EFFICACY OF WASH SOLUTIONS ON *CYCLOSPORA CAYETANENSIS* AND

CRYPTOSPORIDIUM PARVUM RECOVERY FROM BASIL

by

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(Under the Direction of Ynés R. Ortega)

ABSTRACT

In this study, E-Pure water, 3% levulinic acid-3% sodium dodecyl sulfate, 1 M glycine, 0.1 M phosphate buffered saline, 0.1% Alconox®, and 1% HCl-pepsin were evaluated for their effectiveness in recovering *Cyclospora cayetanensis* and *Cryptosporidium parvum* from basil. One hundred and 1,000 oocysts of both parasites were inoculated on top surfaces of basil leaves and stored for 1 h and 24 h. The samples were hand washed for 1 min with each of the wash solutions. DNA was then extracted and amplified using nested PCR. All wash solutions could detect both parasites' oocysts at the concentration of 1,000 oocysts/25 g basil, with the recovery rates of 100%. At the concentration of 100 oocysts/25 g basil, the lowest and highest recovery rates observed were 18.5% and 70.4%, respectively. 1% HCl-pepsin showed a lower variability in recovering both parasites' oocysts in the 24 h post-inoculation time at the 100 oocysts level.

INDEX WORDS: *Cyclospora*, *Cryptosporidium*, recovery, wash solutions, basil

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INTRODUCTION AND LITERATURE REVIEW

Cyclospora cayetanensis

Cyclospora cayetanensis (*C. cayetanensis*) is an emerging human pathogen that has been associated with numerous outbreaks of diarrhea illness. This coccidian parasite causes intracellular infections, primarily in the epithelial cells of the duodenum and jejunum when infectious oocysts are ingested. The routes of transmission can be through the fecal-oral route or ingestion of contaminated food or water. Cases of cyclosporiasis have been mostly associated with imported berries. In 1996, in the United States and Canada, there were 1,500 cases associated with eating raspberries from Guatemala *(27)*. Other outbreaks were also related with lettuce, basil, snow peas imported from countries where *Cyclospora* is endemic.

An oocyst is the encysted zygotic stage of a parasite that is surrounded by a protective wall. Sporulation can occur under ideal temperature and humidity. *C. cayetanensis* oocyst is characterized by its ability to produce green/blue fluorescence when illuminated with ultraviolet light. In addition, it is highly resistant to many disinfectants commonly used in food and water processing *(51)*. Thus, drinking water that lacks adequate sanitation can result in direct infection.

History

This coccidian parasite was first described in 1870 in the intestines of moles, and the genus *Cyclospora* was named by Schneider in 1881 *(15)*. The first cases of human

Cyclospora infection were discovered in the 1980s when the AIDS epidemic emerged and *Cryptosporidium* was identified as an important opportunistic infection. *Cyclospora* oocysts were originally misidentified as *Cryptosporidium* in the acid-fast stains observation. *C. cayetanensis* was initially identified as a blue-green alga or a large form of *Cryptosporidium* and was also referred to as cyanobacterium-like body or coccidian-like body (CLB) *(36)*. The first report of human cyclosporiasis can be dated to 1979 when *Isospora*-like organisms were found in three humans from Papua New Guinea *(1)*. Fifteen years later, studies led by Ortega showed a complete description of this organism, and it was classified as a coccidian parasite belonging to the genus *Cyclospora (45)*. The name *Cyclospora cayetanensis* was proposed, in honor of Peruvian University Cayetano Heredia, where field studies on *Cyclospora*-positive specimens were conducted.

Biology and clinical aspects of *Cyclospora*

The oocysts of *C. cayetanensis* are spherical, measure about 8-10 µm in diameter, and have a bilayered wall, which consists of a 50 nm cell wall and a 63 nm outer fibrillar coat. A study by Eberhard et al. suggested that this parasite only infects humans *(20)*. The oocysts that are excreted in the feces by an infected host are not infectious until they sporulate, which takes about 7 to 15 days under favorable environment (23 to 27°C) *(48)*. The sporulated oocyct has two sporocysts (resistant wall), with each containing two infectious sporozoites. The life cycle of this coccidian parasite begins when food or water contaminated with sporulated oocysts is ingested by a susceptible host. Upon ingestion, the oocysts excyst and release sporozoites, which infect the epithelial cells of the small intestine. Except for sporulation, *Cyclospora* undergoes its life cycle, asexual and sexual stages, in the human host.

C. cayetanensis can cause illness that its severity and duration depend on the immune system of the host. According to Centers for Disease Control and Prevention (CDC), the symptoms include watery diarrhea, loss of appetite, weight loss, abdominal bloating and cramping, increased flatulence, nausea, fatigue, and low-grade fever. In some patients, commonly in the endemic areas, asymptomatic infections can occur as well. The incubation period is typically 7 days and can continue up to a month or more *(41)*. Cyclosporiasis is usually self-limited in immunocompetent hosts; however, much more severe symptoms have been observed in the immunocompromised, as well as in HIV-infected individuals. Following *Cyclospora* infection, acalculous cholecystitis, biliary disease, Guillain-Barré syndrome and Reiter syndrome have all been reported in HIV patients *(47)*. In areas where *Cyclospora* is endemic, such as Haiti, Peru, and Nepal, younger children tend to have mild symptoms or asymptomatic infections as result of repeated infections and appear to develop immunity as they get older *(62)*.

Treatment

Cyclosporiasis can be treated with trimethoprim-sulfamethoxazole (TMP-SMX) *(39)*. A 7-day course of TMP-SMX with a 160-800 mg oral dose is given twice daily to immunocompetent patients and a dosage of 5-25 mg/kg to infected children. The same dosage of 160-800 mg is given four times a day for 10 days to the patients with HIV infection. Therapy with TMP-SMX prophylaxis three times a week for a month is effective in treating relapsed patients *(49)*. Alternative medications, such as ciprofloxacin and nitazoxanide can be administered to those patients who are allergic to sulfa drugs *(63, 66)*.

Diagnosis and detection

The diagnosis of cyclosporiasis can be based on identifying the oocysts in the fecal samples by microscopy techniques. *Cyclospora* oocysts are easily overlooked because they normally are excreted in low numbers, thus concentrating the stool specimens before detection are essential. With their characteristic morphology size and shape, *Cyclospora* oocysts can be examined in wet mounts by phase-contrast microscopy and enhanced with epifluorescence microscopy given that they have the autofluorescent property. The oocysts autofluoresce blue with a 365 nm dichroic mirror excitation filter and green with a 450-490 nm dichroic filter *(62)*.

Detection of *Cyclospora* oocysts can also be done using modified acid-fast staining or modified safranin technique with microwave heating *(64)*. In addition to microscopy, molecular methods, such as polymerase chain reaction (PCR) have also been used to identify *Cyclospora* oocysts *(34, 44, 52)*. PCR has the potential to be more sensitive than microscopy; however, infectious and noninfectious oocysts are indistinguishable from this method. A nested PCR assay amplifying the 18S rRNA gene, designed by Relman et al. *(52)*, has been widely used for the detection. Although sensitive, this PCR is not specific enough for *Cyclospora*. The amplified product does not distinguish *Cyclospora* from *Eimeria* species because they are of the same size when using those primers. A restriction fragment length polymorphism (RFLP) analysis can be used to separate the genera.

Epidemiology

Cyclosporiasis is widely distributed throughout the world, commonly in tropical and subtropical regions *(61)*. According to the epidemiologic studies by Bern et al. in

Guatemala in 1999 *(2)*, the overall prevalence of *C. cayetanensis* was 2.3%, and infection was more frequent during the warmer months of May to August, with prevalence peaking in June (6.7%). Infections were most common among children 1-9 years of age, older adults, and among AIDS patients. Cyclosporiasis is season-dependent. Data from studies conducted in Nepal showed that the highest infection occurrence was during the summer and rainy season in May through August *(31, 57)*. In Peru, *Cyclospora* incidents occurred during warm season in December through May *(3, 48)*. On the contrary, in Haiti infection and illness occurred during the driest and coolest months (January to March) of the year *(19)*.

Before the foodborne outbreaks in 1996, *Cyclospora* was known as a travelers' diarrhea. Infections had been reported in developing countries, such as Southeast Asia, Papua New Guinea, Indonesia, India, Nepal, and others. Aside from international travel, consumption of imported produce has been linked with *Cyclospora* infection as well. Cyclosporiasis can be transmitted by contaminated water supplies or food, particularly fruit or vegetables. The first documented waterborne outbreak in the United States occurred in Chicago in 1990, where 23 cases were associated with contaminated hospital water supply. The epidemiological investigation assumed that sediment was disturbed when the storage tank was refilled following the pump failure *(32)*.

Another waterborne outbreak occurred involving 12 of 14 British soldiers in Pokhara, Nepal in June 1994 *(51)*. The water supply consisted of a mixture of river and municipal water and had been chlorinated at a concentration of 0.3 to 0.8 ppm; however, it appeared that the level of chlorination was not adequate to inactivate *Cyclospora*. It was then advised that water had to be boiled or filtered before consumption to prevent

possible infection. Several studies have also discovered the presence of *Cyclospora* oocysts in drinking water sources in Guatemala and Nepal *(18, 58)*.

Foodborne outbreaks of cyclosporiasis in the 1990s affected approximately 3,600 people in the United States and Canada. It was documented that the outbreaks were linked to imported fresh produce such as raspberries, mesclun, and basil. An epidemiological investigation in the spring of 1996 reported that a large cyclosporiasis outbreak occurred in North America, affecting 1,465 people within 20 states, the District of Columbia, and two additional provinces *(27)*. The investigation stated that the outbreak was associated with 55 events that served raspberries, all of which were imported from Guatemala. Not long after, another multistate outbreak of cyclosporiasis occurred in the spring of 1997, once again linking to the outbreak with raspberries originating from Guatemala, despite the control measures that were made by the Guatemalan Berry Commission. Exportation of Guatemalan raspberries was voluntarily suspended in May 1997, which marked the end of the outbreak *(28)*. Unfortunately, raspberries were implicated yet again in wedding receptions in Georgia and Philadelphia in May and June 2000, respectively. Raspberries used in the wedding cake were the source of an outbreak, and a Guatemalan farm was suspected to be involved in the incident *(29)*.

Other than raspberries, fresh produce has also been linked to *Cyclospora* outbreaks. Mesclun was implicated in several outbreaks that occurred in Tallahassee and Orlando, Florida in March and December of 1997, and the traceback investigation indicated that these implicated mesclun were from Peru *(26)*. The outbreak in March coincided with the seasonal nature of cyclosporiasis in Peru *(46)*. Basil was implicated in

a 1999 outbreak in Missouri, where infected people had consumed chicken pasta salads containing basil and tomato basil salads *(37)*. The possible sources of the implicated basil were provided by farms in Mexico and the United States. The first cyclosporiasis outbreak linked to fresh basil occurred in 1997 in the Northern Virginia, and this Missouri outbreak was the second documented fresh basil outbreak *(7)*. Another outbreak associated with consumption of fresh basil was documented in Canada as well. In May 2001, 17 cases of cyclosporiasis occurred in British Columbia. A case-control study stated that the outbreak was related to Thai basil, which was imported from the United States *(30)*. *Cyclospora* reappeared in 2004. An outbreak implicating consumption of raw Guatemalan snow peas in pasta salads occurred in Pennsylvania, with 50 potential cases of cyclosporiasis *(10)*. Since then, *C. cayetanensis* has gained significant attention due to numerous foodborne outbreaks associated with fruits and vegetables imported from developing countries.

Cryptosporidium parvum

Cryptosporidium parvum (*C. parvum*) is an emerging pathogen that infects gastrointestinal tract of both humans and animals. It is an obligate protozoan parasite that causes intracellular infections mainly in the jejunum and ileum, resulting in a watery diarrhea. Cryptosporidiosis can be transmitted directly from person-to-person through fecal exposure, zoonotic exposure, and by ingestion of contaminated food, drinking and recreational water.

Cryptosporidium was first discovered in the gastric glands of mice by Tyzzer in 1907, and *Cryptosporidium muris* was proposed in 1910. Two years later, he identified another new species, known as *Cryptosporidium parvum*. There are 16 known species of

Cryptosporidium, which infect birds, cats, mice, cattle, fish, reptiles, and humans *(22)*. Cryptosporidiosis of humans was first identified in 1976.

Life cycle of *C. parvum*

The oocysts of *C. parvum* are spherical, measure about 4-6 µm in diameter, and have the characteristic of being thin and thick walled. This parasite undergoes a similar life cycle as *Cyclospora*, with the exception that oocysts sporulate inside the hosts and are infectious when shed into the environment. Upon ingestion, body temperature and digestive enzymes induce the oocysts wall to rupture *(33)*. Within the oocysts, four sporozoites are released, which then attach to the intestinal epithelial cells. The sporozoites undergo asexual and sexual reproduction cycle inside the hosts, in which oocysts are produced in the latter. *C. parvum* is able to autoinfect within the same host when the fully sporulated thin walled oocysts excyst. The thick walled oocysts are excreted in high numbers in the feces (approximately 10^5 oocysts/g of feces) and are environmentally resistant *(22)*.

Clinical aspects

There are several species of *Cryptosporidium* that can infect humans; however, the majority of infections are caused by *C. parvum* and *C. hominis*. *Cryptosporidium* infection has been reported in immunocompetent and immunodeficient individuals. The infection is usually characterized by severe watery diarrhea, weight loss, abdominal pain, fever, nausea, and vomiting, with the severity and duration depending on the immune system of the host. In healthy adults in endemic areas, infection is asymptomatic with short term diarrheal illness, lasting 4 to 14 days, and normally is self-limiting. On the other hand, a prolonged, cholera-like illness could occur in immunodeficient or naive

individuals. The duration of illness can continue up to months, which can be deadly in patients with AIDS. Children, particularly those less than 2 years old and malnourished, and the elderly have greater risk of infection as well *(33)*. Current and Garcia *(14)* reported that other than gastrointestinal tract, *Cryptosporidium* could as well infect respiratory tract, gallbladder and biliary tract, and pancreatic duct.

Cryptosporidiosis is found worldwide and more common in developing countries than in developed countries. Survey studies reported that the prevalence rates were between 1-3% in North America and Europe, 5% in Asia, and 10% in Africa *(14)*. The lower incidence rates in developed countries could be due to good sanitation practices and cleaner drinking water.

Treatment

At this time, no effective therapy has been proven to cure cryptosporidiosis. Since the infection is self-limiting in immunocompetent individuals, treatment is not necessary although supportive treatment, such as fluid and electrolyte may be administered if excessive diarrhea occurs to prevent dehydration. The antiprotozoal agent nitazoxanide, which has been approved by the FDA, is also available to treat cryptosporidiosis in immunocompetent individuals, including children. However, the use of nitazoxanide alone in immunocompromised individuals is not effective. If taken in combination with highly active anti-retroviral therapy, the infection can be resolved *(35)*.

Detection

The most frequent method of diagnosis in cryptosporidiosis is the detection of oocysts in the fecal samples by microscopy techniques. Oocysts can be visualized by bright-field microscopy and enhanced with modified acid fast staining. The oocysts

exhibit bright red against a blue-green background. Food and environmental water samples that contain low numbers of oocysts need to be concentrated in order to maximize the oocysts recovery. Filtration, centrifugation, flocculation, and immunomagnetic separation (IMS) are some of the possible techniques used in concentrating the samples. Unlike *Cyclospora* oocysts, *Cryptosporidium* oocysts do not autofluoresce. Immunofluorescent assay (IFA) has been utilized for better sensitivity and specificity in the detection of *Cryptosporidium* oocysts. This method uses antibody labeled with fluorescent reporters, allowing the monoclonal antibodies to bind to epitopes on the oocysts' surface. When using fluorescein isothiocyanate (FITC), the oocysts have the characteristic of bright apple-green fluorescence outlining the oocysts wall. Other immunological methods, such as enzyme-linked immunosorbent assay (ELISA) and immunochromatographic assay can also be used.

Detection of *Cryptosporidium* by PCR has been widely used. Several studies utilizing this method have successfully detected amount as low as 20 oocysts of *C. parvum*. Laberge et al. had designed a specific PCR assay and managed to detect 1 to 10 oocysts in 20 ml of artificially contaminated raw milk *(33)*. The efficiency of the PCR relies on the oocysts recovery and the DNA extraction. Additionally, the nested PCR assay amplifying the 18S rRNA gene is able to detect *Cryptosporidium* oocysts. The internal primers yield a product of about 826 bp which can be visualized by gel electrophoresis *(65)*. Different choice of primers target the 18S rRNA gene will yield different products. Primers used by Johnson et al. amplify products of about 428 to 455 bp *(22)*.

Epidemiology

Cryptosporidium parvum, a major human pathogen, is the species most accountable for diarrhea illness in mammals and the most probable cause in zoonotic transmission of cryptosporidiosis. One of the major routes of cryptosporidiosis is transmitted by direct contact from person-to-person, particularly in daycare centers, households, and hospitals *(42)*. The other transmission can be animal-to-human, waterborne through drinking water or recreational water, and foodborne. Since 1980s, cattle have been considered as a potentially important source for zoonotic infections. Several studies have revealed that cattle manure is associated with several foodborne and waterborne outbreaks *(5, 24)*.

Water has been recognized as an important vehicle for the transmission of *Cryptosporidium (60)*. Numerous investigations have reported contamination of surface source waters in North America. The first documented waterborne outbreak, affecting 117 people, was in July 1984 in Braun Station, Texas *(16)*. The outbreak was linked to sewage contamination of well water. In 1987, a larger waterborne outbreak occurred in Carrollton, Georgia, where 13,000 people were infected *(25)*. Even though the treated water sources had been filtered and disinfected, the small size of *Cryptosporidium* oocysts (4-6 µm) could have passed through the filters and its resistance to chlorine did not eliminate the oocysts. In the early spring of 1993, the largest waterborne outbreak in the United States took place in Milwaukee, Wisconsin. As many as 403,000 people were infected. The investigation reported that the treated water was highly turbid, and oocysts were detected in ice blocks that were made during the outbreak period *(38)*. A boil-water advisory was then issued.

Other cryptosporidiosis outbreaks linked to drinking water have been documented not only in the United States, but also in the United Kingdom, Canada, Japan, and other European countries *(21)*. All of these were related to contaminated source waters with animal feces or sewage and water treatment deficiencies. This further confirms that *C. parvum* oocysts are able to bypass drinking water purification processes.

Outbreaks of cryptosporidiosis have also been associated with recreational waters, such as swimming pools, water parks, and others. Since 1980s, it has been reported that more than 10,000 people were infected in the outbreaks related to recreational waters *(21)*. The first outbreaks, linked to swimming pools, were documented in 1988 in the United States and the United Kingdom. Swallowing the water while in the recreational water is common, thus it is possible to get infected if *Cryptosporidium* oocysts are present in the water. Some factors account for the contamination includes sewage contamination, poor filtration system, fecal accidents from diaper-aged children, and poor hygiene practices. *Cryptosporidium* has a low infectious dose of 10 to 30 oocysts, and infected humans are able to excrete high amount of oocysts ($\leq 10^9$ oocysts in stool/day). Even after the episode of diarrhea has ceased, excretion of oocysts for up to 50 days is also possible *(22)*.

Although *Cryptosporidium* appears more likely to be associated with waterborne transmission, foodborne outbreaks have been reported as well. This parasite has been detected mainly in fresh produce, fruits, and shellfish *(22)*. In some reported cases, consumption of unpasteurized milk was the suspected source of illness. A cryptosporidiosis outbreak connected to milk was reported in the United Kingdom in 1995. Fifty children were sick from drinking school milk *(23)*. The first foodborne

outbreak of cryptosporidiosis in the United States occurred in October 1993, which was linked to drinking fresh-pressed apple cider. A group of students and staff attending the school agricultural fair in Maine were afflicted. It was discovered that apples from the ground near a cattle pasture were used for cider, and these apples could have been contaminated with *Cryptosporidium* oocysts that were present in the cattle feces. Furthermore, the cider was not pasteurized, which could have killed the oocysts *(43)*. Two years afterwards, 50 people in Minnesota were infected with cryptosporidiosis from the consumption of chicken salad in a social event. The epidemiological investigation revealed that the chicken salad had been prepared by the hostess, who operated a licensed day-care facility and had changed a baby's diaper before the food preparation *(6)*. Following the foodborne outbreaks in Maine and Minnesota, another outbreak linked to unpasteurized apple cider was reported in October 1996 in New York *(8)*.

In the following years, green onions were implicated in the outbreaks in Washington. Affected individuals were reported to have eaten foods containing uncooked green onions during the dinner banquet. Two food workers who were positive for *Cryptosporidium* had involved in the food preparation. Moreover, the green onions were not washed before the preparation *(9)*. Similarly, outbreak involving food handler occurred at a university in Washington, DC. A food handler have had diarrhea illness, but continued to work, preparing fruits and vegetables to be eaten raw. As a result, 88 students and 4 cafeteria workers were infected with cryptosporidiosis *(50)*. In 2003, apple cider was implicated once again in foodborne outbreak cryptosporidiosis in Ohio. It was reported that ozonated apple cider was the suspected mode of transmission, and 12 people were afflicted. Even though the apple cider had been ozonated twice, this procedure was

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ineffective in killing *Cryptosporidium (5)*. Ozone treatment if properly used would have prevented the outbreak.

Water is a significant source of contamination in cryptosporidiosis outbreaks, as the oocysts can be readily transported to food. Other than proper water treatment, implementation of prevention program such as HACCP is essential in reducing the risk of contamination. Good agricultural practices, better food handling and sanitation practices also play an important role in preventing outbreaks.

Recovery of *Cyclospora* and *Cryptosporidium* oocysts in fresh produce

Fresh produce has been implicated in numerous parasitic foodborne outbreaks. The rate of consumption of raw or lightly cooked vegetables has increased over the years. Several studies have reported the occurrence of *Cyclospora* and *Cryptosporidium* oocysts in fruits and fresh produce, particularly leafy greens. These case studies have examined produce from local markets in developing countries. A study by Ortega et al. detected the presence of *Cyclospora* and *Cryptosporidium* oocysts in vegetables collected from local markets in Peru *(46)*. Another study by Robertson and Gjerde also observed the occurrence of *Cryptosporidium* oocysts in lettuce and mung bean sprouts in Norway *(55)*. In 2013, a survey in Nigeria detected *Cryptosporidium* oocysts in 70 out of the 200 vegetables obtained from local markets, with the high occurrence in lettuce *(40)*. Furthermore, *Cyclospora* and *Cryptosporidium* have been detected as well in ready-to-eat packaged leafy greens in Canada. A total of 544 samples were purchased from retail stores; 9 out of these samples were positive for *Cyclospora* and 32 were positive for *Cryptosporidium (17)*.

Detection of *Cyclospora* and *Cryptosporidium* oocysts in fresh produce can be performed with microscopy techniques or PCR. However, the efficacies of these methods are highly dependent on the recovery of oocysts from vegetables. Recovery rates for leafy greens and herbs tend to be low ranging from 12 to 14% *(46, 56)*. Washing fresh produce with elution buffers has, therefore, been designed to remove oocysts. Typically, the fresh produce is washed with wash solutions of choice by manually agitating the sample for a period of time. The resulting washes are concentrated by centrifugation and further analyzed by microscopy or PCR. This washing method was utilized by Ortega et al. in Peru; distilled water was used for the washings. The recovery rates of oocysts found in fresh vegetables (green onions, basil, and lettuce) purchased from local markets were 14.5% and 1.8% for *Cryptosporidium* and *Cyclospora*, respectively *(46)*.

The wash method has been modified to maximize the oocysts recovery. Several different wash solutions and techniques have been tested and evaluated. The U.S. Food and Drug Administration method reports recovery rates of 1% for *Cryptosporidium* from seeded cabbage and lettuce samples (200 oocysts/200 g of sample). The method uses sonication bath and a washing solution composed of distilled water, 1% sodium dodecyl sulfate (SDS), and 0.1% Tween 80 *(4)*. Robertson et al. examined variables such as sample size and food matrices. Bean sprouts (50 g), strawberries (100 g), and iceberg lettuce (100g) were seeded with *Cryptosporidium* oocysts and washed using a detergent solution containing 150 ml water and 50 ml elution buffer that contains salts, detergents, and Antifoam A. The samples were washed by rotation in a drum for either 1 min or 5 min and then sonicated for 3 min. The wash solutions were concentrated by centrifugation, followed by immunomagnetic separation (IMS) technique. The mean

recovery rates of this method were 1-5% for bean sprouts, 12-35% for strawberries, and 18-36% for iceberg lettuce *(53)*. Further evaluation of this method was done by using fresher bean sprouts and reducing the lettuce sample size to 30 g. The recovery efficiencies were higher in smaller size samples, and the age of bean sprouts certainly played a part in maximizing the recovery rates *(54)*. In another study by Robertson et al. *(56)*, lectin-coated paramagnetic beads were used to recover *Cyclospora* oocysts from fruits and vegetables. The oocysts recovery rates were 12% for mushrooms, lettuce, and raspberries and 4% for bean sprouts.

Cook et al. had conducted several comprehensive studies examining a number of wash solutions, such as glycine buffer and phosphate-buffered saline (PBS), and washing techniques *(11-13)*. The studies showed a high recovery efficiencies of 75% when 1 M glycine (pH 5.5) in conjunction with pulsification method was used. Additional wash solutions used in *Cyclospora* and *Cryptosporidium* oocysts recovery have been reported. Shields et al. evaluated several wash solutions containing laboratory glassware detergents. From the assessment of three detergents and other wash solutions, 0.1% Alconox® showed higher recovery rates of 70-80%. The use of this wash solution has significantly increased the recovery rates for *Cyclospora* and *Cryptosporidium* from lettuce, basil, and raspberries *(59)*.

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CHAPTER 2

EFFICACY OF WASH SOLUTIONS ON *CYCLOSPORA CAYETANENSIS* AND *CRYPTOSPORIDIUM PARVUM* RECOVERY FROM BASIL[1]

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[1] Chandra, V., and Y. R. Ortega. To be submitted to Journal of Food Protection

Abstract

In this study, six wash solutions, sterile E-Pure water, 3% levulinic acid-3% sodium dodecyl sulfate, 1 M glycine, 0.1 M phosphate buffered saline, 0.1% Alconox®, 1% HCl-pepsin, were evaluated for their effectiveness in recovering *Cyclospora cayetanensis* and *Cryptosporidium parvum* from basil. One hundred and 1,000 oocysts of both parasites were inoculated on top surfaces of the basil leaves and stored for 1 h and 24 h. The samples were then hand washed for 1 min with each of the wash solutions. DNA was extracted from the washed samples and amplified using nested PCR for the detection of both parasites. All wash solutions could detect both parasites' oocysts at the concentration of 1,000 oocysts/25 g basil, with the recovery rates of 100%. At the concentration of 100 oocysts/25 g basil, the lowest recovery rate observed was 18.5%, and the highest was 70.4%. In this study, 1% HCl-pepsin showed a lower variability in recovering *Cyclospora* and *Cryptosporidium* oocysts in the 24 h post-inoculation time at the 100 oocysts level.

Introduction

Foodborne diseases are a major cause of illness and death in the United States. Over the decades, there has been an increase in the number of reported cases of foodborne illness linked to fresh produce. An epidemiologic investigation revealed that there were a total of 190 produce-associated outbreaks from 1973 through 1997, and the study newly recognized *Cyclospora* and *Escherichia coli* O157:H7 as causes of foodborne illness *(32)*. Parasitic foodborne diseases are now on the increase in parallel with the globalization of the food supply, increased consumption of fresh foods, and increased international travel. Scallan et al. estimated 9.4 million episodes of foodborne illnesses, in which 0.2 million of foodborne illnesses were caused by parasites. Scallan et al. also reports that parasites caused 9% hospitalizations and 25% deaths. *Toxoplasma* is one of the leading causes of hospitalization (8%) and one of the leading causes of deaths (24%) *(30)*.

Cyclospora cayetanensis and *Cryptosporidium parvum* are emerging human pathogens that have been associated with numerous outbreaks of diarrhea illness. Both are coccidian parasites that cause intracellular infections, primarily in the epithelial cells of the intestinal tract when infectious oocysts are ingested. While the oocyst of *Cyclospora* is not infectious when excreted as it requires time to sporulate, *Cryptosporidium* oocyst undergoes sporulation inside the host and is infectious when shed into the environment. Fecal-oral transmission of the oocysts has resulted in outbreaks through contamination of drinking water, food, and recreational water.

Numerous outbreaks associated with contaminated fruits (primarily raspberries) *(13, 14, 17)*, fresh produce (basil, mesclun, green onions, and lettuce) *(3, 4, 12, 19, 22)* or

water *(2, 11, 20, 24)* have been reported since 1990, with recurring cyclosporiasis outbreaks of Thai basil in 2001 *(15)* and snow peas in 2005 *(5)* and cryptosporidiosis outbreaks in 2003 and 2009 implicating not only unpasteurized milk and apple cider, but also salad bars and salad mixtures, and green onions *(1, 6).* According to the U.S. Food and Drug Administration (FDA) 2011 import alert, fresh produce, including basil, from Mexico has been refused for admission to the United States as it appeared to contain *Cyclospora (34).*

Several methods have been studied to remove oocysts from food products. Unlike bacteria, parasites are inert and do not multiply in the environment. Thus, isolation and detection methods are essential because there is no existing technique to enrich parasites. Detection of *Cyclospora* and *Cryptosporidium* oocysts in fresh produce can be performed with microscopy techniques or PCR. However, the efficacies of these methods are highly dependent on the recovery of oocysts from vegetables. Washing fresh produce with elution buffers has, therefore, been designed to remove oocysts. Typically, the fresh produce is washed with wash solutions of choice by manually agitating the sample for a period of time. The resulting washes are concentrated by centrifugation and further analyzed by microscopy or PCR.

The purpose of this study was to determine the effectiveness of several wash solutions on *Cyclospora* and *Cryptosporidium* recovery from basil. In this study, basil was selected because it has been implicated with numerous cyclosporiasis outbreaks.

Materials and Methods

Parasites

      Cyclospora cayetanensis oocysts were obtained from naturally infected individual from Peru, and *Cryptosporidium parvum* oocysts were obtained from infected calves and kindly provided by the Centers for Disease Control and Prevention (CDC).

Basil Inoculation

      Basil sprigs (Tom Pontano & Sons Farms, Vineland, New Jersey; Pontano Farms Inc., Boynton Beach, Florida; Rock Garden, Miami, Florida) were purchased from a local farmers' market in Atlanta, Georgia and stored at 4°C for no longer than 3 days prior to inoculation. Each sample contained 25 g basil leaves and was placed in a sterile stomacher bag (Labplas Inc., Ste-Julie, QC Canada). Basil leaves of each sample were spot inoculated droplets with 100 µl E-Pure water containing either 100 or 1,000 oocysts of *Cyclospora* and *Cryptosporidium*. This was done with caution to ensure that the droplets were set on the surfaces of the leaves and not on the bag itself. Samples were kept at room temperature (21±2°C) for approximately 1 h and 4°C for 24 h prior to recovery protocol. Three samples were inoculated for each wash solution. The experiments were repeated three times.

Wash Solutions

      Six wash solutions were used in this study: Sterile E-Pure water (ThermoScientific, Marietta, OH); 3% levulinic acid (Sigma, St. Louis, MO) plus 3% sodium dodecyl sulfate (SDS) (BioRad, Hercules, CA); 1 M glycine (Sigma), pH 5.5; 0.1 M phosphate buffered saline (PBS) (Sigma), pH 7.0; 0.1% Alconox® (Alconox Inc., White Plains, NY); 1% HCl (Fischer, Fairlawn, NJ), pH 2.0 plus pepsin (Fischer,

Nazareth, PA). PBS solution used in this experiment contained 0.14 M sodium chloride, 0.002 M potassium phosphate monobasic, 0.003 M potassium chloride, 0.01 M sodium phosphate dibasic, and E-Pure water. The ratio of 1% HCl plus pepsin used in this experiment was 1.6 g of pepsin in every 25 ml of 1% HCl.

Recovery of Oocysts

Inoculated samples were manually shaken in the bag for 15 s, hand-rubbed for 30 s, and shaken for another 15 s with 200 ml of the wash solutions, except for 1% HCl plus pepsin, which was 100 ml. Similar volumes of the wash solution was distributed into four 50-ml conical tubes. Stomacher bags containing basil leaves were squeezed by hand to remove the fluid as much as possible, and the bags were then discarded. Oocysts were concentrated by centrifugation (Beckman Coulter, Allegra[TM] 6 Centrifuge, Palo Alto, CA) for 15 min at $2,060 \times g$. The supernatant fluid was decanted leaving 3 to 4 ml in each tube. The resultant pellets from each tube were pooled and transferred to a clean 15-ml conical tube, followed by centrifugation under the same conditions described previously. The supernatant fluid was aspirated, and the final pellet was brought to 1 ml with sterile E-Pure water in a 1.5 ml microcentrifuge tube and stored at 4°C prior to DNA extraction.

For each wash solution, one negative control sample was included and treated as described above but without prior inoculation with oocysts.

DNA Extraction

DNA was extracted from samples using the FastDNA® Spin for Soil Kit (MP Biomedicals, Irvine, CA) with slight modifications to the manufacturer's protocols as follows: two hundred microliters of each sample was added to Lysing Matrix E Tube. Samples were processed in FastPrep® FP120 (Thermo Savant, Holbrook, NY) at speed

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setting 5.5 for 30 s. All samples were centrifuged (Eppendorf Centrifuge 5415 D, Eppendorf AG, Hamburg, Germany) at $15,700 \times g$ instead of $18,200 \times g$ as stated in the manufacturer's instructions. The final volume for the DNA was 80 µl, and it was stored at -20°C. Positive control (oocysts in suspension) was also extracted simultaneously.

PCR

A two-step nested PCR assay was used to amplify fragments of the 18S rRNA gene of *Cyclospora (18)* using a Mastercycler gradient thermal cycler (Eppendorf 5331, Westbury, NY). The template for the primary PCR was 1 µl of DNA extract for each sample. One microliter of this primary reaction was used for the secondary nested PCR with the internal primers. The internal primers yielded a product of about 500 bp. The amplification products were analyzed, along with the positive and negative controls, on 2% agarose gels (BioRad) containing ethidium bromide (Sigma). White/UV TMW-20 Transilluminator (Ultra Violet Products, Upland, CA) was used to visualize the gels. A 100 bp DNA ladder (Quick-Load®, New England Biolabs, Ipswich, MA) was included in all gels run to determine the size of the amplified products.

A nested PCR protocol was used to amplify fragments of the 18S rRNA gene of *Cryptosporidium* using primers described by Xiao et al. *(35)*. The template for the primary PCR was 1 µl of DNA extract of each sample. Two microliters of the primary reaction was used as the DNA template for the secondary nested PCR with the internal primers. The internal primers yielded a product of about 826 bp. The amplification products, along with the positive and negative controls, and the 100 bp DNA ladder were electrophoresed in 1.5% agarose gels (BioRad), containing ethidium bromide (Sigma), and visualized on a White/UV TMW-20 Transilluminator.

DNA Purification and Sequencing

Amplification products were purified using QIAquick® PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. The purified samples were then sent for sequencing (MacrogenUSA, Rockville, MD).

Statistical Analysis

Each wash solution had three inoculated samples. Each sample analysis was performed in triplicate during PCR amplification. All experiments were repeated three times. The PCR results for each wash solution were compiled in Microsoft Excel, and the percentage recovery was evaluated. Mean percentage recovery from three replications was determined for each wash solution. The significant effects between post-inoculation times and among wash solutions were analyzed using R (version 2.15.2) at 95% confidence interval.

Results and Discussion

In this study, efficacy of wash solutions on *Cyclospora* and *Cryptosporidium* recovery from basil was evaluated. DNA was extracted from the washed samples, amplified using nested PCR, and visualized with gel electrophoresis. A preliminary study involving six wash solutions with 100 and 1,000 oocysts of *Cyclospora* was performed. Data in Tables 2.1 and 2.2 indicate that recovery of oocysts using 3% levulinic acid (LA) plus 3% SDS and 0.1 M PBS, pH 7.0 were less than recovery using other wash solutions. The less effective wash solutions (LA-SDS and PBS) were not evaluated further in *Cryptosporidium* experiments. As a result, only four, E-Pure water, 1 M glycine, 0.1% Alconox®, and 1% HCl plus pepsin, were used in the following trials.

Another preliminary evaluation of two different techniques was performed to assess a more effective method in combination with the wash solutions used during the oocysts recovery. One technique utilized manually shaking the inoculated samples for 15 s, hand-rubbing for 30 s, and shaking for another 15 s. The other used a rocker platform, where the inoculated samples were gently agitated for 15 min, inverting the bag after 7.5 min. At the low concentration of 100 *Cyclospora* oocysts, the former method showed recovery rates ranging from 28-52%, whereas in the latter, the recovery rates ranged from 0-44%. The manually shaking method provided more friction on the basil surfaces, which could have possibly facilitated removal of oocysts. Therefore, manually shaking method was selected in this study.

Table 2.1 compares the recovery rates of 1,000 *Cyclospora* oocysts from inoculated basil that was stored for 1 h and 24 h prior to recovery. The recovery rates of 1 h post-inoculation time were consistently higher than those in 24 h. This could have been due to the shorter time for the oocysts to attach onto the basil surfaces, thus more oocysts were removed during the washing process. When the inoculated samples were washed with 1% HCl plus pepsin, the mean recovery rates were 100% in both 1 and 24 h post-inoculation time. Table 2.3 compares the recovery rates of 1,000 *Cryptosporidium* oocysts in 1 h and 24 h post-inoculation time. All wash solutions in both post-inoculation times resulted in 100% recovery, except 88.9% for glycine in the 1 h post-inoculation time. Since all wash solutions could detect both parasites' oocysts at the concentration of 1,000 oocysts/25 g basil and the results were similar for both 1 and 24 h post-inoculation time, only two replicates were performed.

Efficacy of wash solutions using 100 *Cyclospora* oocysts/25 g basil was further evaluated. PCR-positive results of each wash solution in 1 h post-inoculation time in each replicate are shown in Figure 2.1. In the first repetition, only 2 out of 9 readings were PCR positive when inoculated basil samples were washed with E-Pure water, glycine, and 1% HCl plus pepsin, 3 out of 9 readings with PBS, and none was PCR positive with 3% LA plus 3% SDS and 0.1% Alconox®. The large amount of foam produced by LA-SDS and Alconox® during the wash step may have interfered with the recovery of this small number of oocysts, resulting in absence of bands in the PCR readings.

In the following repetition, E-Pure water, glycine and 1% HCl plus pepsin showed a slight increase in the PCR-positive readings. Figure 2.2 illustrates the PCR-positive readings of each wash solution in 24 h post-inoculation time. Recovery rates of 100 *Cyclospora* oocysts were similar for both 1 and 24 h post-inoculation time (Table 2.2), with the highest recovery rate observed at 51.9%. There were no significant differences among the wash solutions when analyzed using R; all *p*-values were greater than 0.05. No *Cyclospora* oocysts were detected in any of the negative control samples.

Table 2.4 shows the recovery efficiencies when 100 *Cryptosporidium* oocysts inoculated samples were washed with E-Pure water, 1 M glycine, 0.1% Alconox®, and 1% HCl-pepsin. The mean percentages of oocysts recovered were slightly higher in 1 h post-inoculation time compared with 24 h, with 0.1% Alconox® resulted in 70.4%. However, no significant differences were observed between both post-inoculation time and among the wash solutions (*p*>0.05). Similar to those in *Cyclospora*, Figure 2.3 and 2.4 exhibit the PCR-positive *Cryptosporidium* readings of each wash solution in 1 and 24

h post-inoculation time, respectively. *Cryptosporidium* oocysts were not detected in any of the uninoculated control samples.

The mean percentages of oocysts recovery in this study were equal to the sum of the number of PCR-positive readings divided by the total possible number of PCR readings in three repetitions, which were 27 since each sample analysis was performed in triplicate during PCR amplification. At the concentration of 100 oocysts, the mean recovery rates ranged from 19-52% for *Cyclospora* and 33-70% for *Cryptosporidium*, with 1% HCl-pepsin and 0.1% Alconox® showing the highest recovery rates for *Cyclospora* and *Cryptosporidium*, respectively. Looking from another aspect, when a sample was considered to be positive if at least one PCR reading was positive (the total possible number of PCR readings in three repetitions would be 9), the mean recovery rates ranged from 50-100% for *Cyclospora* and 56-100% for *Cryptosporidium* at the concentration of 100 oocysts. 1% HCl-pepsin and 0.1% Alconox® also showed the highest recovery rates for *Cyclospora* and *Cryptosporidium*, respectively. Even though the mean recovery rates were higher in this case, the result was in agreement with the previous statement that 1% HCl-pepsin and 0.1% Alconox® had higher recovery rates at the concentration of 100 oocysts.

According to studies done by Robertson et al. *(28)*, between one and six *Cryptosporidium* oocysts per 100 g produce are detected. In our study, the nested PCR assays could detect low numbers of oocysts, ranging from 2 to less than 1 oocyst per 25 g basil leaves. Typical PCR amplification products for *Cyclospora* and *Cryptosporidium* are shown in Figures 2.5 and 2.6 respectively. Primers developed by Relman et al. *(25)* were initially used for *Cyclospora* detection in our trials. However, the secondary primers

were not specific and the targeted gene fragment did not amplify. Thus, primers used by Li et al. *(18)* were selected as an alternative. Our studies demonstrated that these primers were more sensitive than the ones by Relman et al. The secondary primers successfully amplified the targeted gene and yielded a product of about 500 bp. Some of the amplified PCR products were then sent for sequencing and confirmed that the amplification was in fact *Cyclospora cayetanensis*.

There have been a number of studies examining the ability of different wash solutions and techniques to recover *Cyclospora* and *Cryptosporidium* from foodstuffs *(7-9, 26, 27, 29)*. In a recent study, Shields et al. *(31)* evaluated the recovery rates of both parasites using several wash solutions containing laboratory glassware detergents; the food samples (fruit, herbs, and leafy greens) were inoculated with 10^3-10^6 *Cyclospora* or *Cryptosporidium* oocysts. From the assessment of three detergents and other wash solutions, Shields et al. reported high recovery rates of 70-80% when washed with 0.1% Alconox®. In our experiments, the recovery rates of 0.1% Alconox® when using 10^3 oocysts also showed similar results of 78-100% (Table 2.1 and 2.3).

As Shields et al. mentioned in the study, Alconox® contains sodium dodecylbenzenesulfonate ($C_{12}H_{25}C_6H_4SO_3Na$), a surfactant found in laundry detergents and tetrasodium pyrophosphate ($Na_4P_2O_7$), a food additive with emulsifying and dispersing properties. The mixture of two compounds appears useful in aiding the removal of oocysts from the surface. When fewer amount of oocysts were used in our studies, 0.1% Alconox® showed high variability in the recovery efficiencies. Although it worked well, the foam produced during the wash could have contributed to the loss of oocysts during the recovery process.

Data from this study indicates that the recovery efficiencies of *Cryptosporidium* oocysts/25 g basil were higher than those of *Cyclospora*. It is presumed that *Cyclospora* oocysts have some stickiness, thus the oocysts attached better on basil surfaces. Furthermore, the characteristics difference between *Cyclospora* and *Cryptosporidium* oocysts may have also contributed to the higher recovery rates of *Cryptosporidium* oocysts. The wall of *Cyclospora* oocyst is bilayered, and it has two sporocysts. Therefore, the sporozoites are protected by both the oocyst and sporocysts walls, whereas *Cryptosporidium* oocyst does not have sporocysts.

While there have been a number of studies examining the period of time between inoculating food and washing food, there is no standard time frame to use thus far. Cook et al. *(7-9)* and Robertson et al. *(26, 27, 29)* mentioned in their studies that the time between inoculation and washing were between one to three hours. In other study, Ortega et al. *(22)* waited 24 h before washing the inoculated lettuce leaves with distilled water. Our study utilized 1 and 24 h post-inoculation. Since no significant difference was observed between both post-inoculation times, further research is needed to determine the appropriate time frame to mimic the fresh produce/fruits contamination on the farm. The U.S. FDA suggests that the ideal time is 72 h post-inoculation time under refrigerated condition. Increasing the time frame for post-inoculation treatment could possibly allow better oocyst attachment to the food, thus any differences among the wash solutions could be determined as well.

Studies done with human volunteers revealed that *Cryptosporidium* has a low infectious dose of 10 to 30 oocysts *(10, 21)*. Although the infectious dose of *Cyclospora* has not been determined, it is assumed to be low as well *(33)*. Therefore, it is important to

be able to identify one oocyst during the investigation of outbreaks. Moreover, it is assumed that oocysts recoveries can vary, depending on factors, such as type of wash solutions used and different food matrices, as surface differences could influence the oocysts attachment. For example, raspberries have been associated in several outbreaks; the nature of the berries' surfaces aids in the oocysts adherence to the fruit. Raspberries have crevices and fine hair-like projections that may enhance oocysts entrapment *(16, 23)*. Hence, it is important to determine wash solutions that are able to perform better in different food matrices.

Although data from this study indicates that no significant differences were observed among the wash solutions, if used under certain circumstances, one wash solution might perform more effectively than the others. For example, in the case of oocysts recovery from foodstuffs which contain fat or lipid, such as cakes, wash solution that has properties of a detergent (i.e. Alconox®) might be more practical to use in the removal of oocysts.

Acknowledgements

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Table 2.1 Recovery rates of 1,000 *Cyclospora cayetanensis* oocysts per 25 g basil using various wash solutions

Wash solution	Percentage oocysts recovery mean (±STD)[a]	
	1 h[b]	24 h[c]
E-Pure water	88.9 (±15.7)[d]	88.9 (±15.7)
3% levulinic acid-3%SDS	83.3 (±23.6)	72.2 (±7.9)
1 M glycine, pH 5.5	88.9 (±15.7)	94.4 (±7.9)
0.1 M PBS, pH 7	94.4 (±7.9)	66.7 (±47.1)
0.1% Alconox	94.4 (±7.9)	77.8 (±31.4)
1% HCl-pepsin	100.0 (±0.0)	100.0 (±0.0)

[a,d] (Sum of PCR-positive readings/total possible number of PCR readings in two replicates tests) × 100% = (16/18) × 100% = 88.9%

[b] Inoculated basil leaves were stored at 21°C for 1 h prior to recovery.

[c] Inoculated basil leaves were stored at 4°C for 24 h prior to recovery.

Table 2.2 Recovery rates of 100 *Cyclospora cayetanensis* oocysts per 25 g basil using various wash solutions

Wash solution	Percentage oocysts recovery mean (±STD)[a]	
	1 h[b]	24 h[c]
E-Pure water	37.0 (±17.0)[d]	37.0 (±17.0)
3% levulinic acid-3%SDS	22.2 (±31.4)[e]	27.8 (±7.9)[g]
1 M glycine, pH 5.5	40.7 (±17.0)	44.4 (±22.2)
0.1 M PBS, pH 7	22.2 (±15.7)[f]	50.0 (±23.6)[h]
0.1% Alconox	18.5 (±17.0)	37.0 (±12.8)
1% HCl-pepsin	40.7 (±17.0)	51.9 (±6.4)

[a,d] (Sum of PCR-positive readings/total possible number of PCR readings in three replicates tests) $\times$ 100% = (10/27) $\times$ 100% = 37.0%

[b] Inoculated basil leaves were stored at 21°C for 1 h prior to recovery.

[c] Inoculated basil leaves were stored at 4°C for 24 h prior to recovery.

[e-h] Values are the means of two replicates tests

Table 2.3 Recovery rates of 1,000 *Cryptosporidium parvum* oocysts per 25 g basil using various wash solutions

Wash solution	Percentage oocysts recovery mean (±STD)[a]	
	1 h[b]	24 h[c]
E-Pure water	100.0 (±0.0)[d]	100.0 (±0.0)
1 M glycine, pH 5.5	88.9 (±15.7)	100.0 (±0.0)
0.1% Alconox	100.0 (±0.0)	100.0 (±0.0)
1% HCl-pepsin	100.0 (±0.0)	100.0 (±0.0)

[a,d] (Sum of PCR-positive readings/total possible number of PCR readings in two replicates tests) × 100% = (18/18) × 100% = 100.0%

[b] Inoculated basil leaves were stored at 21°C for 1 h prior to recovery.

[c] Inoculated basil leaves were stored at 4°C for 24 h prior to recovery.

Table 2.4 Recovery rates of 100 *Cryptosporidium parvum* oocysts per 25 g basil using various wash solutions

Wash solution	Percentage oocysts recovery mean (±STD)[a]	
	1 h[b]	24 h[c]
E-Pure water	55.6 (±29.4)[d]	59.3 (±12.8)
1 M glycine, pH 5.5	51.9 (±6.4)	33.3 (±11.1)
0.1% Alconox	70.4 (±28.0)	51.9(±35.7)
1% HCl-Pepsin	59.3 (±23.1)	55.6 (±19.2)

[a,d] (Sum of PCR-positive readings/total possible number of PCR readings in three replicates tests) × 100% = (15/27) × 100% = 55.6%

[b] Inoculated basil leaves were stored at 21°C for 1 h prior to recovery.

[c] Inoculated basil leaves were stored at 4°C for 24 h prior to recovery.
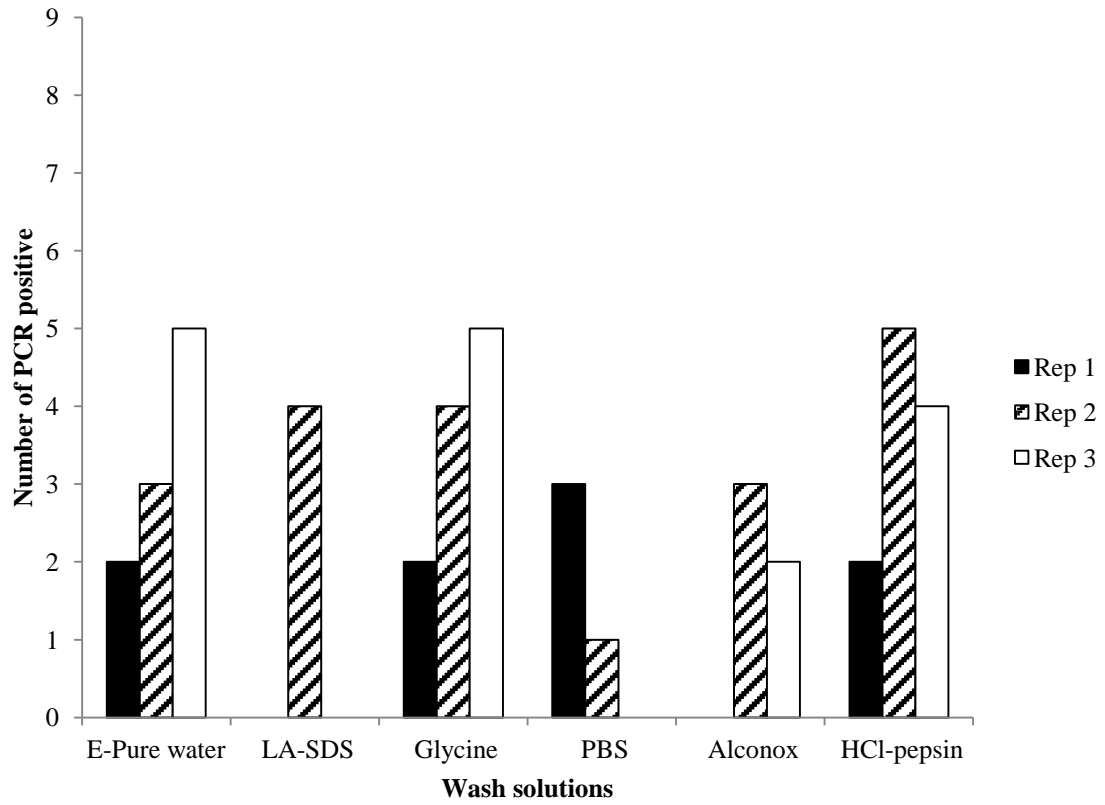
Figure 2.1.   PCR-positive readings of 100 *Cyclospora cayetanensis* oocysts per 25 g

basil in 1 h inoculation time using various wash solutions; n = 9 in each
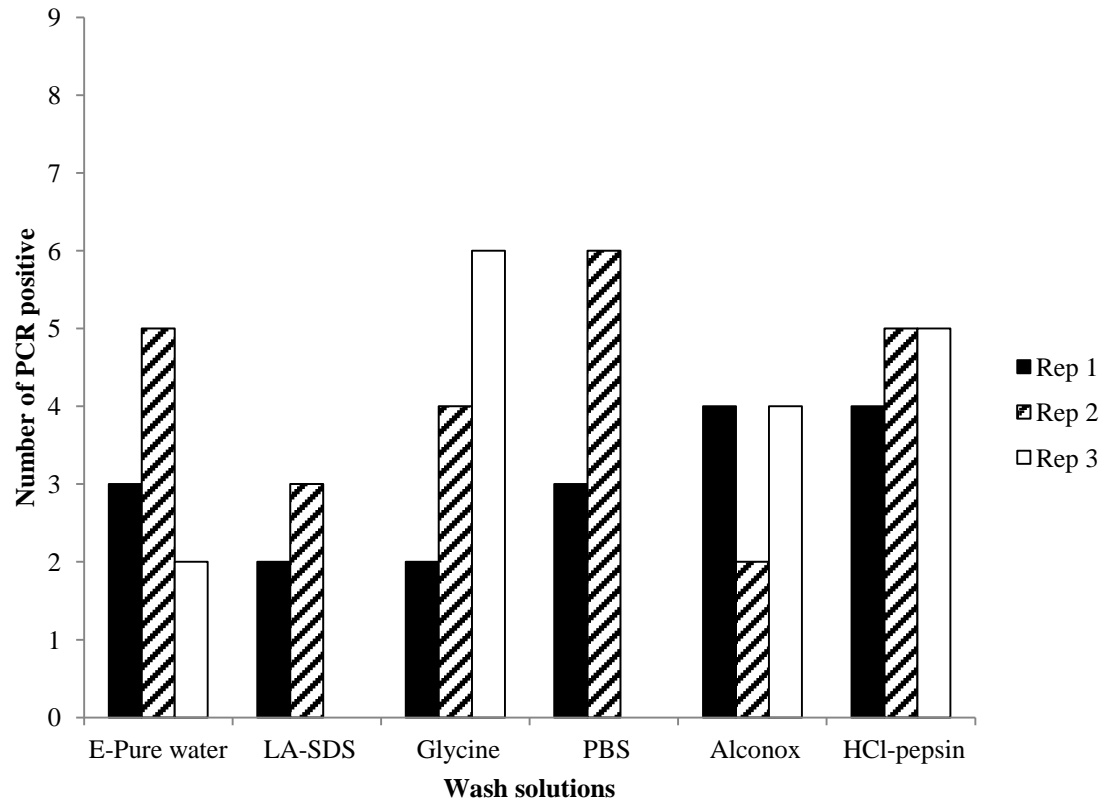
repetition.

Figure 2.2. PCR-positive readings of 100 *Cyclospora cayetanensis* oocysts per 25 g

basil in 24 h inoculation time using various wash solutions; n = 9 in each
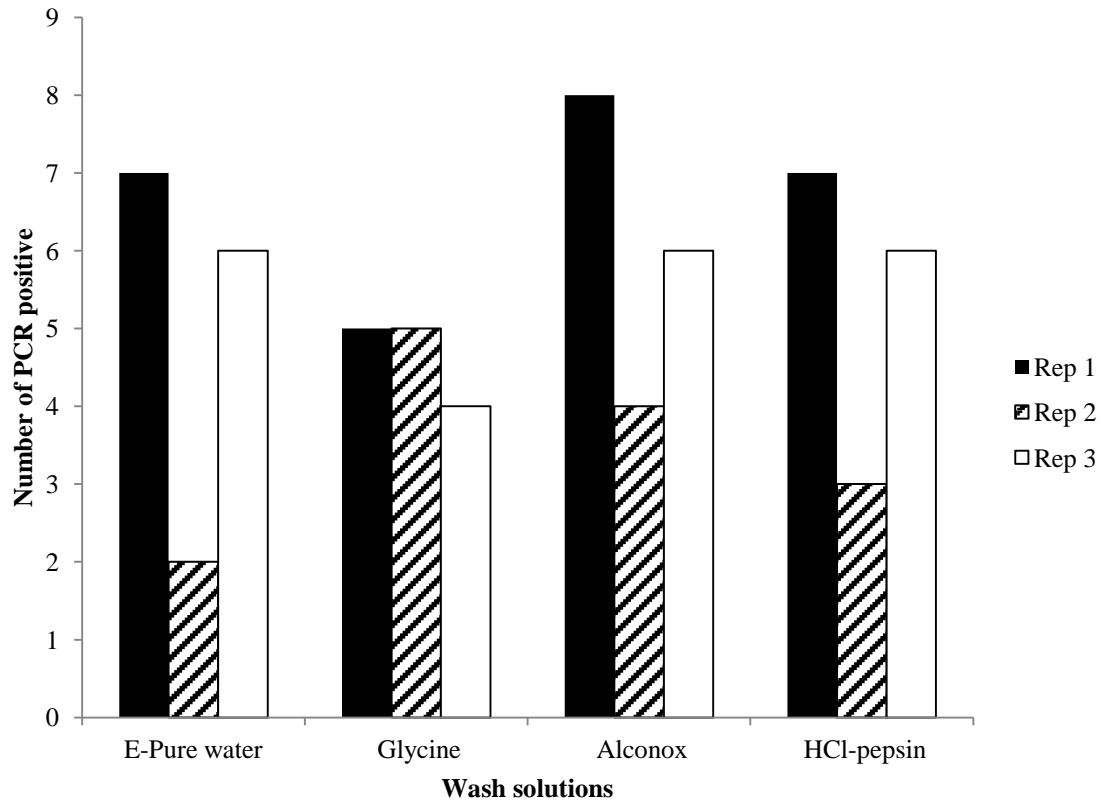
repetition.

Figure 2.3.   PCR-positive readings of 100 *Cryptosporidium parvum* oocysts per 25 g
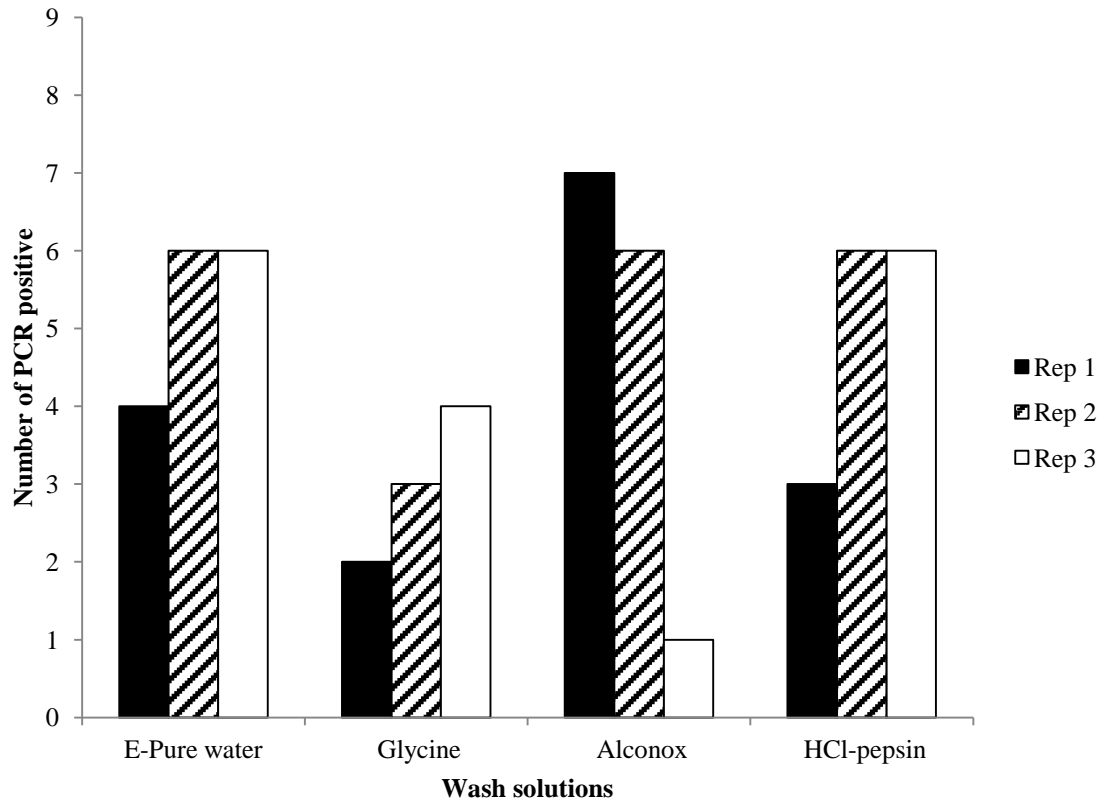basil in 1 h inoculation time using various wash solutions; n = 9 in each
repetition.

Figure 2.4. PCR-positive readings of 100 *Cryptosporidium parvum* oocysts per 25 g

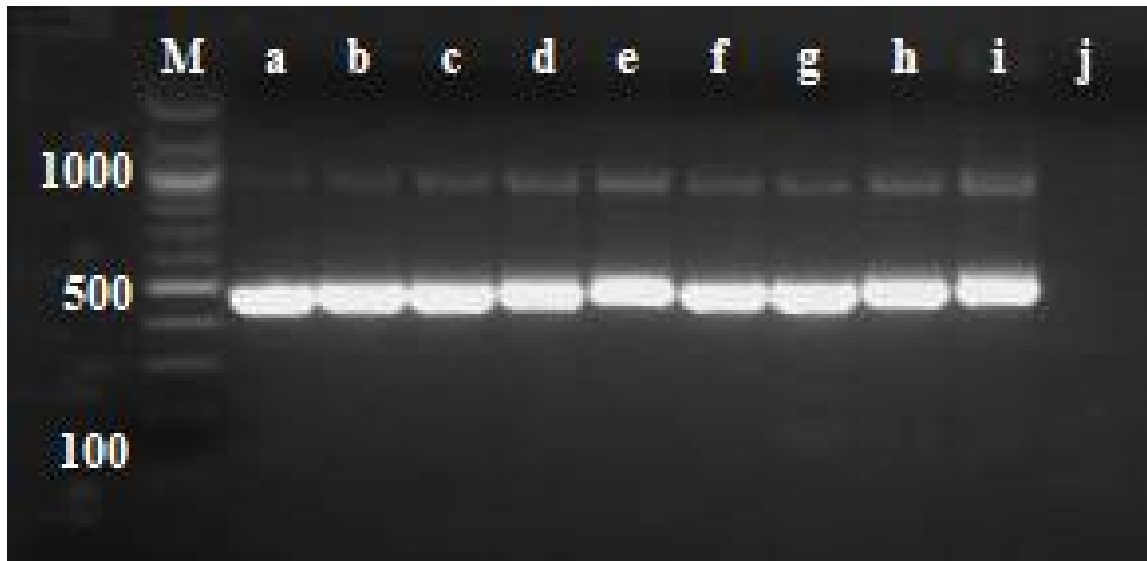basil in 24 h inoculation time using various wash solutions; n = 9 in each

repetition.

Figure 2.5. PCR amplification of 1,000 *Cyclospora cayetanensis* oocysts per 25 g basil
(equivalent to 2 oocysts). *Lane M*: 100 bp DNA ladder, *lanes a-b*: E-Pure
water, *lanes c-d*: 1 M glycine, *lanes e-f*: 0.1% Alconox, *lanes g-h*: 1% HCl-
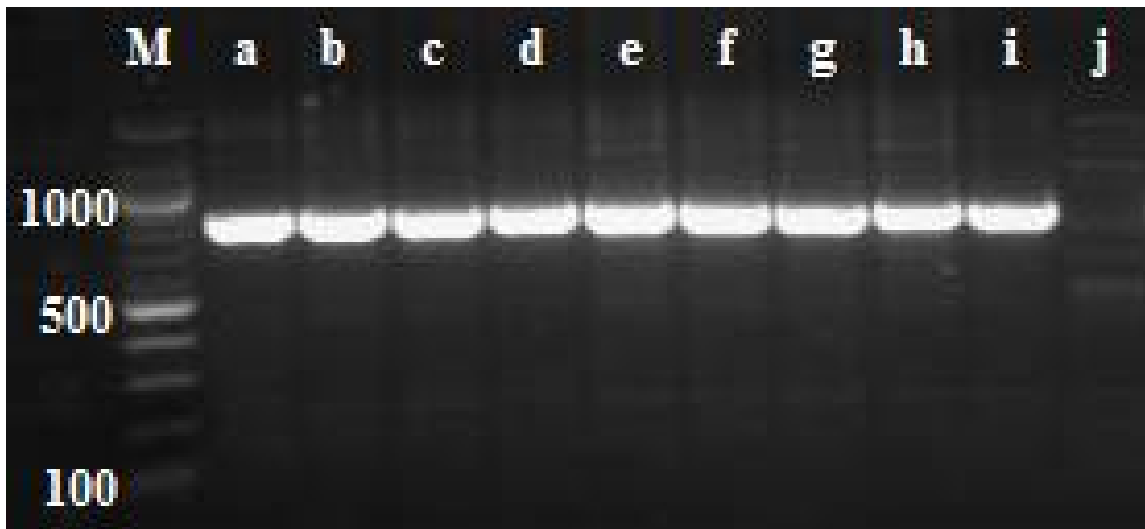pepsin, *lane i*: positive control, *lane j*: negative control

Figure 2.6. PCR amplification of 1,000 *Cryptosporidium parvum* oocysts per 25 g basil

(equivalent to 2 oocysts). *Lane M*: 100 bp DNA ladder, *lanes a-b*: E-Pure

water, *lanes c-d*: 1 M glycine, *lanes e-f*: 0.1% Alconox, *lanes g-h*: 1% HCl-

pepsin, *lane i*: positive control, *lane j*: negative control

CHAPTER 3

CONCLUSION

With the emerging parasitic foodborne illnesses related to fresh produce, an efficient elution method to isolate oocysts from the suspected food is crucial. Since *Cyclospora cayetanensis* and *Cryptosporidium parvum* have low infectious doses and enrichment procedures for these parasites are not available, it is important to maximize the recovery. This study showed that 1% HCl plus pepsin had a lower variability in both 100 *Cyclospora* and *Cryptosporidium* oocysts recovery in the 24 h post-inoculation time, and the nested PCR assays could detect low numbers of oocysts, ranging from 2 to less than 1 oocyst per 25 g basil leaves. The findings of this study can be used to improve the sensitivity of foodborne parasite detection. More studies are needed to determine the recovery efficiencies of both pathogens using other food matrices, such as raspberries, and extended post-inoculation periods prior to recovery.