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Ynés R. Ortega
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Foodborne Parasites

Second Edition

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Ynés R. Ortega • Charles R. Sterling
Editors

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Editors
Ynés R. Ortega, PhD, MPH
Department of Food Science & Technology
and Center for Food Safety
University of Georgia
Griffin, GA, USA

Charles R. Sterling, PhD
School of Animal and Comparative
Biomedical Sciences
University of Arizona
Tucson, AZ, USA

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Amoeba and Ciliates

Ynés R. Ortega and Manuela Verastegui

1.1 Preface

Amoeba and ciliates are two groups of protozoan parasites which have long been known to infect humans. Both are unicellular organisms. The amoeba has pseudopodia, which are cytoplasmic protrusions providing motility to the organism. Amoeba is commonly found in the environment and few are pathogenic to mammals.

The ciliates use the cilia (hair-like structures) on the surface of the organism for high motility. Ciliates such as *Paramecium* are commonly found in environmental waters. The only species pathogenic to humans is *Balantidium coli*, which is also found to infect pigs and nonhuman primates.

Amoeba and ciliates can be acquired either by ingestion of contaminated water or food and by contamination of products or surfaces by food handlers.

1.2 Amoeba

This group of parasites belongs to the phylum Sarcomastigophora, subphylum Sarcodina (Bruckner 1992). The cyst and trophozoite are the two distinct morphological stages of the amoeba. Some amoeba (commensal) can infect humans.

Nonpathogenic amoeba (commensal) that colonize the intestinal tract include *Entamoeba dispar*, *Entamoeba hartmanni*, *Entamoeba moshkovskii*, *Entamoeba*

Y.R. Ortega (✉)
Department of Food Science & Technology and Center for Food Safety,
University of Georgia, Griffin, GA, USA
e-mail: Ortega@uga.edu

M. Verastegui
Departamento de Microbiologia, Universidad Peruana Cayetano Heredia, Lima, Peru

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polecki, *Endolimax nana*, *Entamoeba chattoni*, *Entamoeba invadens*, *Iodamoeba butschlii*, and *Entamoeba coli*.

Amoeba can be identified by observing the morphology of trophozoites or cysts. Trophozoites can be observed only in fresh specimens from an infected individual. The arrangement, size, and pattern of the nuclear chromatin help in the identification of the various species. The size and position of the karyosome is also used in the speciation of the amoeba. The cytoplasm of the trophozoites may contain red blood cells (RBCs), bacteria, yeasts, and molds. The number and size of the nuclei in the cyst are taken into consideration when identifying the genera and species of amoeba, as well as chromatoidal bodies and vacuoles present in the cytoplasm. All of these characteristics are not easily noted in fresh preparations, requiring preparation of permanent stains of fecal smears and examined at 1000x magnification. Mixed infections are very common; therefore, observation of several parasitic structures is necessary for a conclusive diagnosis (Leber and Novak 2005).

The pathogenic amoeba for humans is *Entamoeba histolytica*. It was described by Fedor Losch in 1875 from a Russian patient with dysenteric stools (Lösch 1875). *E. histolytica* has been recovered worldwide and is more prevalent in the tropics and subtropics than in cooler climates. In areas of temperate and colder climates, it can be found in populations living in unsanitary conditions. Poor hygienic conditions, contaminated food and water, and malnourishment contribute to the high prevalence of amoebiasis in certain developing countries, particularly in children. In Bangladesh, a third of the children acquire amoebiasis in their first year of life (Morgado 2016). Amoebiasis is also frequently identified in the Indian subcontinent, Africa, and South America. In developed countries, amoebiasis is also frequently detected in travelers and immigrants.

The pathogenicity of *Entamoeba* has been controversial. In some instances, *E. histolytica* may cause invasive disease and extraintestinal amoebiasis, and in other instances, it may cause mild or asymptomatic infections. The host immune status, strain variability, environmental conditions, and the gastrointestinal microbiota are factors that may influence the clinical presentation of the disease. Axenic cultivation of the amoeba has facilitated the study of isoenzyme profiles including glucophosphate isomerase, phosphoglucomutase, malate dehydrogenase, and hexokinase in various isolates. Sargeaunt (1978) concluded that *Entamoeba* could be characterized based on their isoenzyme analysis and characterized in various zymodemes. Of the amoeba that infects humans, differences between *E. dispar* (nonpathogenic) and *E. histolytica* (pathogenic) are not only genotypic, but phenotypically distinct, although they are morphologically similar.

The life cycle of amoeba starts when the cyst, which is the infectious form, is acquired by ingestion of contaminated materials, such as food and water, or by direct fecal-oral transmission. Once in the intestinal tract, excystation occurs and trophozoites are released and propagate via asexual multiplication. Cyst formation occurs in the colon where conditions are unfavorable for the trophozoite. Cysts are excreted in the feces and can remain viable in the environment for up to several weeks if protected from environmental conditions (Garcia 1999).

Entamoeba gingivalis, an amoeba that infects the buccal cavity, is commonly associated with gingivitis and is localized in the soft tartar between the teeth and the oral mucosa. It does not have a cyst stage, and transmission is considered to be person to person or by contact with buccal secretions.

The free-living amoeba are frequently found in the environment, particularly in surface waters including ponds, lakes, and rivers. Three of these are of public health relevance: *Acanthamoeba*, *Naegleria*, and *Balamuthia*.

1.2.1 Entamoeba histolytica

Entamoeba histolytica has been described worldwide with an estimated 50 million cases annually. In areas of endemicity up to 50% of the population may be infected. It ranks second in worldwide causes of morbidity by parasitic infections (Laughlin and Temesvari 2005). Humans are the primary reservoirs of *E. histolytica*; however, it has been described as also infecting nonhuman primates. This transmission can occur via person-to-person or by ingestion of cysts present in contaminated food or water. The cysts excyst in the intestine and trophozoites are released and start dividing. Some will encyst and be excreted with the feces. In invasive amoebiasis, trophozoites may penetrate the bowel and disseminate to the liver, lungs, brain, pericardium, and other tissues. Invasive amoebiasis tends to affect men predominantly, but asymptomatic infection is equally distributed among both genders (Acuna-Soto et al. 2000). Immigrants from South and Central America and Southeast Asia are two groups with a high incidence of amoebiasis. Travelers are at high risk for acquiring the infection. In areas where *E. histolytica* and *E. dispar* are endemic, *E. histolytica* are more predominant in travelers and *E. dispar* are more predominant in residents. Amoebiasis in homosexual males is frequently transmitted by sexual behavior. Asymptomatic presentation is up to 30% (Walderich et al. 1997).

1.2.1.1 Morphology

Trophozoites are between 12 and 60 μm in diameter. The nucleus is characterized by evenly arranged chromatin on the nuclear membrane and a karyosome that is small, compact, and centrally located (Fig. 1.1). The cytoplasm is granular and has vacuoles containing bacteria or debris. In cases of dysentery, red blood cells may be present in the cytoplasm. Immature cysts are characterized by 1–2 nuclei, a glycogen mass, and chromatoidal bars with smooth round edges. Mature cysts have four nuclei, and the glycogen mass and the chromatoidal bars may disappear as the cyst matures. This process occurs while oocysts migrate in the intestine. The cyst measures 10–20 μm. Once the cyst is ingested by the new host, the gastric enzymes and neutral or alkaline pH in the intestines induce the trophozoites to become active, at which point they are liberated (Fig. 1.2).

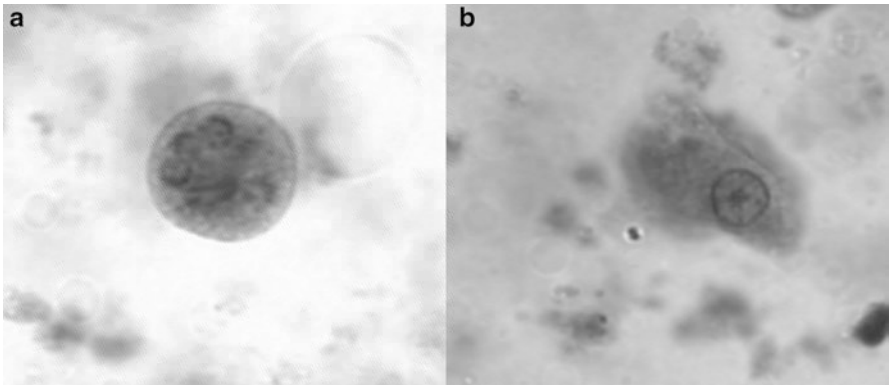

Fig. 1.1 *Entamoeba histolytica* (**a**) cyst (**b**) trophozoite

1.2.1.2 Clinical Significance

The World Health Organization estimates 50 million infections and 100,000 deaths per year (Anonymous 1997). The clinical presentation of *E. histolytica* can be asymptomatic, symptomatic without tissue invasion, and symptomatic with tissue invasion (Zaki and Clark 2001). Approximately 10% of infected individuals will have clinical symptoms such as dysentery, colitis, or, in few instances, amoebomas. Amoebomas are localized granulomatous tissues with tumor-like lesions resulting from chronic ulceration. They may be mistaken for malignancy. Amoebic dysentery is characterized by diarrhea with cramping, lower abdominal pain, low fever, and the presence of blood and mucus in feces. Ulcers start at the surface of the epithelium that deepens into a classic flask-shaped ulcer. Abdominal perforation and peritonitis are rare, but can be serious complications. Amoebic colitis is characterized by intermittent diarrhea over a long period of time and can be misdiagnosed as ulcerative colitis or irritable bowel syndrome (Leber and Novak 2005). In few cases amoebic infections can result in appendicitis. Entamoeba will invade the ileocecal appendix, produce inflammation, necrosis, and perforation. In these cases, symptoms are more severe including fever and pain in the lower right quadrant of the abdomen. Incubation period may vary from days to months.

Entamoeba histolytica can migrate to the liver causing liver abscesses. Symptoms include fever, nausea, weakness, and abdominal pain in the right upper quadrant. The biliary duct system can also be compromised resulting in jaundice. Other complications include hepatomegaly. Infection can also invade the lungs, pericardium, brain, etc. Symptoms may be acute or gradual and may include low-grade fever, pain in the right upper quadrant, and weight loss. In Mexico and Brasil, *E. dispar* has been reported in patients with invasive amoebiasis (Ximenez et al. 2010).

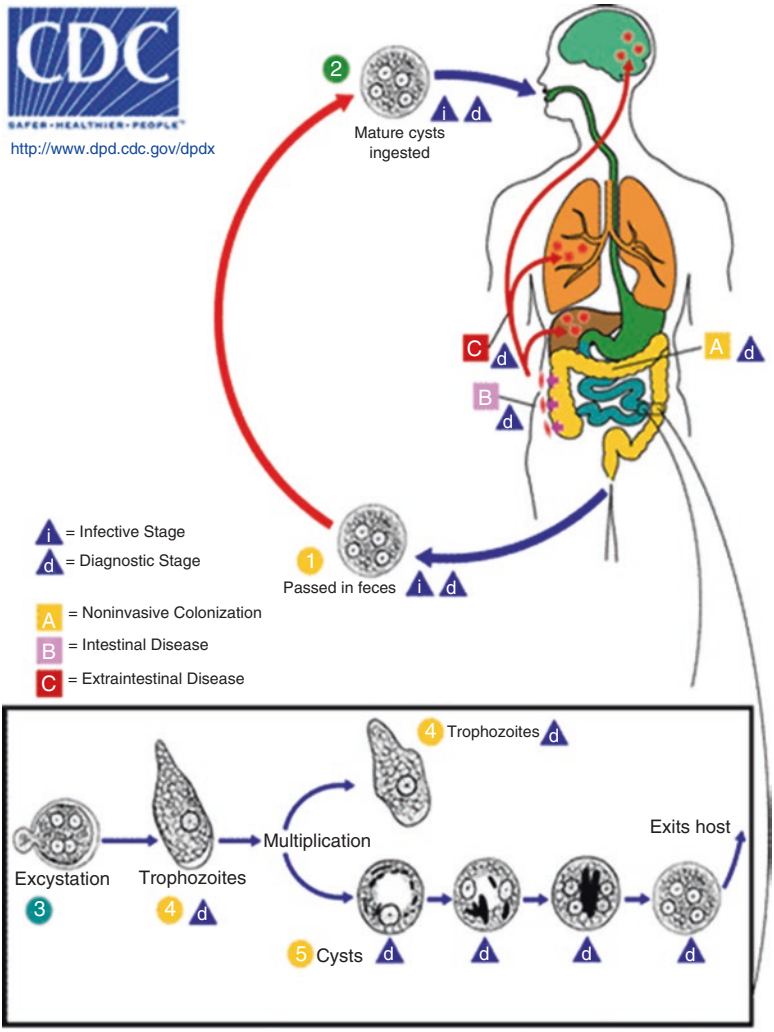

Fig. 1.2 *Entamoeba histolytica* life cycle (Graph obtained from http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary)

1.2.1.3 Pathogenesis and Immunity

Adhesins, amoebapores, and proteases have been associated with lysis of the colonic mucosa in intestinal amoebiasis (Espinosa-Cantellano and Martinez-Palomo 2000). *Entamoeba* has a cell surface protein that has a sensory activity and contributes to the surface adhesion of the trophozoite. The Gal/GalNAc lectin recognizes galactose and N-acetylgalactosamine found on the human colonic mucin glycoproteins. Interaction between this lectin and the host glycoproteins is required for adherence and contact-dependent cytolysis (Petri et al. 1989). This lectin is unique in *E.*

histolytica and has been used to develop the ELISA diagnostic assay produced by TechLab. The trophozoite moves forming the pseudopod in front, the membrane moves to the uroid, which is a posterior foot. The amoeba collects surface antigens including host antibodies on the uroid. Membrane shedding is active at the uroid region eliminating the accumulated ligands including antigens, Gal/GalNAc lectins, and the 96 kDa surface protein with the host antibodies. This process may contribute to the evasion of the host immune defenses. Amoeba with a defective cytoskeleton cannot form a cap or form uroids and cannot cause cell cytolysis, suggesting that the cytoskeleton may play a role in contact-dependent cytolysis (Arhets et al. 1998).

The cysteine proteinases are a major virulence factor. These proteinases can degrade elements of the extracellular matrix including fibronectin, laminin, and type I collagen (Keene et al. 1986). These proteinases also interfere with the complement pathways and the humoral response of the human immune system. Gal/GalNAc lectin inhibits complement mediated lysis because it mimics the CD59, a membrane inhibitor of C5b-9 in human blood cells. The proteinases can degrade and inactivate C3 and C5 to circumvent the host immune response, as well as degrade secretory IgG and IgA; limiting the host humoral immune response (Kelsall and Ravdin 1993). The presence of IgA anti-lectin provides a marker of acquired immunity.

E. histolytica also secretes a pore-forming protein, the amoebapore containing three isoforms: A, B, and C. It works by inserting ion channels into artificial membranes and may be cytolytic to eukaryotic cells (Leippe et al. 1994; Rosenberg et al. 1989).

The mechanisms of defense of the host include production of mucin. The Gal/GalNac lectin binds to it. Whether it serves as defense or as an inducer for colonization needs to be determined (Petri et al. 1989).

The inflammatory response provides another mechanism of defense. In vitro and in vivo studies demonstrated that the presence of trophozoites cause the expression of a variety of cytokines, including IL-1b and IL-8. This production occurred in regions other than those in direct contact with the parasite (Zhang et al. 2000).

A subunit of the Gal/GalNAc lectin of 170 kDa induces production of IL-12 in human macrophages. The IL-12 promotes Th1 cytokine differentiation and, in turn, macrophage protection (Campbell et al. 2000).

Diagnosis of infection can be made by examination of fecal samples, material collected using a sigmoidoscope, tissue biopsy, and abscess aspirates. Conventional ova and parasite examination can be used to detect cysts and trophozoites based on morphological characteristics of the parasite. However, it will not discriminate *E. histolytica* from *E. dispar*. Trophozoites stained with trichrome stain can measure 10–60 μm and the nuclei are clearly noticeable.

Serological testing can be used. Several tests have been developed including ELISA, IHA, IFA, and latex agglutination. Serum antibodies have been identified in 85% of patients with proven amoebiasis (by histology) and in 99% of patients with extraintestinal amoebiasis. Persons with *E. dispar* do not develop detectable levels of antibodies (Leber and Novak 2005). Diagnosis is facilitated by the examination

of permanently stained slides. Diagnostic assays specific for *E. histolytica* in clinical specimens are available on the market (Tech Labs, Blacksburg, VA) (Garcia et al. 2000; Ong et al. 1996; Pillai et al. 1999). Zymodeme analysis has been used to differentiate between *E. histolytica* and *E. dispar*, which, although specific, is also expensive and time consuming.

Molecular assays such as PCR have been developed (Evangelopoulos et al. 2000; Rivera et al. 1996; Zindrou et al. 2001; Sanuki et al. 1997), and more sensitive and specific methods are emerging. Roy and collaborators compared a real-time PCR against the antigen detection tests and SS- rRNA and traditional PCR (72% sensitive and 99% specific). The real-time PCR was more sensitive (79% sensitive and 96% specific) than all the other assays, and the specificity was higher by PCR. Using the TechLab antigen detection kit only detected 49% of positive specimens (Roy et al. 2005). These tools have been very helpful for differentiating species as well as overcoming the need for skilled microscopists.

1.2.1.4 Therapy

If treating asymptomatic infection with cyst excretion, a luminal amoebicide such as iodoquinol or paromomycin is recommended. If tissue invasion has occurred, tissue amoebicides such as metronidazole or tinidazole followed by iodoquinol or paromomycin are recommended (CDC 2017). Oral rehydration should be observed to maintain adequate fluid intake. Follow-up stool examination is important, since these treatments may lead to drug resistance. The multidrug resistance gene EhPgp1is constitutively expressed in drug resistant trophozoites (Ramirez et al. 2005).

1.3 *Dientamoeba fragilis*

Dientamoeba, originally considered an amoeba, is now considered nonflagellate trichomonad parasite and is closely related to *Histomonas* and *Trichomonas* spp.

1.3.1 Morphology and Transmission

The trophozoite measures 5–15 μm and pseudopodia are angular. No flagella are present. The cytoplasm is highly granular, and it is characterized as having 1–2 nuclei without peripheral chromatin and karyosome clusters of 4–8 granules. Cysts have not been identified in *Dientamoeba fragilis*. This parasite does not have a cyst form, and its transmission is less understood. However, transmission is suspected to be associated with helminth eggs such as *Ascaris* and *Enterobius*. Higher incidences have been reported in mental institutions, missionaries, and Indians in Arizona. It has been reported in pediatric populations (Garcia and Bruckner 1993). Symptoms

include fatigue, intermittent diarrhea, abdominal pain, anorexia, and nausea. It has been reported to cause noninvasive diarrheal illness. *Dientamoeba* colonizes the cecum and the proximal part of the colon. Reports of *Dientamoeba* are limited and this may be related to the difficulty in identifying the organisms. Asymptomatic cases of *D. fragilis* have been reported. This may be related to the description of two genetic variants using PCR-RFLP of the ribosomal genes (Johnson and Clark 2000).

1.3.2 Therapy

Tetracycline or iodoquinol are recommended as the drug of choice for individuals with symptomatic infection. If coinfections include helminths such as *Enterobius*, mebendazole is usually included in the treatment (Butler 1996).

1.4 Nonpathogenic Amoeba

1.4.1 Entamoeba hartmanni

Entamoeba hartmanni is morphologically similar to *E. histolytica*/*E. dispar*. The trophozoite measures 5–12 μm and has one nucleus with a peripheral chromatin. The karyosome is small, compact, and centrally located. The cyst measures 5–10 μm. The mature cyst contains four nuclei. Chromatoidal bodies are like those of *E. histolytica*.

1.4.2 Entamoeba coli

It is commonly found in individuals in developing countries. It is characterized for having a cyst of 10–35 μm that may contain up to eight nuclei. Chromatoidal bars are splinter shaped and have rough pointed ends. The nuclei have distinctive characteristics, including the coarsely granular peripheral chromatin. The large karyosome is usually eccentric. The trophozoite measures between 15 and 50 μm and usually bacteria are present in the cytoplasm.

1.4.3 Endolimax nana

The trophozoite measures between 6 and12 μm and has a granulated and vacuolar cytoplasm. The cyst measures between 5 and 10 μm. It is usually oval and when mature may have up to four nuclei. The nuclei have nonvisible peripheral chromatin, and the karyosome is larger than the *Entamoeba.* Morphologically, it is very different than the *Entamoeba* species.

1.4.4 Iodamoeba butschlii

The trophozoite measures between 8 and 20 μm. The cytoplasm is granular and vacuolated. The cyst may be oval or round and measures between 5 and 20 μm. The mature cyst, contrary to the other amoeba, contains only one nuclei characterized by the absence of peripheral chromatin and a larger karyosome. It usually contains a large glycogen vacuole that stains brown when the sample is prepared using iodine. *Iodamoeba* can be easily differentiated from the other amoeba.

Entamoeba coli, *Endolimax nana*, and *Iodamoeba butschlii* can be easily differentiated from *E. histolytica* primarily by their size, followed by the nuclei characteristics and cytoplasmic inclusions.

1.5 Free-Living Amoeba

Naegleria, *Acanthamoeba*, and *Balamuthia* have been identified in the central nervous system of humans and other animals. *Acanthamoeba* can also cause keratitis, and both *Acanthamoeba* and *Balamuthia mandrillaris* may cause cutaneous infection in humans. *Naegleria fowleri* and *Acanthamoeba* spp. are commonly found in soil, water, sewage, and sludge. These amoebae feed on bacteria and multiply in the environment. They may harbor pathogenic bacteria to humans such as *Legionella*, *Mycobacterium avium*, *Listeria*, etc. Whether *Acanthamoeba* serves as a reservoir for human pathogens is unknown. Meningoencephalitis caused by *Naegleria* has been coined primary amoebic meningoencephalitis (PAM). It is an acute and fulminant disease that can occur in previously healthy children and young adults who have been in contact with fresh water about 7–10 days prior to development of clinical signs. It is characterized by severe headache, spiking fever, stiff neck, photophobia, and coma leading to death within 3–10 days after onset of symptoms. The amoeba finds their way through the nostrils, to the olfactory lobes and cerebral cortex.

Acanthamoeba and *Balamuthia* encephalitis is found primarily in immunosuppressed individuals with exposure to recreational fresh water. Chronic granulomatous amoebic encephalitis (GAE) has an insidious onset and is usually chronic. The invasion and penetration to the CNS may be through the respiratory tract or the skin. These amoebae have been predominantly associated with waterborne transmission in recreational waters. Whether these amoebae are associated with foodborne transmission has not been determined.

Naegleria fowleri is susceptible to amphotericin B alone or in combination with miconazole. Few patients infected with *Acanthamoeba* have survived when treated, but in most instances, patients with encephalitis have died. A successful recovery of a patient with GAE included surgery and treatment with sulfadiazine and fluconazole (Seijo et al. 2000). Skin infections have a good prognosis and usually require topical treatment with 2% ketoconazole cream.

1.6 Ciliates

Ciliates are highly motile protozoa. They are characterized by cilia present on the surface. Free-living ciliates can be found in environmental waters. The only species pathogenic to humans is *Balantidium coli*. It was initially identified in dysenteric stools of two patients and was later described by Leukhart in 1861 and Stein in 1862 (Diana 2003). *Balantidium* can exist in reservoirs such as pigs and nonhuman primates. *Balantidium coli* can be found in as many as 45% of pigs from intensive farming to 25% in wild boars (Weng et al. 2005; Solaymani-Mohammadi et al. 2004). In Denmark, 57% of suckling pigs had *Balantidium* (Hindsbo et al. 2000); however, balantidiasis has not been reported in humans. In some regions of Venezuela, balantidiasis was observed in 12% in humans and 33.3% in pigs. Nonhuman primates have also been reported carrying the infection in the tropics. Monkeys, chimpanzees, gibbons, macaques, and gorillas can harbor *Balantidium* (Nakauchi 1999).

Human infections can occur in warmer climates. Sporadic cases have been reported in cooler areas and in institutionalized groups with poor hygienic conditions. In the USA, it is rarely found in clinical specimens. Deficient environmental sanitation favors dissemination of the infection (Devera et al. 1999).

1.6.1 Life Cycle and Morphology

The trophozoite and the cyst are the only two stages of *Balantidium coli*. The trophozoite is large and oval. It measures 50–100 μm long to 40–70 μm wide. The cyst measures 50–70 μm. The movement is rotary. The body is covered with longitudinal rows of cilia, and they are longer near the cytostome. The trophozoite is pear shaped with an anterior end pointed and the posterior end broadly rounded. The cytoplasm contains vacuoles with ingested bacteria and cell debri. The trophozoite and cyst contain two nuclei: one large bean-shaped nucleus and a round micronucleus (Fig. 1.3). The cyst form is the infective stage. It has a thick cyst wall. Trophozoites secrete hyaluronidase which aids in the invasion of the tissues. Cysts are formed as the trophozoite moves down the large intestine (Fig. 1.4).

1.6.2 Clinical Significance

Frequently, *Balantidium* infections can be asymptomatic; however, severe dysentery similar to those with amoebiasis may be present. Symptoms include diarrhea or dysentery, tenesmus, nausea, vomiting, anorexia, and headache. Insomnia, muscular weakness, and weight loss have also been reported. Diarrhea may persist for

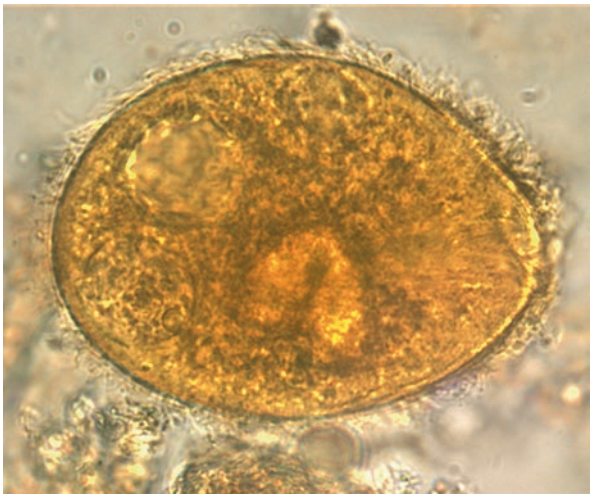

Fig. 1.3 *Balantidium coli* trophozoite. *Arrow* points at the trophozoite cytostome (Picture obtained from http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary)

weeks or months prior to development of dysentery. Fluid loss is similar to that observed in cholera or cryptosporidiosis. Symptomatic infections can occur; resulting in bouts of dysentery similar to amoebiasis. Colitis caused by *Balantidium* is often indistinguishable from E. *histolytica* (Castro et al. 1983).

The organism can invade the submucosa of the large bowel, causing ulcerative abscess and hemorrhagic lesions. The shallow ulcers are prone to secondary infections by bacteria (Knight 1978). In few cases, extraintestinal disease in addition to the liver and the lung, other presentations such as peritonitis, urinary tract infection, and inflammatory vaginitis, have been reported (Karuna and Khadanga 2014). *Balantidium* has been described in the urinary bladder of an infected individual (Maleky 1998; Knight 1978; Ladas et al. 1989). Pulmonary lesions can occur in immunocompromised patients without obvious contact with pigs nor history of diarrhea prior to pulmonary infection (Anargyrou et al. 2003). *Balantidium* pneumonia has been described in a 71 old woman suffering from anal cancer (Vasilakopoulou et al. 2003). Chronic colitis and inflammatory polyposis of the rectum and sigmoid colon and an intrapulmonary mass have been described in a case with balantidiasis (Ladas et al. 1989).

After ingestion, the trophozoite excretes hyaluronidase, which aids in the invasion of the tissue. On contact with mucosa, mucosal invasion is accomplished by cellular infiltration in the area of the developing ulcer. The organism can invade the submucosa of the large bowel. Ulcerative abscesses and hemorrhagic lesions can occur. Some of the abscess formations may extend to the muscular layer. The shallow ulcers and submucosal lesions are prone to secondary bacterial infection. Ulcers may vary in shape and the ulcer bed may be full of mucus and necrotic debris.

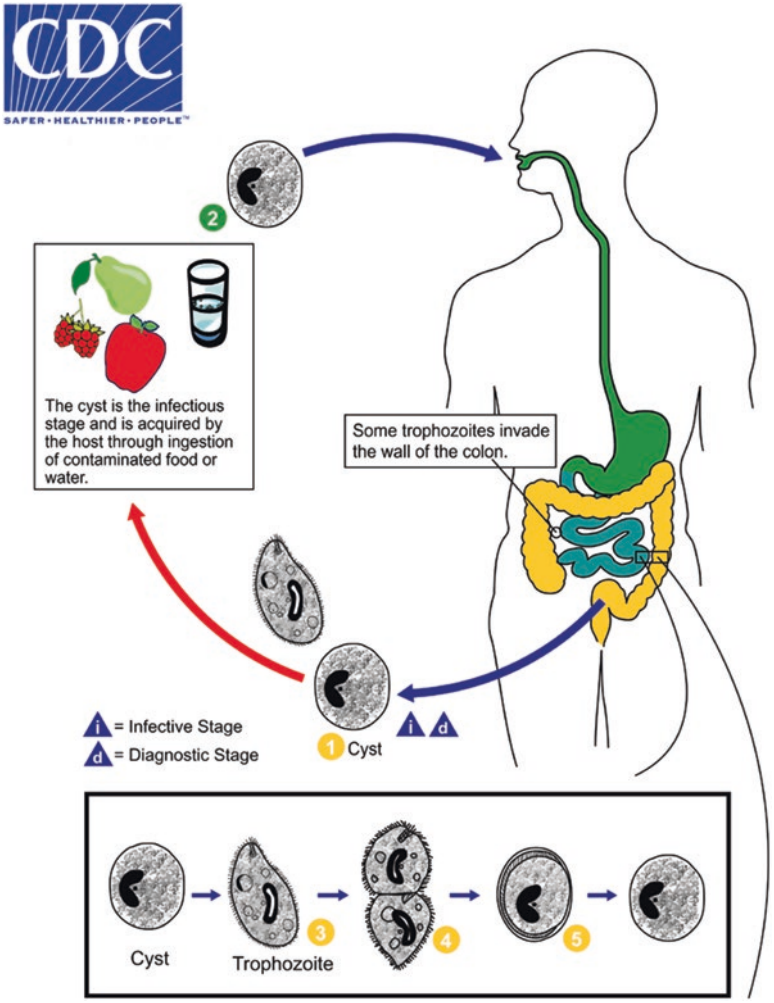

Fig. 1.4 *Balantidium coli* life cycle diagram (Credit goes to CDC. Picture obtained from http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary)

1.6.3 Diagnosis and Treatment

Wet preparation examinations of fresh and concentrated fecal material can determine the organism, as the shape and motility are characteristic of this ciliate. Tetracycline is the drug of choice, although it is considered an investigational drug for this infection. Iodoquinol or metronidazole may be used as alternative drugs.

1.6.4 Epidemiology and Prevention

Several studies have demonstrated the presence of *B. coli* in developing countries. Balantidiasis has been reported in 8% of children of the Bolivian Altiplano (Basset et al. 1986).

Domestic hogs probably serve as the most important reservoir host for balantidiasis. In areas where pigs are the main domestic animal, the incidence of infection is high. Risk factors to acquire this infection include working at pig farms or slaughter houses. Infection can turn into an epidemic if conditions favor propagation in the community. This has been observed in mental hospitals in the USA, where poor sanitary conditions are common. Preventive measures include increased attention to personal hygiene and sanitation measures, since the mechanisms of transmission are via contaminated water or foods with *Balantidium* cysts.

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Chapter 2
Foodborne *Giardia duodenalis* and *Typanosoma cruzi*

Charles R. Sterling

2.1 *Giardia duodenalis*

2.1.1 Introduction

Antonie van Leeuwenhoek (1632–1723), widely known for his work on the improvement of the microscope, and one of his disciples, yours truly, share something in common. We both have witnessed the flagellated "animalcule," also known as *Giardia*, from our own diarrheic feces under a microscope. While Van Leeuwenhoek made no connection of his observation to what might have been going on within him, that was certainly not the case with me. It is also certain that he had no idea as to how he might have acquired his infection, while I am most certain that my infection was foodborne.

My case history began circa May of 1990 while on a research trip to Peru to work with colleagues on another intestinal parasite, *Cryptosporidium*. Being a parasitologist, I had been careful on previous trips to Peru not to imbibe tap water or to eat leafy vegetables. On the last day of my trip in 1990, my colleagues and I ventured off to a restaurant well known to locals for some pisco sours and anticucho de corazon. I alone was hankering for a salad and assured by my friends that if there was one place in Lima where it was safe to eat one, it was here. A week later, back in the sanctuary of my office at the University of Arizona (UA), the symptoms of eructation, bloating, and diarrhea commenced. Putting two plus two together, I examined my own stool sample, only to witness the tumbling leaf motility of this flagellated demon. I immediately contacted an infectious disease specialist at the UA medical center who just also happened to be an expert on *Giardia* and obtained a prescription for Flagyl, the only drug for this infection available at that time in the United

C.R. Sterling, PhD (✉)
School of Animal & Comparative Biomedical Sciences, University of Arizona, Tucson, AZ 85721, USA
e-mail: csterlin@email.arizona.edu

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States. I continued with my duties at the UA, not knowing that my graduate students were recording my multiple daily voyages to the bathroom and delighting in the fact that I, a parasitologist, had defined myself as one who sits on one stool while looking at another! Within 5–6 days of treatment, I was more or less back to normal, vowing to avoid salads while traveling abroad. This case brings me to the point of thinking about how the salad components might themselves have come to bear this parasite: (1) fecal material was used to fertilize the fields in which the salad components were grown, (2) the field was contaminated by irrigation water bearing cysts, or (3) the food handler that prepared the salad was infected and did not practice good sanitation before passing his *Giardia* on to me. I don't even like to think about the latter possibility, but it certainly fits in with known *Giardia* transmission patterns. Enough about my very personal experience with *Giardia* and on to what we know about this organism and its foodborne transmission.

2.1.2 Overview of Importance, Life Cycle, and Biology

Several recent excellent reviews have been written about the biology of *Giardia* and form the basis for what follows (Adam 2001; Carranza and Luhan 2010; Ankarklev et al. 2010). *Giardia duodenalis* (synonymous *Giardia lamblia* and *Giardia intestinalis*) is a ubiquitous enteric protozoan pathogen inhabiting the upper portions of the small intestines and affecting man and a variety of domestic and wild animals. It is one of the most commonly reported parasitic infections of humans in developed countries but extracts its greatest clinical impact in disadvantaged and developing countries, causing an estimated 3×10^8 cases per year (Lane and Lloyd 2002). Symptoms of intestinal discomfort and diarrhea can be persistent and pervasive, particularly in the young. Asymptomatic infections are more frequently encountered but may serve as a reservoir from which others can acquire infection, especially in endemic developing countries. Very few virulence factors have been identified for *Giardia*, and overall disease mechanisms are probably multifactorial.

Giardia possesses two morphologically distinct developmental stages in its life cycle: the motile flagellated trophozoite that inhabits the small intestine of its host and is capable of dividing by binary fission and the environmentally protected cyst stage that allows *Giardia* to survive outside the host's body once passed in feces (Fig. 2.1). In stained preparations, trophozoites have the appearance of a sting ray with two transcriptionally active nuclei and centrally positioned parabasal bodies that give it the appearance of a face staring back at you through the microscope. Trophozoites typically measure 12–15 μm in length by 5–9 μm in width. Their ventral surface is shaped as a concave disc which, with the aid of flagellar motion, assists trophozoites in adhering to the intestinal epithelium. Transit from the small intestines and under the right circumstances causes the trophozoites to form cysts. Trophozoites predominate in diarrheic stools and cannot survive for extended periods once passed from the host. Unique biological features of the trophozoite among eukaryotic organisms include the two already mentioned active nuclei, the lack of

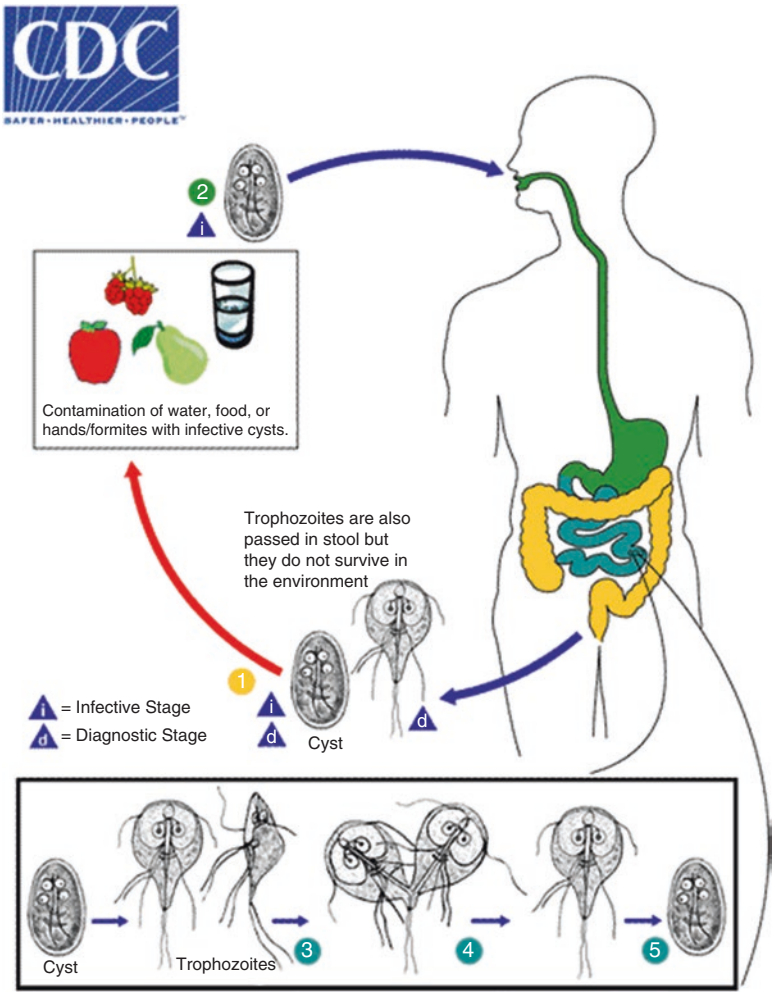

Fig. 2.1 *Giardia duodenalis* life cycle. Image courtesy of DPDx (http://www.cdc.gov/dpdx/)

mitochondria, peroxisomes and Golgi, and the appearance of a "mitochondrial remnant" called the mitosome which probably has a function in anaerobic respiration. Another unique feature of the trophozoite is its ability to exhibit antigenic variation through the exchange of ~200 variant surface proteins (Prucca et al. 2011). In *Giardia*, the possibility of antigenic variation was first suggested by the marked difference in molecular sizes of the surface antigens (Nash and Keister 1985) among isolates that were quite similar genetically (Nash et al. 1985). These surface antigens are secreted into culture medium in large quantities and are the dominant molecules found when trophozoites are surface labeled. They were initially called excretory-secretory products (Nash et al. 1983; Nash and Keister 1985) and are now called variant-specific surface proteins (*VSP*) (Mowatt et al. 1991). This property of

Giardia probably plays a role in helping the organism evade host immune responses and also in expanding host diversity. In addition to the foregoing, the *Giardia* genome contains many genes required for meiosis (Ramesh et al. 2005), and recombination among isolates of one sub-assemblage, AII, genotype A2, has been reported from infected individuals of Peru (Cooper et al. 2007, 2010). Recombination also has been reported among assemblage B isolates of humans and also between assemblages previously considered independently evolving lineages (Lasek-Nesselquist et al. 2009; Siripattanapipong et al. 2011). Whether such reports of recombination represent true meiotic events remains to be established but is mentioned because *Giardia* has long been thought to be asexual.

Cyst formation is induced by host specific factors including high bile and low cholesterol levels and a basic pH (Lauwaet et al. 2007). Mature cysts possess a thick cyst wall composed of interlinking carbohydrates, sugars, and proteins and an enclosed zoite form with four nuclei. They typically measure 9 by 12 μm in size. Cysts allow the parasite to survive in the external environment in water and soil for months. Cysts are moderately susceptible to the action of commonly used disinfectants such as chlorine. Effective filtration methods remain the most effective barrier to preventing *Giardia* cysts from getting into potable water supplies (Plutzer et al. 2010). That said, many developing countries lack both conventional disinfection or filtration methodologies. Because cysts constitute the infective stage in the life cycle they play a major role in the foodborne transmission of giardiasis.

2.1.3 Speciation and Zoonotic Potential

Currently, there are six recognized species of *Giardia.* These include two distinct species from birds, *G. ardeae* and *G. psittac*; two from rodents, *G. muris* and *G. microti*; one from amphibians, *G. agilis*; and a large taxonomic grouping termed *G. duodenalis* (Filice 1952) and meant to specify all *Giardia* from mammalian hosts other than mice (Thompson 2004). Numerous studies have clearly identified *G. duodenalis* as a species complex whose members have been divided into eight distinct genetic assemblages (Ryan and Cacciò 2013) (Table 2.1).

Table 2.1 *Giardia doudenalis* genetic assemblages

Assemblage	Host range
A	Humans, livestock, cats, dogs, beavers, guinea pigs, other primates
B	Humans, other primates, dogs, beavers, other wildlife
C/D	Dogs
E	Cattle, sheep, pigs, goats, alpaca
F	Cat
G	Rats
H	Marine mammals

Giardia duodenalis isolates that commonly infect humans fall into two major genotypes or assemblages, each with a number of genetic subgroups (Adam 2001). One of the major controversies surrounding *Giardia* is related to its true zoonotic potential (Bauer 1994; Bemrick and Erlandsen 1988; Connaughton 1989; Faubert 1988; Monis and Thompson 2003; Thompson 1998). It should be pointed out that the finding of different genotypes in humans and animals indicates the lack of zoonotic transmission but that the finding of similar genotypes in different hosts, however, is not prima facie evidence that zoonotic transmission is occurring. (However, the finding of identical genotypes in two hosts would suggest that cross-species transmission had occurred in the recent past.) In other words, similarity of genotypes is a necessary but not sufficient condition to indicate epidemiologically significant transmission from one host to another. An important aspect of this controversy is not whether zoonotic transmission can ever occur but whether it is important in terms of biology and epidemiology. It is important to point out that human infectivity studies using isolates from animals infected with genotypes A and B have not been conducted. Such studies would likely put the zoonotic issue to rest as has been done for *Cryptosporidium* (DuPont et al. 1995).

In spite of the foregoing statements, investigators have isolated the same *G. duodenalis* assemblage from beavers and humans living within the same geographical area while investigating waterborne disease outbreaks, implying that zoonotic transmission was occurring (Isaac-Renton et al. 1993; Baruch et al. 1996; McIntyre et al. 2000). In one case, an infected beaver had an organism of the same genotype as that contaminating the water and causing human infection. When the beaver was removed, the water contamination was eliminated and the epidemic stopped (Isaac-Renton et al. 1993). More recent studies involving the use of molecular tools in endemic foci where the *Giardia* transmission frequency is high have also strongly implicated zoonotic transmission involving dogs, livestock, other primates, and humans (reviewed in Thompson and Ash 2015). If conclusively proven that zoonotic transmission of *Giardia* occurs with regular frequency, then this would importantly impact on the water and foodborne transmission of *Giardia* to humans.

2.1.4 Epidemiology and Foodborne Transmission

There is little controversy over the fact that *Giardia* is transmitted via the fecal-oral route, either directly or indirectly. Transmission routes include direct person to person, animal to animal, potential zoonotic, potential zooanthroponotic, waterborne, and foodborne. The latter, which is the topic of this chapter, could include use of contaminated water in irrigation of food crops or in food preparation, as well as contamination due to poor sanitary habits used by food handlers. Most reports of the potential for foodborne transmission of *Giardia*, with perhaps the exception of my own experience, come from either reports of the presence of *Giardia* cysts on or in food, epidemiological studies of foodborne outbreaks, reports of poor personal hygiene practices used by food handlers, surveys of *Giardia* infection in food

handlers, and risk assessments for the risk of foodborne outbreaks due to the presence of *Giardia* in water used to irrigate fresh produce.

The finding of *Giardia* cysts on foods that humans consume is likely a good indication that the individual food product may, and I emphasize may, lead to *Giardia* infection and disease. It has already been demonstrated that a dose as low as 25 cysts may lead to infection (Rendtorff 1954). Foods from which cysts have been identified include dill, lettuce, bean sprouts, radish sprouts, and strawberries (Robertson and Gjerde 2001). Lettuce and leafy greens far and away lead the pack of vegetables shown to be positive (Cook et al. 2007; Dixon et al. 2013). The unanswered question is how did these foods become contaminated in the first place although many possibilities do exist. In addition to vegetables, shellfish, such as oysters and clams, through filter feeding, has been shown to harbor *Giardia* cysts (Graczyk et al. 1998, 1999a, b, 2003a, 2006; Gómez-Couso et al. 2005a, b; Hänninen et al. 2005; Shets et al. 2007). Theoretically, at least, consumption of raw shellfish could lead to infection and illness. Potential mechanical transmission of *Giardia* to food sources via synanthropic flies is also suggested by the finding of cysts on and within their bodies (Graczyk et al. 2003b).

Epidemiological investigations of possible foodborne disease outbreaks have provided some of the most compelling evidence for foodborne transmission of *Giardia*. Keep in mind that investigations of such outbreaks usually have involved multiple reported cases among individuals attending a single event where affected individuals who became sick all reported ingesting a common food. One of the first reported linkages to such an outbreak came from a nursing home in which evidence of foodborne transmission included a significant association ($P = 0.04$) deduced from sandwich consumption and illness (White et al. 1989). This type of association was not seen in individuals who ate cooked food. As it turned out, one of the food handlers had an infected child who attended a day care center and later became infected herself. She was the first, time-wise, of nine employees in the food service area who ultimately tested positive for *Giardia*. This same outbreak also provided compelling evidence for person-person transmission in this type of setting. Another outbreak occurred at a family party in which 9 of 25 people consumed fruit salad prepared by a mother who had a diapered child and pet rabbit that tested positive for *Giardia* (Porter et al. 1990). Yet another outbreak of *Giardia* came from a case-controlled investigation of employees in a corporate office setting who ate at a cafeteria whose food was prepared by a food handler who had giardiasis (Mintz et al. 1993). In this study, odds ratio and confidence interval data among 26 ill of 188 total individuals linked illness to the consumption of raw sliced vegetables prepared by this food handler. Additional overviews of such outbreaks are well summarized in the literature (Rose and Slifko 1999; Dawson 2005; Smith et al. 2007; Plutzer et al. 2010).

As pointed out in the above-noted outbreak situations, not only food but also food handlers were implicated in association with foodborne transmission. It's not surprising, therefore, that studies have been undertaken to screen food handlers for *Giardia* and other enteric parasites. One such screening report from Heathrow Airport, where a detailed medical history was mandated on all individuals prior to hiring of ground catering staff, showed that about 4% of applicants tested positive

and that many of these showed the presence of *Giardia* (McGirr 1969). Other such studies among food handlers have been reported from southern Brazil and western Iran (Takizawa et al. 2009; Colli et al. 2014; Kheirandish et al. 2014). The Brazilian studies, in particular, pointed to an association of infection with working conditions and improper hygiene. The take-home message, as in my own case, is traveler be safe rather than sorry.

Giardia duodenalis has frequently been described from numerous animal sources, and several studies have been conducted in both domestic and wild animals to assess whether or not feces from these animals possess a risk to human infection via contaminated food or water. In one study, conducted on dairy cattle in Ontario, Canada, *Giardia* was detected in 42% of cattle. Genotype assemblage B was isolated from 24.5% of cattle, while cattle-specific assemblage E was detected in 17.5% of cattle tested, suggesting that fecal material from cattle could pose a potential risk of zoonotic transmission, including through contaminated water or food (Coklin et al. 2007). The use of livestock waste bearing *Giardia* cysts, and its increasing environmental burden, likewise has called attention to such a source as contributing to human foodborne illness (Budu-Amoako et al. 2011). In a study conducted in California, *Giardia* genotypes were assessed from wild and domestic animals and linked to environmental loading that could impact on transmission of the parasite to humans. Interestingly, and in contrast to the study from Canada, most of the *Giardia* from cattle was of the host-specific E assemblage, while wild canids have a preponderance of *Giardia* from the A and B assemblages. The conclusion from this study was that cyst shedding from cattle may pose a lower risk to humans than that from other animals, such as wild canids (Oates et al. 2012). Another interesting study where genotyping studies were not conducted showed that 23.5% of rodents collected from produce fields of a major agricultural region of the Central California Coast harbored *Giardia*. Other potential human pathogens were also isolated from these rodents. The point of the study was to drive home the point that feces from these animals could contaminate preharvest crops (Kilonzo et al. 2013). Keep in mind, however, that rodent species of *Giardia* have not been implicated in causing human infections. In a longitudinal cohort study conducted in urban and rural settings of Vellore, India, an association was made of fly densities and infectious diarrhea. Fly densities were six times higher in the rural settings due largely to garbage disposal close to homes. Likewise, diarrheal episodes and duration were higher in individuals under the age of 5 included in the study. Pathogens isolated from the children included *Giardia*, and the point was made that mechanical transmission of such pathogens to food could likely occur in such a setting and be mediated by flies (Collinet-Adler et al. 2015).

A matched case-controlled study conducted in Southern England to investigate risk factors for sporadic giardiasis showed that consumption of lettuce had a positive and independent association with infection and disease (Stuart et al. 2003). This study was conducted using postal questionnaires. Another study in an agricultural area of Mexico identified the presence of *Giardia* cysts (range < 17–1633 per 100 liters) in irrigation water heavily impacted by humans and animals. Therefore,

ample opportunity existed for contaminated water to find its way onto produce crops (Mota et al. 2009). This study went one step further, and a risk assessment study was performed to assess the potential of infection from produce items of this area. The risk assessment for *Giardia* from lettuce was determined to be 2×10^{-1} at the highest detectable concentration. Much of the produce, including lettuce, from this area is imported into the United States on an annual basis. The authors were quick to point out the need for mitigation strategies that would help minimize the risk of human infection.

2.1.5 Detection

Standardized methods for detecting *Giardia* cysts in water, based on entrapment by filtration, elution, concentration by immunomagnetic separation (IMS), and identification by specific morphometric criteria and immunofluorescence, have been developed and standardized (US-EPA Method 1623 and 1623.1 1999, 2012). Use of these methods can be enhanced by following with PCR-RFLP or sequencing to determine specific parasite species or genotype (reviewed in Koehler et al. 2014). Such standardized methodologies, however, have not been established for *Giardia* isolation or detection from foods. As already noted, however, *Giardia* has been detected on food products. The incorporation of IMS, following more conventional concentration and separation methods used for foods, led to recovery of 46% ± 19% *Giardia* cysts on artificially contaminated leafy green products (Cook et al. 2007). Another study involved cyst recovery involving a washing procedure by drum rotation and sonication followed by centrifugation and IMS led to an average recovery of 67% (Robertson and Gjerde 2001). Lately, a technique employing inertial microfluidic separation, which does not use IMS and is reported to be less costly and time-consuming to techniques that use IMS, had an average recovery of 68% for *Giardia* cysts from spiked lettuce samples. This technique utilized initial concentration methods previously refined and developed (Dixon et al. 2013) and depends on the presence of a microfluidic *G. duodenalis* inertial separation chip that can channel eluates containing cysts into recovery and waste reservoirs followed by epifluorescent detection of cysts following staining with specific FITC-labeled monoclonal antibodies (Fig. 2.2). Alternatively, PCR can be used to detect *Giardia* cysts from vegetables (Dixon et al. 2013), and a recent study has shown this method to be ten times more sensitive than light microscopy (Ramirez-Martinez et al. 2015). Bear in mind that the abovementioned methods for *Giardia* cyst detection in produce have largely been made on artificially seeded samples. With a minimum detection of 10^3 cysts by PCR in 50 g of seeded lettuce (Ramirez-Martinez et al. 2015), one can only wonder what the minimum detection level will be in field harvested or store bought produce. Also bear in mind that cyst numbers on such produce are likely to be low, but that human infection can be produced by low cyst numbers.