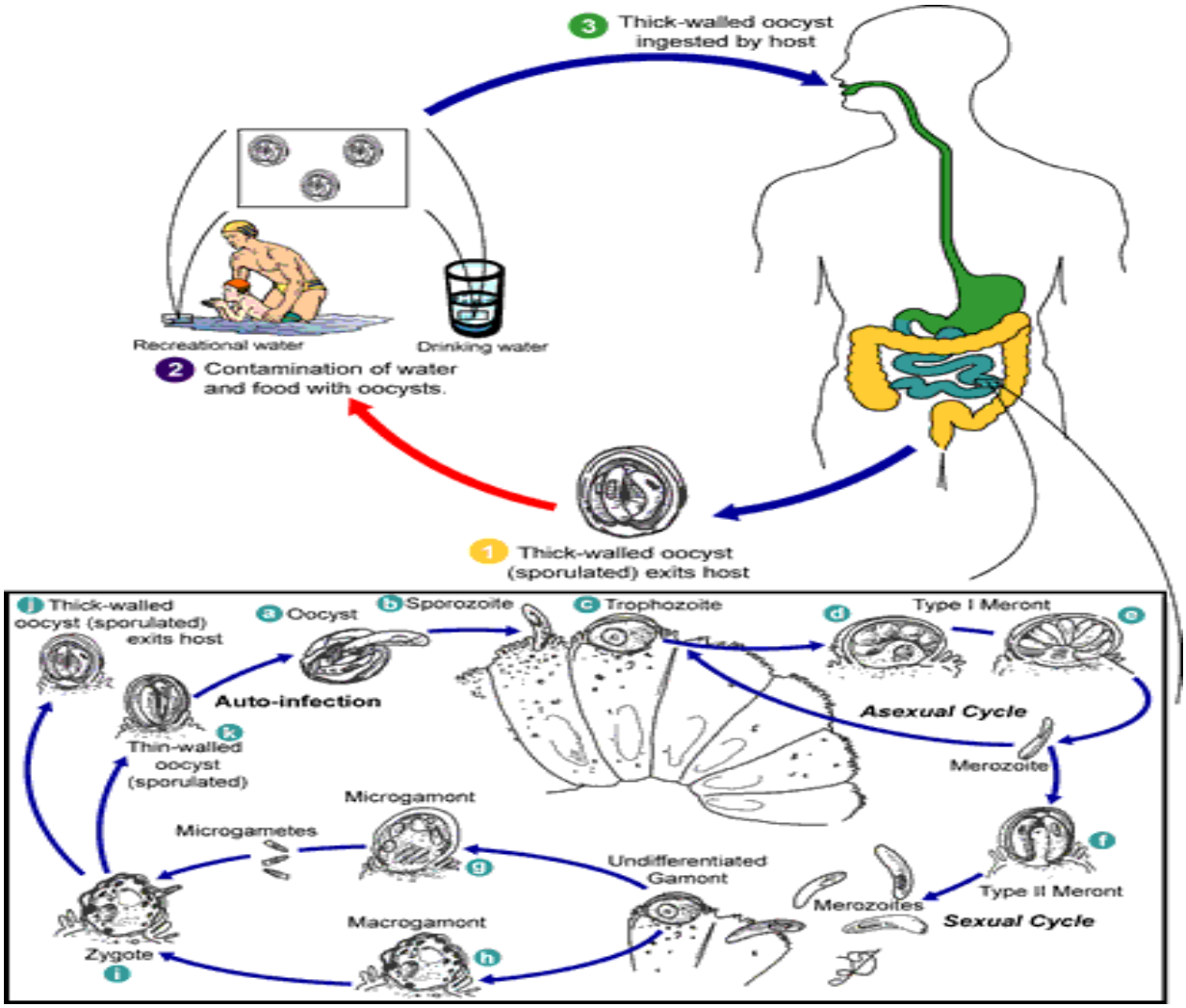


Figure 2: Diagrammatic representation of the *Cryptosporidium* oocysts life cycle and route of infection in humans (Fayer 1997).

In the above figure 2, A: oocysts (infective stage); B: sporozoite released after the oocysts wall-break; C: trophozoite develop from sporozoite; D and E: asexual cycle takes place, merozoites are produced; F: sexual cycle-two merozoites are produced (namely microgament and macrogament); G and H: Microgament and macrogament under-go fertilization to form zygote (I);

J: sporulated oocysts from the zygote exist in the host; K: sporulated oocyst re-infect the host.

The most vital stage in the life cycle of *Cryptosporidium* is the infective stage known as oocysts. The oocysts are excreted in the faeces of infected animals or humans. After the host has ingested oocysts they interact with stomach acid and bile salts and then the life cycle commences as explained in the order below:

Excystation – the thick wall of the oocysts breaks open to release about four sporozoites.

Merogony - involves two cycles of asexual reproduction, which results in production of structures called meronts that contain either 8 (1^{st} generation) or 4 (2^{nd} generation) merozoites.

Gametogeny – merozoites from the merogony stage invades new cells, which are, microgametes (“male”) and macrogametes

Fertilization - when the micro gametes mature they break out of the host cell and invade a cell containing a macro gamete and fertilize them to form zygotes.

Oocysts wall formation - the majority of the zygotes develop a thick cell while a small number of zygotes retain a thin cell wall and excyst while still in the host, to start another cycle of infection.

Sporogony - zygotes develop four sporozoites to become oocysts, which are then passed out in the faeces, which infect the epithelial cells of the small intestines mainly in the jejunum and ileum of the next host.

Oocysts are infective after they are excreted, thus permitting immediate faecal-oral transmission.

2.2.3 Transmission of oocysts

Oocysts may be transmitted by:

Eating raw unwashed vegetables.

Eating vegetables or fruits washed with contaminated hands.

Drinking untreated water.

Swallowing contaminated recreational water (swimming pools).

Changing child's diaper that is infected.

Working with young animals with diarrhoea.

Unhygienic practices and poor sanitation (Centers for Disease Control and Prevention: Cryptospodiosis October 2007).

2.2.4 Human health effect (*Cryptosporidium*)

Cryptosporidium parvum is a species responsible for causing a gastrointestinal illness known as cryptosporidiosis. The symptoms are mentioned below (Centers for Disease Control and Prevention: Cryptospodiosis October 2007):

Watery diarrhoea

Abdominal cramps

Nausea

Weight loss

Low grade fever

Vomiting

Dehydration due to diarrhoea.

It is not known exactly how many oocysts are sufficient to cause Cryptosporidiosis. Care should be taken to avoid even low numbers of oocysts that may be present in any (potable or untreated) water to cause infection.

Depending on the immune response of the host, the duration of symptoms and outcome vary typically. The symptoms usually develop 4-6 days after infection but may appear anytime from 2 - 14 days after infection (Betts *et al*. 1995). The incidence of infection is the greatest during childhood. Two weeks is the maximum period for infected people with healthy immune system to be ill, whereas other healthy individuals may not even get sick. However, cryptosporidiosis poses a significant threat to immunocompromised

patients (patients whose immune system is not competent and strong enough for example AIDS patients, HIV infected people, cancer patients on chemotherapy, transplant patients or others taking medications that suppress the immune system, diabetic, alcoholic or pregnant individuals (Fayer 1997). Cryptosporidiosis is self-regulatory and there are currently no drugs developed for its treatment. Healthy people with competent immune systems may recover on their own, some even develop some immunity to subsequent infections. Many antimicrobial agents have been tested for anticryptosporidial effects and are not safe but an effective therapeutic agent has been discovered thus far to help the immunocompromised patients.

Paromomycin has been tested and used until the symptoms have decreased. Niazoxanide, azithromycin or anticryptosporidical hyper immune bovine colostral antibodies may help some patients. It is advised that anyone with diarrhoea should drink plenty of fluids to prevent dehydration.

2.2.5 Examples of *Cryptosporidium* outbreaks

Around the world there have been waterborne disease outbreaks caused by *Cryptosporidium* oocysts. The following examples indicate different cases of Cryptosporidiosis outbreaks and how different countries, communities and water service authorities handle outbreak cases. The outbreak examples also indicate the impact of the waterborne diseases on communities affected.

Cryptosporidium outbreak: Milwaukee, Wisconsin 1993 (Betts, _et al._ 1995).

During March and April 1993 in Milwaukee, Wisconsin the largest documented waterborne outbreak in history of United States occurred. Milwaukee is the largest city in Wisconsin with a population above 630 000 and is situated on the western shore of Lake Michigan. Milwaukee Water Works supplied the community's drinking water in that area. The Milwaukee Water Works had two water treatment plants, one in the north of the city and

the other in the south both treatments plants obtained their intake water from Lake Michigan.

On the 5th April 1993, high numbers of workers around town were absent from work (different sectors). Most of the people who were absent had the same reason; they suffered from gastrointestinal illness, and immediately an epidemiologic investigation began.

On the 7th April 1993, *Cryptosporidium* oocysts were detected in the stools of seven Milwaukee area residents and the oocysts were linked to water supply. Boil water advisory was issued on the evening of 7th April 1993 and the South plant was temporarily closed on the 9th April 1993. Reports from the investigation done, recorded by Betts *et al*. (1995:59) indicated high levels of turbidity in treated water from the south plant. Based on the reports of high turbidity levels the possibility of waterborne cryptosporidiosis was suggested.

South Water Treatment plant at the time of the outbreak obtained its water from an intake pipe that extends 7600 feet offshore and rests on the bottom of Lake Michigan under 50 feet of water. Inside the plant, during water treatment processes, chlorine and the coagulant polyaluminium chloride was added. Six months before the outbreak the coagulant was changed from alum to polyaluminium chloride in an attempt to reduce levels of lead and copper leaching into the drinking water.

Large fluctuations in the turbidity of the raw water for South Plant were observed with no major increase in turbidity of treated water. The turbidity of treated water on the 23rd March 1993 was high, it exceeded 0.4 NTU and peak daily turbidity reached unprecedented levels of up to 1.7 NTU about the 28th March 1993. Coagulant was immediately changed back to alum.

The source of oocysts in Lake Michigan remained speculative. Natural conditions, heavy rainfall during early spring likely resulted in higher than

usual levels of organic material, including cow manure from fields being washed into streams that flow into rivers and lakes. Other possible sources of oocysts were the slaughterhouse and meat packing plant in central Milwaukee and sewage treatment plant located at the confluence of Milwaukee's three rivers, as they flowed into the lake.

Changing the coagulant before testing the polyaluminium chloride effectiveness compromised a water treatment process of South Plant. Any change in the treatment process can disrupt the effectiveness of the treatment process allowing oocysts not to be trapped effectively. This could have played a major role in the waterborne Cryptosporidiosis outbreak in Milwaukee.

***Cryptosporidium* outbreak: Madison 2005 (Turabeladze, Lin, Weiser & Zhu 2007).**

On the 8th August 2005 a child was diagnosed with cryptosporidiosis. An investigation determined that the ill child swam in the public swimming pool while symptomatic. More cases identified with diarrhoea were among people who used the public swimming pool during that period. The public swimming pool was voluntarily closed for disinfection.

Investigation continued while the swimming pool was closed and at least four more cases were identified. Those four cases were linked to the public swimming pool. Reports indicated that diarrhoea cases identified date as far back as 27th July 2005; more tests were conducted by the Missouri State Public Health laboratory for the presence of *Cryptosporidium* oocysts.

During the outbreak investigation no confirmed cases were reported as severe or requiring hospitalisation. Few confirmed diarrhoea cases were treated. When more tests were conducted on more samples, laboratory tests indicated that the samples from municipal water and the disinfected town pool tested negative for *Cryptosporidium* oocysts. The results proved that the source of the outbreak in Madison was the public pool; although the outbreak

was not severe maybe some patients were just carriers of the protozoan parasite, which may reduce diarrhoea positive cases.

***Cryptosporidium* outbreak: South West England 2006 (Hoek, M.R., Oliver, I., Heard, L., Chalmers, R. & Paynter 2008).**

When Cornwall Health Protection Unit was notified about an outbreak of diarrhoea and vomiting among children and teachers in a primary school, an investigation commenced. The investigation was done on all the children in the school and it showed a clear increase in the number of children with diarrhoea in those children who participated in the school excursion to an outdoor adventure farm in South West England.

Stool samples were tested and four of the samples from the children who visited the farm in South West England were found to be positive for *Cryptosporidium parvum.* A cohort study was implemented of the twenty-eight students and eight teachers according to Hoek *et al.* (2008). Environmental and epidemiological investigation was done tracing back to the school field trip to the farm during 22nd May and 26th May 2006.

Investigation indicated that the most likely transmission route of infection was contact with feacally contaminated surface water from the private well. The well was not protected from livestock as there was no fence around it and there were visible cracks at the ground level of the well through which plants roots could be seen growing into the well according to Hoek *et al*. (2008). One of the observations: around the well there were livestock faeces present beside the well wall and rabbit faeces on the well cover. This clearly indicated the access of livestock to the well and possible causes of contamination to the well.

Action was taken immediately to perform maintenance on the well. A maintenance schedule was followed, the well was emptied, cleaned and disinfection was done by adding chlorine. Samples were collected from the

well on the 12th June and 19th June, tested and the results were negative for the presence of *Cryptosporidium* oocysts.
The investigation indicated that the outbreak might have been caused by various sources. High rainfall experienced, the private well used to supply water that is exposed to livestock and poor maintenance of the well.

***Cryptosporidium* outbreak: Utah 2007 (Rolfs, Beach, Hlavsa, Calanan & EIS officer 2007).**

The Utah Department of Health received 1 902 case reports from the laboratory during June and December 2007, and were confirmed as cryptospodiosis. An outbreak was suspected since most of Utah residents were suffering from diarrhoea during that period (May to December 2007) and then an investigation was carried out (Rolfs *et al*. 2007).

Investigation indicated that about 1 506 patients were infected; most of the infected patients were somehow exposed to recreational water venues and being in contact with persons ill with diarrhoea. The outbreak spanned a geographic area with multiple drinking water sources, so contaminated water was deemed to be an unlikely mode of transmission.

Utah Department of Health held a press conference in July and instructed the public not to swim while ill with diarrhoea. On the 28th August 2007 intensified control measures were implemented in 10 of the 12 health districts: They were:
1) banning all children less than five years and anyone needing diapers from swimming in public recreational venues,
2) requiring all public recreational venues to be hyper chlorinated,
3) finally asking all child care programs to suspend all water activities and enforce diarrhoea exclusion policies.

Slowly the cryptosporidiosis incidence rate decreased after the implementation of intensified control measures and further decreased after

many outdoor pools were closed in September, reported by Rolfs *et al*. (2007). The cause of the outbreak in Utah might have been contamination from the recreational water venues, which was being contaminated by cattle or by other carriers of the parasites.

***Cryptosporidium* outbreak: Northamptonshire 2008 (Health Protection Agency July 2008).**

Anglian Water found traces of *Cryptosporidium* in a water sample from the water supplies to Northamptonshire, Daventry and surrounding villages. On the 25th June 2008, for the first time since Anglian Water Treatment plant was built fifty years ago, a protozoan parasite was detected in the final product – drinking water.

More than 700 people reported symptoms of cryptosporidiosis, which included watery diarrhoea, abdominal cramps, nausea and dehydration. By the 14th July 2008 only thirteen cases of cryptosporidiosis were confirmed and another thirteen cases were still under investigation (Health Protection Agency July 2008). A 'boil water' alert was issued after low levels of the protozoan parasite were detected through routine monitoring on the 25th June 2008. Vehicles equipped with loudspeakers were used to warn people and advised to boil water before use. Tests were carried out to identify the type of *Cryptosporidium* found in Pitsford. Continuous water samples were tested to check if there were still traces of *Cryptosporidium*.

Investigation was conducted and Anglian Water's chief operating officer reported that based on the investigation they concluded that the occurrence was due to a combination of unusual circumstances. It was confirmed that an animal was linked to the source of *Cryptosporidium* at Pitsford Water treatment works. A small rabbit was responsible for water contamination; somehow it managed to gain access to the treatment process through a remote ancillary tank and secreted some parasites into the water before the protozoan parasite was detected.

Due to contaminated water around the area, about twenty schools that rely on tap water supplied by Anglian Water for drinking were shut down immediately. Some supermarkets in Daventry ran out of bottled water because of panic buying by surrounding residents.

Tests indicated that Pitsford water had been free from *Cryptosporidium* since early hours of Thursday 26th June 2008, less than 24 hours after boil water alert was issued to the public. The boil water alert was lifted on Friday 4th July 2008.

***Cryptosporidium* outbreak: Australia 2008 (Australia Healthcare newsletter 2008).**

The latest outbreak documented occurred in Australia. There was a surge in gastroenteritis caused by the microorganism *Cryptosporidium.* There were two hundred and twenty eight cases reported. It was discovered during investigation that children sharing baths and swimming pools were the children with cryptosporidiosis symptoms.

Health authorities gave guidelines to reduce the risk of infection as:

- Washing hands with soap after using the toilet, before eating and after handling animals
- Not sharing bath water with others
- Staying out of swimming pools when infected and not using swimming pools for fourteen days after symptoms cease
- Avoiding child care, school or work until symptoms have ceased.

The public swimming pools were contaminated and people were using the pools they infected.

2.3 *Giardia*

2.3.1 General description

Giardia is a flagellated protozoan clonal parasite that has an oval shape and is thought to be predominantly asexual. The cytoskeleton of cysts includes a median body, four pairs of flagella (anterior, posterior, caudal, and ventral), and a ventral disk shown in Figure: 3. The protozoan parasite *Giardia* cysts can be transmitted from host to host in the same way as the *Cryptosporidium* oocysts.

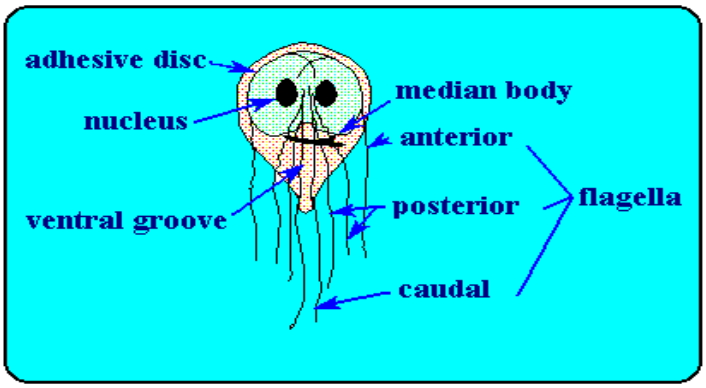


Figure 3: Diagram of a tear-drop flagellated *Giardia* trophozoites showing different structures, it is composed of and some of which it uses when infecting the host cell (Monis & Thompson 2003).

Giardia consists of the following known species, each with a specific host:

- *Giardia agilis;* host is amphibians
- *Giardia muris;* host is rodents, birds and reptiles
- *Giardia duodenalis;* host is humans and other mammals and birds
- *Giardia psittaci;* host is budgerigars (kind of an Australian bird)
- *Giardia ardeae;* host is herons (long-legged long-necked wading bird) (Mara & Horan 2003)

Giardia cysts infect the proximal small intestines in humans and other mammals causing giardiasis (Smith, Robertson & Ongerth 1995). The

Giardia cyst is oval and on average about 10–15 μm in length, its unique characteristics make *Giardia lamblia* one of the easiest intestinal protozoans to diagnose as shown in Figure 4.

A: FITC B: DAPI fluorescence C: DIC

Figure 4: *Giardia* cysts microscopy, A: FITC examination; B: DAPI fluorescence examination and C: DIC examination, American Water Works Association 1995.

2.3.2 Life cycle of *Giardia*

Giardia posseses of a very simple and direct life cycle and it usually starts after ingestion of *Giardia* cysts by the host via contaminated drinking water or food as illustrated in Figure 6. The cysts then inhabit the intestine of humans where they reproduce and cause infection. After the host has ingested cysts they interact with stomach acid and bile salts and then the life cycle commences (Figure 5 and 6).

Different stages in a life cycle of *Giardia* are explained in order below:

Excystation - takes place in the small intestine with the emergence of trophozoites.

Attachment to intestinal epithelial cell - trophozoites make use of the flagella to migrate to a given area of the small intestine and adhere closely to the lining of the small intestine by means of an adhesive disk to epithelial cells and thus maintain their position despite peristalsis.

Attachment depends on active metabolism and is inhibited by temperatures below 37°C, increased oxygen levels, or reduced cysteine concentrations. At this stage the host may experience diarrhoea.

Binary fission – where the organism splits directly into two equal-sized offspring (trophozoites), each with a copy of the parent's genetic material. These trophozoites have two nuclei that are nearly identical in appearance and they undergo nuclear replication cytokinesis at approximately the same time, therefore the cysts contain four nuclei.

Encystation – takes place, which is the formation of the cysts. A wall that is 0.3 to 0.5 µm thick and composed of an outer filamentous layer and an inner membranous layer with two membranes that covers the four nuclei. A web of 7- to 20-nm filaments covers the outer portion of the cyst wall.

Cysts that are free in the lumen of the intestine can pass out with faeces.

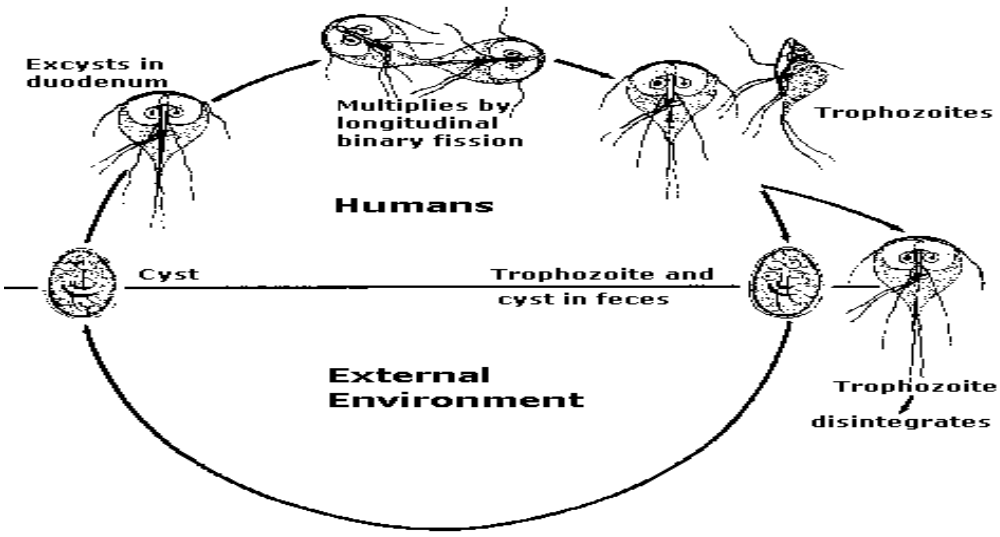


Figure 5: A schematic representation of the life cycle of *Giardia* shown from the environment into the host (human), Centre for disease control and prevention, October 2007.

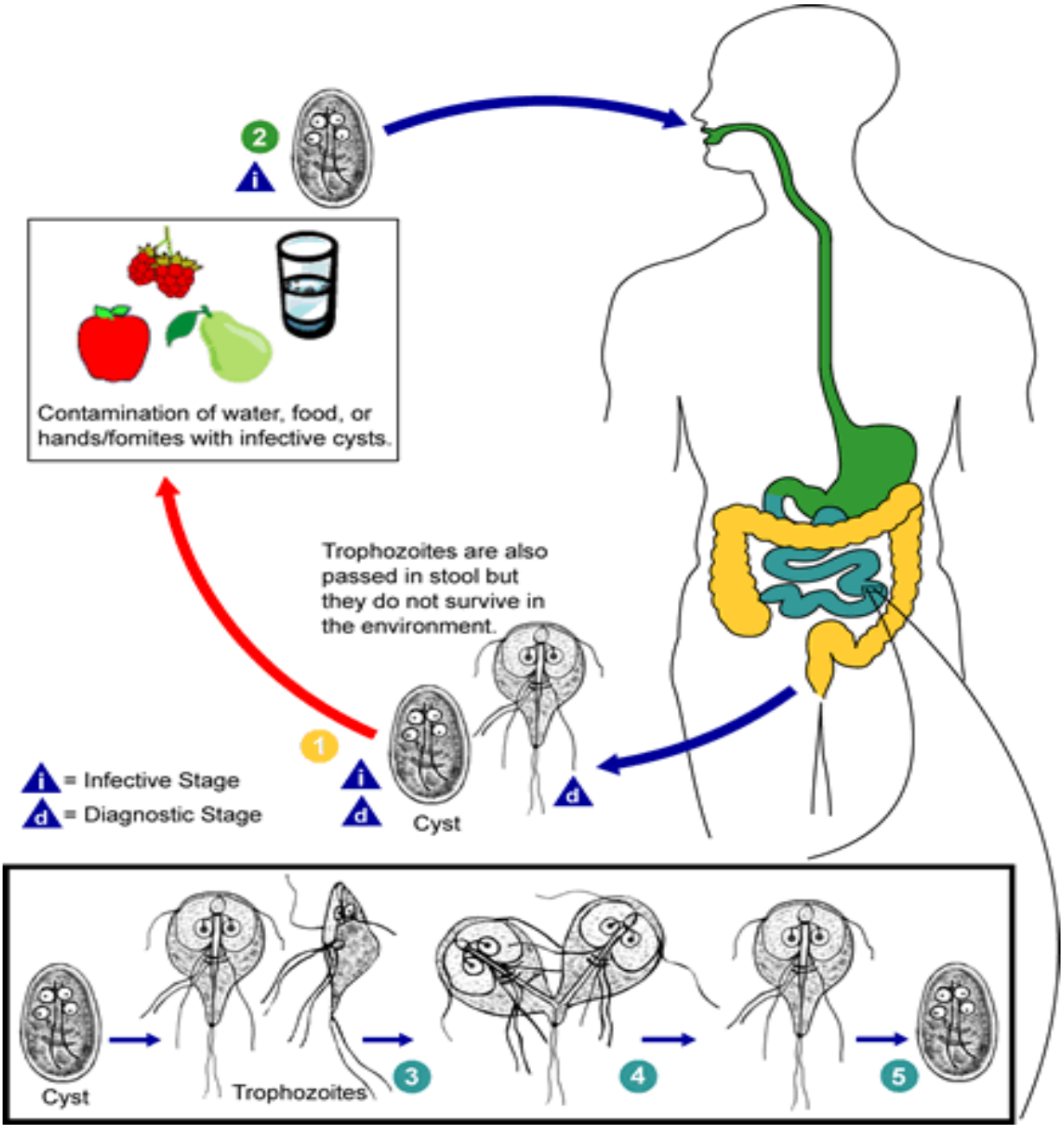


Figure 6: A visual representation of the life cycle of *Giardia* and its infectivity to humans. Centre for disease control and prevention, October 2007.

2.3.3 Human health effect (Giardiasis)

Giardiasis is a gastrointestinal illness caused by the parasite *Giardia lamblia* or *Giardia duodenalis,* this illness has sometimes been referred to as “beaver fever”. Based on a giardiasis waterborne outbreak in Banff National Park

(Canada) where the untreated drinking water fed by the mountain streams was thought to be contaminated by beavers (and hence beaver fever). *Giardia* infection may be asymptomatic or it may cause diseases ranging from self-limiting diarrhea to a severe chronic syndrome.

Giardiasis symptoms (Centers for Disease Control and Prevention: Giardiasis, October 2007):

Chronic diarrhoea
Abdominal cramps
Bloating
Fatigue
Malaise
Vomiting
Excessive flatulence
Weight loss
Dehydration
Frequent loose and pale greasy stools (steatorrhea).

Infection can be asymptomatic but in most cases symptoms occur in one to two weeks post infection. The acute phase of giardiasis is short and characterized by flatulence, abdominal distension and cramps. Some patients infected with giardiasis may develop a severe disease that is not self-limited with signs and symptoms that include:

Interference with the absorption of fat and fat-soluble vitamins.
Retarded growth.
Weight loss.
Celiac-disease-like syndrome.

Giardiasis is self-regulatory but an untreated person can continue to shed the parasite for quite some time. Unlike cryptosporidiosis, giardiasis can be treated with metronidazole and quinacrin hydrochloride and furazolidone can also be used. Metronidazole is an antimicrobial agent with a broad spectrum

of activity against anaerobic bacteria and protozoa (Centers for Disease Control and Prevention: Giardiasis, October 2007).

2.3.4 Example of *Giardia* outbreaks

***Giardia* outbreak: South Worcestershire 1991-1992 (Betts *et al*. 1995).**

A doctor covering a rural area of South Worcestershire noticed six cases of severe unexplained diarrhea. Routine microbiological analyses were done and results were negative but an intense investigation was conducted. All the patients treated in the clinic had some or all of the following symptoms: diarrhea and steatorrhea with one or more of the following: abdominal pains, bloating, fatigue, anorexia and flatulence for more than 48 hours. *Giardia lamblia* was identified as the protozoan parasite responsible for the giardiasis and appropriate treatment with metronidazole was recommended.

A boil water alert was issued and an improved filtration system on the water treatment plant was installed. Common factors or aspects that linked the infected people were investigated. Investigation results indicated that all 28 cases identified lived in the same area and a private water supplier supplied their water.

Private water supplier obtained the source water from a shallow spring. Information gathered during the investigation, indicated that the source of water from the spring was vulnerable to contamination. Animals were grazing in the field where the spring arose and the water treatment plant was inadequate.

Appropriate control measures were applied, which was that while the boil water alert was still applied, and the treatment plant was upgraded. The animals were removed from the area where they were grazing to another location (Betts *et.al*. 1995:59).

2.4 Examples of both *Cryptosporidium* and *Giardia* outbreaks

In some waterborne outbreaks both *Cryptosporidium* oocysts and *Giardia* cysts can be responsible for the waterborne outbreak.

***Cryptosporidium* and *Giardia* outbreak: Norway 2004 (Nygard, Schimmer, Sobstad, Walde, Tveit & Langeland, 2006).**

Bergen is the second largest city in Norway located in Hordaland County. The population was estimated to be around 240 000 people. The university hospital alerted the municipal officer on the 29th October 2004 about an increase of patients diagnosed with giardiasis. The outbreak investigation team (representatives from municipal health, local food safety and the water and sewage authorities) conducted an investigation immediately.

An investigation was conducted mainly to describe the outbreak, identify the sources of contamination and to implement short and long-term control measures. Daily the laboratory of parasitology at the university hospital confirmed cases of giardiasis, stools tested were *Giardia* positive. Metronidazole was prescribed as the suitable treatment for infected patients.

Data for water supplied to people from August to November 2004 was collected, reviewed and compared with results of 2003. Turbidity, total bacterial count, thermotolerant coliform bacteria (*Escherichia coli* bacteria) count and *Clostridium perfringens* spores were the parameters investigated. Water samples were collected from different locations, in the water source area and from several small streams that went into the lake to be tested for the presence of *Giardia* cysts.

Results indicated high numbers of thermotolerant coliform bacteria and *Escherichia coli* in raw water in late August and September, with the highest values in samples taken on 31st August of 64 *Escherichia coli* / 100ml. The results were not suspicious, as it was expected during that time of the year to have high *Escherichia coli* bacteria count.

Due to the presence of *Clostridium perfringens* spores in routine samples of treated water in September, water samples were also analysed to investigate for the presence of *Cryptosporidium* and *Giardia*. One presumptive *Giardia* cyst / 10 litres was detected. Later in November samples of treated water showed five presumptive *Giardia* cysts / 10 litres and also raw water samples showed maximum of five presumptive *Giardia* cysts and *Cryptosporidium* oocysts / 10 litres. The presence of 1 *Cryptosporidium* oocyst or 1 *Giardia* cyst in water to be consumed by people indicate that the purification treatment procedure was compromised since 1 *Cryptosporidium* oocyst or 1 *Giardia* cyst in water can cause waterborne diseases.

Heavy rain falls experienced during a short period in September may have contributed to the outbreak by overloading the old sewage system and causing leakage to the lake, which was used as source water.

2.5 South Africa - *Cryptosporidium* and *Giardia,* 1996 –1998 (Jarmey-Swan, Bailey & Howgrave-Graham, 2001)

A study done in Kwa-Zulu Natal was based on the data obtained from two different laboratories: government hospital pathology laboratories and a private pathology laboratory. No cryptosporidiosis or giardiasis waterborne outbreaks were reported in South Africa, the study was done on individual cases. The period investigated was from January 1996 to March 1998 concentrating on confirmed cases of cryptosporidiosis and giardiasis.

The study population varied with respect to race and income as the private pathology laboratory tested non-formed stools from patients of a higher income bracket, while government hospitals tested non-formed stools from patients of various socio-economic backgrounds within Kwa-Zulu Natal. Government hospital laboratories used different criteria to test stools for *Cryptosporidium* oocysts and *Giardia* cysts compared to the private pathology laboratory.

Results obtained from the data collected were evaluated with regard to rainfall, race, gender, and distribution of occurrence in children younger than five years of age. Results were also evaluated based on patients who were infected with human immunodeficiency syndrome who had diarrhoea or did not have diarrhoea to check the presence of protozoan parasites.

The main findings of the study were:

1. *Cryptosporidium* and *Giardia* prevalence did not appear to correlate statistically with rainfall, month or year.
2. Infection could be due to factors like poor personal hygiene, lack of potable water supply, poor sanitation and lack of education.
3. Increase in the number of samples submitted for *Cryptosporidium* and *Giardia* analysis was independent of the prevalence of either protozoan parasite.
4. Female and male patients tested for cryptosporidiosis had similar positive percentages while giardiasis was more prevalent in female patients than in male patients. Difference of both diseases cryptosporidiosis and giardiasis only appeared where there was age difference between females and males.
5. In children younger than five years the data indicated that *Cryptosporidium* was most prevalent in the less than one-year age group, while *Giardia* was most prevalent in the three to four-year age group.
6. A low percentage of *Cryptosporidium* and *Giardia* positive cases were recorded for both HIV patients with or without diarrhoea.

In South Africa the occurrence of cryptosporidiosis and giardiasis is probably higher than recorded, an estimate that about one in fourteen people with diarrhoea according to Jarmey - Swan *et al*. (2001) seek formal treatment from a health practitioner, clinic or hospital every year. Some people use

traditional home remedies while others seek treatment from traditional or spiritual healers.

Even though in South Africa there has not been a major outbreak of cryptosporidiosis and giardiasis, this does not imply that we are safe from these waterborne diseases. Besides being a requirement by the government to test drinking water for the presence of protozoan parasites, it also helps in monitoring the water purification method used.

According to Curriero, Patz, Rose & Lele (2001), prevalence worldwide of *Cryptosporidium* outbreaks are 1- 4% in Europe and 3 - 20% in Africa, Asia and South America and *Giardia* outbreaks are 3-7% in developed countries and average of 20% (range 5- 43%) in undeveloped countries. In conclusion during the waterborne outbreak of cryptosporidiosis and giardiasis the affected community's lives are disrupted in different ways. People panic and react differently, loose the trust of the water suppliers and it is difficult to gain the customer's trust. It is important to monitor different sources of water used in the water treatment plants which is purified and supplied to customers

2.6 Removal of *Cryptosporidium* oocysts and *Giardia* cysts from water

Cryptosporidium oocysts and *Giardia* cysts can be detected in water by using different methods but how can they be removed from potable water or source water? Removal of oocysts and cysts depend mainly on the effectiveness of the water purification system used in water purification plants. The removal and inactivation of *Cryptosporidium* oocysts and *Giardia* cysts from raw water are complicated by their small size and resistance to commonly used oxidants in the water purification treatment processes.

Maintenance and good operation of the water treatment plants is highly recommended as it plays a major role in removal of protozoan parasite's oocysts and cysts, and can also prevent unnecessary outbreak of waterborne diseases. There are different steps in the water purification process and the

following steps are used by Rand Water and are explained in brief in annexure B, Rand water purification process.

Coagulation
Flocculation
Sedimentation
Stabilization or Carbonation
Filtration
Disinfection

The sedimentation and filtration stages play a major role in removal of protozoan parasite's oocysts and cysts by trapping them, since chlorine at low concentration levels is ineffective against bacterial spores, oocysts and cysts. Filtration with the aid of coagulation / flocculation followed by disinfection is the most practical method to achieve high removal or inactivation rates of oocysts and cysts. Ultraviolet light and ozone are effective against *Cryptosporidium* oocysts and *Giardia* cysts. Factors influencing the use of ultraviolet light and ozone in the large purification plants are:

Relatively expensive
Require complex operations
Maintenance
Still require secondary disinfection
Ultraviolet light requires high electrical demand (Hall & Glysson, 1991).

2.7 Method developments for detection of *Cryptosporidium* and *Giardia*

The development of methods for detection of *Cryptosporidium* oocysts and *Giardia* cysts was triggered by the outbreaks of giardiasis in 1965 and cryptosporidiosis in 1985. Early methods used for detecting *Giardia* according to Hall & Glysson, (1991:483) used membrane filtration for collection of cysts from sewage raw water sample as summarized in figure7.

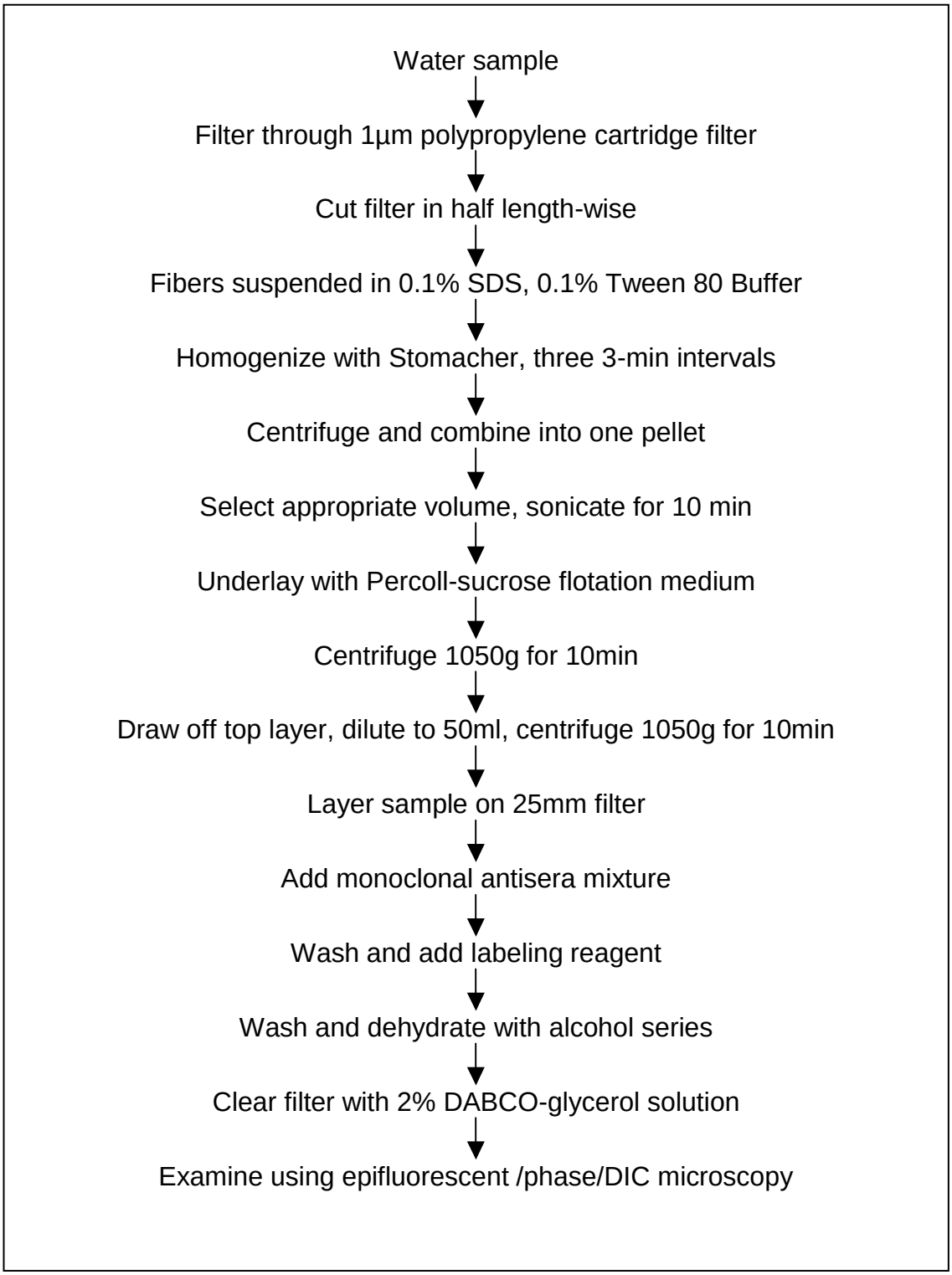


Figure 7: Flow diagram Method for simultaneous detection of *Cryptosporidium* and *Giardia* (Hall & Glysson, 1991)

This method was however not suitable for other types of water (other than sewage). The method proved to be complicated and laborious. Improvements were made and later the yarn-mound orlon cartridge filter for cysts was introduced and it proved to be usable for large volumes of water samples. Initially immunofluorescence staining described by Riggs, Dupuis, Nakamura & Spath 1983, and proposed by Sach 1985, in conjunction with membrane filtration was used to detect *Giardia* from water samples.

In 1985 the methods to detect *Giardia* were adapted for use in the recovery of *Cryptosporidium*. Three basic steps of the method were:

1. Filtration and concentration of the water sample
2. Purification
3. Immonofluorescent staining.

Cysts were collected by propylene – wound filters and a sucrose gradient centrifugation method was used to identify and quantify oocysts. However, there were limitations to the method; immunofluorescence technique used did not clearly differentiate between targeted organisms (oocysts and cysts), algae and other debris interfering with identification. To try and improve identification, antibodies were added to bind with oocysts and cysts (Hall & Glysson, 1991).

Early in the 1990's, the American Society for Testing and Materials (ASTM) proposed a test method for detection of *Cryptosporidium* oocysts and *Giardia* cysts in low turbidity water. During that time the United Kingdom Standing Committee of analysis provided a tentative method for detecting *Cryptosporidium* oocysts and *Giardia* cysts, they called it the "blue book method". When the two methods, ASTM method or cartridge filter system and the method involving membrane filtration were compared, advantages and disadvantages of the method were observed and are summarized in Table 3.

Table 3: Advantages and disadvantages of ASTM method and Membrane method

Advantages	
ASTM Method	**Membrane method**
Ability to sample large volumes of sample	Less expensive
Identify fluorescing oocysts and cysts	Less time required to complete analysis
Disadvantages	
ASTM Method	**Membrane method**
Expensive method	Method was limited
Takes time to complete analysis	Did not differentiate oocysts and cysts from algae

The United States Environmental Protection Agency (US-EPA) approved US-EPA Method 1622 for detection and quantification of *Cryptosporidium* oocysts in water samples during 1990's by filtration, immunomagnetic separation and fluorescence assay identification. The method was a part of a monitoring rule set by US-EPA known as "Information Collection Rule" and it was called ICR Protozoan Method for Detecting *Giardia* cysts and *Cryptosporidium* oocysts in water by a fluorescent Antibody Procedure (US-EPA, Method 1623: 1999).

In addition, more work was done by US-EPA on the method that existed. Then in 1996 a research program was initiated to identify new and innovative technologies for protozoan monitoring analysis and draft US-EPA Method 1622 for detection of *Cryptosporidium* was developed. August 1998 the *Cryptosporidium* method was validated through interlaboratory testing study and it was revised and approved in January 1999.

US-EPA Method 1623 was also developed for detection of both *Cryptosporidium* and *Giardia*; it was validated in October 1998 and was then officially used from mid-July 1999. The US-EPA Method 1623 (1999) had the following advantages:

Sample collection filters permitted 100% capture of oocysts and cysts.

Subsequent elution provided ≥80% removal of target organisms from the filter.

The entire packed pellet volume could be separated and analyzed, eliminating sub-sample analysis.

The addition of 4'-6'-diamidino-2-phenylindole (DAPI) stain permitted easier confirmation of putative oocysts and cysts.

Both US-EPA Method 1622 (1996) and US-EPA Method 1623 (1999) require filtration, immunomagnetic separation of the oocysts and cysts, and an immunofluorescence assay for determination of oocysts and cysts concentrations, with confirmation through dye staining 4'-6'-diamidino-2-phenylindole (DAPI) stain and the use of differential interference contrast microscopy (DIC).

The immunomagnetic separation procedure was followed as described by the manufacturer of the immunomagnetic separation kit used (Dynal A.S. Norway). US-EPA Method 1622 and US-EPA Method 1623 performed better than the other method ICR by having high recoveries. Modifications and alternatives to both methods were proposed as part continuous improvement plan.

US-EPA Method 1622 and US-EPA Method 1623 were the best methods available for monitoring protozoan parasites in water samples, but they have limitations as mentioned below:

Detection relies upon direct examination of water sample.

Methods are limited to 10 litres, 50 litres and 1000 litres sample volumes which can be tested.

Both methods are expensive.

Processes are time consuming.

Only trained and competent analysts can perform the analysis.

Low numbers of samples can be examined.

Method detects presence or absence of *Cryptosporidium* (US-EPA Method 1622, 1996) and both *Cryptosporidium* and *Giardia* (US-EPA Method 1623, 1999) but does not identify the species.

Further improvements were done by US-EPA on the existing method and US-EPA Method 1623, 1999 was updated and the latest version was published in 2005. Refer to annexure C for the latest version of US-EPA 1623 Method, 2005 and is summarized in Figure 8.

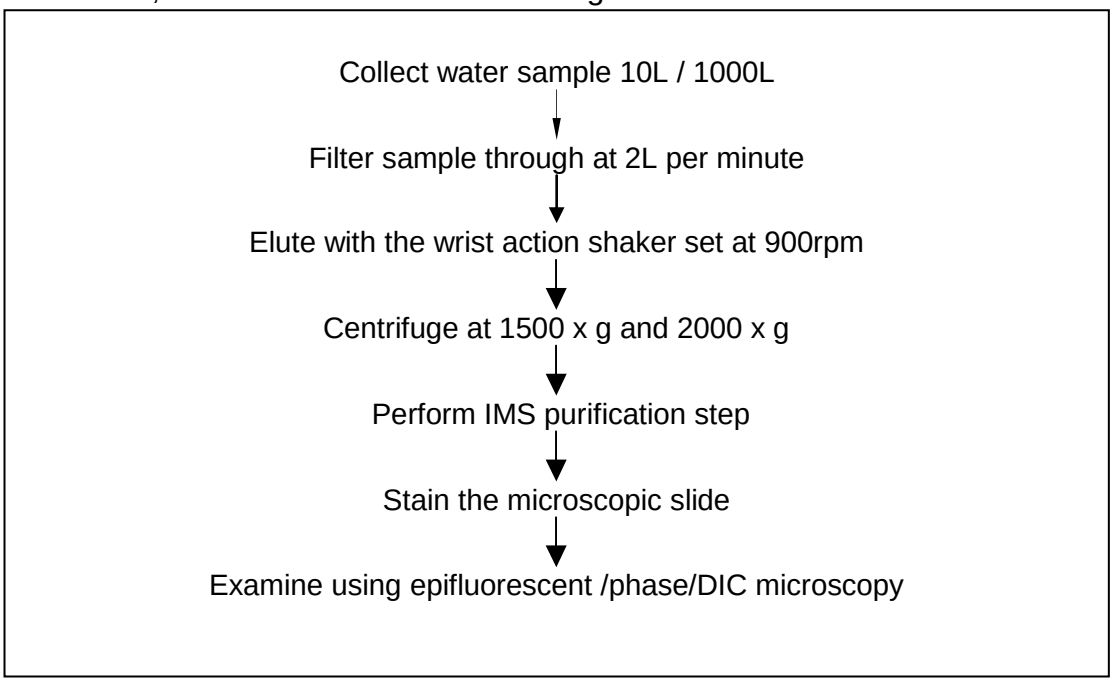


Figure 8: Summarized flow diagram of US-EPA Method 1623, 2005

The method used by Rand Water to analyse water samples for the detection of *Cryptosporidium* oocysts and *Giardia* cysts in water was based on the US-EPA 1623 Method (1999); the method was accredited by an accreditation body of South Africa, South African National Accreditation System (SANAS). The standard operating procedure for the method published by the Drinking Water Inspectorate (2003) differed from the US-EPA Method 1623 (2005) and the Rand Water Method 06 (2007) at a certain step of the method to be discussed later. The following are the steps of the method which were different from the three methods:

Elution - Wrist shaker speed

Inclusion of pre-treatment buffer

Concentration – centrifuge speed

Separation - immunomagnetic separation procedure

2.8 Key identified different steps

Table 4: Summarised difference of key identified method steps.

Method / Method step	Rand Water Method 06 (2007)	US-EPA Method 1623 (2005)	Drinking Water Inspectorate (2003)
Elution: wrist action shaker speed	600rpm	900rpm	900rpm
Elution; inclusion of pre-treatment buffer	Not done	Done	Done
Elution; pre-treatment buffer used	Not done	US buffer	DWI buffer
Concentration: Centrifuge speed	1100XG	1500XG	2000XG
Separation: IMS –two acid dissociation	Done only on spiked samples	Done on all samples	Done on all samples
Separation: IMS-second dissociation added to the same well	Two different microscope slide wells used	One microscope slide well used	One microscope slide well used

Drinking Water Inspectorate, standard operating procedures (2003), pre-treatment buffer inclusion in the elution step: The buffers to be used were prepared in advance and warmed to reach the temperature of 37 ± 2 °C. After the filtration step before the concentration step the Envirocheck filter capsules were rinsed with the warmed buffer (refer to annexure D) to remove excess debris and reduce turbidity of the water sample, especially untreated water samples.

The three methods compared had minor differences among them, which can have a major impact in the recovery of cysts and oocysts. Therefore it is important to evaluate each step separately to determine the effect it has on the recovery of cysts and oocysts. This will be dealt with in the following chapters.

CHAPTER 3

3 METHODOLOGY

3.1 Introduction - Verification of key identified different steps

Methods used to perform microbiological tests should be able to offer the required accuracy. According to ISO/IEC 17025:2005 well-trained and only competent analysts can perform the accredited methods. All the accredited methods used in an accredited laboratory or the methods submitted by microbiological laboratories for accreditation by the accreditation body must always be validated.

The main differences between the currently used Rand Water Method 06 (2007) for isolation and detection of *Cryptosporidium* oocysts and *Giardia* cysts, US-EPA 1623 (2005) and DWI methods were identified and discussed in Chapter 2. Verification was done on the key identified steps in the different methods by performing the following experiments.

Experiment 1: Elution of the sample in a filter capsule using different wrist action shaker speeds.
Experiment 2: Concentration of sample using different centrifuge speeds.
Experiment 3: Purification and separation of oocysts and cysts by comparing two almost similar IMS procedures.
Experiment 4: Comparing two pre-treatment buffers and including the pre-treatment step in eluation step.

The Rand Water Method 06 (2007) was used to determine if the difference in the steps improved the recoveries of *Cryptosporidium* oocysts and *Giardia* cysts.

3.2 Materials

3.2.1 Apparatus and equipment

Watson-Marlow Peristaltic pump connected to the drain

Laboratory wrist action shaker with arms for agitation of sampling capsules

Centrifuge

Incubator set at 37 ± 2 °C

Sample Dynal Mixer

Balances

pH meter

Vortex mixer

Vacuum source

Olympus epi-fluorescence microscope

Timer

Immunomagnetic separation (IMS) apparatus

Magnetic particle concentrator for 10-ml Leighton tubes (MPC-1/ 6)

Magnetic particle concentrator for microcentrifuge tubes (MPC-S)

Micropipette (various volumes)

Fridges

3.2.2 Consumables

Capsule filter, ENVIROCHECK for high volume sampling.

Easyseeds: Easyseeds is a vial containing a known number of *Cryptosporidium* oocysts and *Giardia* cysts.

Tubing: PVC Tygon tubing (12mm) and Silicon tubing (10mm).

10L carboys

Centrifuge tubes Conical, graduated 250ml tubes.

1.5ml Conical-shaped microcentrifuge tube.

10ml pipettes

Pipette tips

Plastic or glass beakers

Measuring cylinders, 50 ml, 100 ml and 2000 ml.

Flat-sided sample tubes 16 x 125mm Leighton-type tubes with 60 x 10 mm flat-sided magnetic capture area.

Appropriate pipetting aid

Suction flask

Well slides PTFE coated well slides, 10mm well diameter

Glass coverslips 22 x 22mm or 24 x 60mm

Immersion oil

Waste containers

3.2.3 Media and Reagents

Elution buffer

Tween 20

Pre-treatment buffer

Reagent grade water: water used from the ELIX UV system.

Drinking water: drinking water from the rising mains at Vereeniging Purification Works.

Raw water: Vaaldam water

1.0 M NaOH

0.1 M HCl

Methanol

Cryptosporidium and *Giardia* FITC-mAb stains

4'-6'-diamidino-2-phenylindole (DAPI) stain

Mounting media

Nail polish

3.3 Method

Rand Water method 06 (2007) for isolation and detection of *Cryptosporidium* oocysts and *Giardia* cysts in water was used to conduct the different experiments.

3.3.1 Sample collection and spiking the sample

Water samples (reagent grade water, drinking or raw water) were collected in red-colour coded 10 litre carboys. The samples were spiked with Easyseeds vial containing a known number of *Cryptosporidium* oocysts and *Giardia* cysts.

For the preparation, the cap of the vial of Easyseeds was removed and 2 ml of 0.05% (v/v) Tween 20 was added to the vial. The vial cap was replaced and vial shaken vigorously. The contents were poured into the carboy with the water sample. About 3ml of reagent grade water was added to an empty vial and shaken vigorously to rinse the vial. The contents of the vial were added to the same carboy with the sample and mixed well by shaking.

3.3.2 Sample filtration

The water sample was filtered through the Envirocheck sampling filter capsule. One end of Envirocheck filter capsule was connected (refer to Figure 9), to the inlet valve of the Tygon tubing (12mm) (refer to Figure 9) and the clamp Envirocheck capsule and held in upright position by a retort stand.

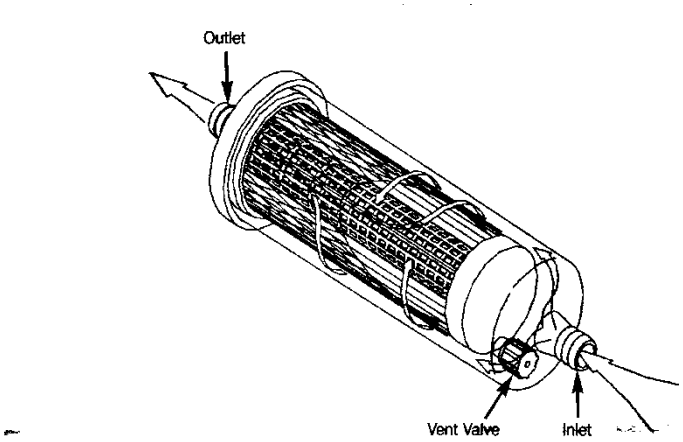


Figure 9: Envirocheck filter capsule (Rand Water Method 06, 2007)

The peristaltic pump was adjusted to pump 2 litres of water per minute. After connecting the filter capsule, the peristaltic pump was switched on and the

water sample was filtered through the filter capsule at the speed of 2L per minute. When 10 litres of the sample had been filtered, 2 litres of reagent grade water was added to rinse the carboy and the reagent grade water was filtered through the same filter capsule to ensure complete recovery of the oocysts and cysts from the Envirocheck filter capsule used to filter water sample. The outlet of the filter capsule was sealed with the blue vinyl end cap of the Envirocheck filter capsule.

3.3.3 Elution

Approximately 150ml of the elution buffer was poured through the inlet fitting of Envirocheck capsule to cover the pleated white membrane of the filter capsule (to remove trapped oocysts and cysts from the membrane of the filter capsule). The filter capsule was clamped in one of the clamps on the laboratory shaker (refer to Figure 10) with the bleed valve positioned at the top on a vertical axis (in the 12 o'clock position) as indicated in Figure 10. The laboratory shaker set at 600 rpm and the capsule shaken for 5 minutes. The filter capsule was then removed from the shaker and inlet cap removed then the contents were poured into an empty 250ml conical centrifuge tube.

Elution buffer was once again added to the Envirocheck filter capsule to cover the pleated white membrane of the filter capsule. The filter capsule was clamped in one of the clamps on the laboratory shaker with the bleed valve positioned on a horizontal axis (4 o’clock position). The laboratory shaker was set at 600rpm and the capsule shaken for 5 minutes.

After 5 minutes, only the position of the capsule was changed from the 4 o’clock position to the bleed valve in the 8 o'clock position. The laboratory shaker set at 600 rpm and the capsule once more shaken for 5 minutes. The inlet end cap was then removed and the contents poured into the same 250ml conical centrifuge tube.

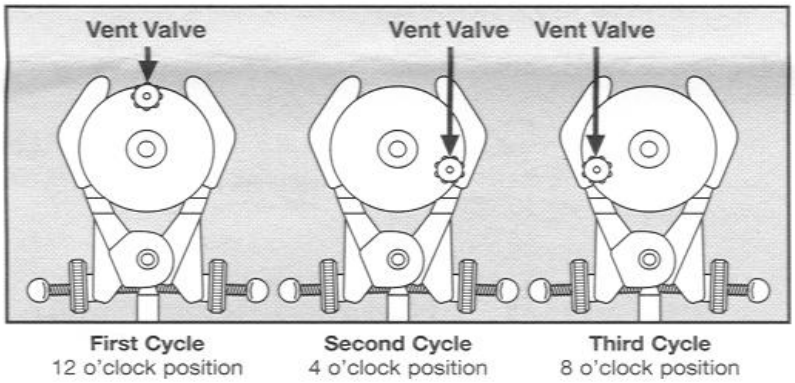


Figure 10: Different shaking positions of capsule for maximum elution on wrist action shaker (Rand Water Method 06, 2007)

3.3.4 Concentration

The conical centrifuge tube containing the filter capsule elute was centrifuged at 1100xg for 15 minutes. The oocysts and the cysts settle at bottom forming a pellet. Carefully the supernatant was aspirated to about 3 - 5ml above the pellet.

3.3.5. Separation (purification)

All the immunomagnetic separation materials to be used were removed from the refrigerator before use and allowed to reach room temperature. From the 10 x SL™-Buffer-A supplied (clear colourless solution) in the IMS kit a 1-x dilution SL™-buffer-A, using reagent grade water as the diluent was prepared. The dilution solution was retained in a separate container to be used later. 1ml of the 10 x SL-buffer - A (clear colourless solution) and 1ml of the10 x SL-buffer- B (supplied - magenta solution) was added to the Leighton tubes labelled with the name of the samples analysed.

The water sample concentrate (after centrifugation) was transferred from the 250ml conical centrifuge tube to the flat-sided labelled (sample name) Leighton tubes containing the SL-buffers (A + B). The Dynabeads® Crypto-Combo vial from the IMS kit was mixed using a vortex to re-suspend the beads and 100µL of the re-suspended Dynabeads® Crypto-Combo were

added to the sample tubes containing the water sample concentrate and SL-buffers (A + B). The Dynabeads®Giardia-Combo vial from the IMS kit was also mixed using a vortex to re-suspend the beads and 100µL of the re-suspended Dynabeads®Giardia-Combo were added to the same sample Leighton tubes containing the water sample concentrate, Dynabeads®Crypto-Combo, and SL-buffers (A + B).

The labelled Leighton tubes with the sample and Dynabeads (both *Cryptosporidium* and *Giardia*) were attached to the rotating mixer and rotated at approximately 18 rpm for 60 minutes at room temperature. After 60 minutes the Leighton tubes containing the sample were removed from the mixer and placed in the magnetic particle concentrator (MPC-1/ 6), with the flat side of the tube toward the magnet.

Without removing the sample tube from the MPC-1/ 6, the magnet side of the MPC-1/ 6 was placed downwards, so that the tubes were horizontal and the flat sides of the tubes were facing downward. Gently the sample tubes were rocked and rolled by hand at an angle of approximately 90°, tilting the cap-end and base-end of the tube up and down in turn for two minutes. Then immediately after two minutes the caps were removed from the Leighton tubes and, keeping the flat side of the tubes on top, all of the supernatant from the tubes held in the MPC-1/ 6 was discarded into a suitable liquid waste container.

The Leighton tubes were removed from the MPC-1/ 6 and the already prepared 1-mL 1X SL-buffer-A was added to the Leighton tubes to re-suspend all material in the tubes by gently mixing the sample with the 1X SL-buffer. All the liquid from the sample Leighton tubes was transferred to 1.5 ml conical-shaped micro centrifuge tube labelled with the corresponding sample name. The micro centrifuge tubes were placed into the second magnetic particle concentrator (MPC-S), with the magnetic strip removed.

The magnetic strip was placed into the MPC-S with the 1.5ml conical-shaped micro centrifuge tubes containing the sample and mixed well by gently rocking and rolling at 180 °C for one minute. The contents of the micro centrifuge tube(s) end to end were agitated, to ensure that all magnetic beads and debris in each tube are fully re-suspended. Without removing the magnet and touching the sides of the micro centrifuge tube, the supernatant from the tube was aspirated using a Pasteur pipette (plugged) and then the magnetic strip was removed.

100 µl of 0.1 M HCl (to separate the beads and the oocyst and cysts) was added to each tube and mixed for 10 to 15 seconds using a vortex mixer. The micro centrifuge tubes were placed in the MPC-S without the magnetic strip and allowed to stand at room temperature for 10 minutes without being disturbed. After 10 minutes the contents of the micro centrifuge tubes were mixed by vortex, for 10-12 seconds. The micro centrifuge tubes were then placed in the MPC-S without the magnetic strip and the magnetic strip was replaced in MPC-S to capture the beads. The tubes were allowed to stand for 10 seconds without being disturbed; this allowed the beads to be captured by the magnet at the back wall of micro centrifuge tubes.

10 µl of 1.0 N NaOH (to neutralize the acid added) was added to each well on of the sample microscope slides to be used. All the liquid from the micro centrifuge tube placed in the MPC-S was transferred to the corresponding microscope slide well containing the 1.0 N NaOH. This was done without disturbing the beads at the back wall of the tube. A second dissociation was performed by repeating the same steps in the method, starting from the step of adding 100 µl of 0.1 M HCl to each corresponding micro centrifuge tube, this step is done to recover any remaining *Cryptosporidium* oocysts and *Giardia* cysts.

The microscope slides were dried in the 37 ± 2 °C incubator. After the drying step a drop of methanol was added to each microscope slide well containing the dried sample. Methanol was allowed to air dry, to fix the *Cryptosporidium* oocysts and *Giardia* cysts to the microscope slide.

3.3.6 Sample staining

Microscope slide wells were overlaid with FITC stain, 25 µl of *Crypto* FITC-mAb and 25µl of *Giardia* FITC-mAb. The microscope slides with the FITC stain were then placed in a humidity chamber and incubated at 37±2 °C for at least 30 minutes. The microscope slides were removed from the incubator and gently aspirated to remove any excess FITC-labelled mAb from each well of the slides.

One drop of an already prepared 4', 6-diamidino-2-phyenylindole (DAPI) working solution was added to each well of the microscope slides, to stain the nucleus of oocysts and cysts. The microscope slides were allowed to stand at room temperature for 2 minutes with the DAPI. The excess DAPI was carefully removed without disturbing the sample on the microscope slides. A drop of reagent grade water was added to each well to rinse off DAPI. The excess reagent grade water was aspirated carefully and the microscope slides were once more dried in an incubator at 37 ± 2 °C.

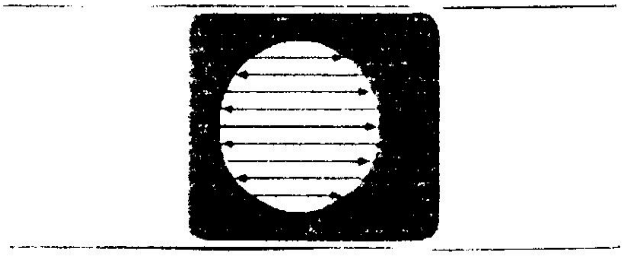
A drop of mounting medium was added to each well and a cover slip applied to the microscope slide. Four corners of the cover slip were sealed off with nail polish. Once the nail polish was dry the microscope slides were viewed under the microscope.

3.3.7 Microscopy

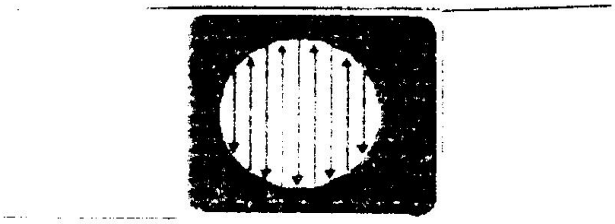
Examination of fluorescent sample preparations:

Slide scanning

Firstly the microscope was focused and systematically each well of the microscope slide was scanned, using a side-to-side or top-to-bottom pattern as in Figure 11.



Side-to-side pattern



Top-to-bottom pattern

Figure 11: Microscope slide examination pattern (Rand Water Method 06, 2007)

3.3.8 Identification of *Cryptosporidium* oocysts and *Giardia* cysts: Fluorescein isothiocyanate (FITC)

The suspected organisms labelled with FITC-mAb when examined using epifluorescence microscopy (FITC, filter block); should exhibit the characteristics in Table 5.

Table 5: Characteristics of *Cryptosporidium* oocysts and *Giardia* cysts labelled with FITC-mAb (Rand Water Method 06, 2007)

Cryptosporidium oocysts	*Giardia* cysts
○ Apple green fluorescence (often with bright edges) ○ Spherical or slightly ovoid in shape, some oocysts deviate from this description ○ Some oocysts will exhibit creases, splits and suture lines ○ Diameter of 4-6μm	○ Apple green fluorescence (often with bright edges) ○ On occasion cysts may appear spherical, or ovoid in shape. Some cysts will exhibit creases and folds ○ Dimensions of 12 -15μm x 8 -10μm

When an apple green fluorescent event was observed which was a characteristic of a *Cryptosporidium* oocyst or *Giardia* cyst, the object was examined with the UV filter block for DAPI and subsequently with Differential Interference Contrast DIC.

3.3.9 Identification of *Cryptosporidium* oocysts and *Giardia* cysts by use of Differential Interference Contrast (DIC)

Once an object/particle which exhibited characteristics typical of an FITC oocysts or cysts was isolated, the microscope was switched to the UV filter block for DIC (position O), the light stop for the UV light was closed and the transmitted light source switched on ensuring that the sub stage condenser was in place. It is important that the light from the mercury vapour lamp was blocked as UV light can damage the DIC filter. The DIC filter and prism were positioned and enhanced the image by: adjusting the light intensity and/or turning the adjustment screw on the prism. All the oocysts and cysts, which identified as *Cryptosporidium* oocysts and *Giardia* cysts, were enumerated and recorded on reporting sheets.

3.3.10 Reporting of results

The results of the matrix spiked sample were calculated to determine the recovery rate of *Cryptosporidium* and *Giardia* as stated in the certificate of analysis supplied with each batch of Easyseeds

$$\text{Recovery (\%)} = \frac{\text{oocysts or cysts detected} \times 100}{\text{Number of oocysts or cysts in Easyseeds as per Certificate of Analysis.}}$$

Different experiments were conducted by following the Rand Water Method 06 (2007) and comparing the different steps identified.

3.4 Experiments for verification of key identified different steps.

3.4.1 Experiment 1. Elution of the sample in a filter capsule using different wrist action shaker speeds

Aim: To compare two different speeds of the wrist action shaker for the elution step (600rpm and 900rpm) in the method.

Procedure: (The same technique as described in section 3.3 was used with the differences as indicated in these procedures).

Duplicate 10 litre samples of reagent grade water were collected, spiked with Easyseeds, filtered through the Envirocheck filter capsules and eluted. On one set of samples, the elution step was done by agitation of the Envirocheck filter capsules with the wrist action shaker set at 600rpm according to Rand Water Method 06 (2007) and the other set of samples were agitated with the wrist action shaker set at 900rpm according to US-EPA 1623 Method, (2005). After elution step, all samples were processed according to the Rand Water Method for isolation and detection of *Cryptosporidium* and *Giardia* as described in section 3.3. Statistical analyses were done to determine if there was a significant difference between the two wrist action shaker speeds.

3.4.2 Experiment 2. Concentration of sample using different centrifuge speeds

Aim: To compare the recovery rate of *Cryptosporidium* oocysts and *Giardia* cysts when three different centrifuge speeds: 1100×g, 1500×g and 2000×g speed were used.

Procedure: (The same technique as described in section 3.3 was used with the differences as indicated in these procedures).

Three sets of 250ml reagent grade water samples were spiked with Easyseeds and processed by setting the centrifuge at different speeds. First set of samples was centrifuged at 1100×g for 15 minutes (Rand Water Method 06, 2007), the second set of samples was centrifuged at 1500×g for 15 minutes (US-EPA Method 1623, 2005) and the last set of samples was centrifuged at 2000×g for 15 minutes (DWI Method, 2003). After the concentration step (centrifugation), all samples were processed according to the Rand Water Method 06, (2007) for isolation and detection of *Cryptosporidium* oocysts and *Giardia* cysts as described in section 3.3. Statistical analyses were done to determine if there was a significant difference between the three centrifuge speeds used.

3.4.3 Experiment 3. Purification and separation of oocysts and cysts by comparing slightly different IMS procedures

Aim: To compare the recovery rate of *Cryptosporidium* oocysts and *Giardia* cysts when two slightly different immunomagnetic separation procedures were followed: Rand Water Method 06, (2007) and US-EPA 1623 Method, (2005) IMS procedures.

Procedure: (The same technique as described in section 3.3 was used with the differences as indicated in these procedures).

Duplicate 10ml reagent grade water samples were spiked with Easyseeds. One set of samples was analysed according to Rand Water IMS method as described in section 3.3 and the other set of samples was analysed

according to US-EPA IMS Method 1623 (2005) refer to annexure C for a detailed description of US-EPA Method 1623, (2005). Microscope slides were stained and examined under the microscope and all *Cryptosporidium* oocysts and *Giardia* cysts were enumerated. Statistical analyses were done to determine if there was a significant difference between the two IMS procedures.

3.4.4 Experiment 4. Comparing two pre-treatment buffers and including the pre-treatment step in elution step.

Aim: To compare recovery rates of *Cryptosporidium* oocysts and *Giardia* cysts from spiked water sample when two different pre-treatment buffers were used.

Procedure: (The same technique as described in section 3.3 was used with the differences as indicated in these procedures).

Two types of water (drinking water and untreated water) were tested to determine the effect of rinsing the Envirocheck filter capsules with buffers. For the experiment two sets of 10 litre water samples were spiked with Easyseeds and filtered through the Envirocheck filter capsule. After filtration the pre-treatment of filter capsules was done with two different buffers according to Drinking Water Inspectorate SOP (2003) discussed in Chapter 2 section 2.8 paragraph 1.

The sodium poly-phosphate buffer was prepared by dissolving 5g sodium polyphosphate in one litre of reagent grade water. The sodium hexametaphosphate buffer was prepared by dissolving 50g sodium hexametaphosphate in one litre of reagent grade water. The sodium polyphosphate buffer (referred to as DWI buffer) was heated to 37 °C before use and the sodium hexametaphosphate buffer (referred to as US buffer) was kept at room temperature for analysis to be performed.

One set of samples was pre-treated with 5% sodium hexametaphosphate buffer and the other set of samples pre-treated with Sodium poly-phosphate buffer. Concentration, IMS procedure, microscope slide staining and microscope examination were done according to Rand Water Method 06 (2007) as described in section 3.3. Statistical analyses were done to determine if there was a significant difference between the buffers and the results of filter capsules where pre-treatment was not done.

Raw data for all the above experiments (3.4.1 to 3.4.4) was collected and evaluated using the basic statistical analyses to determine the significant difference of the different steps compared. Microsoft Excel 2003 programme was used and the Paired t -Test: paired two samples for means was also performed on the data collected.

CHAPTER 4

4 EXPERIMENTAL RESULTS AND DISCUSSION

4.1 Introduction

The data collected from different experiments was analysed statistically using the Paired t - test (test used when two data sets are not independent) a better tool for comparison than the normal t – test. The Paired t – test results gave the probability that the null hypothesis is true. A value of 5% (0.05) or greater suggests that there was no significant difference between the means, any actual difference was likely to be due to chance. If the value was less than 5% (0.05), an alternative hypothesis was made since the means appeared to be significantly different, as they did not overlap significantly.

For this study hypothesis 1 and hypothesis 0 were used: if the P(T< =t) one tail results were more than 0.05 statically H_0 was accepted which indicated the results were the same and if the P(T< =t) one tail results were less than 0.05 statically H_0 was rejected which indicated the results compared had a significant difference.

4.2 Elution of the sample in a filter capsule using different wrist action shaker speeds

The wrist action shaker shakes the Envirocheck filter capsule with the elution buffer inside to wash off the oocysts and cysts from the membrane of the filter capsule that were trapped during the filtration step. Table 6 shows the raw data results of the *Cryptosporidium* oocysts and *Giardia* cysts recovered from the two sets of reagent grade water samples analysed, see page 58.

Table 6: Different wrist action shaker speeds (rpm) recovery results

Cryptosporidium				*Giardia*		
600rpm	900rpm	Difference 900-600rpm		600rpm	900rpm	Difference 900-600rpm
29	51	22		20	59	39
38	51	13		24	60	36
38	59	21		20	46	26
59	59	0		46	26	-20
42	50	8		26	48	22
22	53	31		19	56	37
37	52	15		22	52	30
32	57	25		24	33	9
31	45	14		28	25	-3
21	49	28		25	25	0
45	61	16		22	46	24
40	53	13		48	43	-5

4.2.1 *Cryptosporidium* - Statistical analyses

Table 7: Statistical analyses (t-Test: Paired Two Samples for Means) comparing recoveries for *Cryptosporidium* on different wrist action shaker speeds

	600 rpm	900 rpm
Mean	36.16667	53.33333
Variance	107.4242	22.60606
Observations	12	12
Pearson Correlation	0.546669	
Hypothesized Mean Difference	0	
df	11	
t Stat	-6.81455	
P(T<=t) one-tail	0.0000145	
t Critical one-tail	1.795885	
P(T<=t) two-tail	0.000029	
t Critical two-tail	2.200985	

H_o Recoveries for 600 rpm and 900 rpm are the same

H_1 Recoveries for 900 rpm are better than recoveries for 600rpm

$\alpha = 0.05$

$0.0000145 < 0.05$

Reject H_o, that is recoveries for 900 rpm are better than 600rpm

Table 8: Statistical analyses (95% Confidence interval of differences) comparing recoveries for *Cryptosporidium* on different wrist action shaker speed

Average difference	15.8
SD differences	11.1
95% Confidence interval for the average difference; 900 rpm minus 600 rpm	
Lower confidence limit	7.6
Upper confidence limit	24.1

The recovery of *Cryptosporidium* oocysts when 600rpm speed was used ranged from 21% to 59% and when 900rpm was used the range was from 45% to 61% (Table 6).

The recoveries of *Cryptosporidium* oocysts when 900rpm speed was used were higher when compared to 600rpm speed. A significant difference was statistically observed, the t-Test results in Table 7 were -6.81455. H_o was rejected as P(T< =t) = 0.0000145 one tail results were less than 0.05. The paired t-Test and 95 percent confidence interval in Table 7 and Table 8, show that there was a statistical significant difference between the wrist action shaker speeds. The recoveries of *Cryptosporidium* oocysts were better at 900rpm than 600rpm at a 95 percent confidence level.

The evaluated results indicated the recoveries of *Cryptosporidium* oocysts when the wrist action shaker was set at 900rpm speed were better than recoveries of *Cryptosporidium* oocysts when wrist action shaker was set 600rpm speed.

4.2.2 *Giardia* - Statistical analyses

Table 9: Statistical analyses (t-Test: Paired Two Samples for Means) comparing recoveries for *Giardia* on different wrist action shaker speeds

	600 rpm	900 rpm
Mean	27	43.25
Variance	94.36364	170.3864
Observations	12	12
Pearson Correlation	-0.45024	
Hypothesized Mean Difference	0	
df	11	
t Stat	-2.89177	
P(T<=t) one-tail	0.00733	
t Critical one-tail	1.795885	
P(T<=t) two-tail	0.014659	
t Critical two-tail	2.200985	
H_o Recoveries for 600 rpm and 900 rpm are the same		
H_1 Recoveries for 900 rpm are better than recoveries for 600rpm		
α = 0.05		
0.00733 < 0.05		
Reject H_o, that is recoveries for 900 rpm are better than 600 rpm		

Table 10: Statistical analyses (95% Confidence interval of differences) comparing recoveries for *Giardia* on different wrist action shaker speed

Average difference	23.3
SD differences	22.3
95% Confidence interval for the average difference; 900 rpm minus 600 rpm	
Lower confidence limit	6.8
Upper confidence limit	39.9

The paired t-Test and 95 percent confidence interval in Table 9 and Table 10, show that there was a statistical significant difference between the wrist action shaker speeds. The recoveries of *Giardia* cysts were better at 900rpm than 600rpm at 95 percent confidence.

In addition the recovery of *Giardia* cysts at 600rpm ranged from 19% to 48% and at 900rpm the recovery rate ranged from 25% to 60% (Table 6). The recoveries of *Giardia* cysts when 900rpm speed was used were higher when compared to the recoveries when 600rpm speed was used. Statistical analysis also indicated in Table 9 that t-Test results were –2.89177. H_o was rejected as P(T< =t)= 0.00733 one tail results were less than 0.05. There was a significant difference between the 900rpm speed and 600rpm speed.

Therefore the evaluated results indicated the recoveries of *Giardia* cysts when the wrist action shaker was set at 900rpm speed were better than recoveries of *Giardia* cysts when wrist action shaker was set at 600rpm speed. Increasing the speed of the wrist action during the elution step enabled more oocysts and cysts to be trapped in the membrane of the Envirocheck filter capsule to be released to the elution buffer during agitation, thus higher recoveries.

4.3 Concentration of samples using different centrifuge speeds

Centrifuging the samples is the concentration step that allows the pellet suspected to contain oocysts and cysts, to settle down at the bottom of the centrifuge bottles. Table 11 below shows the results of the *Cryptosporidium* oocysts and *Giardia* cysts recovered from three sets of samples where different centrifuge speeds were used

Table 11: Different centrifuge speeds recovery results

Cryptosporidium				*Giardia*		
1100xg	1500xg	2000xg		1100xg	1500xg	2000xg
47	61	56		48	42	50
35	55	58		40	60	53
54	68	37		39	45	34
32	61	55		21	47	29
37	48	47		24	38	50
45	63	43		53	68	34
45	68	53		58	60	46
47	63	58		49	51	52

Table 11: continued						
48	59	47		51	58	49
45	61	53		53	55	46
45	65	45		43	49	51
50	56	46		44	49	51

4.3.1 *Cryptosporidium* - Statistical analyses

Table 12: Statistical analyses (t-Test: Paired Two Samples for Means) comparing recoveries for *Cryptosporidium* at centrifuge speed 1100xg versus 1500xg

	1100xg	*1500xg*
Mean	44.16666667	60.66667
Variance	40.6969697	32.24242
Observations	12	12
Pearson Correlation	0.493563677	
Hypothesized Mean Difference	0	
df	11	
t Stat	-9.37366127	
P(T<=t) one-tail	7.03E-07	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	1.41E-06	
t Critical two-tail	2.200985159	

H_o Recoveries speeds 1100xg and 1500xg are the same

H_1 Recoveries for speed 1500xg are better than recoveries for 1100xg

$\alpha = 0.05$

0.000001< 0.05

Reject H_o, that is recoveries for speed 1500xg are better than 1100xg

Table 13: Statistical analyses (t-Test: Paired Two Samples for Means) comparing recoveries for *Cryptosporidium* at centrifuge speed 1100xg versus 2000xg

	1100xg	*2000xg*
Mean	44.16666667	49.83333
Variance	40.6969697	43.9697
Observations	12	12
Pearson Correlation	-0.517208299	
Hypothesized Mean Difference	0	
df	11	
t Stat	-1.732187034	
P(T<=t) one-tail	0.055573977	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	0.111147954	
t Critical two-tail	2.200985159	

H_o Recoveries speeds 1100xg and 1500xg are the same

H_1 Recoveries for speed 1500xg are better than recoveries for 1100xg

$\alpha = 0.05$

0.056> 0.05

Accept H_o, that is recoveries for speed 1100xg and 2000xg are the same

Table 14: Statistical analysis (95% Confidence interval of differences) comparing recoveries for *Cryptosporidium* at different centrifuge speeds

		1500xg minus 1100xg	2000xg minus 1100xg
Average difference		17.7	7.7
SD differences		6.4	15.4
95% Confidence interval for the average difference	Lower confidence limit	12.9	-3.7
	Upper confidence limit	22.4	19.1

The recovery of *Cryptosporidium* oocysts at the centrifuge speed of 1100xg ranged from 32% to 54%, at the centrifuge speed of 1500xg ranged from 48% to 68% and at the centrifuge speed of 2000xg recoveries ranged from

37% to 58% (Table 11). When the centrifuge was set at 1500xg speed recoveries were better than at 1100xg and 2000xg centrifuge speed.

Statistical analyses indicated that the centrifuge speed of 1500xg was better than 1100xg and 2000xg. The t-Test results in Table 12 for 1100xg and 1500xg centrifuge speed were 9.37366 and the H_o was rejected as the P(T<=t) = 0.0000007 one tail results were less than 0.052, which indicated the significant difference in the results compared. The paired t-Test and 95 percent confidence interval in Table 12 and Table 14, show that there was a statistical significant difference between 1100xg and 1500xg centrifuge speeds with the centrifuge 1500xg giving better recoveries.

The paired t-Test and 95 percent confidence interval in Table 13 and Table 14, show that there was no statistical significant difference between 1100xg and 2000xg centrifuge speeds. The t-Test results in Table 12 for 1100xg and 2000xg speed were 1.733218 and the H_o was accepted as the P(T<=t) = 0.05557 one tail results were greater than 0.05, therefore the recoveries for 1100xg centrifuge speed and 2000xg centrifuge speed were the same.

The recoveries of *Cryptosporidium* oocysts when the centrifuge was set for 1500xg speed were better than when centrifuge was set at 1100xg or 2000xg centrifuge speed.

4.3.2 *Giardia* - Statistical analyses

Table 15: Statistical analyses (t-Test: Paired Two Samples for Means) comparing recoveries for *Giardia* at centrifuge speed 1100xg versus 1500 x g

	1100xg	*1500xg*
Mean	43.58333	51.83333
Variance	128.8106	74.33333
Observations	12	12
Pearson Correlation	0.63191	
Hypothesized Mean Difference	0	

Table 15: continued

df	11
t Stat	-3.2057
P(T<=t) one-tail	0.004185
t Critical one-tail	1.795885
P(T<=t) two-tail	0.008369
t Critical two-tail	2.200985

H_o Recoveries speeds 1100xg and 1500xg are the same

H_1 Recoveries for speed 1500xg are better than recoveries for 1100xg

$\alpha = 0.05$

0.004< 0.05

Reject H_o, that is recoveries for speed 1500xg are better than 1100xg

Table 16: Statistical analyses (t-Test: Paired Two Samples for Means) comparing recoveries for *Giardia* at centrifuge speed 1100xg versus 2000xg

	1100xg	*2000xg*
Mean	43.58333	45.41667
Variance	128.8106	68.08333
Observations	12	12
Pearson Correlation	0.285484	
Hypothesized Mean Difference	0	
df	11	
t Stat	-0.5303	
P(T<=t) one-tail	0.303222	
t Critical one-tail	1.795885	
P(T<=t) two-tail	0.606444	
t Critical two-tail	2.200985	

H_o Recoveries speeds 1100xg and 2000xg are the same

H_1 Recoveries for speed 2000xg are better than recoveries for 1100xg

$\alpha = 0.05$

0.303> 0.05

Accept H_o, that is recoveries for speed 1100xg and 2000xg are the same

Table 17: Statistical analyses (95% Confidence interval of differences) comparing recoveries for *Giardia* at different centrifuge speeds

		1500xg minus 1100xg	2000xg minus 1100xg
Average difference		12.5	4.2
SD differences		11.2	15.5
95% Confidence interval for the average difference	Lower confidence limit	4.2	-7.3
	Upper confidence limit	20.8	15.6

The recovery of *Giardia* cysts at the centrifuge speed of 1100xg ranged from 21% to 58%, at the centrifuge speed of 1500xg ranged from 38% to 68% and at the centrifuge speed of 2000xg recoveries ranged from 29% to 53% (Table 11). When the centrifuge was set at 1500xg speed recoveries were better than at 1100xg and 2000xg centrifuge speed.

The paired t-Test and 95 percent confidence interval in Table 15 and Table 17, show that there was a statistical significant difference between 1100xg and 1500xg centrifuge speeds. The t-Test results in Table 15 for 1100xg and 1500xg centrifuge speed were –3.2057 and the H_o was rejected as the P(T< =t) = 0.004185 one tail results were less than 0.05, therefore the recoveries for 1500xg centrifuge speed were better than 1100xg centrifuge speed cause of the significant difference observed statistically.

The paired t-Test and 95 percent confidence interval in Table 16 and Table 17, show that there was no statistical significant difference between 1100xg and 2000xg centrifuge speeds. The t-Test results in Table 16 for 1100xg and 2000xg centrifuge speed were –0.5303 and the H_o was accepted as the P(T< =t) = 0.303222 one tail results were greater than 0.05, therefore the recoveries for 1100xg centrifuge speed and 2000xg centrifuge speed were the same.

Though 1500xg centrifuge speed yielded better recoveries of *Cryptosporidium* oocysts and *Giardia* cysts, 2000xg centrifuge speed can be used for reagent grade water and drinking water samples only as the pellets from these water matrices are very small and there are hard particles in the samples that can damage the oocysts and cysts. The higher centrifuge speed of 2000xg cannot be used if the sample contains sand or other gritty material, for example untreated raw water sample that may degrade the condition of any oocysts and / or cysts present in the sample, lower centrifuge speed to be used for all untreated and raw water samples, preferably 1500xg.

4.4 Purification and separation of oocysts and cysts by comparing two slightly different immunomagnetic separation procedures

Immunomagnetic separation (IMS) is the separation and purification step, where oocyst and cysts in the sample are separated from other particulates and Dynabeads used. Optimising the steps within the IMS procedure can play a role in increasing the recovery of oocysts and cysts.

According to currently used Rand Water Method 06 (2007) acid dissociation was done only on spiked matrix samples. The US-EPA Method 1623 (2005) IMS procedure stated that first and second acid dissociation was performed on all samples analysed. The second acid dissociation when performed according to Rand Water Method 06 (2007) was done in two separate microscope wells while in US-EPA Method 1623 (2005) one microscope well was used for both dissociations. Raw data of recoveries of *Cryptosporidium* oocysts and *Giardia* cysts when two slightly different IMS procedure where performed are indicated in Table 18.

Table 18: IMS results of two slightly different procedures (Rand Water 06, 2007 and US-EPA Method 1623, 2005)

Cryptosporidium			*Giardia*	
RW -IMS	USEPA-IMS		RW-IMS	USEPA-IMS
73	89		75	98
71	89		72	91
81	73		84	92
89	86		85	81
24	76		27	70
68	60		52	70
71	46		72	48
75	67		68	50
58	74		70	75
58	60		72	67
73	77		77	61
50	77		57	86

4.4.1 *Cryptosporidium* - Statistical analyses

Table 19: Statistical analyses (t-Test: Paired Two Samples for Means) comparing recoveries for *Cryptosporidium* on current Rand Water 06 (2007) Method and the US-EPA 1623 (2005) and DWI (2003)

	Current method	USEPA 1623
Mean	65.91666667	72.8333333
Variance	284.9924242	165.969697
Observations	12	12
Pearson Correlation	0.087710328	
Hypothesized Mean Diff/	0	
df	11	
t Stat	-1.179266729	
P(T<=t) one-tail	0.131584733	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	0.263169466	
t Critical two-tail	2.200985159	

Table 19: continued

H_o Recoveries for current Rand Water method 06 (2007) and US-EPA Method 1623 (2005) and DWI (2003) are the same

H_1 Recoveries for current Rand Water method 06 (2007) are better than and US-EPA Method1623 (2005) and DWI (2003)

$\alpha = 0.05$

0.13> 0.05

Accept H_o, that is recoveries current Rand Water Method 06 (2007) and US-EPA Method 1623 (2005) and DWI (2003) are the same

Table 20: Statistical analyses (95% Confidence interval of differences) comparing recoveries for *Cryptosporidium* on current Rand Water Method 06 (2007) and the US-EPA Method 1623 (2005) and DWI (2003)

Average difference	6.916667
SD differences	20.3177
95% Confidence interval for the average difference: US-EPA Method 1623 (2005) / DWI (2003) minus current Rand Water Method 06 (2007)	
Lower confidence limit	-8.16
Upper confidence limit	21.99

The recovery of *Cryptosporidium* oocysts when Rand Water Method 06 (2007) was performed ranged from 24% to 89% and when US-EPA Method 1623 (2005) was performed recoveries ranged from 46% to 89% (Table 18).

The paired t-Test and 95 percent confidence interval in Table 19 and Table 20 show that there was no statistical significant difference between the current Rand Water Method 06 (2007) and US-EPA Method 1623 (2005) at confidence level of ninety five percent. The t-Test results in Table 18 for Rand Water IMS and US-EPA IMS were –1.179266 and the H_o was accepted as the P(T< =t) = 0.13158 one tail results were greater than 0.05,. therefore the recoveries for the Rand Water Method 06 (2007) and US-EPA Method 1623 (2005) were the same.

4.4.2 *Giardia* - Statistical analyses

Table 21: Statistical analyses (t-Test: Paired Two Samples for Means) comparing recoveries for *Giardia* on current Rand Water Method 06 (2007) and US-EPA Method 1623 (2005) and DWI Method (2003)

	Current method	*USEPA 1623 (2005)*
Mean	67.58333333	74.08333
Variance	252.9924242	262.2652
Observations	12	12
Pearson Correlation	0.173786488	
Hypothesized Mean Difference	0	
df	11	
t Stat	-1.091285145	
P(T<=t) one-tail	0.149236871	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	0.298473742	
t Critical two-tail	2.200985159	

H_o Recoveries for current Rand Water Method 06 (2007) and US-EPA Method 1623 (2005)) and DWI (2003) are the same

H_1 Recoveries for current Rand Water Method 06 (2007) are better than US-EPA Method 1623 (2005) and DWI (2003)

$\alpha = 0.05$

0.149> 0.05

Accept H_o, that is recoveries current Rand Water Method 06 (2007) and US-EPA Method 1623 (2005) and DWI (2003) are the same

Table 22: Statistical analyses (95% Confidence interval of differences) comparing recoveries for *Giardia* on current Rand Water Method 06 (2007) and the US-EPA Method1623 (2005) and DWI (2003)

Average difference	6.5
SD differences	20.63
95% Confidence interval for the average difference: US-EPA 1623 (2005) / DW (2003)I Method minus current Rand Water Method 06 (2007)	
Lower confidence limit	-8.81
Upper confidence limit	21.81

The recovery of *Giardia* cysts when Rand Water Method 06 (2007) was performed ranged from 27% to 85% and when US-EPA Method 1623 (2005) was performed recoveries ranged from 48% to 98% (Table 18).

The paired t-Test and 95 percent confidence interval in table 21 and table 22, show that there was no statistical significant difference between the current Rand Water Method 06 (2007) and US- EPA Method 1623 (2005) and DWI (2003) at confidence level of ninety five percent. The t-Test results in Table 21 for Rand Water IMS and US-EPA IMS were –1.09129 and the H_o was accepted as the P(T< =t) = 0.1492369 one tail results were greater than 0.05, therefore there was no significant difference between the recoveries of *Giardia* cysts when the Rand Water Method and the US- EPA Method 1623 (2005) were performed.

Although there was no significant difference on the recoveries of *Cryptosporidium* oocysts and *Giardia* cysts when the two slightly similar IMS procedures were performed, the two rinses with the 1X buffer step was adopted to rinse any oocysts and cysts remaining in the Leighton tube and time spent when examining the microscope slide was reduced when US-EPA Method 1623 (2005) was followed because *Cryptosporidium* oocysts and *Giardia* cysts were enumerated in one well instead of two microscope wells

(reducing time spent working under microscope). Spending long hours on a microscope can cause one to loose concentration especially if a high number of microscope slides must be examined and oocysts and cysts be enumerated.

4.5 Comparing two pre-treatment buffers and including the pre-treatment step in eluation step.

During the eluation step, the eluation buffer when added to filter capsule loosens the trapped oocysts and cysts from the filter membrane. The pre-treatment when added to the filter capsule did not loosen the oocysts and cysts from the filter membrane because of the different ingredients used for both buffers. Table 23 summarizes the results for recoveries of *Cryptosporidium* oocysts and *Giardia* cysts recovered from the spiked (treated) drinking water samples.

Table 23: Different pre-treatment buffers results (treated water)

Cryptosporidium				*Giardia*		
No buffer	US Buffer	DWI Buffer		No buffer	US Buffer	DWI Buffer
50	46	49		51	30	35
54	49	51		40	39	42
39	34	42		31	46	42
44	46	35		34	46	49
53	41	45		53	37	33
45	50	46		36	43	45
24	43	46		19	35	36
41	35	38		36	34	37
34	41	49		20	28	33
25	46	45		22	42	39
21	38	42		20	39	34
51	42	45		22	43	46

4.5.1 Drinking water matrix: *Cryptosporidium* - Statistical analyses

Table 24: Statistical analyses (t-Test: Paired Two Samples for Means) on the drinking water matrix comparing recoveries for *Cryptosporidium,* no buffer versus US buffer

	No buffer	US Buffer
Mean	40.08333333	38.6666667
Variance	136.9924242	34.4242424
Observations	12	12
Pearson Correlation	0.435975951	
Hypothesized Mean Difference	0	
df	11	
t Stat	0.464672646	
P(T<=t) one-tail	0.325614062	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	0.651228124	
t Critical two-tail	2.200985159	

H_o Recoveries for Rand Water Method 06 (2007) no buffer and US buffer are the same

H_1 Recoveries for US buffer are better than recoveries for Rand Water Method 06 (2007) no buffer

α = 0.05

0.326> 0.05

Accept H_o, that is recoveries Rand Water Method 06 (2007) no buffer and US buffer are the same

Table 25: Statistical analyses (t-Test: Paired Two Samples for Means) on the drinking water matrix comparing recoveries for *Cryptosporidium,* no buffer versus DWI buffer

	No buffer	DWI Buffer
Mean	40.08333333	39.5833333
Variance	136.9924242	33.719697
Observations	12	12
Pearson Correlation	0.443293693	
Hypothesized Mean Diff	0	
df	11	
t Stat	0.164804411	
P(T<=t) one-tail	0.436043723	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	0.872087445	
t Critical two-tail	2.200985159	

H_o Recoveries for Rand Water Method 06 (2007) no buffer and DWI buffer are the same

H_1 Recoveries for Rand Method 06 (2007) no buffer are better than recoveries for DWI buffer

$\alpha = 0.05$

0.436 > 0.05

Accept H_o, that is recoveries Rand Water Method 06 (2007) no buffer and DWI buffer are the same

Table 26: Statistical analyses (t-Test: Paired Two Samples for Means) on the drinking water matrix comparing recoveries for *Cryptosporidium* on US buffer versus DWI buffer

	US Buffer	*DWI Buffer*
Mean	38.66666667	39.5833333
Variance	34.42424242	33.71969697
Observations	12	12
Pearson Correlation	0.985489028	
Hypothesized Mean Diff	0	
df	11	
t Stat	-3.187523387	
P(T<=t) one-tail	0.004322285	

Table 26:continued	
t Critical one-tail	1.795884814
P(T<=t) two-tail	0.008644571
t Critical two-tail	2.200985159
H_o Recoveries for US buffer and DWI buffer are the same	
H_1 Recoveries for DWI buffer are better than recoveries for US buffer	
α = 0.05	
0.004 < 0.05	
Reject H_o, that is recoveries for DWI buffer are better than US buffer	

Table 27: Statistical analyses (95% Confidence interval of differences) on the drinking water matrix comparing recoveries for *Cryptosporidium,* DWI buffer versus US buffer

		US buffer	DWI buffer
Average difference		-7.3	-6.8
SD differences		9.9	9.5
95% Confidence interval for the average difference	Lower confidence limit	-14.7	-13.8
	Upper confidence limit	0.0	0.2

The recovery of *Cryptosporidium* oocysts when no buffer was used ranged from 21% to 54%, when US-buffer was used recoveries ranged from 34% to 50% and when the DWI buffer was used recoveries ranged from 35% to 49% (Table 23). The paired t-Test results at 95 percent confidence interval in Table 24 where no buffers were used in one set of samples and US buffer was used in another set of samples were 0.46467 and the H_o was accepted as the P(T< =t) =0.32561 one tail results were greater than 0.05, therefore the recoveries for the Rand Water Method 06 (2007) where no buffer was used and where the US buffer was used had no significant difference.

Table 25 shows that there was no statistical significant difference between the Rand Water Method 06 (2007) where no buffer was used and when DWI buffer was used, t-Test results were 0.1648 and the H_o was accepted as the P(T< =t) = 0.43604 one tail results were greater than 0.05, therefore the

recoveries when no buffer was used and when DWI buffer was used had no significant difference.

However the paired t-Test in Table 26 and the ninety five percent confidence interval in Table 27 showed that there was a statistical difference between the US buffer and the DWI buffer. The t-Test results for DWI buffer and US buffer were –3.1875 and the H_o was rejected as the P(T< =t) = 0.004322 one tail results were less than 0.05. Therefore the recoveries of *Cryptosporidium* oocysts where DWI buffer was used were better than when US buffer was used.

4.5.2 Drinking water matrix: *Giardia* - Statistical analyses

Table 28: Statistical analyses (t-Test: Paired Two Samples for Means) on the drinking water matrix comparing recoveries for *Giardia,* no buffer versus US buffer

	No buffer	US Buffer
Mean	32	33.75
Variance	141.8181818	45.29545
Observations	12	12
Pearson Correlation	0.770165076	
Hypothesized Mean Difference	0	
df	11	
t Stat	-0.759798541	
P(T<=t) one-tail	0.231674106	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	0.463348212	
t Critical two-tail	2.200985159	

H_o Recoveries for Rand Water Method 06 (2007) no buffer and US buffer are tl same

H_1 Recoveries for US buffer are better than recoveries for current Rand Water Method 06 (2007) no buffer

α = 0.05

0.232 > 0.05

Accept H_o, that is recoveries for Rand Water Method 06 (2007) no buffer and US buffer are the same

Table 29: Statistical analyses (t-Test: Paired Two Samples for Means) on the drinking water matrix comparing recoveries for *Giardia,* no buffer versus DWI buffer

	No buffer	DWI Buffer
Mean	32	34.16667
Variance	141.8181818	39.9697
Observations	12	12
Pearson Correlation	0.784854743	
Hypothesized Mean Difference	0	
df	11	
t Stat	-0.941095021	
P(T<=t) one-tail	0.183432158	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	0.366864315	
t Critical two-tail	2.200985159	

H_o Recoveries for Rand Water Method 06 (2007) no buffer and DWI buffer are the same

H_1 Recoveries for Rand Water Method 06 (2007) no buffer are better than recoveries for DWI buffer

$\alpha = 0.05$

$0.183 > 0.05$

Accept H_o, that is recoveries for Rand Water Method 06 (2007) no buffer and DWI buffer are the same

Table 30: Statistical analyses (t-Test: Paired Two Samples for Means) on the drinking water matrix comparing recoveries for *Giardia* on US buffer versus DWI buffer

	US Buffer	*DWI Buffer*
Mean	33.75	34.16667
Variance	45.29545455	39.9697
Observations	12	12
Pearson Correlation	0.862100933	
Hypothesized Mean Difference	0	
df	11	
t Stat	-0.41838707	
P(T<=t) one-tail	0.341857985	
t Critical one-tail	1.795884814	
P(T<=t) two-tail		
Table 30:continued	0.683715971	
t Critical two-tail	2.200985159	

H_0 Recoveries for US buffer and DWI buffer are the same

H_1 Recoveries for DWI buffer are better than recoveries for US buffer

$\alpha = 0.05$

$0.342 > 0.05$

Accept H_0, that is recoveries for DWI buffer and US buffer are the same

Table 31: Statistical analyses (95% Confidence interval of differences) on the drinking water matrix comparing recoveries for *Cryptosporidium,* DWI buffer versus US buffer

		US buffer	DWI buffer
Average difference		-3.2	-2.7
SD differences		6.1	5.3
95% Confidence interval for the average difference	Lower confidence limit	-7.7	-6.6
	Upper confidence limit	1.3	1.3

Giardia cysts recoveries when no buffer was used ranged from 19% to 53%, when US-buffer was used ranged from 28% to 46% and when the DWI buffer was used recoveries ranged from 33% to 49% (Table 23). The paired t-Test results at 95 percent confidence interval in Table 28 where no buffers were used in one set of samples and US buffer was used in another set of samples were –0.759799 and the H_o was accepted as the P(T< =t) = 0.23167 one tail results were greater than 0.05. Therefore the recoveries for the Rand Water Method 06 (2007) where no buffer was used and where the US buffer was used were the same, which means there was no significant difference.
The paired t-Test results in Table 29 indicated that there was no statistical significant difference observed between the Rand Water Method 06 (2007) where no buffer was used and DWI buffer. The t-Test results were -0.9411 and the H_o was accepted as the P(T< =t) =0.18343 one tail results were greater than 0.05. Recoveries of *Giardia* cysts when no buffer was used on samples and when DWI buffer was used to process the samples were the same, without significant difference.

In addition, Table 30 shows no statistical difference on recoveries of *Giardia* cysts when the two buffers namely US buffer and the DWI buffer were used to process the samples. The t-Test results were -0.41839 and the H_o was accepted as the P(T< =t) =0.34186 one tail results were greater than 0.05, therefore the recoveries for US buffer and the DWI buffer were the same, without significant difference.

4.5.3 Source water matrix: *Cryptosporidium* - Statistical analyses

Table 32: Statistical analyses (t-Test: Paired Two Samples for Means) on the source water matrix comparing recoveries for *Cryptosporidium,* no buffer versus US buffer

	No buffer	US Buffer
Mean	5.75	42.58333
Variance	4.022727273	26.26515
Observations	12	12
Pearson Correlation	-0.090652739	
Hypothesized Mean Diff	0	
df	11	
t Stat	-22.50250798	
P(T<=t) one-tail	7.51772E-11	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	1.50354E-10	
t Critical two-tail	2.200985159	
H_o Recoveries for Rand Water Method 06 (2007) no buffer and US buffer are the same		
H_1 Recoveries for US buffer are better than recoveries for Rand Water Method 06 (2007) no buffer		
	α = 0.05	
	0.000 < 0.05	
Reject H_o, that is recoveries for US buffer are better than Rand Water Method 06 (2007) no buffer		

Table 33: Statistical analyses (t-Test: Paired Two Samples for Means) on the source water matrix comparing recoveries for *Cryptosporidium,* no buffer versus DWI buffer

	No buffer	DWI Buffer
Mean	5.75	44.41667
Variance	4.022727273	21.17424
Observations	12	12
Pearson Correlation	0.16991518	
Hypothesized Mean Difference	0	
df	11	
t Stat	-28.51797197	
P(T<=t) one-tail	5.78212E-12	

Table 33: continued

t Critical one-tail	1.795884814
P(T<=t) two-tail	1.15642E-11
t Critical two-tail	2.200985159

H_0 Recoveries for Rand Water Method 06 (2007) no buffer and DWI buffer are the same

H_1 Recoveries for DWI buffer are better than recoveries for Rand Water Method 06 (2007) no buffer

$\alpha = 0.05$

$0.000 < 0.05$

Reject H_0, that is recoveries for DWI buffer are better than current Rand Water Method 06 (2007) no buffer

Table 34: Statistical analyses (t-Test: Paired Two Samples for Means) on the source water matrix comparing recoveries for *Cryptosporidium* on US buffer versus DWI buffer

	US Buffer	*DWI Buffer*
Mean	42.58333333	44.41667
Variance	26.26515152	21.17424
Observations	12	12
Pearson Correlation	0.432070343	
Hypothesized Mean Difference	0	
df	11	
t Stat	-1.220852785	
P(T<=t) one-tail	0.12383256	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	0.24766512	
t Critical two-tail	2.200985159	

H_0 Recoveries for US buffer and DWI buffer are the same

H_1 Recoveries for DWI buffer are better than recoveries for US buffer

$\alpha = 0.05$

$0.124 > 0.05$

Accept H_0, that is recoveries for DWI buffer and US buffer are the same

Table 35: Statistical analyses (95% Confidence interval of differences) on the source water matrix comparing recoveries for *Cryptosporidium* on DWI buffer versus US buffer

		US buffer	DWI buffer
Average difference		39.5	39.8
SD differences		6.3	5.4
95% Confidence interval for the average difference	Lower confidence limit	34.8	35.8
	Upper confidence limit	44.2	43.9

The paired t-Test and 95 percent confidence interval in Table 32 and Table 35 show that there was a statistical significant difference between the Rand Water Method 06 (2007) where no buffer was used and US buffer. The t-Test results were –22.5025 and the H_o was rejected as the P(T< =t) = 7.51772E-11 one tail results were less than 0.05, therefore the recoveries of US buffer were better than Rand Water Method 06 (2007) where no buffer was used. The paired t-Test in Table 33 and the ninety five percent of confidence interval in Table 35 showed that there was a statistical difference between the Rand Water Method 06 (2007) where no buffer was used and the DWI buffer.

The t-Test results were –28.51797 and the H_o was rejected as the P(T< =t) = 5.78212E-12 one tail results were less than 0.05, therefore the recoveries where DWI buffer were better than Rand Water Method 06 (2007) where no buffer was used. Again the paired t-Test in Table 34 showed that there was no statistical difference between the US buffer and the DWI buffer with the results indicating that the t-Test results were for DWI buffer and US buffer were –1.22085 and the H_o was accepted as the P(T< =t) = 0.12383 one tail results were greater than 0.05, therefore the recoveries of DWI buffer and US buffer were statistically the same.

4.5.4 Source water matrix: *Giardia* - Statistical analyses

Table 36: Statistical analyses (t-Test: Paired Two Samples for Means) on the source water matrix comparing recoveries for *Giardia,* no buffer versus US buffer

	No buffer	US Buffer
Mean	3.75	37.66667
Variance	5.659090909	45.87879
Observations	12	12
Pearson Correlation	-0.005641934	
Hypothesized Mean Diff	0	
df	11	
t Stat	-16.33713134	
P(T<=t) one-tail	2.31215E-09	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	4.6243E-09	
t Critical two-tail	2.200985159	
H_o Recoveries for Rand Water Method 06 (2007) no buffer and US buffer are the same		
H_1 Recoveries for US buffer are better than recoveries for Rand Water Method 06 (2007) no buffer		
	α = 0.05	
	0.000 < 0.05	
Reject H_o, that is recoveries for US buffer are better than from current Rand Water Method 06 (2007) no buffer		

Table 37: Statistical analyses (t-Test: Paired Two Samples for Means) on the source water matrix comparing recoveries for *Giardia,* no buffer versus DWI buffer

	No buffer	DWI Buffer
Mean	3.75	39.25
Variance	5.659090909	29.84091
Observations	12	12
Pearson Correlation	-0.169644466	
Hypothesized Mean Difference	0	
df	11	
t Stat	-19.46628415	

Table 37: continued	
P(T<=t) two-tail	7.14217E-10
t Critical two-tail	2.200985159
H_0 Recoveries for Rand Water Method 06 (2007) no buffer and DWI buffer are the same	
H_1 Recoveries for DWI buffer are better than recoveries for Rand Water Method 06 (2007) no buffer	
α = 0.05	
0.000 < 0.05	
Reject H_0, that is recoveries for DWI buffer are better than recoveries for Rand Water Method 06 (2007) no buffer	

Table 38: Statistical analyses (t-Test: Paired Two Samples for Means) on the source water matrix comparing recoveries for *Giardia* on US buffer versus DWI buffer

	US Buffer	*DWI Buffer*
Mean	37.6666667	39.25
Variance	45.87878788	29.84091
Observations	12	12
Pearson Correlation	0.842731917	
Hypothesized Mean Difference	0	
df	11	
t Stat	-1.500803339	
P(T<=t) one-tail	0.080776496	
t Critical one-tail	1.795884814	
P(T<=t) two-tail	0.161552992	
t Critical two-tail	2.200985159	
H_0 Recoveries for US buffer and DWI buffer are the same		
H_1 Recoveries for DWI buffer are better than recoveries for US buffer		
α = 0.05		
0.081 > 0.05		
Accept H_0, that is recoveries for DWI buffer and US buffer are the same		

Table 39: Statistical analyses (95% Confidence interval of differences) on the source water matrix comparing recoveries for *Giardia,* DWI buffer versus US buffer

		US buffer	DWI buffer
Average difference		36.5	39.0
SD differences		8.1	6.3
95% Confidence interval for the average difference	Lower confidence limit	30.5	34.3
	Upper confidence limit	42.5	43.7

The paired t-Test results in Table 36 and the ninety five percent confidence interval in Table 39 were -16.33713 and the H_o was rejected as the P(T< =t) =2.31215E-09 one tail results were less than 0.05, therefore the recoveries of US buffer were better than Rand Water Method 06 (2007) where no buffer was used. There was a statistical significant difference between the Rand Water Method 06 (2007) where no buffer was used and DWI buffer the t-Test results in Table 37 and the ninety five percent confidence interval in Table 38 were –19.466284 and the H_o was rejected as the P(T< =t) =3.57109E-10 one tail results were less than 0.05, therefore the recoveries of DWI buffer were better than Rand Water Method 06 (2007) where no buffer was used.

Between the US buffer and DWI buffer there was no statistical significant difference, in Table 38 the t-Test results were for DWI buffer and US buffer were –1.5008 and the H_o was accepted as the P(T< =t) = 0.08.78 one tail results were greater than 0.05, therefore the recoveries of DWI buffer and US buffer were statistically the same.

The eluation buffer is designed to remove the oocysts and cysts from the membrane and the pre-treatment loosen or remove large particles for example soil particles, debris and excess turbidity from the membrane of the filter which can affect recovery of the oocysts and cysts. The excess debris or other particles present in the sample especially raw or untreated water

sample can damage *Cryptosporidium* oocysts and *Giardia* cysts during eluation step and concentration steps of the method.

Consequently when pre-treatment was done to the Envirocheck filter capsule, the filter capsule was cleared with less “debris” which plays a major role when examining the oocysts and cysts under microscope. The pellet size after centrifugation decreased thus minimizing the number of IMS procedures done per sample as the test depends on the pellet size. Examination of microscope slides was also done with more ease because there was less background interference.

4.6 Overall summary of the experimental results

Table 40: Summarized experimental results

Experiment number	*Cryptosporidium* oocysts recoveries		*Giardia* cysts recoveries	
	Improved recoveries	Not improved recoveries	Improved recoveries	Not improved recoveries
1: Comparing two different wrist action shaker speeds	**X**		**X**	
2:Comparing three different centrifuge speeds	**X**		**X**	
3: Comparing two almost similar immunomagnetic separation procedures		**X**		**X**
4: Comparing two pre-treatment buffers and including the pre-treatment step (Treated water)		**X**		**X**
4: Comparing two pre-treatment buffers and including the pre-treatment step (Raw/source water)	**X**		**X**	

Overall there was a significant difference in the recoveries of *Cryptosporidium* oocysts and *Giardia* cysts in some experiments as shown in Table 40. Even though in experiment 3 where different steps immunomagnetic separation procedures in two methods were compared the recoveries of oocysts and cysts did not improve but the time spent on enumeration of oocysts and cysts was reduced.

The experiments conducted were successful as there was statistical significant difference observed in different steps evaluated by comparison. The main aim of the experiments to determine if the recoveries of oocysts and cysts will increase was achieved. The next was to conduct secondary validation of the Rand Water Method 06 (2007) by implementing the findings obtained from the experiments in Chapter 3 and 4, which will be dealt in the next chapter.

CHAPTER 5

5 SECONDARY VALIDATION OF RAND WATER METHOD

5.1 Introduction

Validation is the process that is followed to demonstrate with the provision of objectives that a specific method is suitable for the intended purpose.

Primary validation is carried out when the method has been developed and has never been used before and is the responsibility of the laboratory developing the method. Secondary validation or verification is carried out when a laboratory wants to implement a standard method that has been developed and validated elsewhere.

Guidelines on validation and quality assurance in Microbiological testing are mentioned in SANAS guideline TG 28-02. The laboratory adapting the standard method must prove that the method is fit for the intended purpose in their environment and the laboratory should also demonstrate its competence to meet the performance characteristics of the method.

5.2 Secondary validation of the modified Rand Water Method 06

Aim: To validate the performance of the modified Rand Water Method with the incorporation of new changes, verified in different experiments (Chapter 3) to determine the overall performance and improvement of the method.

Procedure

The Rand Water Method 06 (2007) discussed in chapter 3 section 3.3 was used with inclusion of the following steps:

Wrist action speed 900 rpm,

Use of DWI buffer for pre-treatment in the elution step for source or raw water

Centrifuge speed of 2000xg to be used for drinking water and quality control (reagent grade water) samples

Centrifuge speed of 1500xg to be used for untreated or raw water samples

IMS step: US-EPA Method 1623 (2005) IMS procedure to be adopted.

Three different water matrices were used for secondary validation namely reagent grade water, drinking water and untreated water.

Reagent grade water samples were collected from the Elix UV system

Drinking water samples were from different sample points, one from Vereeniging purification works (MA-21) and the other one from Zuikerbosch purification works (MB-10). The two samples were selected from different purification works as the plants run independently and both supply potable water to customers.

Untreated water samples were also collected at two different sampling points. Two source samples were selected which feed the two different purification plants. M-A18 from Vereeniging purification works and M-Canal from Zuikerbosch purification works.

Duplicate sets of water samples (ten samples per analyst) were collected, analysed by two different competent analysts and two different competent analysts enumerated the same microscope slides.

All sets of water samples were spiked with Easyseeds and filtered through the Envirocheck filter capsule as has been described before and processed as follows:

Elution

Reagent grade water samples and drinking water samples were eluted immediately and for agitating the filter capsule wrist action shaker was set at 900rpm. Raw water samples after filtration were pre-treated before the concentration step. Sodium phosphate buffer (DWI buffer) was used in the

pre-treatment step. Samples were also eluted with the wrist action set at 900rpm for shaking the filter capsule.

Centrifuge Speed

Centrifuge speed was selected according to the type of water analysed. Drinking water and reagent grade water samples 2000xg speed was used and for raw water 1500xg was used.

IMS Procedures

USEPA IMS procedure was performed on all samples. Microscope slides were stained and examined under microscope; Figure 12 summarizes process flow of the optimized method used.

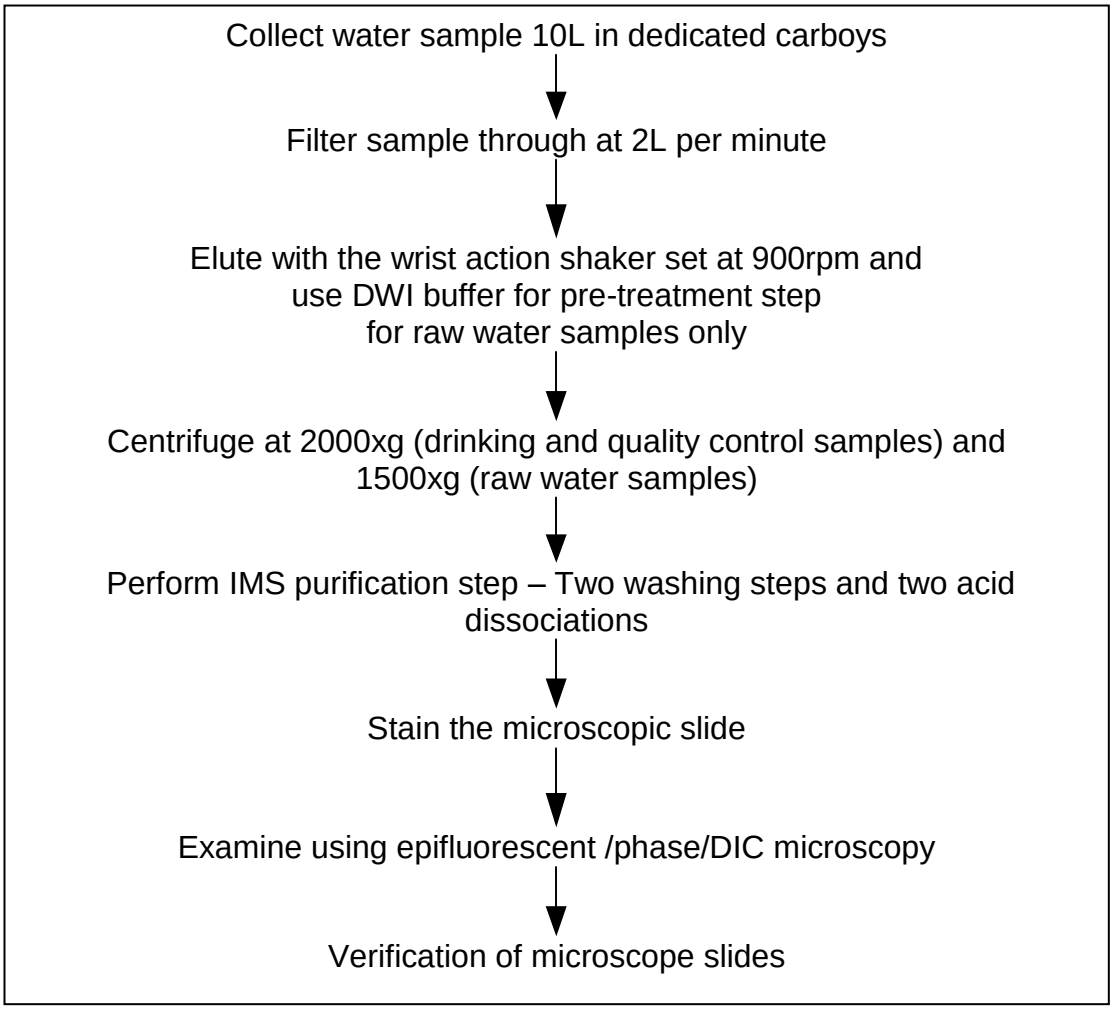


Figure 12: Summarized flow chart of method used for secondary validation

The results of the secondary validation of the method were compared with the acceptance criteria as stipulated in US – EPA 1623 (2005), Table 41 as well as the findings from Validation Report 2007 in Table 42.

Table 41: Quality Control Acceptance Criteria for *Cryptosporidium* and *Giardia* (US-EPA 1623. 2005)

Performance test	Acceptable criteria
Matrix spike/ matrix spike duplicate (for method modifications)	
Mean recovery (as percent) *Cryptosporidium*	13 - 111
Mean recovery (as percent) *Giardia*	15 - 118

Table 42: Quality Control Acceptance Criteria for *Cryptosporidium* and *Giardia* (Validation report 2007)

Performance test	Minimum Recovery	Mean Recovery
Mean recovery Reagent grade water	26%	31%
Mean recovery drinking water	20%	28%
Mean recovery raw water	25%	42%

5.3 Results and Discussion

Data collected was analysed by performing basic statistics (minimum, maximum, mean and standard deviation) and deriving the reproducibility. The accuracy of results for secondary validation method was calculated for each sample matrix.

5.3.1 *Cryptosporidium*

The mean recovery for all water matrices were above 20% refer to Figure 13, 14 and 15. The percentage that the analyst verifying the results differed from the analyst doing the analysis varied between -59% and 91%, which is acceptable for a biological test as shown in Table 43. The minimum recoveries for the optimized steps had improved for the reagent grade water recoveries improved 9%, drinking water recoveries increased by 4% and no

increase for raw water sample, -15% when compared to previous available Rand Water validation report (2007) mentioned in Table 43. The minimum recovery for the source water had significantly lower recovery of oocysts and cysts than the minimum recovery recommended by the US-EPA 1623 (2005) also mentioned in Table 43.

Figure 13: Graph of reagent grade water results, *Cryptosporidium* oocysts

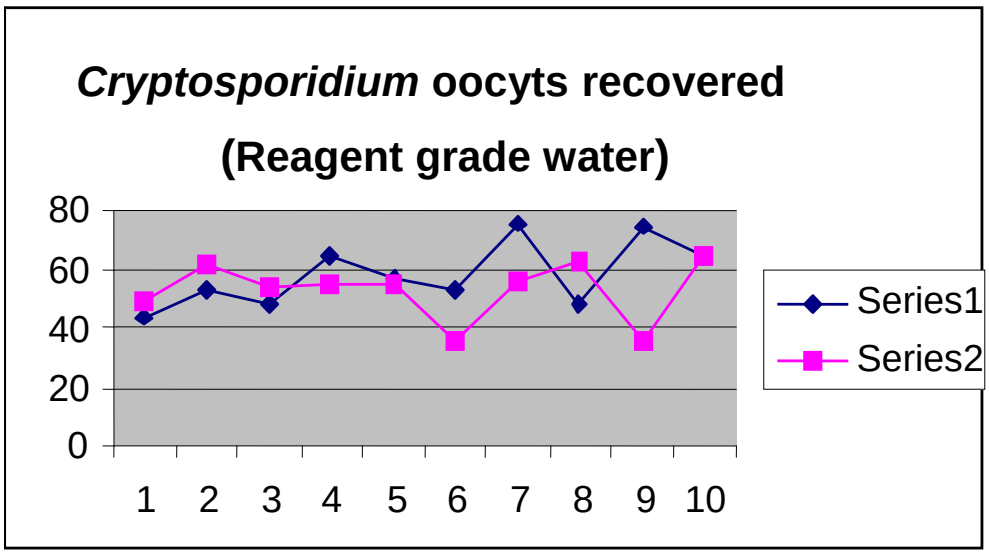


Figure 14: Graph of drinking water results, M-A15 and M-B10

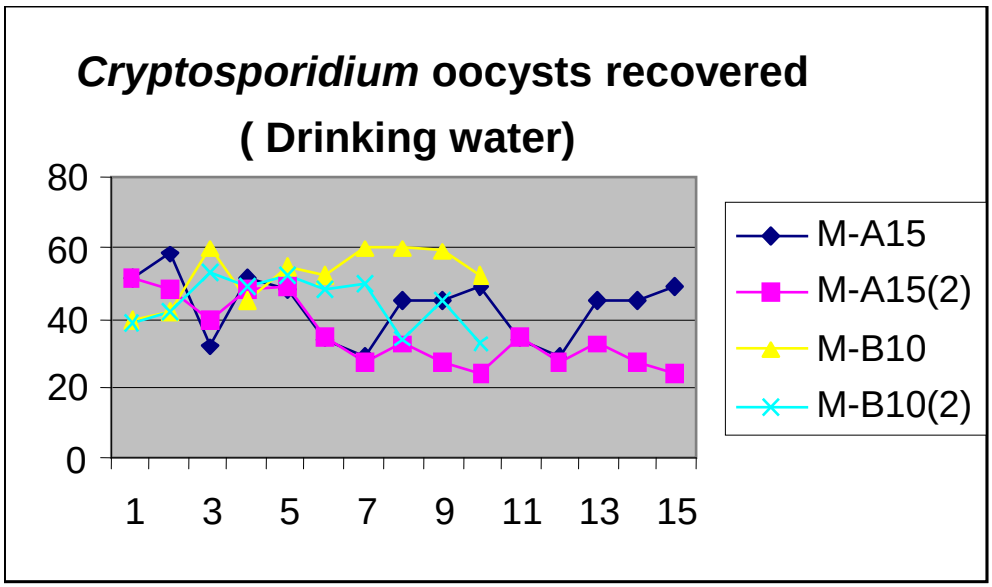


Figure 15: Graph of raw water results, M-A18 and M-Canal

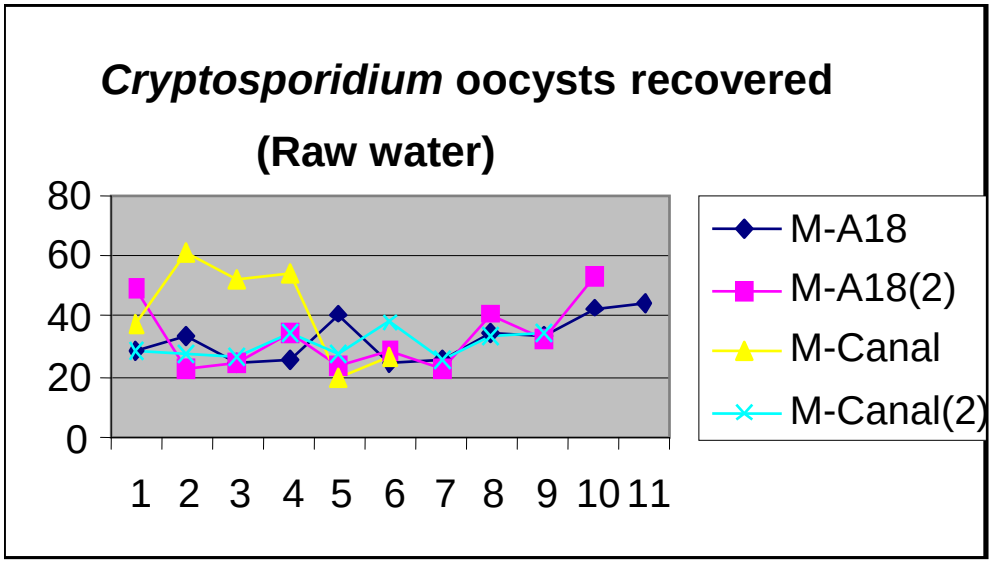


Table 43: Statistical analyses (percentage difference between counts) on the different water matrices for *Cryptosporidium*

Water matrix	Minimum % difference between counts	Maximum % difference between counts
Reagent grade water	-59	26
Drinking water	-21	25
Raw water	-29	91

Table 44: Summary results for *Cryptosporidium* oocysts on different water matrices

Water Matrix	**USEPA**	**Validation 2007**	**Optimized steps**
Reagent grade water minimum Recovery	13%	26%	35%
Reagent grade water mean Recovery	49%	31%	55%
Drinking water minimum Recovery	13%	20%	24%

Table 44: continued			
Drinking water mean Recovery	49%	28%	44%
Raw water minimum Recovery	13%	25%	10%
Raw water mean Recovery	49%	42%	29%

Recovery of *Cryptosporidium* oocysts when reagent water was used for validation increased from a mean recovery of 31% to 55%, for drinking water recovery of *Cryptosporidium* oocysts mean recovery increased from 28% to 44% and for raw water the mean recovery decreased from 42% to 29% as shown in Table 44. This implies that validation was successful.

5.3.2 *Giardia*

The mean recoveries for all water matrices were above 20% (refer to graph 16, 17, and 18) except for few raw water samples. The percentage that the analyst verifying the results differed from the analyst doing the analysis varied between -41% and 30%, which is acceptable for a biological test as shown in Table 46. The minimum recoveries for the optimized steps have improved significantly for the reagent grade water and drinking water when compared to previous Rand Water validation (2007) data as shown in Table 46. The minimum recovery for the source water was significantly lower than the minimum recovery recommended by the US-EPA 1623 (2005) as shown in Table 46.

Figure 16: Graph of reagent grade water results, *Giardia* cysts

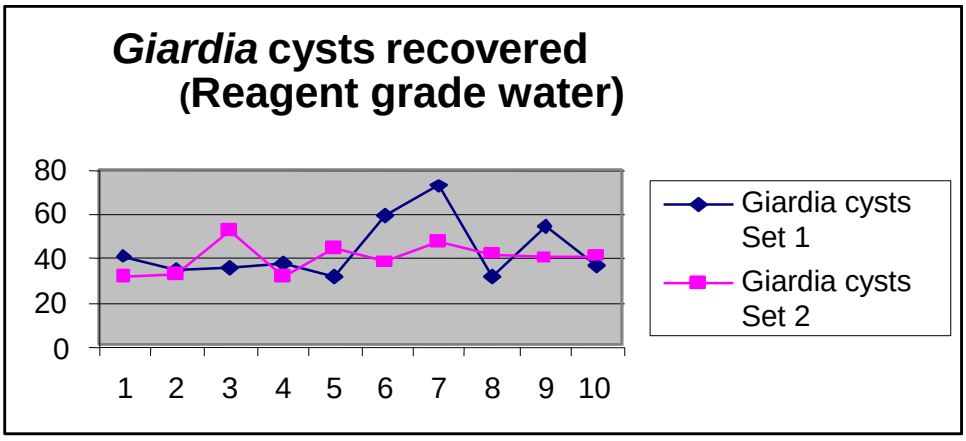


Figure 17: Graph of drinking water results, M-A15 and M-B10

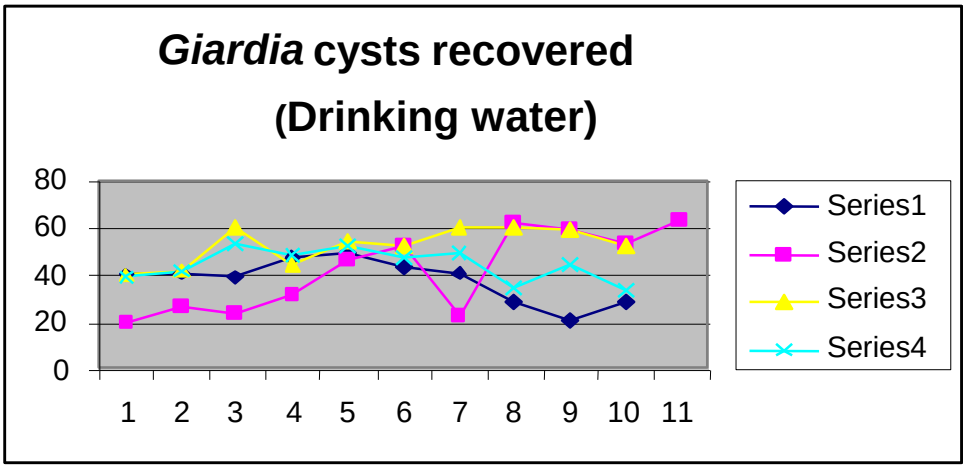


Figure 18: Graph of raw water results, M-A18and M-Canal

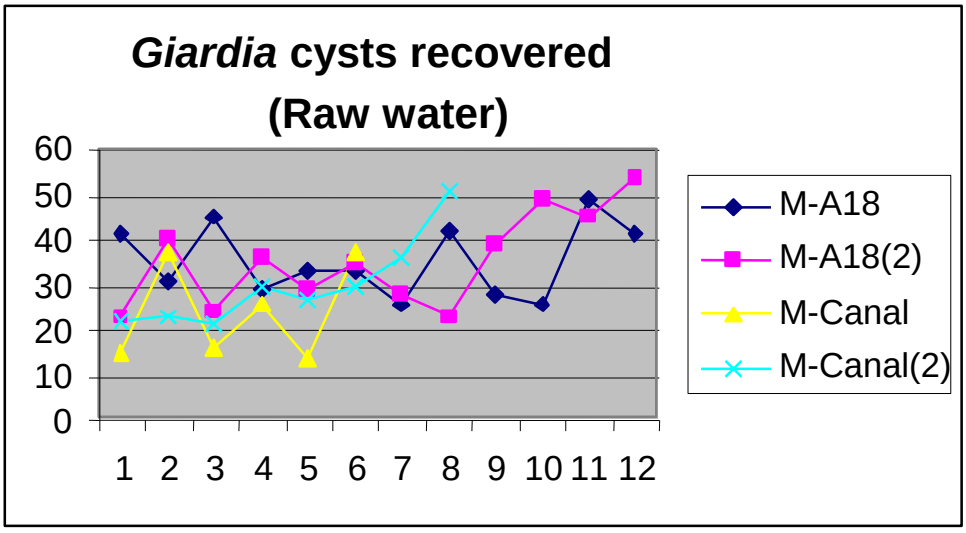


Table 45: Statistical analyses (percentage difference between counts) on the different water matrices for *Giardia*

Water matrix	Minimum % difference between counts	Maximum % difference between counts
Reagent grade water	-41	19
Drinking water	-10	15
Raw water	-43	30

Table 46: Summary results for *Giardia* cysts on different water matrices

Water Matrix	**USEPA**	**Validation 2007**	**Optimized steps**
Reagent grade water minimum Recovery	13%	26%	31%
Reagent grade water mean Recovery	49%	31%	41%
Drinking water minimum Recovery	13%	20%	16%
Drinking water mean Recovery	49%	28%	46%
Raw water minimum Recovery	13%	25%	13%
Raw water mean Recovery	49%	42%	32%

Recovery of *Giardia* cysts when reagent water was used for validation increased from a mean recovery of 31% to 41%, for drinking water mean recovery increased from 28% to 46% and for raw water the mean recovery decreased from 42% to 32% as shown in Table 46.

The secondary validation on the Rand Water Method 06 was successful as there was an improvement on the recoveries of *Cryptosporidium* oocysts and *Giardia* cysts when the complete method was carried out. The recovery rates of *Cryptosporidium* oocysts and *Giardia* cysts increased when compared to historical validation data. The results obtained indicated that optimized method was fit for the intended purpose under the laboratory environment and the laboratory demonstrated that it was competent to meet the performance characteristics of the method.

CHAPTER 6

6 CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to optimize and verify the changes made to US-EPA Method 1623 (2005) to improve the performance of the Rand Water Method 06 (2007). The Rand Water 06 method is used in the laboratory to analyse water samples for isolation and detection of *Cryptosporidium* oocysts and *Giardia* cysts. US-EPA Method 1623 (2005), Rand Water Method 06 (2007) and DWI Standard Operating Procedure (2003) were compared and key steps identified and verified in different experiments.

The results obtained from different experiments indicated that there was an improvement in recovery of *Cryptosporidium* oocysts and *Giardia* cysts. Later secondary validation of the method was done with the implementation of changes verified in the different experiments. Results of secondary validation when compared to historical data, indicated that there was an increase in recovery rate of *Cryptosporidium* oocysts and *Giardia* cysts in different water matrices. The results and conclusions reached are explained in brief below.

6.1 Elution of the sample in a filter capsule using different wrist action shaker speeds

The statistical evaluation of the data indicated that the wrist action shaker set at 900rpm yielded high recovery rates of *Cryptosporidium* oocysts and *Giardia* cysts when compared to the agitation speed of 600rpm. The 900rpm speed was used later for the method secondary validation and the final validation results indicated an increase in the recovery rate of *Cryptosporidium* oocysts and *Giardia* cysts.

It is thus advantageous to use an agitation speed of 900rpm for the elution step in the method for detecting *Cryptosporidium* oocysts and *Giardia* cysts

in water as more oocysts and cysts are loosened from the filter capsule membrane after being trapped during the filtration step.

6.2 Concentration of sample using different centrifuge speeds

The statistical evaluation of the data indicated that when the centrifuge speed was set at 1500xg recoveries of oocysts and cysts were better than when the centrifuge was set at 1100xg and 2000xg. However the use of a centrifugation speed of 2000xg for drinking water is still preferred, as the centrifugal force will pull down any oocysts and cysts that may be present in the drinking water and reagent grade water samples.

A centrifuge speed of 2000xg speed is to be used for drinking water and reagent grade water samples and a centrifugation speed of 1500xg should be used for raw water samples. The particles present in raw or untreated water samples can damage the oocysts or cysts which might be present in water when high centrifugation speed is used, this can lead to poor recoveries of oocysts and cysts present in the water sample analysed.

6.3 Purification and separation of oocysts and cysts by comparing two almost similar IMS procedures

No statistical significant difference was obtained when the Rand Water Method 06 (2007) IMS procedure and the US-EPA 1623 (2005) IMS procedure were compared. However, to align the current method and the international acceptable procedures, the two rinses with the 1X buffer step to rinse off any remaining oocysts or cysts remaining in the Leighton tube and performing two acid dissociations must be included in the optimized method. Two acid dissociations will increase the confidence of the method as more oocysts and cysts will be rinsed off from the Dynabeads and time spent enumerating the oocysts and cysts under the microscope will be decreased as one well of the microscope slide will be counted instead of two wells of the microscope slide.

6.4 Comparing two pre-treatment buffers and including the pre-treatment step in eluation step.

The statistical evaluation of the data indicated that the recoveries of *Cryptosporidium* oocysts and *Giardia* cysts improved when sodium poly-phosphate buffer (referred to as DWI buffer) was used instead of sodium hexametaphosphate buffer (referred to as US buffer). The results indicated that there was no significant difference in recoveries on drinking water when the pre-treatment step was included in the method, therefore the pre-treatment step is to be excluded for the analysis of drinking water and reagent grade water samples. When raw and untreated water samples are to be analysed a pre-treatment step must be included to rinse the filter capsule, reducing any debris or other particles which can increase turbidity of the sample.

During the study the Envirocheck capsule membrane of raw or untreated water samples which were treated before the concentration step with pre-treatment buffer, most of the dirt from the sample was removed and the pellet sizes of samples known to have larger pellets were reduced thus decreasing the number of IMS (immunomagnetic separation) sets done on raw or untreated water.

6.5 Secondary validation of the Rand Water method

The increase in recovery rate results of *Cryptosporidium* oocysts and *Giardia* cysts from different water matrices (reagent grade water and drinking water) used for secondary validation of the Rand Water Method 06, for detection and isolation of *Cryptosporidium* oocysts and *Giardia* cysts in water indicated that secondary validation was successful.

The mean recovery rate of reagent water and drinking water samples increased respectively with more than 40% recovery on average when compared to validation done in 2007. The mean recovery of *Cryptosporidium* oocysts in reagent grade water samples increased from 31% to 55%,

drinking water samples increased from 28% to 44% and raw water decrease from 42% to 29%. The mean recovery of *Giardia* cysts in reagent grade water samples increased from 31% to 41%, drinking water samples increased from 28% to 46% and raw water decreased from 42% to 32%.

Even though the recovery rate of raw water decreased, the use of a pre-treatment buffer reduced the number of IMS performed per sample by reducing the pellet size. The enumeration of microscope slides was also easier as there was less background interference from the sample. The time spent on the microscope enumerating the oocysts and cysts from the microscope slides was decreased.

The success of secondary validation indicated that the optimized method met the requirements of SANAS guideline TG 28-02, that the method was fit for the intended purpose in the Rand Water laboratory environment. The laboratory also demonstrated their competence to meet the performance characteristics of the optimized method.

6.6 Recommendations

The optimized Rand Water Method 06 (2009); should be implemented by the laboratory to analyse water samples for isolation and detection of *Cryptosporidium* oocysts and *Giardia* cysts. There are still a few steps in the method that can still be optimized, for example, the aspiration step (decrease the speed of aspiration as high speed can disturb the sample pellet), the pre-treatment step with buffer (optimize the step) and the staining of the slides needs to be looked at; especially the aspiration step. An investigation should be done on how to improve the recovery rate of *Cryptosporidium* oocysts and *Giardia* cysts in raw or untreated water samples.

Secondary validation should be carried out more often, it is suggested once in three years to improve the performance of the method. In addition when secondary validation is conducted, more sets of water samples should be

analysed to gather enough data for statistic analysis and reduce the time spent when experiments needed to be repeated with more samples because limited data was not sufficient to be used.

Currently the method can only detect the presence of *Cryptosporidium* oocysts and *Giardia* cysts in water, further investigation and experiments need to be done using the polymerase chain reaction (PCR) to identify the species present in water. Already studies have been done to compare microscopic method to the PCR based method (Robertson, Hermansen & Giede 2008). Polymerase chain reaction based method offer the solutions to the identification of *Cryptosporidium* oocysts and *Giardia* cysts and also offer the potential for increased sensitivity and specificity for detecting waterborne *Cryptosporidium* and *Giardia* on the microscope slide.

The optimization of the Rand Water Method 06 (2007) was successful as the recovery rate of *Cryptosporidium* oocysts and *Giardia* cysts from water increased. All the changes that were verified and increased the recovery rate oocysts and cysts were incorporated into the improved method. Rand Water Method 06 (2009) was acceptable and can be recommended for future use of isolation and detection of *Cryptosporidium* oocysts and *Giardia* cysts in the types of water: drinking water, raw water, untreated water, source water or reagent grade water

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ANNEXURES

ANNEXURE A – 1

SANS 241:2006, Microbiological safety requirements

Table 1 — Microbiological safety requirements

1	2	3	4	5
Determinand	**Unit**	**Allowable compliance contribution[a]**		
		95 % of samples, min.	**4 % of samples, max.**	**1 % of samples, max.**
		Upper limits		
E. coli[b] or	count/100 mL	Not detected	Not detected	1
Thermotolerant (faecal) coliform bacteria[c]	count/100 mL	Not detected	1	10

[a] The allowable compliance contribution shall be at least 95 % to the limits indicated in column 3, with a maximum of 4 % and 1 %, respectively, to the limits indicated in column 4 and column 5. The objective of disinfection should, nevertheless, be to attain 100 % compliance to the limits indicated in column 3.

[b] Definitive, preferred indicator of faecal pollution.

[c] Indicator of unacceptable microbial water quality, could be tested instead of *E. coli* but is not the preferred indicator of faecal pollution. Also provides information on treatment efficiency and aftergrowth in distribution networks.

ANNEXURE A – 2

SANS 241:2006, Physical, organoleptic and chemical requirements

Table 2 — Physical, organoleptic and chemical requirements

1	2	3	4	5
Determinand	**Unit**	**Class I (recommended operational limit)**	**Class II (max. allowable for limited duration)**	**Class II water consumption period,[a] max.**
Physical and organoleptic requirements				
Colour (aesthetic)	mg/L Pt	< 20	20 – 50	No limit[b]
Conductivity at 25 °C (aesthetic)	mS/m	< 150	150 – 370	7 years
Dissolved solids (aesthetic)	mg/L	< 1 000	1 000 – 2 400	7 years
Odour (aesthetic)	TON	< 5	5 – 10	No limit[b]
pH value at 25 °C (aesthetic/ operational)	pH units	5,0 – 9,5	4,0 – 10,0	No limit[c]
Taste (aesthetic)	FTN	< 5	5 – 10	No limit
Turbidity (aesthetic/operational/ indirect health)	NTU	< 1	1 – 5	No limit[d]
Chemical requirements — macro-determinand				
Ammonia as N (operational)	mg/L	< 1,0	1,0 – 2,0	No limit[d]
Calcium as Ca (aesthetic/operational)	mg/L	< 150	150 – 300	7 years
Chloride as Cl^- (aesthetic)	mg/L	< 200	200 – 600	7 years
Fluoride as F^- (health)	mg/L	< 1,0	1,0 – 1,5	1 year
Magnesium as Mg (aesthetic/ health)	mg/L	< 70	70 – 100	7 years
(Nitrate and nitrite) as N (health)	mg/L	< 10	10 – 20	7 years
Potassium as K (operational/health)	mg/L	< 50	50 – 100	7 years
Sodium as Na (aesthetic/health)	mg/L	< 200	200 – 400	7 years
Sulfate as $SO_4^=$ (health)	mg/L	< 400	400 – 600	7 years
Zinc as Zn (aesthetic/health)	mg/L	< 5,0	5,0 – 10	1 year

ANNEXURE B – 1

Water purification treatment process

Water purification process involves seven stages:

- Coagulation
- Flocculation
- Sedimentation
- Stabilization or Carbonation
- Filtration
- Disinfection
- Chloramination

Each stage in the purification process is accompanied by changes in the physical and chemical composition of the water. Competent trained people constantly monitor these changes and corrective action is taken to prevent the water quality from deviating from the prescribed limits.

Coagulation

Screening is done before the coagulation stage takes place. Water passes through metal screens to trap large living organisms, sticks, papers, tree leaves, plastic wrappers and litter are removed from raw water. The clumping of particles which results in the settling of impurities takes place, that process is coagulation. The water from the Vaal Dam contains highly dispersed particles, which, because they are colloidal, tend to remain suspended for a long period. For Rand Water treatment systems raw limestone (calcium carbonate) from the Northern Cape is fired in a shift kiln at Zwartkopjes at 1200Â°C to convert it to calcium oxide and carbon dioxide gas. The burnt limestone is crushed and slaked with water in rotating slakers to produce slaked lime or calcium hydroxide, Figure 1.

Figure: 1. Raw limestone (calcium carbonate) from the Northern Cape

The slaked lime is then added to the water as the main coagulant to destabilize the electrostatic charges of suspended particles in the water. A small quantity of activated sodium silicate is also added to the raw water to enable the suspended particles to clump together to form larger clusters or flocs which can then be settled out by a coagulation systems process, which destabilizes the particles during flash mixing periods in about 20 to 30 seconds. In this process sodium silicate and slaked lime are added to the water and rapidly mixed to achieve instantaneous and complete homogenization of the coagulant with the specific volume of raw water being dosed.

Flocculation

The next step is flocculation, which is the clumping together of the suspended particles that have been destabilized by coagulation to form heavier visible particles called floc. The formed floc remains in suspension as the water flows at high velocity through either spiral flocculators or baffled channel conditioning bays, Figure 2. Orthokinetic flocculation predominates in Rand Water treatment systems resulting from the fluid motion at higher velocity gradients and larger particle sizes. During this stage in the water purification system most oocysts and cysts are removed.

Figure: 2. Flocculators or baffled channel conditioning bays used in Rand Water.

Sedimentation

One of the oldest known steps in water treatment processes is sedimentation. The process consists of depositing sediment from suspension in water. The floc settles at the bottom of these sedimentation tanks by gravity during treatment. It is aided by the addition of chemical coagulants to produce flocs, which are allowed to settle in specially designed tanks called sedimentation tanks, also engineered to remove sludge (floc at the bottom of tank), Figure 3.

This stage also removes the oocysts and cysts in water. Rand Water uses horizontal flow tanks with retention times of 4 hours and produces water with a turbidity of 5 NTU at the outlet weirs, which is considered acceptable for filtration. Depending on the turbidity of the incoming raw water, between 95 percent and 97 percent of the suspended particles are removed during sedimentation.

Figure: 3 Sedimentation tank

Figure: 4. Removal of dry sludge

Between 500 and 1300 tons of dry sludge are produced each day during the purification process. This is removed from the sedimentation tanks as shown in Figure 4 at Zuikerbosch and Vereeniging in thin slurry containing 3 percent mass by volume of dry sludge. The sludge consists mainly of calcium carbonate, magnesium hydroxide and complex silicates containing aluminium and iron. Phase separation of the sludge takes place in a thickening plant. The separation is enhanced through adding between 0.3 and 0.8 kg of

polyacrylaride per ton of sludge and the thickened sludge is then spread in 200 mm layers over an area of 200 hectares and sun dried.

Stabilisation or Carbonation

After sedimentation, the water flows into carbonation bays where it is stabilised by bubbling carbon dioxide gas, obtained from the lime-burning kilns, through the water. The use of lime as a coagulant raises the pH of the water to about 10.5, which is very unstable, conducive to scale forming and makes water feel and taste soapy. Carbonation reduces the pH to between 8.0 and 8.4, which is necessary to produce chemically stable water that will not cause excessive scaling or corrosion.

Filtration

During the filtration process the suspended matter is removed from a liquid through a medium which is permeable to the liquid but not to the suspended material. The water passes into the filter houses where it flows through rapid gravity sand filter beds which contain finely graded silica sand pebbles of different grain size. The remaining suspended particles, small water plants, animals and germs are removed at this stage including the oocyts and cysts if present. If the filters are not of high quality and maintained properly, because of their size oocysts and cysts still have the possibility to go through the filters. Filters are covered to exclude light to less than 25 lux to prevent algal growth on the filters. After filtration, the water has a residual turbidity of 0.5 NTU or less. The filter backwash water is treated to remove organics, heavy metals, bacteria and other contaminants, which concentrate in this water before being recycled back to the inlet of the filters.

Disinfection

Primary disinfection

Chlorine is the most commonly used disinfectant to kill microorganisms and most bacteria that may be present in the water, but it is ineffective against bacterial spores, oocysts and cysts. The main purpose of primary disinfection

is to kill or render harmless microbiological organisms that cause diseases. There are no chlorine contact chambers and the mixing takes place in the pipelines.

Secondary disinfection by chloramination

Chlorine, although an excellent disinfectant, does not remain active for much longer than 6 to 8 hours. Disinfection needs to be repeated with a less powerful agent that will remain active for long periods so that the water is be protected right up to the end consumer. This is achieved by dosing chlorine and ammonia at the booster pumping station in the correct mass ratio of not less than 4:1 and forming the monochloramine in situ. The monochloramine, although less active than chlorine, protects the water against bacteria for periods of up to 8 days.

ANNEXURE C - 1

US-EPA Method 1623 (2005)

Sample collection and filtration

- Water samples are collected in ten litres carboys. For quality control purposes one sample is spiked with a known number of oocysts and cysts suspensions.
- Filter cartridge is connected to the pump and carboy using appropriate tubing. The pump is switched on to pump water through filter cartridge and is set to pump two litres per minute.

Elution

- Sufficient quantity of elution buffer is prepared in advance to elute all samples analysed. Elution buffer is poured through the inlet fitting of the filter cartridge to cover the pleated white membrane of the cartridge.
- The capsule is securely clamped in one of the clamps on the laboratory wrist shaker with the bleed valve positioned at the top on a vertical axis (in the 12 o'clock position). Agitate the capsule for approximately 5 minutes at a speed of approximately 600rpm.
- After the first agitation the filter is removed from the shaker, the inlet cap is also removed, and the contents are poured into the 250ml conical centrifuge tube.
- Elution buffer is added again to cover the pleated white membrane of the cartridge filter and the filter is returned to the wrist action shaker with the bleed valve positioned at the 4 o'clock position. Filter capsule is then agitated in that position for five minutes.
- After five minutes change the position of the capsule to the 8 o’clock position and the filter is agitated for a final 5 minutes.
- The contents are poured into the 250ml centrifuge tube.

Pre-treatment buffer

- Pour the 5% HaHMP buffer through the inlet fitting. Add enough buffer to cover the pleated membrane.
- Replace the inlet cap and clamp the cap in place.
- Clamp capsule in wrist action shaker with the bleed valve positioned in the 12 o'clock position.
- Turn the shaker on and set the speed to maximum for approximately 5 minutes.
- Remove the filter module and secure in a vertical position with the white pressure release valve uppermost position.
- Remove the end caps and allow the pre-treatment buffer to drain completely. Attached to vacuum source to completely remove buffer from filter.
- Fill the capsule with DI water through the inlet port. Immediately remove the DI water through the outlet port.
- Replace the lower cap and pour elution buffer into the filter module to a depth of approximately 13mm above the white pleated filter element. Replace the upper end cap. Place the filter into the wrist action shaker with the white pressure release valve in the 12 o'clock position and tighten the clamp
- Shake the filter at 900 ± 25rpm for 5 minutes.
- Remove the filter module and decant the elution buffer from the inlet end into 250ml centrifuge tube.
- Return the capsule to the shaker with the bleed valve positioned at the 4 o'clock position. Turn on the shaker and agitate the capsule for approximately 5 minutes.
- Remove the filter from the shaker, but leave the elution buffer in the capsule. Re-clamp the capsule to the shaker at the 8 o'clock position. Turn on the shaker and agitate the capsule for a final 5 minutes.
- Remove the filter from the shaker and pour the contents into the 250ml centrifuge tube.

- Rinse down the inside of the capsule filter walls with reagent water or elution buffer using a squirt bottle inserted in the inlet end of the capsule.
- Invert the capsule filter over the centrifuge tube and ensure that as much of the eluate as possible has been transferred.

Sample Concentration and Separation (Purification)

- Centrifuge the 250ml centrifuge tube containing the capsule filter elute at 1100×g for 15 minutes.
- Do not use the brake of the centrifuge. Immediately when the centrifuge stops remove the centrifuge tubes from the centrifuge and aspirate.
- Prepare a 1X dilution of SL-buffer-A from the 10X SL-buffer-A (clear, colourless solution) supplied. Use reagent grade water as the diluent.
- For every 1ml of 1X SL-buffer-A required, mix 100µL of 10X SL-buffer-A and 0.9ml diluent water to make 1ml. For each 10ml sample or subsample to be processed through IMS, add 1mL of the already prepared 10X SL-buffer-A to a flat-sided tube (Leighton-type tubes).
- Transfer the water sample concentrate (after centrifugation) to the flat-sided tube(s) containing the SL-buffers. Label the flat-sided tube(s) with the sample number or name.
- Vortex the Dynabeads®Crypto-Combo vial from the IMS kit to suspend the beads and add 100µL of the re-suspended Dynabeads®Crypto-Combo to the sample tube(s) containing the water sample concentrate and SL-buffers A +B.
- Vortex the Dynabeads®Giardia-Combo vial from the IMS kit to suspend the beads and add 100µL of the re-suspended Dynabeads®Giardia-Combo to the sample tube(s) containing the water sample concentrate, Dynabeads®Crypto-Combo, and SL-buffers A + B.

Affix the Leighton-type tubes with the sample and Dynabeads to the rotating mixer and rotate at approximately 18rpm for 60 minutes at room temperature.

After 1 hour of rotation, remove each sample tube from the mixer and place the flat side tubes in the magnetic particle concentrator (MPC-1 or MPC-6) with flat side of the tube toward the magnet. Without removing the sample tube from the MPC-1, place the magnet side of the MPC-1 downwards, so the tube is horizontal and the flat side of the tube is facing down. Gently rock the sample tube by hand at approximately 90° angle; tilting the cap-end and base-end of the tube up and down in turn for two minutes. Then immediately remove the cap and, keeping the flat side of the tube on top, pour off all of the supernatant from the tube held in the MPC-1 into a suitable liquid waste container and do not remove the tube from MPC-1 during the step.

- Remove the sample tube from the MPC-1 and resuspend the sample in 1 ml 1X SL-buffer-A.
- Mix very gently to resuspend all material in the tube. Transfer all the liquid from the sample tube to a labeled, 1.5ml microcentrifuge tube and ensure that all of the liquid and beads are transferred.
- Place the microcentrifuge tubes in MPC-M and gently rock/roll the tube through 180° by hand for approximately 1 minute. Immediately aspirate the supernatant from the tube held in the MPC-M.
- Add 100µL of 0.1N HCl to the microcentrifuge tubes and vortex.
- Allow the microcentrifuge tubes placed in the MPC-M without the magnetic strip to stand undisturbed in a vertical position for at least 10 minutes at room temperature.
- Prepare microscope well slide. Add 10µL of 1.0N NaOH to the slide wells and label the slide with sample number or name. Without removing the microcentrifuge tube from the MPC-M, transfer the entire sample from the microcentrifuge tube in the MPC-M to the slide well with the NaOH.
- Air-dry the well slide or dry them in a slide warmer set at 35 °C to 42 °C.

Sample Staining

- Fixate the slides by adding a drop of methanol and allowed to air-dry.
- Follow manufacturer's instructions in applying stain to slides and then place the slides in a humid chamber and incubate at room temperature (or incubate can be used) for approximately 30 minutes.
- Gently aspirate the excess reagent from below the well using a clean Pasteur pipette or absorb with paper towel or other absorbent material placed at edge of slide. Add a drop of 4'-6'-diamidino-2-phenylindole (DAPI) staining solution to each well and allow to stand at room temperature for approximately 1 minute.
- When sides are dry add mounting medium and cover with the cover slip.

Microscope examination for *Cryptosporidium* oocysts

FITC examination

Use epifluorescence to scan the entire well for apple-green fluorescence of oocyst and cyst shapes. When brilliant apple-green fluorescent ovoid or spherical objects 4 to 6µm in diameter are observed with brightly highlighted edges, increase magnification to 400X and switch the microscope to the UV filter block for DAPI then to DIC at 1000X.

DAPI fluorescence examination

Using the UV filter block for DAPI, the object will exhibit one of the following characteristics:

(a) Light blue internal staining (no distinct nuclei) with a green rim

(b) Intense blue internal staining

(c) Up to four distinct, sky-blue nuclei

Look for atypical DAPI fluorescence, for example more than four stained nuclei, size of stained nuclei, and wall structure and colour.

DIC examination

Using DIC, look for external or internal morphological characteristics atypical of *Cryptosporidium* oocysts (for example spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.) If atypical structures are not observed, then categorize each apple-green fluorescing object as:

(a) An empty *Cryptosporidium* oocyst

(b) A *Cryptosporidium* oocyst with amorphous structure

(c) A *Cryptosporidium* oocyst with internal structure (one to four sporozoites/oocyst)

Microscope examination for *Giardia* cysts

FITC examination

When brilliant apple-green fluorescing round ovoid objects (8 - 18 µm long by 5 - 15 µm wide) are observed with brightly highlighted edges, increase magnification to 400X and switch the microscope to the UV filter block for DAPI then to DIC at 1000X.

DAPI fluorescence examination

Using the UV filter block for DAPI, the object will exhibit one or more of the following characteristics:

(a) Light blue internal staining (no distinct nuclei) and a green rim

(b) Intense blue internal staining

(c) Two to four sky-blue nuclei

Look for atypical DAPI fluorescence, e.g., more than four stained nuclei, size of stained nuclei, and wall structure and color.

DIC examination

Using DIC microscopy, look for external or internal morphological characteristics atypical of *Giardia* cysts (for example spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.). If atypical structures are not observed,

then categorize each object meeting the criteria specified as for the following, based on DIC examination:

(a) An empty *Giardia* cyst

(b) A *Giardia* cyst with amorphous structure

(c) A *Giardia* cyst with one type of internal structure (nuclei, median body, or axonemes)

(d) A *Giardia* cyst with more than one type of internal structure

A positive result is a *Giardia* cyst that exhibits typical IFA fluorescence, typical size and shape and exhibits nothing atypical on IFA, DAPI fluorescence, or DIC microscopy.

ANNEXURE D - 1

Drinking Water Inspectorate Standard Operating Procedure – 2003 (pre-treatment buffer)

Drinking Water Inspectorate SOP – pre-treatment buffer step in the method. Prepare pre-treatment buffer and elution buffer in advance. For analysis heat the pre-treatment buffer, elution buffer and reagent grade water to 37 ±1 ºC. Filter the sample through the Envirocheck filter capsule and remove excess water from the sample. Pour the warmed pre-treatment buffer into the filter cartridge to a depth of approximately 13mm above the white pleated filter element.

Replace the vinyl cap and place the filter into the wrist action shaker in the 12 o'clock position and shake the filter at 900rpm for 5 minutes. Remove the filter from the shaker and remove the end caps to drain the pre-treatment buffer. Replace the lower cap and pour warmed reagent grade water into the filter to the depth of approximately 13mm above the white pleated filter element. Replace the caps and gently rotate the filter manually for 30 seconds to rinse off the pre-treatment buffer. Remove the end caps to drain reagent grade water completely.

Replace the lower cap and pour warmed elution buffer into the filter to the depth of approximately 13mm above the white pleated filter element. Replace the caps and place the filter into the wrist action shaker in the 12 o'clock position and shake the filter at 900rpm for 5 minutes. Remove the filter and decant the elution buffer from the inlet end into the 250ml centrifuge tube.

Pour warmed elution buffer again into the filter to the depth of approximately 13mm above the white pleated filter element. Replace the caps and place the filter into the wrist action shaker in the 4 o'clock position and shake the

filter at 900rpm for 5 minutes. Change the position of the filter to 8 o'clock position and shake the filter at 900rpm for 5 minutes.

Remove the filter from the wrist action shaker and pour the contents into the same 250ml centrifuge tube. Rinse down the inside of the capsule filter with reagent grade water or elution buffer using a squirt bottle inserted in the inlet of the capsule. Invert the capsule filter over the centrifuge tube and ensure that as much of the eluate as possible has been transferred to the same 250ml centrifuge tube.