EFFECTS OF PESTICIDES AND TEMPERATURE ON SPORULATION AND VIABILITY OF *CYCLOSPORA CAYETANENSIS*

by

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(Under the direction of YNES ORTEGA)

ABSTRACT

Outbreaks of *Cyclospora cayetanensis* had been associated with raspberries, basil and lettuce. The leading hypotheses in the raspberry associated outbreaks were that fungicidal spray water used on raspberry fields was contaminated with the parasite. Three different fungicides including captan, benomyl and zineb and two different insecticides including diazinon and malathion were tested on *C. cayetanensis* at different dilutions and exposure time. Sporulation was observed at all exposure times starting from the lowest of 30 minutes to highest of 1 week from low to high concentrations of pesticides. A reduction in sporulation was observed only with benomyl. At one week benomyl decreased sporulation at the highest concentration of fungicide (0.03 g/10mL i.e. 2.5 lb/100gal). The temperature of exposure and the length of time affected sporulation of *Cyclospora cayetanensis* on food substrates like basil and dairy produce. In dairy products as food substrate e.g., milk, diluted milk and whipped cream, sporulation was not observed at -15°C for 24 hrs. With basil as a food substrate, sporulation was observed until 48 hrs at -20°C, 1 hr at 50°C and 4 days at 37°C, however no sporulation occurred at temperatures of -70°C, 70°C and 100°C. Temperature studies on oocysts in water showed similar results except that basil protected oocysts at freezing and heating temperatures more than water, thereby having higher sporulation percentages.

INDEX WORDS: *Cyclospora cayetanensis*, pesticides, temperature, basil, raspberries.

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DEDICATION

To Karthick, Mom and Dad. Thanks for supporting me and encouraging me all through out the way.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Cyclospora cayetanensis is a coccidian parasite that has just begun to emerge and has been a cause of numerous food borne outbreaks in the 1990s. The parasite *Cyclospora* is widely distributed throughout the world and infection with it is associated with prolonged watery diarrhea in travelers to endemic countries (Soave, 1996).

Eimer first described Cyclosporan organisms in 1870 and the genus *Cyclospora* has been named by Schneider in 1881 (Ortega, Gilman, and Sterling, 1994). It was first noted in the intestines of moles in 1881 and was identified as a cause of human infection in 1977 in Papua, New Guinea (Ashford, 1979). Ashford provided the first description of human-associated Cyclosporan oocysts, based on detection in stool specimens collected in 1977, from individuals living in Papua New Guinea. The unfamiliar and nonspecific features of the parasite led to confusion in classification suggesting it may be an *Isospora.* Beginning in mid 1980s, *Cyclospora* oocysts were given different names by different researchers.

Popularization of acid-fast staining technique, in early 1980s used for detecting *Cryptosporidium* in stool specimens, set the stage for recognition of the *Cyclospora* species. There were various reports of diarrheal illness in immunocompromised and immunocompetent children and adults from 1986 to 1993, which were linked to large Cyanobacterium-like bodies (Ashford, 1979 and Bendall et al., 1993). The newly identified parasite had a prokaryotic appearance and was also felt to resemble blue-green algae or Cyanobacterium and the internal organelles autofluoresced under ultraviolet epifluorescence resembling the photosynthesizing organelles of the blue-green algae. However it did not have all the characteristics of algae, so it was referred to as a "Cyanobacteria-like body" (Long et al., 1991). Definitive identification of the organism as a coccidian was made in 1993, when Ortega et al. succeeded in inducing the organism to sporulate and demonstrated that there were two sporocysts inside the oocysts, each having two sporozoites. Thus the organism was documented as a member of the genus

Cyclospora (Ortega et al., 1993). The investigators also provided additional evidence in support of this taxonomic designation by showing it had the characteristics of Apicomplexan coccidia (Ortega, Gilman, and Sterling, 1994). The researchers proposed the name " *Cyclospora cayetanensis*" after the university in Lima, Universidad Peruana Cayetano Heredia, where this research had been conducted. Relman et al. provided molecular evidence that this organism is a coccidian by using Polymerase Chain Reaction (PCR) analysis. It was demonstrated that *Cyclospora cayetanensis* was phylogenetically related to species of the coccidian genus *Eimeria* (Relman et al., 1996).

Biology of *Cyclospora*. *Cyclospora* is in the subclass Coccidiasina and in the sub-phylum Apicomplexa. It is related to four other coccidian parasite in humans: *Cryptosporidium*, *Isospora*, *Sarcocystis* and *Toxoplasma* (Nunez Fernandez, Galvez, and Finlay Villalvilla, 1995; Soave, 1996). Coccidian species can be differentiated by their shape, size and morphology (Soave, Herwaldt, and Relman 1998; Marshall et al., 1997). *Cyclospora* oocysts are spherical and are 8-10 µm in diameter. *Cyclospora* appears as nonrefractile spheres that contain a cluster of refractile membrane bound globules (Connor, 1997; Bern et al., 2002 and Ortega et al., 1993). After sporulation the entire globule divides into two ovoid structures which are called sporocysts, each containing two sporozoites and each sporozoite measuring 1.2-9.0 microns (Mota, Rauch, and Edberg, 2000 and Ortega et al., 1993). Fourteen species of *Cyclospora* have been identified, however the oocysts isolated from moles, snakes, rodents and myriapods are ovoid and slightly larger than the spherical forms found in humans (Mohamed and Molyneux, 1990 and Ortega, Gilman, and Sterling, 1994). The host specificity of *Cyclospora cayetanensis* seems to be exclusively for humans. No other animal species have been identified with this coccidian.

The life cycle of *Cyclospora* in the human host has not been fully characterized but it does appear that it undergoes all the life cycle stages except sporulation in the human host

(Figure 1.1). Various forms of the asexual stages have been identified in human intestinal epithelial cells by various researchers (Sifuentes-Osornio et al., 1995; Sun et al., 1996 and Nhieu et al., 1996). The gametocyte stage which is part of the sexual multiplication was also found in the tissue sections (Ortega et al., 1997 and Sun et al., 1996). Like *Isospora*, human *Cyclospora* oocysts also require time outside the host to sporulate therefore person-person transmission is not possible unlike that of *Cryptosporidium.* The amount of time it requires to sporulate in nature is unknown but under laboratory conditions, sporulation occurs after 5-11 day incubation in either distilled water or in 2.5% potassium dichromate at temperatures of 25 to 32° C (Ortega et al., 1993).

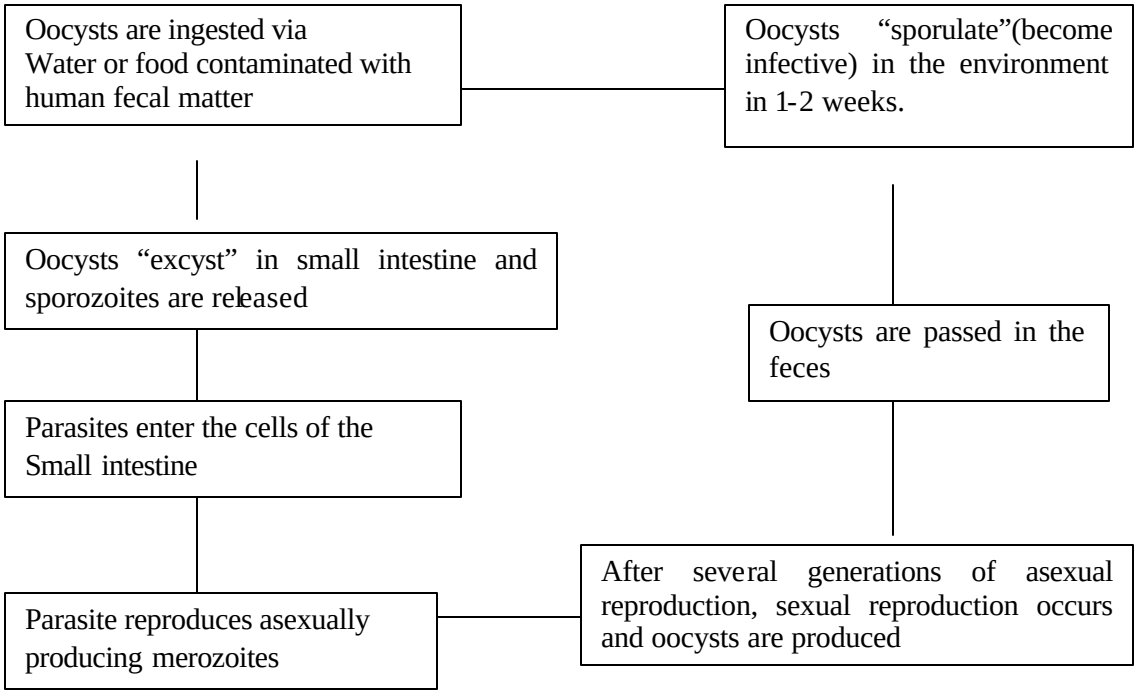

Figure 1.1. Life cycle of *Cyclospora cayetanensis.*

Diagnosis and detection. The *Cyclospora* oocysts can be diagnosed based on microscopic observations of fecal samples or in biopsy specimens. Clinical laboratories do not naturally examine for presence of *Cyclospora* oocysts unless it is specifically requested. *Cyclospora* oocysts are easily overlooked, because they are usually excreted in low numbers thereby necessitating concentrating the stool specimens before detection. *Cyclospora* oocysts are acid-fast, thus simple inexpensive staining techniques like Ziehl-Neelsen are commonly used. The use of acid-fast staining for parasitologic application dates back to a report published in 1981 about its use for identification of *Cryptosporidium* (Henriksen and Pohlenz, 1981). *Cyclospora* demonstrates great variability to acid-fast staining technique and may appear unstained, pink or red. Care should be taken to measure the oocysts as they could be easily confused with *Cryptosporidium (Cyclospora* is 8-10µm – twice the size of *Cryptosporidium*). In a recent study with six different staining techniques, it was found that uniform and enhanced staining of *Cyclospora* could be achieved with a modified safranin technique applied to microwave-heated fecal smears (Visvesvara et al., 1997). *Cyclospora* could be detected in the tissues by histological examination of tissues stained with hematoxylin and eosin (Nhieu et al., 1996). The autofluorescing property of *Cyclospora* makes detection easier compared to staining techniques. They can be seen as neon blue circles under an ultraviolet fluorescence microscope fitted with a 365nm excitation filter. The oocysts autofluoresce green under ultraviolet epi-illumination using a 450-490 nm dichroic mirror excitation filter (Relman et al., 1996 and Ortega, Gilman, and Sterling, 1994). In a comparative study in Guatemala, it was observed that the estimated sensitivity of acid-fast staining versus fluorescence microscopy was 78% (Moreno-Camacho et al., 1998 and Berlin et al., 1998). Molecular techniques such as PCR could be used to detect the parasite DNA (Relman et al., 1996; Visvesvara et al., 1997; Eberhard, Pieniazek, and Arrowood, 1997; Orlandi and Lampel, 2000 and Pieniazek et al., 1996). These techniques are useful because it adds to specificity and sensitivity. Evaluation of nested PCR using specific primers for amplification demonstrated a sensitivity and specificity of 62% and 100% respectively (Garcia-

Lopez, Rodriguez-Tovar, and Medina-De la Garza, 1996). PCR has the potential to be more sensitive than microscopy but it does not differentiate live from dead *Cyclospora* oocysts and also the PCR primers used for amplification of *C. cayetanensis* have crossed-amplified other coccidians especially those belonging to genus *Eimeria* (Garcia-Lopez, Rodriguez-Tovar, and Medina-De la Garza, 1996). No monoclonal antibodies are currently available commercially.

Clinical manifestations. Clinical features of *C. cayetanensis* can present as acute, self-limiting illness lasting for few days, or can develop chronic diarrhea lasting over weeks. Diarrhea is usually watery and lacks blood or inflammatory cells and often occurs in cyclical patterns alternating with constipation (Soave, Herwaldt and Relman, 1998). The other symptoms include profound fatigue, anorexia, weight loss, abdominal bloating or gas, abdominal cramps, and nausea. Fever, chills, headache and vomiting are less common. The incubation period for *Cyclospora* infection ranges from 2 to 11 days (Hoge, Shlim, and Echeverria, 1993). A flu-like prodrome with myalgias and arthralgias may precede the onset of diarrhea. Although illness is self-limiting, it may be prolonged and may last for weeks (Wurtz 1994; Sifuentes-Osornio et al., 1995; Chiodini, 1994; Berlin et al., 1994). Diarrhea typically lasted longer in immunocompromised patients with a mean duration of 4 months (Hoge et al., 1995; Rabold et al., 1994; Madico et al., 1993 and Ortega et al., 1993). Among most of the immunocompromised hosts, *Cyclospora* infections have occurred primarily in HIV-infected individuals. Clinical illness in these patients are similar to that of cryptosporidiosis wherein the illness can be prolonged, severe and is associated with high rates of recurrence that can be attenuated with long-term suppressive therapy (Pape et al., 1994 and Sifuentes-Osornio et al., 1995). Abnormal xylose absorption has been documented in a small number of patients (Bendall et al., 1993 and Connor et al., 1993).

Treatment. Oral trimethoprim-sulfamethaxazole (TMP-SMX) is the only effective treatment for *Cyclospora* infection available until recently (Soave and Johnson, Jr., 1995 and Madico et al.,

1993). The first report studied five patients in Peru who were successfully treated with TMP-SMX (Madico et al., 1993). Wurtz (1994) reported that oral TMP-SMX attenuated symptoms in an AIDS patient with cyclosporiasis. The efficacy of the drug was also confirmed in an open-label study in Haiti in which HIV patients with cyclosporiasis were treated with TMP-SMX four times a day for 10 days. Recurrent *Cyclospora* infection was frequent, but responded to the second course of treatment. TMP-SMX prophylaxis three times a week was effective in preventing relapse in most patients (Pape et al., 1994). Hoge et al. (1995) conducted a double blind, placebo-controlled trail of TMP-SMX for *Cyclospora* infections among immunocompetent patients in Nepal. Symptoms cleared after 7 days of treatment in 94% of patient receiving TMP-SMX and 91% of patients in placebo group also cleared infection after a crossover treatment for 7 days with TMP-SMX. An alternate drug Ciprofloxacin could be used to cure patients suffering from *Cyclospora* infection (Verdier et al., 2000).

Geography and seasonality of *Cyclospora* outbreaks. *Cyclospora* infections appear to be most common among tropical and subtropical areas (Soave, 1996 and Soave, Herwaldt and Relman, 1998). Data from studies conducted in Nepal and Peru confirmed the seasonality of *Cyclospora* infection. In Nepal the incidence of infections is highest during the rainy and warm season from May to October and in Peru from April to June (Sherchand and Cross, 2001 and Ortega et al., 1993). Studies conducted by Bern et al. (1999) in Guatemala showed that the infection is most common in spring, peaks in June, and lasts until August. Most of the *Cyclospora* infection cases in developed countries have been observed in travelers to endemic areas or eating imported foods. Jelinek et al. (1997) had reported that 1% of 469 German travelers returning from developing countries were infected with *Cyclospora* and 3% were infected with *Cryptosporidium.* Laboratory reporting of *C. cayetanensis* started in England and Wales in 1993, since then 284 cases have been reported with most of the cases occurring in June and July. Recent foreign travel

had been reported in 61% of the cases. The countries most often implicated in descending order were Nepal, Indonesia, India, Turkey and the Dominican Republic (Cann et al., 2000).

Transmission and outbreaks. There have been numerous water-borne and food-borne outbreaks of *Cyclospora*. The oocysts when excreted are noninfectious. Infection occurs only when the sporulated oocysts are ingested either with contaminated food or water. Direct person-to-person transmission is unlikely (Soave et al., 1998). To date no animal hosts have been identified for *Cyclospora*. *Cyclospora* has been isolated (Garcia-Lopez, Rodriguez-Tovar, and Medina-De la Garza, 1996) from chickens, dogs, ducks and primates (Yai et al., 1997 and Smith et al., 1996). However a surveillance study conducted in Lima in 1995 with samples from 443 animals such as chicken, ducks, parrots, pigs, doves, rats, goats, dogs, and cats failed to prove that animals were reservoir for *C. cayetanensis* (Ortega et al., 1993). Studies done in Haiti obtained the same results (Eberhard et al., 1997). The *Cyclospora* isolated from monkeys are morphologically similar as the *C. cayetanensis* but molecularly different. Therefore 3 other species names were introduced based on the animal species they infect (Eberhard et al., 1997).

Waterborne outbreak. The first waterborne outbreak in the U.S occurred in 1990 in a physician's dormitory. The epidemiological investigation concluded that drinking tap water was associated to the cases of cyclosporiasis. Researchers reported that stagnant water in the bottom of the tank of either of 2 storage tanks presumably containing *C. cayetanensis* contaminated the water supply after a broken water pump was fixed (Huang et al., 1995).

In June 1994, a waterborne outbreak occurred among British expatriates in a military detachment in Pokhara, Nepal (Robald et al., 1994). The water supply was a mixture of river and municipal water, which was chlorinated and then kept in a sealed storage tank to be supplied to the homes in the camp. This water had been chlorinated. Chlorination of 1-3 ppm appeared to not inactivate *Cyclospora*. This outbreak suggested that water had to be either boiled

or filtered before consumption. Hoge et al. (1995) conducted a case-control study among travelers and expatriates at 2 outpatient clinics in Kathmandu, Nepal. Consumption of untreated water was identified as the risk factor and structures morphologically similar with *Cyclospora* oocysts were found in tap water from the home of a case patient who had drunk untreated tap water. Several isolated cases of cyclosporiasis were reported in United States and were associated with exposure to drinking water or recreational water or sewage (Ooi, Zimmerman and Needham, 1995 and Wurtz, 1994).

Foodborne outbreaks. Numerous foodborne outbreaks in the 1990s have been associated with *Cyclospora* in North America and Canada. *Cyclospora* outbreaks were associated with ingestion of several fresh fruits and vegetables such as raspberries, mesclun, basil etc, which in many instances were imported. For some of these outbreaks, the vehicle or source could not be exactly identified. Complication of these investigations included the fact that potentially implicated fresh produce was often used as garnishes or several types of produce were often served together.

In spring of 1995, two outbreaks were documented in the United States. The first was an outbreak at two social events in Florida and the second had been in a country club in New York. In Florida, the commonalities between two social events were raspberries imported from Guatemala and strawberries from California. Strawberries were later excluded and raspberries were considered as the probable source (Koumans et al., 1998). The outbreak in New York was thought to be related to water from coolers on the country clubs golf course. Re-evaluation of data suggested that the outbreak could be associated to a fruit; fresh raspberries of undetermined source were among the fruits served (Koumans et al., 1998). Other reports of cases in New York, Texas and Canada in the end of May were reported. The commonalities in the 55 event-related clusters implicated raspberries to be the most probable source of outbreak except one at which Guatemalan blackberries were served. Another multistate, multicluster outbreak occurred in April and May 1997 even after the Guatemalan Berry Commission instituted control measures on

the farms (Bern et al., 1999). The outbreak ended after exportation of Guatemalan raspberries was voluntarily suspended. The shipments were allowed to resume again in mid-August 1997. In the spring of 1998, FDA strictly prohibited importation of raspberries in to the United States but then Canada continued importation. The outcome was a multicluster outbreak in Ontario, Canada, thus continuing the pattern of springtime related outbreaks (CDC, 2000).

In September 1997 illness at an Inn in Virginia was associated with a fruit plate, which had raspberries, not imported from Guatemala. An outbreak in Georgia in mid-May 1998 was of importance in the sense that it occurred during the spring when the United States did not import raspberries from Guatemala. In 1999, the implicated vehicle for an outbreak in Canada was a dessert that included fresh Guatemalan blackberries, frozen Chilean raspberries and fresh U.S strawberries. The evidence that blackberries caused illness was suggestive but not conclusive (Health and Social Services Committee of the Regional Municipality of York, 2000).

Cyclospora outbreaks have also been associated with basil, mesclun and berries. In 1997, multiple outbreaks caused by mesclun occurred in Florida, including at least one outbreak that began in March and another that occurred in December. Mesclun was implicated in an outbreak in Tallahassee and trace back studies suggested these were from Peru (Herwaldt, 2000). The timing of the outbreak in April and in March was consistent with the seasonality of cyclosporiasis in Peru (Madico et al., 1997). Two outbreaks linked to fresh basil were also reported, one occurred from mid-June through mid-July in the Northern Virginia-Washington D.C (CDC, 1997 and Levy et al., 1998) and another occurred in late July 1999 in Missouri (Lopez et al., 2001). In the 1997 outbreaks, the source of contamination was pesto sauce and the manner by which it became contaminated was not known. Two possible ways of contamination was put forth. Either the gourmet food store, which received 10% of the distributor's basil, had poor food handling practices (e.g. sub optimal refrigeration), or the contamination had occurred during harvesting. In the latter it was possible that oocysts had sufficient time to sporulate

because the interval from when the basil was handled until it was eaten, averaged at least several days (Lopez et al., 2001). The mechanisms of disinfections and inactivation for *Cyclospora* are not known. This may be due to the lack of an animal model where the parasite is propagated. All research related to *Cyclospora* has to be done with the oocysts obtained from naturally infected individuals.

Cryptosporidium parvum. *Cryptosporidium parvum* belongs to the sub-Phylum Apicomplexa, to the sub-Class Coccidiasina, and the Family Cryptosporidiidae. It is an obligate enteric coccidian parasite that infects the gastrointestinal tract and is one of the most important enteric pathogens in both humans and animals. Within the genus *Cryptosporidium*, there are currently 10 recognized species: *Cryptosporidium baileyi* and *C. meleagridis* found in birds, *C. felis* in cats, *C. muris* found predominantly in mice, *C. wrairi* in guinea pigs, *C. andersoni* in cattle, *C. nasorum* found in fish, *C. serpentis* found in reptiles, *C. parvum* found in humans and *C.saurophilum* in skunk (Fayer et al., 2000). *Cryptosporidium* has been first diagnosed in humans in 1976. They had been well recognized as the cause of watery diarrhea lasting several days to a week (Dubey et al., 1990). *C. parvum* infections could be found on the brush border membrane of the enterocytes of the small bowel, from the ileum to the colon and had been reported on a number of different organs and tissues (Dubey et al., 1990). The sexual and asexual replication occurs in the host intestine and already infectious oocysts are shed in the feces (Current and Garcia, 1991). *C. parvum* oocysts vary in the size range of 4-6µm in diameter and contain four slender, fusiform sporozoites that escape after excystation.

Effects of commercial disinfectants on *Cryptosporidium* oocysts viability have been investigated. Commercial disinfectants and chlorination at normal drinking water treatment levels are not effective (Fayer and Ungar, 1986). A study conducted by Fayer in 1995 showed that, 3% chlorine as sodium hypochlorite for up to 18 h did not affect the viability and oocysts

kept in undiluted Clorox ® bleach (5.25% aqueous sodium hypochlorite) for up to 2 hrs still initiated infection in neonatal BALB/ c mice. Disinfection by ozone treatment and UV irradiation seemed to be effective in inactivating the oocysts of *Cryptosporidium* (Korich et al., 1990 and Smith et al., 1993). In one of the study conducted by Korich et al. (1990), long- term exposure of *Cryptosporidium* to 70-100% bleach, 10% formalin or 5-10% ammonia was able to completely eliminate infectivity. Campbell et al. (1982) determined that, oocysts treated with ammonia (5%) and formol saline (10%) for 18 hrs was infectious to pathogen-free mice. Robertson et al. (1992) investigated the effect of water treatment processes on the viability of oocysts. Lime, ferric sulphate and alum inactivated oocysts because of the high alkalinity or acidity; however when pH, contact time and concentration were corrected to those used in water treatment plants, the compounds had no significant impact on the oocyst viability.

Effect of environmental conditions on oocyst viability has also been studied (Robertson, Campbell, and Smith, 1992). Desiccation is 100% lethal after drying for 1- 4 days at ambient temperatures (Heuschele et al., 1986). Robertson et al. (1992) demonstrated that snap-freezing oocysts in liquid-nitrogen caused 100% death, whereas oocysts survived slow freezing at -22° C. In another study as demonstrated with neonate mice, oocysts were still infectious after 7 days at -10° C, 24 h at -15° C, and 5-8 h at -20° C, but became non-infectious after 7 days at -15° C, 24 h at -20° C or 1 h at -70° C (Fayer and Nerad, 1996).

Heat inactivation studies conducted by Anderson (1985) concluded that oocysts of *Cryptosporidium* spp. in calf feces and ileal scrapings were rendered non-infective to mice when warmed from 9-55° C over a period of 15-20 min exposure or when held at 45° C for 5 min (Anderson, 1985). According to Blewett (1988), just 5 min exposure at 50-55° C was lethal to oocysts. Fayer (1994) demonstrated that, when water containing *C. parvum* oocysts reached temperature of 72.4° C or higher within 1 min or when the temperature was held at 64.2° C or

higher for 2 min of a 5-min heating cycle, infectivity was lost. Fujino et al. (2002) observed that, the infectivity of *Cryptosporidium* oocysts to mice and chickens was lost by heating to 55° C for 30 sec, 60° C for 15 sec and 70° C for 5 sec. This difference in the result is may be due to the different procedures used in the determination of infectivity.

Waterborne and food borne outbreaks of *Cryptosporidium*. Waterborne outbreaks of cryptosporidiosis have been documented in countries around the world. Between the years of 1986 to 1996, 16 cryptosporidiosis outbreaks in drinking water were reported in Europe with the majority in England and Wales, and 14 in North America with the majority in the USA. According to Smith et al. (1998) and Kramer et al. (2001), rainfall was associated to outbreaks related to drinking water and fecal accidents in recreational water (Smith, 1998; Kramer et al., 1998 and Levy et al., 1998). In the U.S there have been 10 drinking water outbreaks of cryptosporidiosis documented from 1984 to 1996. In Milwaukee in 1993, there was a well-publicized outbreak, wherein about 419,000 people suffered from watery diarrhea and it was the largest waterborne outbreak ever recorded in United States (Mackenzie et al., 1995). In Canada from 1993 to 1996, four drinking water outbreaks occurred and around 31,900 people were affected. Many of the outbreaks were due to surface water contamination with human sewage discharge and runoff, which occurred during heavy rainfalls (Curriero et al., 2001). The first recreational outbreak of cryptosporidiosis occurred in 1988 in Los Angeles (CDC, 2000; CDC, 2001 and Kaye, 2001). The outbreaks were mostly linked to swimming pools, water fountains, water parks, etc.

The reservoirs and transmission routes of *Cryptosporidium* spp. suggested a risk for human infections through contaminated food. Young farm animals such as calves, lambs, goats and swine had been commonly infected with *C. parvum* (Tzipori et al., 1983). Bovine cryptosporidiosis is prevalent worldwide. The high prevalence of *C. parvum* in beef and dairy

farms increased the awareness of the food industry that milk, meat and other animal products, contaminated by feces, may be potential routes of transmission. Outbreaks associated to contaminated food, especially unpasteurised milk, fresh sausage, and salad have been reported (Moore et al., 1993 and Laberge, Griffiths and Griffiths, 1996). Contaminated hand-pressed cider was the vehicle in a large foodborne outbreak that occurred in 1993 at a county fair in Maine (Millard et al., 1994).

Fungicides and insecticides. The outbreaks of *Cyclospora* in the U.S associated to possibly contaminated raspberries were thought to be due to contamination of pesticidal spray water. The common diseases caused in raspberries by fungi are anthracnose, botrytis fruit rot, cane blight, crumbly berry, orange rust, powdery mildew, raspberry leaf spot and spur blight. The insects causing disease in raspberries are cane borers, Japanese beetles, potato leafhopper, raspberry aphid, raspberry crown borer, sap bettle tarnished plant bug, yellow jackets etc. The most common insecticides used are malathion and diazinon. The commonly used fungicides on raspberries were captan, benomyl and zineb (Markle et al., 1998 and Pritts et al., 1989).

The effects of the fungicides benomyl, captan and chlorothalonil on soil microbial activity have been tested. These three fungicides had some inhibitory effects on soil microbial activity and the soil dehydrogenase activity was increased by the three fungicide treatments. Fungicides could inhibit or stimulate certain groups of microorganisms in the soil. The extent of the influence of fungicides on soil microorganisms depends on many factors. Some of the major factors included physical, chemical and biochemical properties of the soil and the nature and concentration of fungicide (Chen et al., 2001). Blank et al. (2000) observed that food poisoning organisms thrived in crop sprays. Addition of strains of *Shigella*, *Salmonella*, *Listeria* and *Escherichia coli* 0157:H7 to the formulations of dozens of commonly used herbicides, fungicides and insecticides showed that, bacteria thrived in approximately a third of the pesticides, growing

best in the fungicide chlorothalonil, the weed killer linuron and the insecticide permethrin and chlorpyrifos. *Salmonella*, *E.coli* and *Shigella* grew best in chlorothalonil (Blank et al., 2000).

Captan. Captan is a non-systemic fungicide used to control diseases of many fruits, ornamental and vegetable crops. Captan is rapidly degraded in near neutral water. Half-lives of 23 to 54 hours and one to seven hours have been reported under various acidities and temperatures (USDA handbook No.661). The effective residual life in water is two weeks (Chemical information systems, inc, 1988). The solubility of captan is 5.1 mg/L and melting point is 178ºC. The mouse LD50 is 7,000 mg/kg. The lowest dose for humans that can cause death is 1,071 mg/kg (National Library of Medicine, 1987).

Benomyl. Benomyl is a systemic, benzimidazole fungicide that is selectively toxic to microorganisms and to invertebrates, especially earthworms. It is used against a wide range of fungal diseases of field crops, fruits, nuts, ornamentals, mushrooms, and turf. Formulations include wettable powder, dry flowable powder, and dispersible granules. Benomyl completely degrades to carbendazim within several hours in acidic or neutral water. The half-life of carbendazim is 2 months (Kidd et al., 1991). Since benomyl is a systemic fungicide, plants absorb it. Once it is in the plant, it accumulates in veins and at the leaf margins. The metabolite carbendazim seems to be the fungicidally active agent. Benomyl residues are quite stable; with 48 to 97% remaining as the parent compound 21 to 23 days after application (USDA, 1984). Water solubility is 2 mg/L.

Diazinon. Diazinon is a nonsystemic organophosphate insecticide used to control scavenger yellow jackets in the western U.S. It is used in home gardens and farms to control a wide variety of sucking and leaf eating insects. It is used on rice, fruit trees, sugarcane, corn, tobacco, and potatoes as well as on horticultural plants. The breakdown rate is dependent on the acidity of water. At highly acidic levels, one half of the compound disappeared within 12 hours, while in a

neutral solution the pesticide took 6 months to degrade to one half of the original concentration (Howard et al., 1991). In plants, low temperatures and high oil content tend to increase the persistence of diazinon. Generally the half-life is short in leafy vegetables, forage crops and grass. The range is from 2 to 14 days (Bartsch, 1974). Water solubility is 48mg/L at 20°C (Kidd et al., 1991).

***Malathion*.** Malathion is a nonsystemic, wide-spectrum organophosphate insecticide. It was one of the earliest organophosphate insecticides developed (introduced in 1950). Malathion is suited for the control of sucking and chewing insects on fruits and vegetables. In raw river water, the half-life is less than 1 week, whereas malathion remained stable in distilled water for 3 weeks (Howard PH et al., 1991). The breakdown products in water are mono and dicarboxylic acids. The water solubility is 130 mg/L (Kidd et al., 1991).

***Zineb*: (ethylene (bis) dithiocarbamate pesticides (EDBCs)).** The EBDCs are dithiocarbamate fungicides used to prevent crop damage in the field and to protect harvested crops from deterioration during storage or transport. Zineb is used to protect fruit and vegetable crops from a wide range of foliar and other diseases. It is available in the U.S. as wettable powder and dust formulations. Zineb is poorly soluble in water, unstable and hydrolyzes rapidly, producing ethylene thiourea (ETU) and other compounds (Howard, 1989; Kidd et al., 1991). The water solubility is 10 mg/L at 25°C.

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CHAPTER 2

EFFECTS OF PESTICIDES ON SPORULATION AND VIABILITY OF *CYCLOSPORA CAYETANENSIS* AND *CRYPTOSPORIDIUM PARVUM* [1]

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EFFECTS OF PESTICIDES ON SPORULATION AND VIABILITY OF *CYCLOSPORA CAYETANENSIS* AND *CRYPTOSPORIDIUM PARVUM*

ABSTRACT

Foodborne outbreaks caused by *Cyclospora cayetanensis* in raspberries have been linked with usage of raspberries contaminated through the exposure to water containing human feces, specifically when berries were sprayed with insecticides and fungicides. Three different fungicides captan 50% W.P, benomyl 50% W.P, and zineb 75% W.P and two different insecticides malathion 25% W.P and diazinon 4E 47.5% were used at five different concentrations for time intervals starting from 30 min to 1 week. No considerable decrease in sporulation was observed for all the pesticides from time periods of 30 min to 24 hrs at all concentrations. Considerable decrease in percentage sporulation was observed for fungicide benomyl at the time period of 1 week. In the case of *Cryptosporidium parvum,* the percentage decrease for benomyl 50% W.P was from 100 to 70%, for captan 50% W.P it was from 100 to 80% and for diazinon 4E 47.5% it was from 100 to 50% at the maximum pesticide concentration and time period of 1 week. In conclusion the research showed that pesticides used in raspberry farms at recommended concentration levels did not affect the sporulation of the parasite.

Cyclospora cayetanensis is a coccidian parasite that causes gastroenteritis. It is not spread directly from person to person but can be spread via contaminated food or water. There have been various food related outbreaks in the U.S. In the 1990's at least 11 definite and probable foodborne outbreaks of cyclosporiasis, affecting around 3600 persons were documented, all of which occurred in North America and Canada (Herwaldt, 2000). Several types of fresh produce like raspberries, basil and lettuce have been vehicles for such outbreaks (Chalmers, Nichols, and Rooney, 2000). For many outbreaks vehicle or source has not been identified. In the spring and early summer of 1996, the largest ever reported outbreak of cyclosporiasis, affecting more than 1400 people in North America was associated with eating raspberries; traced back data indicated

the source to be Guatemalan raspberry farms (Herwaldt and Ackers 1997; Caceres et al., 1998). A year later despite the improvements in water quality and sanitation in berry farms, another outbreak occurred related to Guatemalan raspberries (Herwaldt and Beach, 1999). In spring 1997, fresh raspberries were implicated as source of outbreak in Ontario, Canada (Osterholm, 1999).

At this time, neither the specific source nor the period at which contamination of raspberries occurred was clear. The potential sources considered were soil, irrigation water, human contact or animals. The possibility that bird droppings might have contaminated the berries was suspected, but no natural or experimental infection with *C. cayetanensis* has been demonstrated in animals (Ortega et al., 1997; Eberhard, Nace, and Freeman, 1999). The only other source could be water that was either used for irrigation or water used in pesticide sprays.

Raspberries had been introduced into Guatemala in the late 1980s as an export crop, and the volumes exported to United States markedly increased in the mid 1990s (Katz et al., 1999). Guatemala has been exporting raspberries over the last decade. Raspberries are grown as annuals and harvested between July and September. Raspberry plants are 4-5 feet off the ground and are usually drip irrigated. The possibility of contamination with this type of irrigation was unlikely since the fruits do not make contact with water. Raspberries are handpicked and kept in cold chain until exported to the U.S. The leading hypothesis was that raspberries were contaminated through exposure to water; specifically insecticides and fungicides used to spray berries were mixed with contaminated water containing human feces (Herwaldt, 2000). The only other possible source of the organism was infected persons, who either directly or indirectly picked up or sorted raspberries. Surveillance of outpatient health care clinics in Guatemala showed that, seasonality of human cyclosporiasis overlapped with Guatemala's export season for raspberries (Bern et al., 2000).

The main objective of this study is to determine the effects of different concentrations of fungicides and insecticides on sporulation of *C. cayetanensis* and *Cryptosporidium parvum* at various incubation periods.

MATERIALS AND METHODS

Preparation of *Cyclospora cayetanensis* oocysts. *Cyclospora cayetanensis* oocysts were obtained from naturally infected individuals from Pampas de San Juan, Lima, Peru. Feces were sieved and stored in 2.5% potassium dichromate. Modified ethyl acetate method was used for initial concentration. Pellets were diluted in distilled water and layered over a primary discontinous sucrose gradient and centrifuged at 4300 g for 20 min. Oocysts were stored in 2.5% potassium dichromate solution at 4º C.

Unsporulated *Cyclospora* oocysts were washed three times with distilled water and sedimented by centrifugation at 4300 g for 5 min to remove all the potassium dichromate. Two isolates, A and B, of *Cyclospora* were used in this study. Oocysts were enumerated using a Neubauer hemocytometer from the stock preparation and were diluted with distilled water to prepare inocula of 3000-4000 oocysts/50µl. The oocysts preparation of isolate A had 1.19×10^6 oocysts/ml and isolate B had 2.24×10^6 oocysts/ml. Duplicates (D1, D2) were prepared for each isolate and were subjected to varying concentrations of pesticides starting from the lowest concentration to highest. Incubation periods of 30 min, 1 hr, 4 hr, 8hr, 24 hr and 1 week were chosen for each pesticide treatment.

Pesticide treatment for Cyclospora cayetanensis. The fungicides chosen for the study were captan 50% W.P (EPA Reg no. 16-151), Dragon® ,NY, U.S.A, benomyl 50 % W.P sold as Benlate® (EPA Reg no.352-564), DuPont, DE, U.S.A, and zineb 75% W.P (EPA no.01812-00414-AA-00000), DuPont, DE, U.S.A. The insecticides were malathion 25% W.P (EPA Reg no.51036-104), Micro Flo, Florida, U.S.A and diazinon 4E 47.5% (EPA Reg no.51036-GA-1), Micro Flo, Florida, U.S.A. The pesticides were obtained from U.S.D.A, Griffin Experimental

Station, UGA, Griffin, GA. Various concentrations of pesticides were made with reference to spray dilution chart for small quantities of pesticides (Table 2.1). For this study, small quantities of pesticide solution (10ml) were prepared with the recommended application concentration as the standard value. Five different concentrations of pesticide solutions were prepared (T1, T2, T3, T4, and T5).

Procedure. Pesticide treatment of 50 µL was added to 50 µL of distilled water containing *Cyclospora* oocysts. The mixture was incubated at 23° C for required test time interval, after which the oocysts were washed three times with distilled water and sedimented by centrifugation at 4300 g for 5 minutes to remove the pesticide. *C. cayetanensis* oocysts kept in distilled water were the control. Potassium dichromate of 50 µL was added, left at 23° C incubator for two weeks to sporulate. The oocysts were counted individually using a 20× magnification of light microscope. The number of sporulated and unsporulated *Cyclospora* oocysts in a 3 µL sample was recorded to find percentage sporulation (Figure 2.1).

Preparation of *Cryptosporidium parvum* oocysts. *Cryptosporidium* oocysts (Iowa isolate) were obtained from the Parasitology Laboratory, University of Arizona. Oocysts were purified using primary and secondary sucrose gradients followed by an isopycnic Percoll gradient, Sigma®. Purified oocysts were stored at 4° C in 0.01 % Tween containing 100 µL /mL penicillin, 100 µL /mL streptomycin and 100µl /mL gentamycin to retard bacterial growth. Stock solutions of oocysts were enumerated with a hemocytometer and diluted with distilled water. Each treatment had 10^6 oocysts/ mL in distilled water (Arrowood and Sterling, 1987).

Pesticide treatment for *Cryptosporidium parvum*. Pesticide solutions were prepared similar to the *Cyclospora* experiment. Two fungicides captan 50% W.P (EPA Reg no. 16-151), Dragon®, NY, U.S.A and benomyl 50% W.P, Benlate® (EPA Reg no.352-564), DuPont, DE, U.S.A, and one insecticide diazinon 4E 47.5% (EPA Reg no.51036-GA-1), Micro Flo, Florida, U.S.A were evaluated at two highest concentrations (T4 and T5) (Table 2.1). Incubation periods selected

were 30 min, 1 hr, 4 hr, 8 hr, 24 hr, and 1week. The test incubation periods for captan and diazinon were 1hr, 8 hrs, 24 hrs and 1 week, and 1hr, 4 hrs, 8 hrs, 24 hrs and 1 week respectively and for benomyl it was 1 hr, 8 hrs, 24 hrs and 1 week. Pesticide treatments were incubated with C*ryptosporidium* at 23° C for the required time period and then stored at 4° C. Oocysts were washed three times with distilled water and centrifuged at 4300 g for 4 minutes to completely remove the pesticide and to inoculate mice litters.

Table 2.1. Recommended dosage of pesticides and different dilutions of treatment prepared.

Pesticide	Recommended application concentration	Treatments [a]				
		T1	T2	T3	T4	T5
Captan 50%W.P	2 lb/100 gal	0.5 lb/100 gal	1lb/100 gal	1.5 lb/100 gal	2 lb/100 gal	2.5 lb/100 gal
Benomyl 50% W.P	1 lb/100 gal	0.5 lb/100 gal	1lb/100 gal	1.5 lb/100 gal	2 lb/100 gal	2.5 lb/100 gal
Diazinon 47.5%	4 teaspoons/gallon	1 tsp/gal	2 tsp/gal	3 tsp/gal	4 tsp/gal	5 tsp/gal
Malathion 25% W.P	3 lb/100 gal	1.5 lb/100 gal	2 lb/100 gal	2.5 lb/100 gal	3 lb/100 gal	3.5 lb/100 gal
Zineb 75% W.P	2 lb/100 gal	0.5lb/100 gal	1 lb/100 gal	1.5 lb/100 gal	2 lb/100 gal	2.5 lb/100 gal

[a]Treatments are the different concentrations of pesticide solution prepared with values lower and higher than the recommended dosage, starting with the lowest T1 to highest T5. W.P in the table stands for wettable powder.

Bioassay for infectivity*:* Twenty-nine timed pregnant CD-1/ICR dams were obtained from Harlan Sprague Dawley. The animals were housed in an approved facility according to NIH guidelines. Fresh water and pelleted feed were available at all times; each of the pregnant mice was housed separately in a cage. Five-day-old neonate mice were inoculated with pesticide treated oocyst. Each mice pup was orally inoculated with a 50 µL aqueous suspension containing 100,000 oocysts of C. *parvum.* Ten to twelve pups housed in 29 cages, received different time-concentration of pesticide treatment. Five days after inoculation, the pups were euthanised by inhalation of CO_2 and one centimeter segment of terminal ileum was removed, fixed in 10% formalin, embedded in paraffin, sectioned and hematoxylin-eosin stained. Each 3-µm sections were examined by light microscopy at 40× magnification. Evidence of infection was defined as the observation of *Cryptosporidium* parasite developmental stages in the microvilli of prepared ileal tissue sections. Tissues from each mice were scored plus (infected) or minus (not infected) by microscopic observation and the proportion of animals infected at each dose was calculated and recorded (Figure 2.2).

RESULTS AND DISCUSSION

Effect of pesticide on *Cyclospora cayetanensis*

Captan. Fungicide captan was used in varying concentrations starting from the lowest (T1) to highest of (T5) (Figure 2.3). The mean percentage sporulation values of treatment concentrations T2, T3, T4, T5 are compared with treatment concentration T1 and control samples. The time of exposure varied from minimum of 1 hr to maximum of 1 week (Figure 2.6). With increase in time of exposure there was no change observed in percentage sporulation. Similarly, at low and high concentrations of fungicide, no change in percentage sporulation was observed. Exposure of oocysts to fungicide captan for a period of one week did not affect the sporulation of the oocysts for both isolate A and B.

Benomyl. Fungicide benomyl of varying concentrations T1, T2, T3, T4 and T5 had no effect on sporulation for up to exposure time of 24 hrs. The results were similar for both isolate A and B. However, exposure of oocysts for 1 week with the highest treatment concentration (T5) resulted in decreased sporulation (Figure 2.8). Comparison of mean percentage sporulation of oocysts in all the treatments with treatment T1 and control did not show any changes in percentage sporulation with increase in time and concentration (Figure 2.4).

Zineb. Fungicide zineb did not affect the sporulation of *Cyclospora* for upto the time period of 24 hrs for treatment concentrations T1, T2, T3, T4 and T5 (Figure 2.7). No difference in sporulation was observed in any of the treatments including the 7-day exposure (Figure 2.8).

Diazinon. Insecticide diazinon from lowest (T1) to highest concentration (T5) did not prevent sporulation of *Cyclospora* oocysts. The mean percentage sporulation values of T2, T3, T4 and T5 were compared with the mean percentage sporulation values of T1 and control. The various incubation times and concentration of the insecticide did not affect the sporulation of *C. cayetanensis* (Figure 2.5). One week of exposure time did not decrease the percentage sporulation (Figure 2.8).

Malathion. Insecticide malathion from lowest concentration (T1) to highest concentration (T2) did not have any effect on the sporulation of *Cyclospora*. Percentage sporulation values of T2, T3, T4 and T5 were compared with T1. Sporulation did not change with any of the exposure times and treatment concentrations including exposure time of one week (Figure 2.6 and Figure 2.8).

Effect of pesticide on *Cryptosporidium parvum.* All the three pesticides (captan, benomyl and diazinon) at the highest concentration evaluated had an effect on oocyst infectivity but only at 7-days of exposure. Time periods of 1 hr to 24 hrs did not affect the infectivity of the parasite, 100% infectivity was achieved until 24 hrs (Figure 2.7). Fungicides captan and benomyl and insecticide diazinon at the highest concentration (T5) decreased the infectivity of

Cryptosporidium with one-week exposure time. The percentage decrease for benomyl was from 100 to 70%, for captan it was from 100 to 80% and for diazinon it was from 100 to 50%.

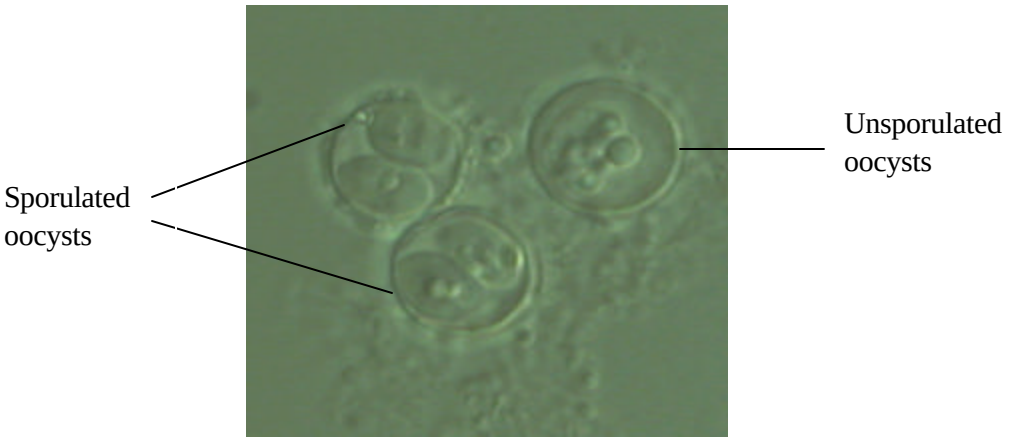

Figure 2.1. Sporulated and unsporulated oocysts of *Cyclospora cayetanensis.* Each sporulated oocysts have two sporocysts.

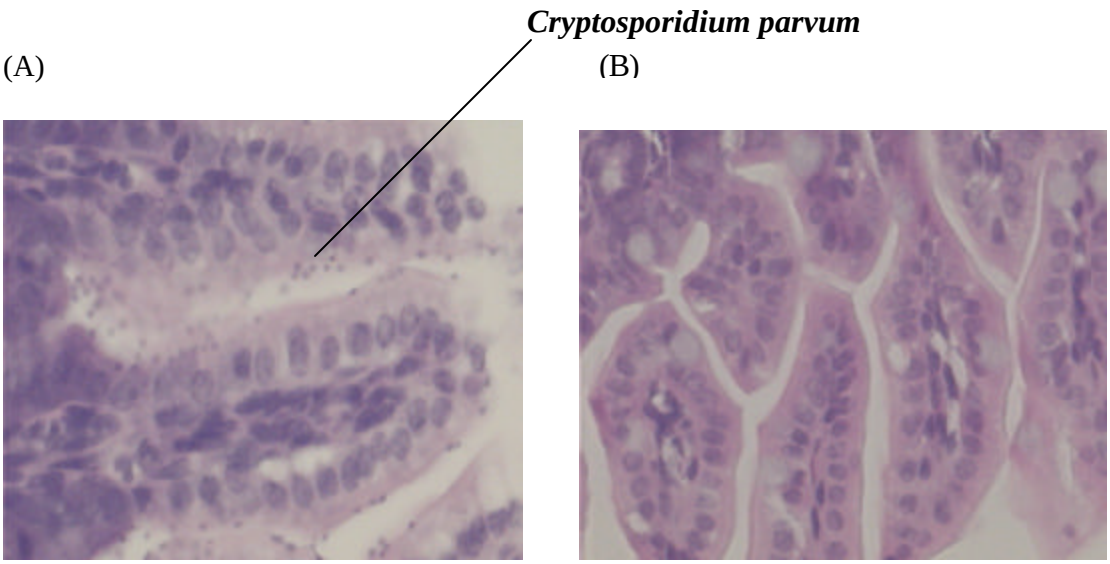

Figure 2.2. Hematoxylin-eosin stained mice ileal tissue. Picture on the left (A) shows the infected tissue with C. *parvum* and picture to the right (B) shows an uninfected ileal tissue of the mice.

CAPTAN FUNGICIDE

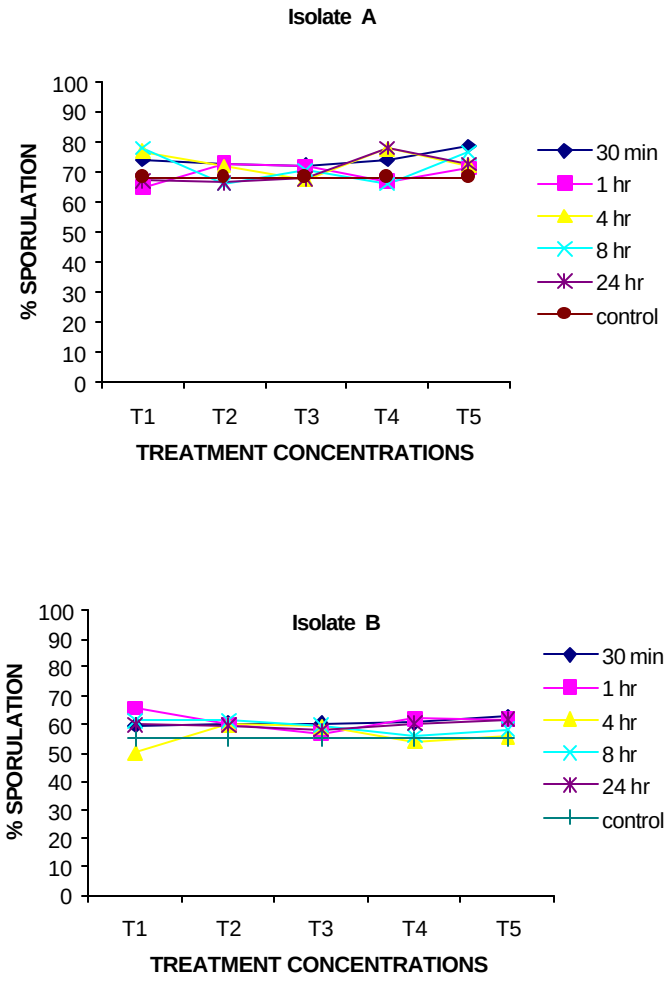

Figure 2.3. Percentage sporulation of *Cyclospora* isolate A and isolate B. Different concentrations of fungicide captan are T1 = 0.6 mg/ mL (0.005 lb/ gal), T2= 1.2 mg/ mL (0.001 lb/ gal), T3 = 1.8 mg/ mL (0.015 lb/ gal), T4 = 2.4 g/ mL (0.02 lb/ gal), T5 = 3 mg/ mL (0.025 lb/ gal).

BENOMYL FUNGICIDE

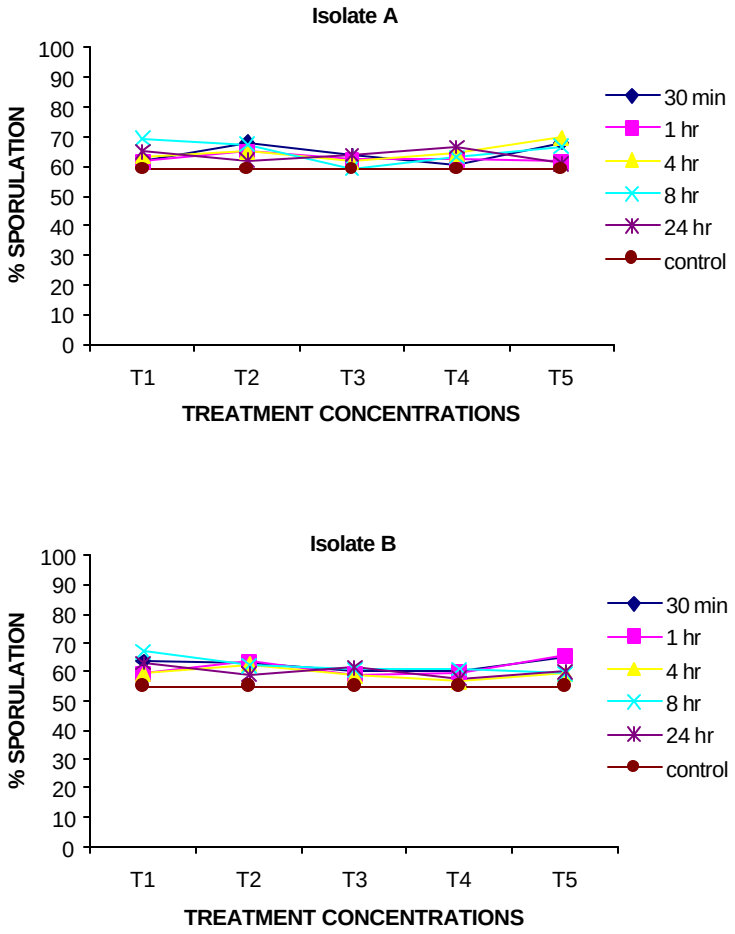

Figure 2.4. Percentage sporulation of *Cyclospora* isolate A and isolate B. Different concentrations of fungicide benomyl are T1 = 0.6 mg/ mL (0.005 lb/ gal), T2= 1.2 mg/ mL (0.001 lb/ gal), T3 = 1.8 mg/ mL (0.015 lb/ gal), T4 = 2.4 g/ mL (0.02 lb/ gal), T5 = 3 mg/ mL (0.025 lb/ gal).

DIAZINON INSECTICIDE

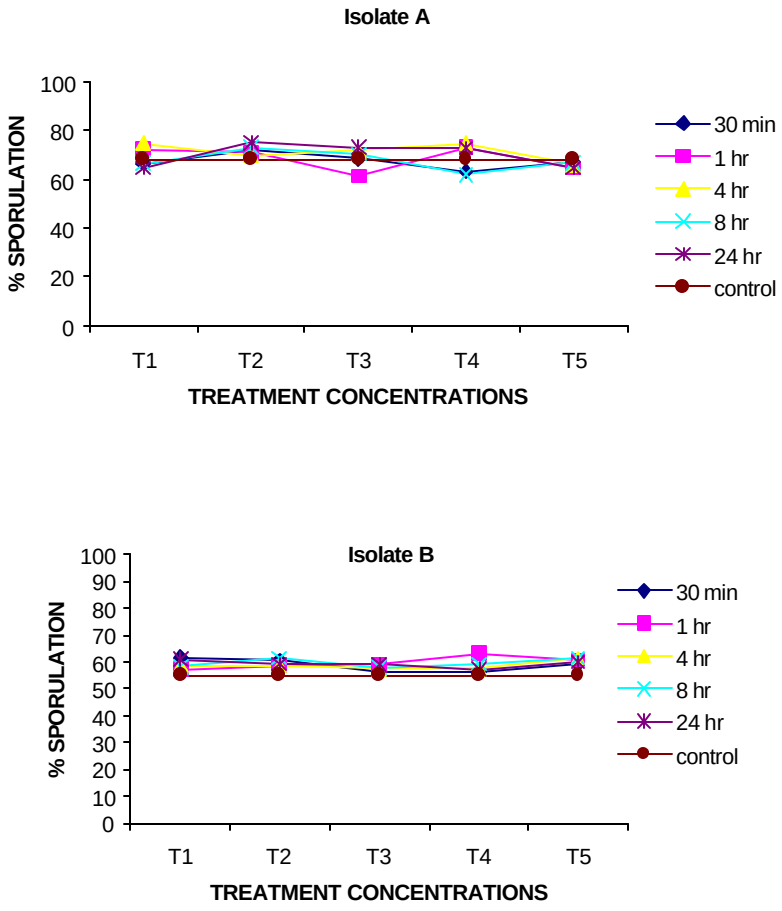

Figure 2.5. Percentage Sporulation of *Cyclospora* isolate A and isolate B. Different concentrations of insecticide diazinon are T1 = 1.32 µL/ mL (1 teaspoon /gal), T2 = 0.264 µL/ mL (2 teaspoons/gal), T3 = 3.96 µL/ mL (3 teaspoons/gal), T4 = 5.28 µL/ mL (4 teaspoons/gal), T5 = 6.6 µL/ mL (5 teaspoons/gal).

MALATHION INSECTICIDE

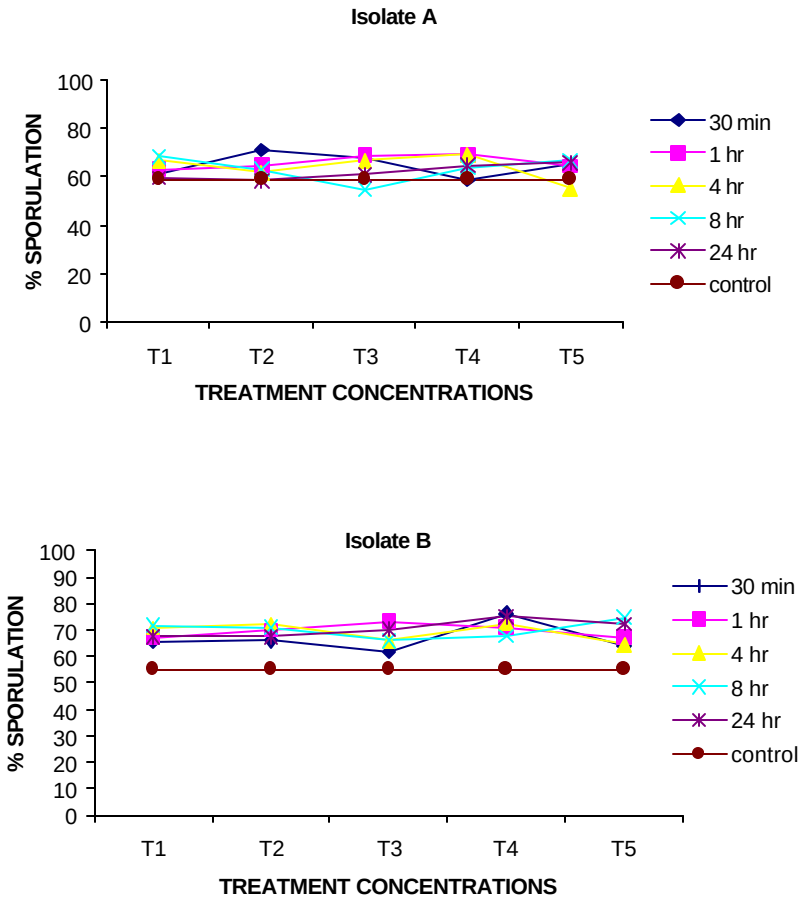

Figure 2.6. Percentage sporulation of *Cyclospora* isolate A and isolate B. Different concentrations of insecticide malathion are T1 = 1.8 mg/ mL (0.015 lb/ gal), T2 = 2.4 mg/ mL (0.02 lb/ gal), T3 = 3 mg/ mL (0.025 lb/ gal), T4 = 3.6 mg/ mL (0.03 lb/ gal), T5 = 4 mg/ mL (0.035 lb/ gal).

ZINEB FUNGICIDE

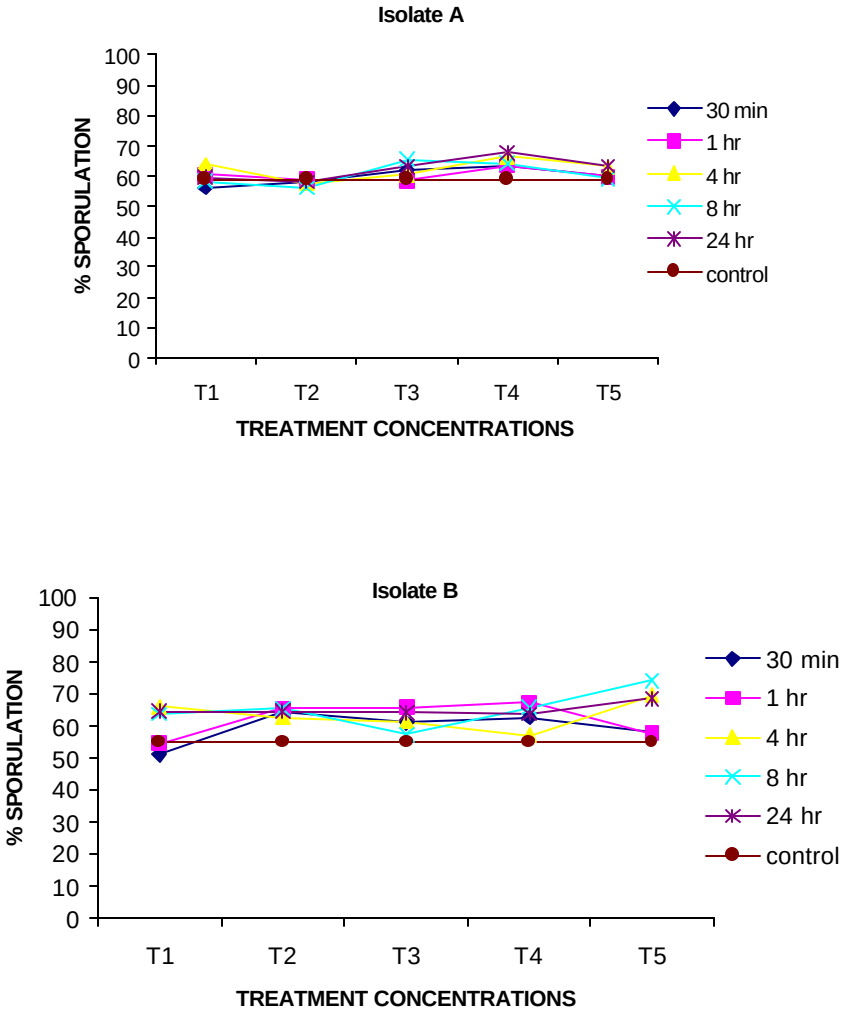

Figure 2.7. Percentage sporulation of *Cyclospora* isolate A and isolate B. Different concentrations of fungicide zineb are T1 = 0.6 mg/ mL (0.005 lb/ gal), T2= 1.2 mg/ mL (0.001 lb/ gal), T3 = 1.8 mg/ mL (0.015 lb/ gal), T4 = 2.4 g/ mL (0.02 lb/ gal), T5 = 3 mg/ mL (0.025 lb/ gal).

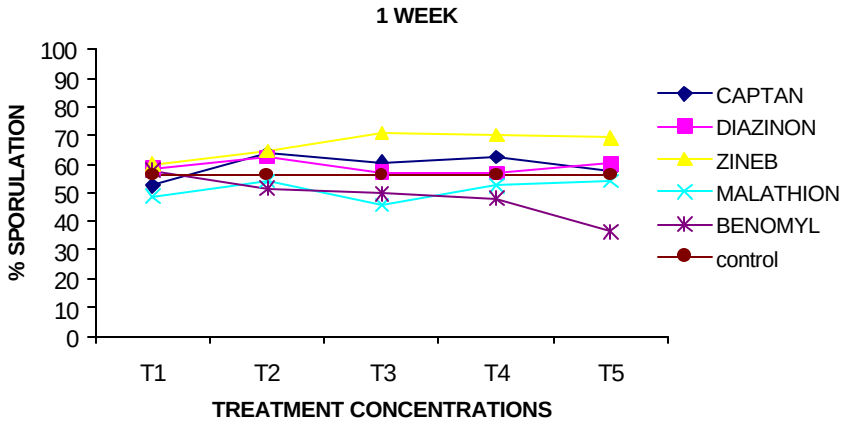

Figure 2.8. Percentage Sporulation of *Cyclospora* with treatment time 1 week. Different concentrations T1, T2, T3, T4 and T5 values of captan, benomyl and zineb are same, T1 = 0.6 mg/ mL (0.005 lb/ gal), T2= 1.2 mg/ mL (0.001 lb/ gal), T3 = 1.8 mg/ mL (0.015 lb/gal), T4 = 2.4 mg/ mL (0.002 lb/ gal), T5 = 3 mg/ mL (0.025 lb/ gal). For diazinon T1 = 1.32µL/ mL (1 teaspoon /gal), T2 = 2.64 µL/ mL (2 teaspoons/gal), T3 = 3.96 µL/ mL (3 teaspoons/gal), T4 = 5.28 µL/ mL (4 teaspoons/gal), T5 = 6.6 µL/ mL (5 teaspoons/gal). For malathion T1 = 1.8 mg/ mL (0.015 lb/ gal), T2 = 2.4 mg/ mL (0.02 lb/ gal), T3 = 3 mg/ mL (0.025lb/ gal), T4 = 3.6 g/ mL (0.03 lb/ gal), T5 = 4 mg/ mL 0.035 lb/ gal).

This study has examined the hypothesis that *Cyclospora* could survive in a pesticide solution and plays a significant role in association with the outbreak caused by Guatemalan raspberries. This might be due to the contamination of irrigation waters with human feces containing *Cyclospora*. By testing different commonly used pesticides on *Cyclospora*, we determined that percentage sporulation of oocysts was not affected until the time period of 1 week, moreover the fungicide benomyl, considerably decreased the percentage sporulation as the concentration of the fungicide was increased but did not completely prevent the sporulation. The exposure of *Cyclospora* to pesticides normally does not occur for more than few days, as fungicidal sprays are used within a week after preparation hence sporulation could not be affected by the presence of pesticides. Even after one week of exposure of *Cyclospora* to pesticides, it was seen that percentage sporulation decreased only for benomyl and the other pesticides evaluated had no effect on sporulation of *Cyclospora.* Of all the fungicides and insecticides used, benomyl was least soluble. Benomyl completely degrades to carbendazim within several hours in acidic or neutral water (Kidd, 1991). Studies conducted on microorganisms with respect to fungicides showed that benomyl, captan and chlorothalonil had some inhibitory activity on soil microorganisms but depended on factors like physical, chemical and biochemical properties of the soil. A mechanistic explanation for some of the results was that fungicide applications killed or inhibited the activity of certain groups of fungi. This could lead to immediate reduction in microbial respiration and biomass. However the dead microorganisms might serve as a substrate for the living microorganisms, which in turn may have lead to a rapid flush in microbial activity (Chen, 2001). Blank et al. (2000) determined that few microorganisms actually flourish in the formulations of insecticide, herbicide and fungicide typically used in agriculture. According to Blank et al. (2000) *Salmonella*, *E. coli* and *Shigella* grew best in chlorothalonil.

The effects of disinfectants or sanitizers on viability of *Cyclospora cayetanensis* is not known. One of the waterborne outbreaks demonstrated that *C. cayetanensis* is persistent to chlorination in normal concentrations. There had been outbreaks of *Cylospora* in chlorinated

drinking water, showing that safe chlorine concentration (0.3-0.8 ppm) used in drinking water had no effect on sporulation and was very liable to cause outbreaks.

Sporulation of *C. cayetanensis* oocysts is a process of differentiation to form sporozoites that is infectious for susceptible hosts. Since to date there is no animal model for *C. cayetanensis*, determination of sporulation is a good indicator of viability, but this does not evaluate the infectivity of the already differentiated or sporulated oocysts. Another difficulty in working with *Cyclospora* is that, the oocysts are obtained from naturally infected individuals and procuring large amounts of oocysts for the studies is difficult.

In the case of *Cryptosporidium parvum*, a substantial percentage of oocysts were rendered uninfective by the usage of high concentration of the fungicide benomyl and captan and insecticide diazinon at 1 week of exposure. However, the highest concentration did not have any effect from time of exposure of 1 hr to 24 hrs, which is normally the time period the parasite might be in contact with the pesticide in the farms.

The raspberries imported from Guatemala could have harbored *Cyclospora* since oocysts could adhere to the hairy follicles of the outer skin of raspberry. Research has revealed that washing raspberries does not completely remove *Cyclospora* that adheres to the surface of the fruit (Herwaldt and Beach, 1999). Coccidial contamination of raspberries by artificial contamination with *Eimeria acervulina* was used as a model for decontamination treatment studies. Results indicated that washing of raspberries was generally not adequate in removing coccidial contamination, but freezing and heat treatment appeared to be effective in killing the parasite. Gamma irradiation of E. *acervulina*-sporulated oocysts at a dose of 0.5 kGy was partially effective, but it was completely effective at 1.0 kGy and higher (Lee and Lee, 2001). The only way by which *Cyclospora* oocysts can be inactivated is by cooking the food product, which is not commonly practiced with fresh fruits and vegetables. The only other possibility is irradiating the fresh fruits and vegetables. Research is being done to study the effect of UV rays

on *Cyclospora*. Studies conducted on *C. parvum* in cider showed that, oocysts exposed to 14.32 mJ of UV irradiation/cm ^{2} were rendered uninfective (Hanes et al., 2002).

In conclusion, the results of this study showed that fungicides and insecticides used on raspberry farms does not affect the sporulation of C. *cayetanensis* at recommended dosage levels. Benomyl reduced the percentage sporulation considerably with increase in treatment concentrations (3 mg/ mL) with exposure time of 1 week. Studies done on *C. parvum* as reference showed that fungicides captan and benomyl and insecticide diazinon, decreased the percentage infectivity with one week as treatment time and the highest treatment concentrations of 9.003 g/ mL, 3 mg/ mL and 6.6 µL/ mL respectively.

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CHAPTER 3

EFFECTS OF TEMPERATURE AND DIFFERENT FOOD MATRICES ON SPORULATION AND VIABILITY OF *CYCLOSPORA CAYETANENSIS* [1]

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To be submitted to Journal of Parasitology.

EFFECTS OF TEMPERATURE AND DIFFERENT FOOD MATRICES ON SPORULATION AND VIABILITY OF *CYCLOSPORA CAYETANENSIS*

ABSTRACT

Effects of temperature on the parasite *Cyclospora cayetanensis* have been studied in two different food substrates. The oocysts of the parasite were subjected to freezing and heating conditions. In dairy substrates at -15º C, sporulation was seen until 24hrs, and at 4º C and 23º C, sporulation was observed up to 1 week. In the basil substrate sporulation was observed up to 6 days at 4º C. At -20º C, sporulation was observed up to 2 days, at 37º C, sporulation was observed up to 4 days, at 50º C, sporulation was seen up to 1 hr, no sporulation was seen at temperatures of -70º C, 70º C and 100º C in the basil food substrate.

Cyclospora cayetanensis, a coccidian parasite has increasingly been recognized as a cause of gastrointestinal tract illness. It was responsible for numerous water and food-borne outbreaks in the1990s. *Cyclospora* was initially referred to as "cyanobacteria-like" body because the internal organelles resembled that of photosynthesizing organelles of the blue-green algae (Ungar et al., 1986) described these organisms in the stool of immunocompetent travelers to Mexico and Haiti with gastrointestinal symptoms. As early as in 1983, researchers in Haiti had described similar organisms in AIDS patients (Pape et al., 1994). During studies in Peru from 1985-1987, researchers identified large *Cryptosporidium*-like organism (Ortega et al., 1993).

Cyclospora appears under the microscope as nonrefractile, double-walled spheres, of 8-10 microns in diameter. *Cyclospora*, in contrast to other coccidian species, requires time outside the host for sporulation to occur (Soave, Herwaldt, and Relman, 1998). Accordingly, approximately 40% of unsporulated oocysts sporulate within 7-10 days in favorable environmental conditions.

Fresh produce (raspberries, mesclun and basil) has been associated with numerous outbreaks of cyclosporiasis (Koumans et al., 1998; 1997; Bern et al., 1999). For many of these outbreaks the actual source of *Cyclospora* was not identified, although the possibility that contaminated water was considered a likely source for at least some of the outbreaks (Herwaldt and Ackers, 1997; Herwaldt and Ackers, 1997; Sterling and Ortega, 1999). A foodborne outbreak occurred at a wedding during May 1996 in Boston. In this outbreak a dessert containing raspberries, strawberries, blackberries, blueberries and cream were significantly associated with the illness (Fleming et al., 1998). Unfortunately having a mixture of fruits precludes the determination of which individual fruit may have been the vehicle of transmission but because previous outbreaks implicating raspberries as a vehicle of *Cyclospora*, raspberries were considered as a possible source (Herwaldt and Ackers, 1997). Another outbreak related to a product containing cream was lemon tart with raspberry in it. Epidemiologic investigation revealed that raspberries were possibly the vehicles of contamination in this case as well. This suggested that either the infective dose of *Cyclospora* was low or the number of oocysts per berry was high or possibly both (Herwaldt, 2000).

Two outbreaks have been associated with basil, one in 1997 and another in 1999. The former occurred in Washington, D.C and the latter in late July 1999 in Missouri. In the 1997 outbreak the mode by which basil was contaminated was not identified. There was a good possibility that contamination was by a human food handler. However, food contaminated by unsporulated oocysts may not be infectious if the food had been eaten before the oocysts sporulated. Two possible modes of contamination were put forth. Either the gourmet food store, which received 10% of the distributor's basil, had poor food handling practices (e.g. sub optimal refrigeration), or the contamination had occurred during harvesting. In the latter it was possible that oocysts had sufficient time to sporulate because the interval from when the basil was handled until it was eaten, averaged at least several days. But it is not known whether if this interval, which was somewhat shorter than documented sporulation times under laboratory conditions, was

sufficiently long for sporulation to occur and whether the ingredients in the pesto sauce could have accelerated sporulation (Ortega et al., 1993; Smith et al., 1997). In the 1999 outbreak that occurred in Missouri, local contamination by food handlers was completely ruled out because the implicated food was eaten soon after it was prepared and the food could not be associated with a specific food provider because since two large clusters of cases in this outbreak were linked to parties catered by different establishments in different counties (Lopez et al., 2001). The possible sources of contamination could have been either a Mexican farm or a U.S farm. On Mexican farm basil was handled barehanded during harvest and 15-fold more basil was supplied by the Mexican farm than the U.S farm. The actual source of contamination remains unknown. This outbreak was the first in the U.S for which *Cyclospora* was detected in epidemiologically implicated food item, apart from the fact that it had been previously detected on various types of produce in various countries (Ortega et al., 1997; Sherchand et al., 1999). In this outbreak, the hostess had frozen the leftovers of the implicated chicken pasta salad, which helped in detecting *Cyclospora* by PCR and microscopy (Lopez et al., 2001).

The objectives of this study were to determine the effect of temperature on sporulation of *Cyclospora cayetanensis* on different food matrices. The first set of food substrate selected was milk, whipped cream, diluted milk due to the outbreaks related to cream and dairy products. The oocysts were subjected to freezing, refrigeration and room temperature. The second food substrate selected was basil in which inoculated oocysts were subjected to heating and cooling conditions and the percentage sporulation of the oocysts were recorded.

MATERIALS AND METHODS

Preparation of *Cyclospora cayetanensis* oocysts. *Cyclospora cayetanensis* oocysts were collected from feces of naturally infected individuals from Pampas de San Juan, Lima, Peru. Feces were sieved and stored in 2.5% potassium dichromate, Sigma®, MO, U.S.A at 4° C. An initial concentration of oocysts was done with modified ethyl acetate method. Pellets were diluted in distilled water and layered over a primary discontinous sucrose gradient and

centrifuged at 4300 g for 20 min. Supernatant was discarded and the pellet containing oocysts were stored in 2.5% potassium dichromate solution at 4º C until use.

Milk, diluted milk and whipped cream as food substrate. Oocysts were washed three times with distilled water and sedimented by centrifugation at 4300 g for 5 min to remove all the potassium dichromate. One isolate of *C. cayetanensis* at a concentration of 2.47×10^6 oocysts/ mL was used for the experiment with milk, diluted milk and whipped cream as food substrate. Nestle carnation nonfat dry milk powder was reconstituted by adding 0.479 g of milk powder to 5 mL of distilled water. Diluted milk was prepared as half the concentration of milk by adding 0.239 g of milk powder to 5 mL of distilled water. Kroger® brand ultrapasteurised sweetened light cream whipped cream was used for the experiment. Whipped cream contained total fat content of 2 grams, saturated fat 1 gram, cholesterol 5 mg, total carbohydrate less than 1 gram. Oocysts were enumerated using a Neubauer hemocytometer from the stock solution and was diluted to prepare inocula at pre-selected oocyst dose of 200 oocysts/ µL. *Cyclospora* oocysts of concentration 10,000 oocysts/50 µL was added to 1 mL Eppendorf tubes containing 50 µl of milk, diluted milk, water and whipped cream. Triplicates (D1, D2, D3) of the isolate were prepared for this food substrate and were subjected to varying temperatures. The exposure temperatures for the dairy based food substrate were 23º C, 4º C, -15º C and treatment time periods of 1 hr, 24 hrs, 48 hrs and 1 week were tested. Oocysts in water at 23º C was used as a control sample. After exposing the oocysts to the required temperature and time interval, it was incubated for two weeks at 23º C in potassium dichromate. Fisher Scientific refrigerator was used for temperature studies at 4º C and -15º C. Lab-line Inc, IL, U.S.A, incubator was used for this study. Samples were examined by light microscopy at 20× magnification. The percentage sporulation was calculated using the number of sporulated and unsporulated oocysts in a 3 µL test sample and were compared to the control.

Basil as food substrate. Two isolates (A and B) of *C. cayetanensis* at concentration of 8.8×10^5 oocysts/mL and 7.5×10^5 oocysts/mL were used for the experiment with basil as the food substrate. Oocysts were enumerated using a Neubauer hemocytometer from the stock solution and was diluted to prepare inocula at pre-selected oocyst dose of 200 oocysts/ µL. Sweet basil plants (Ocimum basilicum) grown from Ferrys® sweet basil seeds were cut to a diameter of 0.5 cm were placed on the inner side of the lids of 200 µL Eppendorf tubes and were inoculated with 2 µl of *Cyclospora* oocysts containing 1000 oocysts and allowed to dry on the leaf surface for 20 minutes. After which it was tapped into the tube containing 50 µL of distilled water. Triplicates (D1, D2, D3) of the isolate were prepared for this food substrate and were subjected to varying temperatures. Exposure temperatures for basil substrate were 4º C, -20º C, -70º C, 37º C, 50º C, 70º C, 100º C and treatment time intervals were as low as 15 minutes to as high as 6 days. Fisher Scientific refrigerator was used for temperature studies at 4º C and -15º C and Revco refrigerator, NC, U.S.A, was used for the temperature study at -70º C. Incubators manufactured by Lab-Line instruments, Inc, IL, U.S.A were used for 37º C and 23º C temperature study. For maintaining temperatures of 50º C, 70º C and 100º C, Eppendorf® mastercycler was used. The time required to reach and control the temperature of 4º C, -15º C and -70º C in the refrigerator were 10 sec, 15 sec and 27 sec respectively. Temperature was determined by a thermocouple, Omega®. Oocysts in water at same temperatures and times intervals were used as a control for comparison with basil. After exposing the basil leaves with *Cyclospora* oocysts for the required time-temperature intervals, the leaves were washed by dispensing distilled water using a pipette and vortexed to remove the oocysts from the surface of the basil leaves and incubated at 23º C in 2.5% potassium dichromate. After a period of two weeks, sporulation rates were determined.

STATISTICAL ANALYSIS

Data analysis was done using "students t test" with $\alpha = 0.05$ using Microsoft Excel. The test samples were compared with the control using this test.

RESULTS AND DISCUSSION

Sporulation of C. *cayetanensis* in water, milk, diluted milk and whipped cream as food substrate

Percentage sporulation at -15º C. Freezing the water samples containing *Cyclospora cayetanensis* oocysts influenced significantly the sporulation of the oocysts (Figure 3.1). Exposure of *C. cayetanensis* in water at different test times showed a significant ($\alpha = 0.05$) difference in percentage sporulation. Similarly *C. cayetanensis* in milk, diluted milk and whipped cream showed significant ($\alpha = 0.05$) decrease in percentage sporulation as the exposure time was increased. As the exposure time increased from 60 minutes to 7 days, percentage sporulation decreased significantly for all the food substrates. No sporulation was observed at exposure time of two days or more.

Percentage sporulation at 4º C. Refrigeration temperature (4º C) did not affect the sporulation of *C. cayetanensis* in the dairy food substrate. No statistically significant difference was observed in percentage sporulation of *C. cayetanensis* oocysts at different times in water, milk, diluted milk and whipped cream (Figure 3.1).

Percentage sporulation at 23ºC. Water, milk, diluted milk and whipped cream at 23º C proved as a suitable substrate for oocysts to sporulate. Comparison of water samples at different times showed no significant difference ($\alpha = 0.05$) in sporulation. The results were similar in the case of milk, diluted milk and whipped cream (Figure 3.1).

Sporulation of C. *cayetanensis* in basil as food substrate.

Percentage Sporulation of *C. cayetanenesis* at 4º C. Oocysts in water and basil samples at 4º C showed no significant ($\alpha = 0.05$) difference from their respective controls incubated at 23º C

(Figure 3.2). The results were similar with both isolate A and isolate B (Table 3.1, Table 3.2, Table 3.3 and Table 3.4). Refrigeration temperature at various incubation periods did not prevent sporulation of *C. cayetanensis.*

Percentage sporulation of *C. cayetanenesis* at –20º C. The percentage sporulation of *C. cayetanensis* at -20º C in water sample was very low when compared to the percentage sporulation of oocysts in water at 23º C (Figure 3.3). The percentage sporulation also decreased as the time of exposure increased for both oocysts in water samples and oocysts in basil samples. A significant difference ($\alpha = 0.05$) was noted when the oocysts in water and oocysts in basil samples were compared with their respective controls. Results were similar for both isolate A and isolate B (Table 3.1, Table 3.2, Table 3.3 and Table 3.4).

Percentage sporulation of *C. cayetanenesis* at -70º C. No sporulation was observed for both oocysts in water and oocysts in basil at -70º C (Table 3.1, Table 3.2, Table 3.3 and Table 3.4). The results were similar for both isolate A and isolate B.

Percentage sporulation of *C. cayetanensis* at 37º C. No significant difference ($\alpha = 0.05$) was observed between oocysts at 23º C water and oocysts at 37º C water and oocysts at 23º C basil and oocysts at 37º C basil samples until 60 minutes. Significant difference was observed for time periods starting from four hours to 4 days when the test samples were compared with their respective controls (Figure 3.4). There was no sporulation at 6 days incubation time for both the isolates and for both water and basil samples (Table 3.1, Table 3.2, Table 3.3 and Table 3.4).

Percentage sporulation of *C. cayetanensis* at 50º C. The percentage sporulation of the test samples for both water and basil dropped significantly even at an exposure time of 15 minutes (Figure 3.5). Significant difference ($\alpha = 0.05$) in percentage sporulation of the oocysts was observed when the water and basil test samples were compared with their respective controls. At 1hr exposure at 50º C, sporulation of oocysts was observed (Table 3.1, Table 3.2, Table 3.3 and Table 3.4). Except for test basil sample kept at 15 minutes (isolate A), all the other samples

showed significant decrease in percentage sporulation when compared with their respective controls.

Percentage sporulation of *C. cayetanensis* at 70º C and 100º C. *C. cayetanensis* oocysts did not sporulate both at 70º C and 100º C (Table 3.1, Table 3.2, Table 3.3 and Table 3.4). Even at low exposure time of 15 minutes, no sporulation was observed for both the isolates A and B.

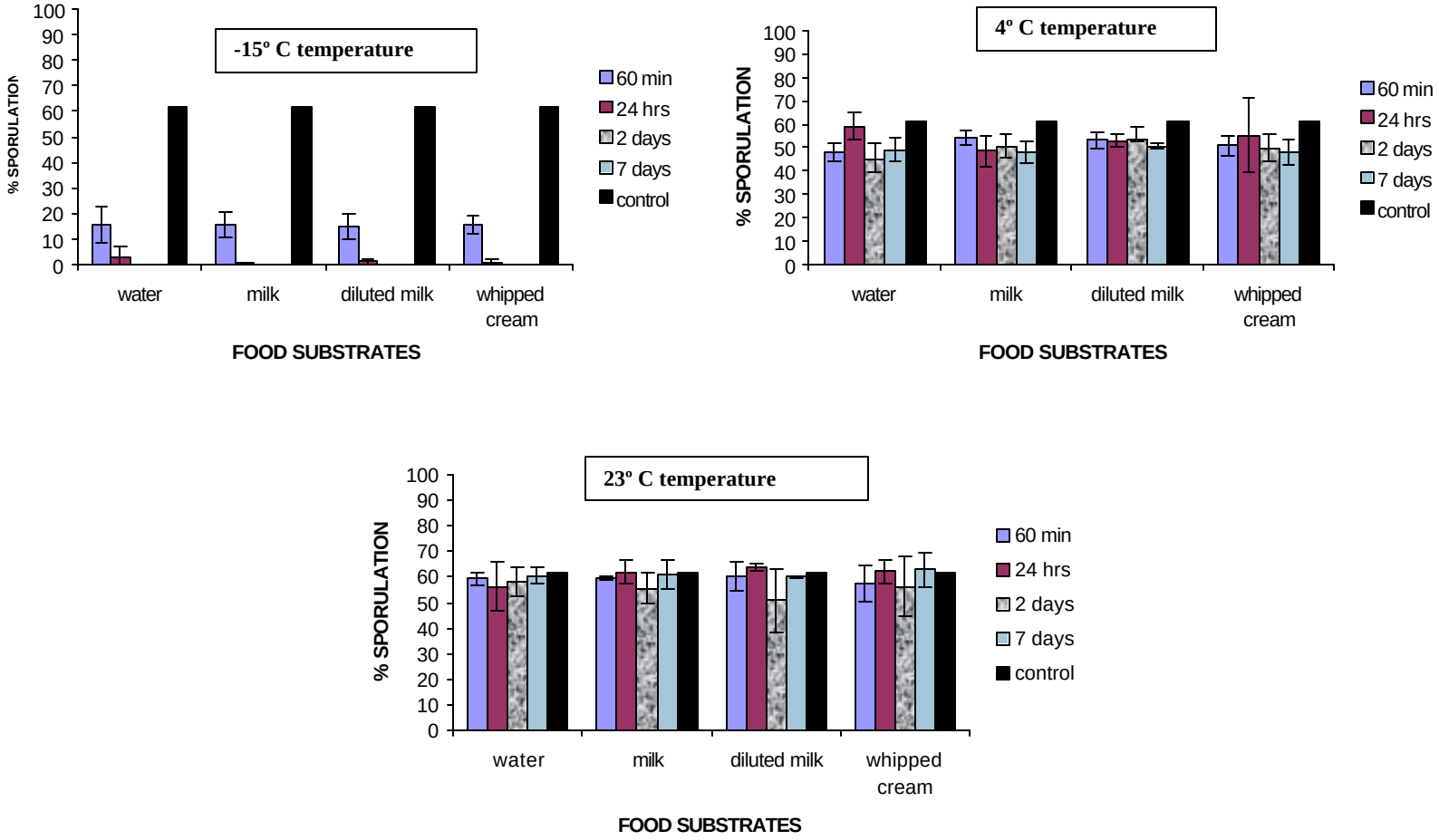

Figure 3.1. Percentage sporulation of C. *cayetanensis* at temperatures of -15º C, 4º C and 23º C in different dairy substrates.

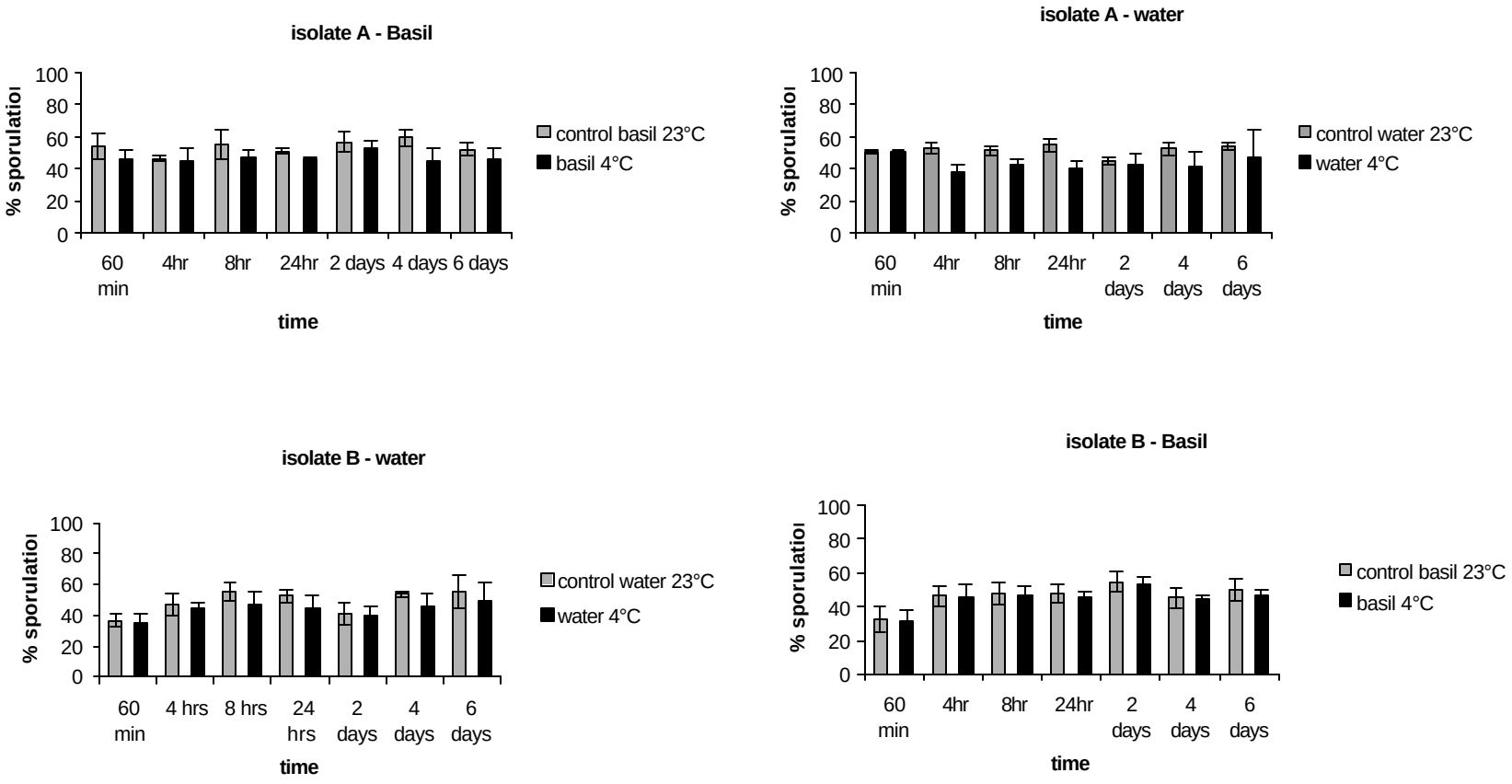

Figure 3.2. Percentage sporulation of C. *cayetanensis* in water and basil at 4º C. Water control sample and basil control samples incubated at 23º C

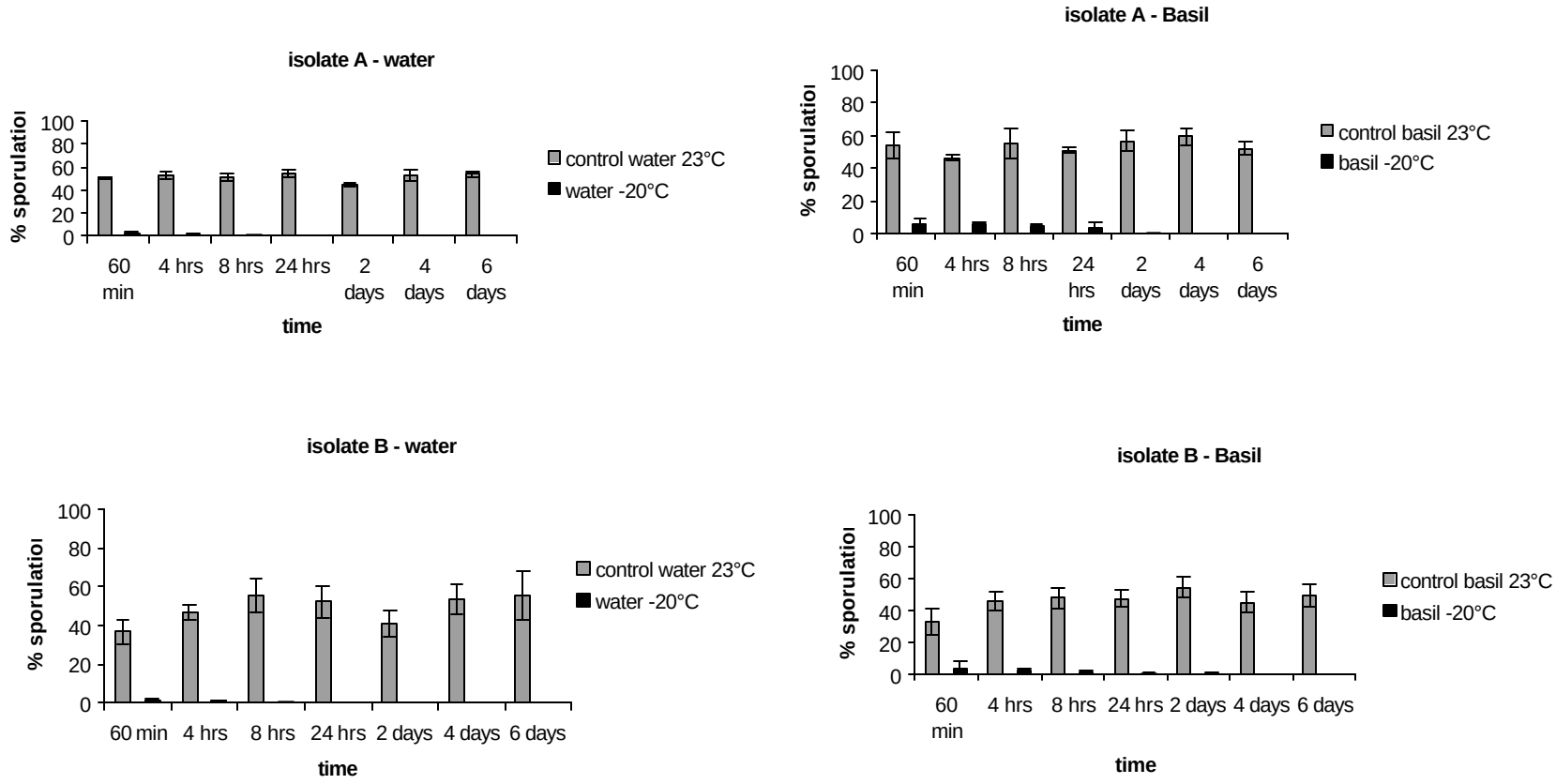

Figure 3.3. Percentage sporulation of *C. cayetanensis* in water and basil at -20º C. Water control samples and control basil samples incubated at 23º C

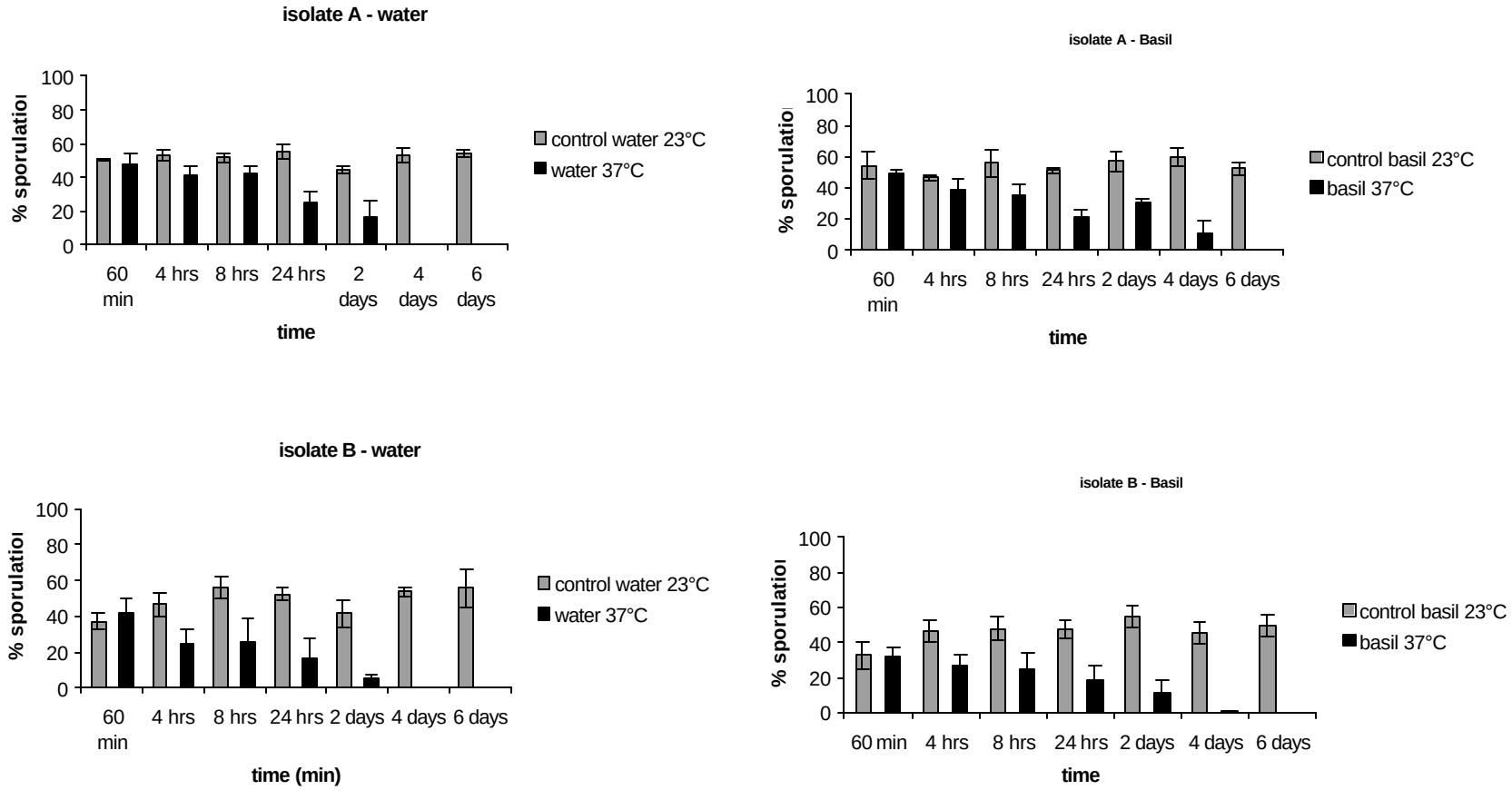

Figure 3.4. Percentage sporulation of *C. cayetanensis* in water and basil at 37º C. Water control sample and control basil sample incubated at 23º C.

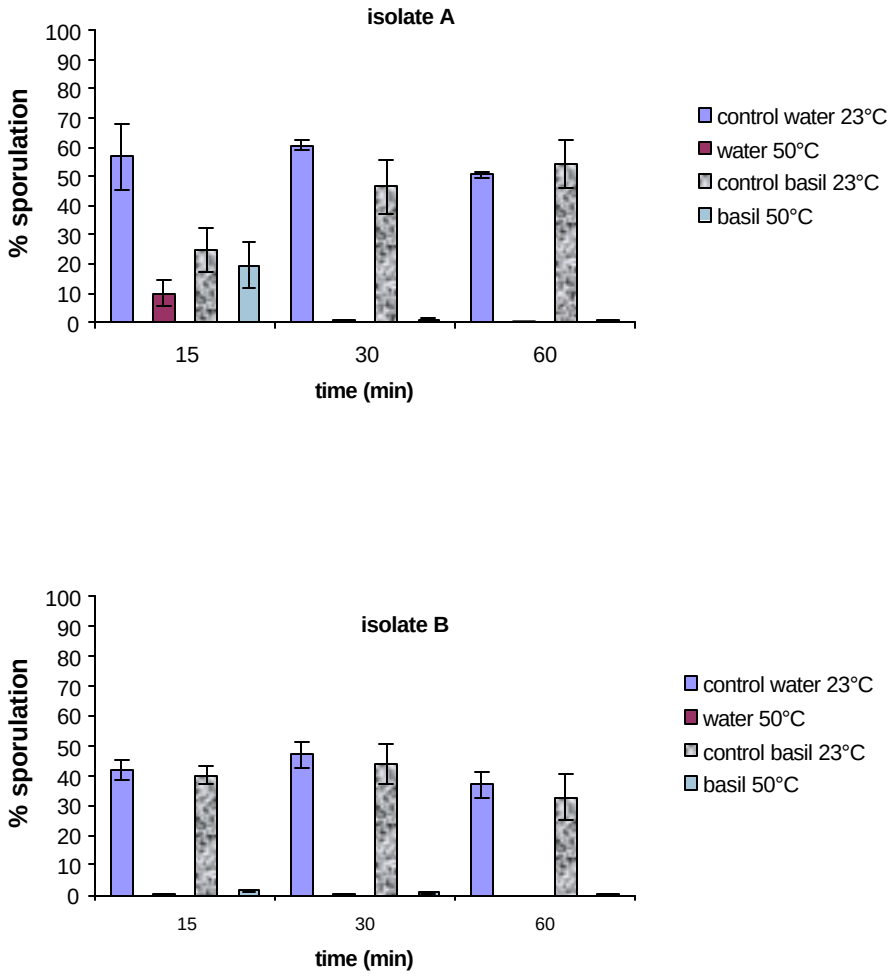

Figure 3.5. Percentage sporulation of *C.cayetanensis* in water and basil at 50º C. Water control sample and basil control sample incubated at 23º C.

Two types of food substrates were evaluated in this study, dairy products, which included milk, diluted milk and whipped cream and the second one was basil. These food substrates were used in this study because of the outbreaks of *Cyclospora cayetanensis* implicated with such foods. *Cryptosporidium parvum*, a parasite belonging to coccidia, has also been implicated in outbreaks of dairy products such as milk consumed unpasteurized. Studies were conducted on the survival characteristics of those parasites in ice cream and it showed that parasites were not viable after 24 hrs at -20º C (Deng and Cliver, 1999). *Cyclospora* is also inactivated at -15º C on dairy substrate. For all the three dairy substrates chosen i.e. milk, diluted milk and whipped cream, the percentage sporulation of *Cyclospora* dropped to zero as the exposure time was increased from 1hr to 1 week. Sporulation was seen until 24 hrs for all the three dairy substrates thereafter no sporulation was observed, indicating that high freezing temperatures inactivate the oocysts after a day of exposure. At 4º C and 23º C, percentage sporulation did not change when exposed for various periods of time. Survival of *C. cayetanensis* oocysts at 4º C for a period of 1 week showed that, this parasite could cause an outbreak if accidental contamination takes place in milk stored in a refrigerator with sporulated oocysts. Studies conducted by Smith H.V et al. (1997), demonstrated that up to 12% of human and baboon-derived oocysts previously stored at 4º C for 1 to 2 months sporulated when stored for 6-7 days at 30º C. At -15º C, sporulation of oocysts in dairy substrates for up to 24 hours was observed suggesting that freezing the fresh produce for a day followed by consumption could lead to infection. Oocysts sporulation in basil as food substrate was dependent on the temperature. Percentage sporulation of oocysts was higher at -20º C, 37º C and 50º C for basil test samples than water test samples. Contrary to the experiment with dairy substrates, C*yclospora* sporulated even after 48 hrs in water and basil test samples, when exposed to -20º C.

In conclusion, the dairy substrate study showed that temperatures as low as -15º C did not inhibit sporulation until 24 hrs. Temperatures of 4º C and 23º C did not change the percentage sporulation of oocysts in milk, diluted milk and whipped cream. The study conducted

with basil as food substrate showed that 4º C did not inhibit sporulation of oocysts up to incubation period of 1week. Sporulation was observed even at 48 hrs in basil as well as water sample kept at a temperature of -20º C. Sporulation was inhibited at -70º C even at 1 hr of exposure time for both basil and water test samples. Sporulation was seen for 4 days when the water and basil samples were exposed at 37º C. At 50ºC sporulation was observed until the time period of 1hr both in water and basil test samples. No sporulation was observed at 70º C and 100º C for both water and basil test samples. Both the studies show that at various temperatures, oocysts remained viable and were able to sporulate.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

The objectives of the research presented in the thesis were:

1. To determine the effects of fungicides and insecticides on sporulation and viability of *Cyclospora cayetanensis* oocysts and *Cryptosporidium parvum.*

2 To determine the effect of low and high temperatures on sporulation and viability of *Cyclospora cayetanensis* oocysts in different food matrices i.e. milk, diluted milk, whipped cream and basil.

Our results indicate that commonly used fungicides and insecticides on raspberry plants did not suppress the sporulation of *C. cayetanensis* even at the high concentrations of the pesticides. Except for one fungicide benomyl, others did not decrease percentage sporulation of *C. cayetanensis* even at a highest concentration of T5 (2.5 lb/100 gallon) and maximum exposure time of one week. Insecticides (Diazinon and Malathion) and fungicides (Captan and Zineb) did not seem to reduce the sporulation. Viability of the oocysts was not affected; however the infectivity of the oocysts could not be determined.

Cryptosporidum parvum oocysts treated with pesticides captan, benomyl and diazinon did reduce the infectivity only at the highest concentration (T5) and longest time of exposure (1week). The infectivity levels decreased as the concentration of the pesticide Benomyl, Captan and Diazinon increased to 2.5 lb/100 gallon (T5), 2.5 lb/100 gallon (T5) and 5 teaspoons/gallon (T5) respectively. But the oocysts were 100% infective for the treatment times of 1hr to 24 hrs.

From this study it could be concluded that if the water used for preparing pesticide treatments for raspberry fields were contaminated with *Cyclospora*, the pesticides would not inactivate the oocysts. Raspberries contaminated with the parasite could cause outbreaks if consumed. Previous studies have shown that even washing does not remove all the parasites from the surface of fruits and vegetables, and that oocysts on the surface can still be infectious provided that post harvest factors are favorable for their persistence.

Our studies on effect of temperature on sporulation and viability of *Cyclospora* oocysts gave the following results. Sporulation of the oocysts on dairy substrate i.e. milk, diluted milk and whipped cream, can occur at 4° C and 23° C from 1 hr to 1 week. But at -15° C, sporulation was observed only until 24 hrs. The results of dairy substrate agreed with that of water used as control sample, showing that dairy based substrate did not give protection for oocysts. In the case of basil as food substrate, oocysts sporulated at maximum exposure time of 6 days at temperatures of 4° C, 23° C and 37° C. At 4° C, sporulation was observed upto 6 days. This was expected since previous studies have shown that oocysts kept at 4° C until 2 months sporulated when incubated at 23° C. At 37° C, sporulation decreased with increasing time until 4 days and was not observed at 6 days. Temperatures of -70° C, 70° C and 100° C were not favorable for sporulation of the parasite. Sporulation was observed until 2 days when incubated at -20° C and until 1 hr at 50° C. The results were similar for both water sample as well as basil substrate, except that in the case of basil, the percentage sporulation was higher at low and high temperatures than that in water. Basil may provide more protection to oocysts against heating and freezing than water.