CHAPTER 7

Molecular Tools for the Identification of Foodborne Parasites

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INTRODUCTION

Parasites have long been associated with food and waterborne outbreaks. Although parasites have been consistently reported in developing and endemic countries, the number of parasites present in the food supply of Americans has multiplied by more than a factor of 8 during the past 15 years. This is partly due to the increase of international travel and population migration. Rapid and refrigerated food transportation from foreign countries facilitates consumer contact with emerging parasites. Cultural habits have also changed towards the

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consumption of (raw or undercooked) fresh produce. These conditions have increased the probability that parasites infect naive populations and cause gastrointestinal illness (91).

Most parasites are obligate intracellular organisms. In contrast with bacteria, parasites are inert and do not multiply in the environment. Any isolation and detection procedures are crucial because an enrichment process for parasites is not available. Molecular assays overcome these difficulties and specific limitations per organism will be discussed.

Based on their morphological attributes, parasites are classified into two groups: protozoa and helminths. The protozoa are single-celled organisms and the helminths are metazoans with a rudimentary digestive and reproductive tract. The helminths are grouped as the nematode or roundworms, the cestoda or tapeworm, and the trematoda or flukes. The primary objectives in developing diagnostic molecular assays have been focused on protozoa because of the limitations of conventional parasitological methods to identify them in foods and the environment.

DNA EXTRACTION PROCEDURES

Many protocols have been described for the isolation of parasite DNA. Some are protocols prepared at individual laboratories. The trend is now to use DNA extraction kits, as this will reduce the possible variables among procedures and laboratories. The recovery efficiency of the extraction procedures, particularly with parasites, is important because of the potentially low number of parasites in environmental samples and food matrices. The sensitivity of the PCR or any molecular assay is dependent on the DNA extraction methodology. Enzyme digestion of the protozoal oocysts has been done using proteinase K in lysis buffer (10 mg proteinase K/ml, 120 mM $NaCl_2$, 10 mM Tris and 0.1% SDS) followed by phenol chloroform-isoamyl alcohol (25:24:1) separation, and DNA precipitation using salts such as 0.3 M sodium acetate with 10 μg glycogen, a DNA carrier, and 2 volumes of 100% ethanol. This method extracts DNA efficiently from parasites, but the most important step in this process is to break open the oocysts to release the protozoal DNA. Not all protozoal cysts or oocysts will be effectively digested; therefore other means of oocyst rupture have been described. One of the commonly used methods for cyst/oocyst breakage is the freeze/thaw method with cycles of freeze/thaw that vary from 3 to 12 cycles. The freezing is done in dry ice/ethanol slurry and thawed at 55°C. This process may change accordingly to the parasite and the laboratory (114). Oocysts can also be disrupted using a bath sonicator (101), keeping under consideration the possible denaturation of the DNA. Based on forensic studies, an extraction free, filter based preparation of DNA has been used and can successfully rupture the parasite cysts or oocysts. This method uses an FTA filter, which is impregnated with denaturants, chelating agents, and free radical traps. The sample is placed on the filter and cut with a hole puncher. The membrane is then rinsed and used

directly for PCR amplification (92). The use of this methodology needs to be evaluated for the processing and evaluation of large sample sizes and the potential for cross-contamination.

PROTOZOAL INFECTIONS

Protozoan parasites relevant to public health and associated with foodborne infections include the ciliates (i.e., *Balantidium coli*), amoeba (i.e., *Entamoeba histolytica)*, flagellates (i.e., *Giardia lamblia*), and coccidia (i.e., *Toxoplasma gondii*, *Cryptosporidium parvum* and *Cyclospora cayetanensis*). Taxonomical placement of some protozoan pathogens has been changing as we learn more about the genetic makeup of these organisms. Such is the case with the Microsporidia, which are now considered to be more closely related to fungi than to the protozoa. *Cryptosporidium*, originally from the phylum *Apicomplexa*, has more characteristics associated to the gregarines than to coccidia. Its taxonomical classification is uncertain and as more evidence is published, these parasites may be reclassified to more appropriate groups.

CRYPTOSPORIDIUM PARVUM

Parasite Description and Identification. *Cryptosporidium* sp. was first recognized by Tyzzer in 1907, from the stomach of a mouse (78). It is currently identified as *C. muris*. Subsequently, other species of *Cryptosporidium* were described and renamed. *Cryptosporidium* species that were morphologically similar were named *Cryptosporidium parvum*, but recent molecular analysis have lead to reclassification into several species: *C. hominis* (*C. parvum* genotype 1 or the human genotype) in humans, *C. andersoni* (*C. muris*-like or *C. muris* bovine genotype) and *C. bovis* (*Cryptosporidium* bovine genotype B) in calves and adult cattle, *C. canis*, (*C. parvum* dog genotype) in dogs, and *C. suis* (*Cryptosporidium* pig genotype I) in pigs. To date, there are 15 established *Cryptosporidium* species in fish, reptiles, birds, and mammals and 8 have been reported in humans (*C. hominis*, *C. bovis*, *C. canis*, *C. felis*, *C. meleagridis*, *C. muris*, *C. suis*, and *Cryptosporidium* cervine genotype). *Cryptosporidium* has been associated with gastrointestinal illness in humans and it is acquired by ingestion of fecally contaminated water or foods. It can also be acquired via person to person.

The life cycle of *Cryptosporidium* starts when mature and infectious oocysts are excreted in the feces of an infected host. The oocysts are ingested along with contaminated water and/or foods and excyst in the gastrointestinal tract. Sporozoites are released and infect the epithelial cells of the small intestine, particularly the ileum. Parasites multiply asexually, producing type I and II meronts containing 8 and 4 merozoites, respectively. Asexual multiplication can continue or differentiate to produce the sexual stages of the parasites. Microgametocytes (male) fertilize the macrogametocyte (female) producing the zygote, which in turn becomes the oocyst. If a thin-walled oocyst is formed, the

life cycle can initiate again. If a thick-walled oocyst (which is environmentally resistant) is formed, it is excreted in the feces (78).

Individuals at risk of acquiring cryptosporidiosis include children in daycare centers, individuals caring for animals, the elderly, immunocompromised individuals, and travelers. Since *Cryptosporidium* is highly resistant to common disinfectants, including chlorine, water parks and fountains have been implicated in numerous outbreaks.

A large waterborne outbreak occurred in 1993 in Milwaukee (76, 146), where more than 400,000 people suffered gastrointestinal illness. Initially, *C. parvum* was considered to be the agent responsible for this outbreak, however, molecular analysis of clinical specimens and water samples demonstrated that it was actually *C. hominis*. Contamination occurred when tap water was contaminated with sewer water back-flow (76, 146).

Two main target antigens of 15-17 and 23 kDa molecular weights have been used for detection of the humoral immune response of individuals with cryptosporidiosis (102). These antigens have been used in ELISA, western blot, or multiplex bead assay for the detection of *Cryptosporidium* antibodies in sera and oral fluids (85, 99, 148).

Stool specimens are the most common clinical samples examined for diagnosis of *Cryptosporidium*. Oocysts are usually concentrated using ethyl acetate concentration methodologies (139). The sample is then examined using microscopy, immunoassays, and molecular techniques. The modified Ziehl-Neelsen acid-fast stain and modified Kinyoun's acid-fast stain are more commonly used in the microscopic identification of *Cryptosporidium* (87, 103).

Immunofluorescence assays (IFA) are more sensitive and specific than the modified acid fast stains and are now being used more frequently in clinical laboratories. They are the gold standard when examining new diagnostic assays (9, 40), but cannot differentiate among the *Cryptosporidium* species (46). Some of these commercial kits include the Merifluor *Cryptosporidium*/*Giardia* kit (Meridian Bioscience; Cincinatti, OH), *Giardia*/Crypto IF kit (TechLab; Blacksburg, VA), Monofluo *Cryptosporidium* kit (Sanofi Diagnostics Pasteur), Crypto/*Giardia* Cel kit (TCS Biosciences; Buckingham, UK), and Aqua-Glo G/C kit (Waterborne; New Orleans, LA). Commercial antigen-capture-based enzyme immunoassays (EIA) available are the Alexon-Trend ProSpecT *Cryptosporidium* Microplate Assay (Alexon-Trend-Seradyn; Ramsey, MN) and Meridian Premier *Cryptosporidium* kit. These assays may not react to *Cryptosporidium* species that are genetically distant from *C. parvum*, such as *C. muris*, *C. andersoni*, *C. serpentis* and *C. baileyi* (46). Lateral flow immunochromatographic assays have also been commercialized for use with stool samples (60)

Parasites can be recovered from foods by washing the samples in 0.025M phosphate buffered saline, pH 7.25 (95). Detergents (1% sodium dodecyl sulfate and 0.1% Tween 80, or the membrane filter elution buffer from EPA method 1623) and sonication (3-10 min) are also used to facilitate the elution of parasites from the food matrices (13, 105). *Cryptosporidium* can then be concentrated by centrifugation and examined by immunofluorescence staining (13, 95). A sucrose flotation step may be included producing a cleaner sample

but at the cost of losing parasites. Moderate recovery rates of 18.2-25.2% were reported for a variety of fresh produce. Immunomagnetic separation has been included in the *Cryptosporidium* recovery procedures from lettuce, Chinese leaves, and strawberries to 42% for *Cryptosporidium* and 67% for *Giardia* (105, 106). *Cryptosporidium* oocysts can be detected by IFA in shellfish gills, gastric glands, and hemocytes from the hemolymph (32, 49).

Identification of *Cryptosporidium* oocysts in water samples is achieved by using IFA after concentration processes (EPA ICR method, EPA method1622/1623, United Kingdom SCA method, and United Kingdom regulatory method) (72). Oocysts are recovered by filtration of 10–100 L or more water, concentrated and stained with FITC-labeled *Cryptosporidium* antibodies. The ICR or SCA methods use nominal 1 μm 10″ cartridge filters and washes concentrated by flotation (using Percoll, sucrose, or potassium citrate). The EPA method 1622 and the United Kingdom regulatory method use capsule filters for filtration followed by immunomagnetic separation (IMS). To determine oocyst viability, 4′, 6-diamidoino-2-phenylindole (DAPI) vital dye is used. The recovery rates of the EPA method 1622/1623 for *Cryptosporidium* oocysts are between 10 and 75% for surface water (67, 115, 137). One of the limitations of these procedures is the cross-reactivity of the monoclonal antibodies used in the IMS and IFA kits. Dinoflagellates (120) and algae (111) may not provide accurate detection and quantification of *Cryptosporidium* oocysts, and may require confirmation by differential interference contrast microscopy.

Surface water samples may contain *Cryptosporidium* oocysts, which are a nonpathogenic species or genotypes for humans. Therefore, identification of oocysts to the species/genotype level is of significant public health importance. The PCR has become a useful tool for determining the genotype *Cryptosporidium* oocysts found in shellfish (32, 44, 45).

Analysis of environmental samples presents several limitations to these assays. The number of parasites in foods is usually small. Other structures morphologically similar may nonspecifically react with antibody-based assays for *Cryptosporidium* and make them less reliable. In addition, the presence of *Cryptosporidium* oocysts, which are not infectious to humans, may be present and could be confused with those of public health relevance. When examining environmental samples (soil, water, or foods), density gradients or antibody-based concentration procedures have been described (i.e., IMS concentration). Concentrates are then used for direct-fluorescent antibody (DFA) or for PCR detection.

Molecular Detection. One of the advantages of using molecular assays on environmental samples is the capability of identifying low numbers of parasites and being able to determine their species and genotype. This allows for parasite fingerprinting in waterborne and foodborne outbreaks, particularly when determining if the parasite is anthroponotic or zoonotic. It also aids in determining the risk factors associated with transmission of cryptosporidiosis in a particular setting (4, 5, 43, 66). Subtyping tools have been useful in the investigation of foodborne and waterborne outbreaks of cryptosporidiosis (43, 71, 124).

Earlier PCR methods (22, 65, 140) have been used only for the identification of *Cryptosporidium* spp. Several PCR-Restriction Fragment Length Polymorphism (RFLP)-based genotyping tools have been developed for the detection and differentiation of *Cryptosporidium* at the species level (6, 62, 70, 74, 86, 120, 146). Most of these techniques are based on the SSU rRNA gene. However, some of the SSU rRNA-based techniques (62, 70) used conserved sequences of eukaryotic organisms, which amplify DNA from organisms other than *Cryptosporidium* (127). Nucleotide sequencing-based approaches have also been developed for the differentiation of various *Cryptosporidium* spp.(82, 84, 125, 126, 136). These techniques use long PCR amplicons, and some amplify other Apicomplexan parasites and dinoflagellates. Because of this lack of specificity and sensitivity they cannot be used for diagnostic purposes (125, 126, 136).

Other genotyping techniques are used specifically to differentiate *C. parvum* from *C. hominis* (15, 83, 97, 118, 141). Out of ten commonly used genotyping tools for *Cryptosporidium* species/genotypes, only the SSU rRNA-based PCR tools can detect all seven *Cryptosporidium* species/genotypes (57).

Microsatellite analysis has been used to characterize diversity between *C. parvum* or *C. hominis* and their subtypes (19, 20, 35, 142). High-sequence polymorphism in the gene of 60 kDa glycoprotein precursor has also been used for subtype analysis (98, 124). Other subtyping tools include sequence analysis of HSP70 (98, 124), heteroduplex analysis and nucleotide sequencing of the double-stranded RNA (71, 146), and single-strand conformation polymorphism (SSCP)-based analysis of the internal transcribed spacer (ITS-2) (41–42).

The SSU rRNA-based nested PCR-RFLP method has been successfully used in conjunction with IMS in the detection and differentiation of *Cryptosporidium* oocysts present in storm water, raw surface water, and wastewater (147, 148).

Two SSU rRNA-based PCR-sequencing tools and one other SSU-based PCR-RFLP tool can differentiate *Cryptosporidium* oocysts in surface and wastewater samples (86, 136), suggesting that humans, farm animals, and wildlife contribute to *Cryptosporidium* oocyst contamination in water. Other genes have also been used for genotyping of *Cryptosporidium*. HSP70 and TRAP-C2-genes have limited use as they do not amplify DNA of *Cryptosporidium* species distant from *C. parvum*(57).

A few PCR related techniques have also been used to quantify and determine viability of *Cryptosporidium* oocysts. Excystation followed by DNA extraction and PCR has been developed to detect viable *C. parvum* oocysts (36, 134). Tissue culture and PCR (26, 67, 108) or reverse transcription-PCR (RT-PCR) (108, 109) has been used to detect viable *Cryptosporidium* oocysts. RT-PCR techniques have been described for the detection of viable oocysts (48, 56, 143), but it may overestimate the viability of oocysts (37). A new integrated detection assay combining capture of double-stranded RNA with probe-coated beads, RT-PCR, and lateral flow chromatography has also been developed, which should shorten detection time (64).

Other molecular tools, such as fluorescence *in situ* hybridization (FISH), or colorimetric *in situ* hybridization of probes to the SSU rRNA have been used in

the detection or viability evaluation of *C. parvum* oocysts (72, 116). Nucleic acid sequence-based amplification (NASBA) has been used in the detection of viable *C. parvum* oocysts (10). More recently, a biosensor technique for the detection of viable *C. parvum* oocysts has also been described (11), and a microarray technique based on HSP70 sequence polymorphism has been developed to differentiate *Cryptosporidium* genotypes (119).

CYCLOSPORA CAYETANENSIS

Parasite Description and Identification. *Cyclospora cayetanensis* was initially identified as a cyanobacteria-like organism. The first reports of gastrointestinal infections in humans go back to 1988, when it was described as a blue-green algae or a large *Cryptosporidium*. It was not until 1992 when Ortega et al. reported a complete description of this parasite as belonging to the coccidian (93, 96). *Cyclospora* has been identified in insectivores, snakes, and rodents. In 1997, three species were identified in nonhuman primates (30, 73). These are morphologically similar to *C. cayetanensis*, but examination of the 18S rDNA demonstrated that they are phylogenetically different. A challenge when working with *Cyclospora* is that is there is no animal model suitable to propagate it in laboratory conditions. The same limitation applies to the nonhuman primate *Cyclospora* species.

Cyclosporiasis is characterized by prolonged watery diarrhea. Nondifferentiated oocysts are excreted in the feces of the infected individual into the environment. Oocysts differentiate after 7-15 days in the environment, becoming fully sporulated and infectious. When a susceptible individual ingests oocysts from contaminated water or foods, the oocyst will excyst and release the sporocysts, which also will undergo excystation (96). Each contains two sporozoites that will infect the epithelial cells of the small intestine (94). *Cyclospora* preferentially colonizes the ileum; however, there are few reports suggesting extraintestinal colonization such as the biliary and respiratory tracts.

Even though there have been few reports of *Cyclospora* associated with drinking or swimming in contaminated water, most of the reported outbreaks in the developed world have been associated with contaminated fresh produce and berries. Lettuce, basil, and raspberries have been the most frequently implicated products (50). In 2004, the first documented outbreak of cyclosporiasis was linked to Guatemalan snow peas (113). Some patients reported consumption of untreated water or reconstituted milk.

Diagnosis of *Cyclospora* in clinical specimens is performed using a modified acid fast stain, or by direct microscopical examination. A procedure to isolate *Cyclospora* oocysts from produce was described by Robertson (107). For mushrooms, lettuce, and raspberries, recovery was 12%; while recovery from bean sprouts was 4% using lectin-coated paramagnetic beads to concentrate the parasite. Although the lectin-coated paramagnetic beads did not significantly improve recovery of the oocysts, it did produce a cleaner and smaller final volume for easier identification of the protozoan under the microscope.

Molecular Detection. The first PCR for *Cyclospora* was developed for clinical specimens. Fecal samples containing oocysts were disrupted using a bath sonicator at 120 W. The product of the nested PCR was a 304 bp amplicon (101). However, this PCR produced amplicons for *Eimeria*; a coccidian that infects a wide range of animals commonly found in environmental samples and is nonpathogenic to humans. This nested PCR was modified using the same primers but without the leader sequence. The template was prepared using 6 freeze/thaw cycles (2 min in liquid nitrogen and heating at 98˚C). To address the issue of PCR inhibitors, Instagene Matrix (Bio-Rad; Hercules, CA) was added during the DNA extraction. Nonfat milk (50 mg/ml) was used in the PCR reaction to overcome the effect of inhibitory effects of the food matrix extracts, soil, and plant matrices. An RFLP PCR was also developed to differentiate between *Eimeria* and *Cyclospora* (58).

An extraction free filter based template preparation was evaluated using *Cyclospora* oocysts from fecal and food matrix samples. Pieces of the FTA filters (Whatman; Florham Park, NJ) were added directly to the PCR mixture after washing with 10 mM Tris (pH8.0) containing 0.1 mM EDTA and heat treatment at 56˚C. A sensitivity of 10 to 30 *Cyclospora* oocyst could be detected in 100 g of fresh raspberries (92).

Quantitative real-time PCR was developed for the identification of Cyclospora, targeting a 83-bp region of the 18s rRNA gene. This analysis was based on one *Cyclospora* isolate. The sensitivity and specificity of the assay will need to be confirmed when examining a larger number of environmental samples (132).

Since other nonhuman primate *Cyclospora* also produced 294 bp amplicon with the SSU-rRNA nested PCR primers, a multiplex PCR was developed. Briefly, PCR is performed first using external, nested PCR primers described by Jinneman, followed with PCR using a series of specific primers that differentiate *Cyclospora* species and *Eimeria* species (90).

A restriction fragment length polymorphism (RFLP) assay was developed using the endonuclease *Mnl* I. RFLP DNA patterns are different between *Cyclospora* and *Eimeria* (58). To simplify the methodology for use at inspection sites, an oligo–ligation assay (OLA) was developed. The target amplicon was detected with an antibody-enzyme conjugate that could be read as a colorimetric assay (59).

Methodologies that could be used for fingerprinting analysis and genotype discrimination are not yet available. The intervening transcribed spacer 1(ITS1) was examined as a potential sequence that will allow discrimination among *Cyclospora* genotypes. When compared, the sequences of 5 isolates from a *Cyclospora* foodborne outbreak were identical. One of two Guatemalan isolates and 2 out of 4 Peruvian isolates were also identical (2). Thirty-six *Cyclospora* samples were examined using ITS1 specific primers. Sequence homology of 460–465 bp from various isolates varied between oocyst samples from 1 to 6.5% and between samples 0 and 5.7%. *Cryptosporidium* ITS is also highly variable (1.1–1.3% within and 0.8–1.6% between oocyst samples). The lack of animal models to obtain a clonal population of *Cyclospora* limits the possibility to

determine if there are different ITS sequence types within one oocyst or if there are infections with various *Cyclospora* strains (88). If intraisolate variation occurs, as with many coccidian parasites, ITS1 may not be a suitable target for genotyping of *Cyclospora*.

GIARDIA INTESTINALIS

Parasite Description and Identification. *Giardia* was initially described by Leeuwenhoek in 1681 (1). *Giardia* infecting humans was renamed in the early 1990s: as *G. intestinalis, G. lamblia,* and *G. duodenalis*. The name *G. lamblia* was well-recognized in the 1970s, but encouraged by other investigators it was changed to *G. duodenalis* and *G. intestinalis* in the 1990s. Over 40 species names have been proposed on the basis of hosts of origin. Now, several species are recognized: *G. muris* from rodents, *G. agilis* from amphibians, *G. psittaci* from parakeets, *G. ardae* from herons, and *G. microti* from voles and muskrats. *Giardia* is the most common cause of waterborne outbreaks; however, there have also been reports of foodborne outbreaks. In developing countries, chronic giardiasis has been associated with long-term growth retardation. *Giardia* can be asymptomatic or cause intermittent or chronic diarrheal complaints. Symptomatic giardiasis is characterized by prolonged and intermittent diarrhea, anorexia, flatulency, weight loss, and malabsorption. Whether *Giardia lamblia* infects humans and other mammals, whether it is a single species, and if so, whether it is a zoonotic infection has been questioned. Foods implicated with giardiasis are fresh produce or foods contaminated by food handlers (1).

The infective stage of *Giardia* is the environmentally resistant cyst and the vegetative stage is the trophozoite. The trophozoite is pear shaped and has 8 flagella and a ventral adhesive disk. When a susceptible individual ingests contaminated water or foods containing the cysts, these cysts will excyst in the intestine aided by bile salts and gastric acids. The trophozoite will multiply asexually. *Giardia* does not invade tissues and propagation occurs on the epithelial surface. The trophozoites attach to the epithelial surface. Some of the trophozoites will encyst in the jejunum and are passed in the feces.

Giardia is the most commonly isolated gastrointestinal parasite worldwide and the U.S., (61) with large waterborne outbreaks having been reported. The cysts are environmentally resistant and survive long periods of time in water at cold temperatures. *Giardia* has been detected in 81% of raw water samples and 17% of filtered water samples (68). Children attending day care centers are at higher risk of acquiring Giardiasis. Waterborne Giardiasis has been associated with unfiltered water; recreational water, such as swimming pools; water fountains; and travel (78).

Molecular classification tools have been used with various *Giardia* isolates and have determined various assemblages or genotypes based on the sequence comparisons of the SS rRNA, triosephosphate isomerase, and glutamate dehydrogenase genes. Genotype A groups 1 and 2 have been isolated from humans and animals. Genotype B has also being isolated from humans and some

animals. Assemblages C and D have been isolated from dogs, F from cats, G from rats, and E from cows, sheep alpaca, goat, and pigs. The significance of these assemblages in the human infections is being examined (1). In the Netherlands, patients with assemblage A isolates presented with intermittent diarrheal complaints, while assemblage B was present in individuals with persistent diarrheal complaints (51).

Diagnosis of *Giardia* in clinical samples is performed using bright field microscopy. Immunofluorescent assay and EIA are commercially available. Merifluor *Cryptosporidium*/*Giardia* kit (Meridian Bioscience), *Giardia*/Crypto IF kit (TechLab), Crypto/*Giardia* Cel kit (TCS Biosciences), and Aqua-Glo G/C kit (Waterborne) are some of the commercially available kits. Detection of *Giardia* cysts in environmental samples presents a significant challenge. Recovery of *Giardia* cysts from surface water to be used by drinking water treatment plants is achieved using the United States EPA Method 1623. Several of these methods have been adapted for detecting parasites in food matrices.

Molecular Detection. However, the low number of cysts typically present in these types of samples requires the use of molecular tools. In clinical specimens *Giardia* and *Cryptosporidium* were detected 22 times more often by PCR than by conventional microscopy (7).

Analysis of nucleotide sequences of glutamate dehydrogenase (GDH), elongation factor 1α (EF1α), SSU rRNA, and triosephosphate isomerase (TPI), and ADP-ribosylating factor genes can discriminate five to seven defined lineages and assemblages of *G. intestinalis* (80, 81, 121). Thus far, the TPI gene has the highest polymorphism in *G. intestinalis* at both intergenotype as well as intragenotype levels, and TPI genotyping has proven very useful in epidemiological investigations of human Giardiasis (121, 123).

Molecular characterization of *Giardia* species in wastewater has been used for community wide surveillance of human Giardiasis (130, 135). The distribution of the *Giardia* species in environmental samples correlates directly with human, agricultural, and wildlife activities. SSU rRNA-based PCR-RFLP (130) and beta-giardin-based PCR-RFLP methods were used to identify and differentiate between *Giardia* assemblages A and B from water samples.

The phylogenetic distance between *G. intestinalis* assemblages A and B is greater than typically used to differentiate two protozoan species (79, 81, 121, 131), suggesting that *G. intestinalis* may be a species complex. Phenotypic differences have also been observed. Assemblage B is more likely found in patients with persistent diarrhea, whereas intermittent diarrhea is mostly observed with assemblage A (51).

TOXOPLASMA GONDII

Parasite Description and Identification. *Toxoplasma* is a coccidian parasite that can cause severe complications for individuals with the infection. It can be asymptomatic or can cause abortion in humans if an acute infection develops

during pregnancy. Healthy individuals may develop encephalitis or be asymptomatic. *Toxoplasma* can be found worldwide and serologically it can be identified in as high as 85% of the population in some European countries. *Toxoplasma* is responsible for 20.7% of foodborne deaths due to known infectious agents. Waterborne outbreaks in Canada and Brazil (12, 54) have been reported as well.

Infection occurs when water or foods are contaminated with cat feces containing *Toxoplasma* oocysts. The oocysts excyst and the sporozoites migrate and preferentially localize in muscle and the brain. The parasites will encyst and form cysts. These contain bradyzoites, which are slow multiplying parasites. Once they become active they are called tachyzoites and multiply quickly. The parasite can cross the placenta to infect the fetal tissues (29).

A large variety of animals can acquire toxoplasmosis, but only felines (domestic and wild) are the definitive hosts. When infected tissues are ingested, the parasites will be released from the tissues and develop to the asexual and sexual stages. Once fertilization occurs, the oocysts are formed and then excreted in the environment. The oocysts are highly resistant even to desiccation and survive on dry surfaces for weeks or even months. *Toxoplasma* causes fatal meningoencephalitis in a variety of marine mammals. Shellfish has been studied during the past several years as indicators of or vectors for transmission of protozoal agents (29). Shellfish can concentrate large volumes of water and it has been demonstrated experimentally that viable *Toxoplasma* oocysts can also be concentrated. *Toxoplasma* has been classified into three different lineages I, II, and III (53). *Toxoplasma* belonging to type I lineage are highly virulent in laboratory animals, whereas the type II and III lineages are nonvirulent. In humans, the type II lineage predominates among *Toxoplasma* associated with infections in AIDS and non-AIDS immunocompromised patients (75–80%), as well as congenital *Toxoplasma* infections (encephalitis, pneumonitis, or disseminated infections). It appears that the type I lineage is more prevalent in congenital *Toxoplasma* infections in Spain (38). Ocular toxoplasmosis is a common sequela of congenital toxoplasmosis, but can be dormant for years and emerge at adulthood; causing severe retinochoriditis. PCR analysis of clinical samples from patients with these conditions determined that most *Toxoplasma* isolates were type I, type IV, or novel types (69). Outbreaks in Canada and Brazil characterized by severe ocular toxoplasmosis were caused by *Toxoplasma* type I strains (16).

Toxoplasma infections can be diagnosed by serological assays examining the antibody response towards the infection. Identification of *Toxoplasma* oocysts can be identified in the environment using conventional microscopy; however, one must consider the limitation to this approach: (1) the small number of parasites present in environmental samples; and (2) the oocysts are indistinguishable morphologically from other coccidians. *Toxoplasma* oocysts have been isolated from mussels, which serve as paratenic hosts assimilating and concentrating oocysts. *Toxoplasma* oocysts can be identified from water samples using the current USEPA method for concentration of *Cryptosporidium* (54). Centrifugation and flocculation procedures using aluminum sulfate and ferric

sulfate can also concentrate *Toxoplasma* oocysts. Sporulated oocysts were recovered more efficiently using aluminum sulfate and unsporulated oocysts could be better recovered using ferric sulfate (63). A TaqMan PCR assay was developed to detect the ssrRNA. Infectious *Toxoplasma* oocysts were detected up to 21 days in mussels as confirmed using the mouse bioassay (8).

Molecular Detection. Most PCR assays used for *Toxoplasma* identification use primers targeting the B1 gene. It is a 35-fold repetitive gene that is highly specific and conserved among strains of *Toxoplasma* (18). A PCR-enzyme immunoassay oligoprobe was developed to detect *Toxoplasma* oocysts. The PCR was directed towards the amplification of the B1 gene. Avidin coated plates were used to capture the biotin-labeled PCR amplicons, and an internal, FITC labeled oligoprobe was allowed to hybridized to the denatured and bound amplicon. The bound FITC-tagged oligoprobe was detected using anti-FITC antibodies tagged with horse radish peroxidase. This assay could detect 50 oocysts in a clean preparation (114). Other assays have focused on the sensitivity of the PCR assay. Jalal designed primers for B1 gene PCR amplification with a sensitivity of 2 parasites/sample (55).

The freeze thaw procedure in Tris-EDTA buffer and proteinase K digestion has been used to break open *Toxoplasma* oocysts. This is followed by DNA extraction using the QIAamp DNA minikit. Again, the B1 gene was used in a real-time PCR using an Icycler device (Bio Rad). Sensitivity of the real-time PCR using experimentally spiked deionized and public drinking water samples was of 1 and 10 oocysts. However, the sensitivity of the PCR was reduced for raw surface waters as only 20%, and 50% were positive by real-time PCR for samples spiked with 100 and 1000 oocysts, respectively (133).

Oocyst heating at 100˚C for 40 min in TE buffer followed with 9 freeze-thaw cycles and proteinase K digestion at 56˚C overnight has been used to break open the oocysts and provide a template for PCR. DNA was subsequently extracted from the broken oocysts using the phenol: chloroform: isoamyl alcohol procedure. DNA amplification using the18S-rRNA gene designed by McPherson and Gajadhar (77) had a theoretical detection limit of 0.1 oocyst if the preparation included oocyst concentration using aluminum sulfate flocculation (63). TaqMan PCR assays were done using B1 and ssrRNA genes to study experimentally inoculated mussels (8). Real-time PCR was more sensitive to nested PCR using B1 and bradyzoites specific genes. LC-PCR also had the advantage to quantify parasites present in serum and peripheral blood mononuclear cells (23). Other targets were used for DNA amplification. These included a 529 bp sequence present at 300 copies in the parasite genome. Using this target sequence, real-time PCR had a tenfold higher sensitivity compared to PCR targeting the 35 copy B1 gene (52). Mobile genetic elements (MGE), which has 100–500 copies/cell was also used for *Toxoplasma* identification followed by RFLP analysis (128). Other single copy genes SAG1-4 and GRA4 genes have been used as targets for *Toxoplasma* characterization and identification.

Characterization of *Toxoplasma* isolates was achieved using PCR-amplified products digested with 13 restriction enzymes and determined the genetic

relationship among *Toxoplasma* isolates and other coccidia (17). RFLP-PCR, random-amplified polymorphic DNA (RAPD), PCR, and sequencing has allowed for genotyping analysis (3, 14, 47). Sequencing of DNA polymerase and the *gra6* genes have also been used to determine strain types (33).

MICROSPORIDIA

Parasite Description and Identification. Microsporidia belongs to the phylum *Microspora*. It contains more than 1000 species and infects a wide range of hosts. Five genera have been implicated in human illness: *Encephalitozoon, Enterocitozoon, Septata, Pleistophora*, and *Vitaforma*. Originally called *S. intestinalis*, it has been reclassified as *Encephalitozoon* and *Nosema cornea* as *Vitaforma cornea. E. bieneusi* causes persistant diarrhea in the immunocompromised, where it is found frequently in the feces.

Microsporidia are obligate intracellular organisms that form highly resistant spores of pirifiorm or ovid shape ranging from 1 to 2 μm in diameter. Spores are ingested along with contaminated water or foods, or inhaled. The spore extrudes its polar filament and injects the sporoplasm (infectious spore material). The sporoplasm undergoes merogony forming multiple primordial forms. Sporogony follows, forming dividing sporonts, which form the sporoblast. They then develop into mature spores. Spores are excreted along with the feces, urine, or respiratory secretions. *E. bieneusi* has been reported in mixed infections with *Cryptosporidium*.

Microsporidial spores can be identified microscopically using Calcofluor white, or modified trichrome with Chromotrope 2R stains. The use of other stains has been reported. Identification of the spores is difficult because of the small size of the spores, which require a well-trained microscopist. Molecular assays have played a significant role, particularly in the identification of particular Microsporidia (78). The various species of Microsporidia can be acquired via various routes. The species acquired by ingestion of contaminated foods or water are *E. bieneusi* and *E. intestinalis*. The latter can be successfully grown *in vitro*.

Molecular Detection. Primers were designed to amplify the SSU rRNA gene of *E. bieneusi* in fecal and biopsy samples, either by PCR or *in situ* hybridization (21). PCR protocols call for rupture of Microsporidia spores using glass beads and overnight digestion with proteinase K to release the template. This is followed by DNA extraction using commercial kits and PCR amplification using SSU rRNA gene (24). PCR protocols seem to work with fresh as well as formalized specimens. Other PCR primers have been developed for detecting the various Microsporidia species.

To confirm the identity of the amplified PCR products from the SSU rRNA gene, restriction endoucleases *HaeIII* and *Pst I* have been used to distinguish between *E. bieneusi* and *E. intestinalis* (34); however, these restriction enzymes do not differentiate *E. intestinalis* from *E. cuniculi*. Primers were also designed to amplify conserved segments of the SSU rRNA of these two Microsporidia.

Results were confirmed by standard staining methods and immunofluorescence assay specific for *E. intestinalis* (89). Orlandi evaluated the filter based protocol described for *Cyclospora* and *Cryptosporidium* in PCR detection of *E. intestinalis*. The PCR assay could identify as few as 10–50 *E. intestinalis* spores (92). PCR amplification followed by *Hinf1* endonuclease restriction could identify *E. intestinalis* from clinical specimens (100). Examination of a fragment of the ITS region suggests the presence of genetically distinct strains of *E. bieneusi* (104). Detection of Microsporidia spores in environmental samples was evaluated using immunomagnetic separation followed by PCR and with the concentration of the Microsporidia spores by immunomagnetic separation, PCR could detect as few as 10 spores per 100 L of tap water (117). Real-time quantitative PCR has been developed using the polar tube protein gene 2 of *E. intestinalis*, which could be used to quantify the number of spores produced *in vitro*, or to determine the effect of inactivation procedures (138). Real-time PCR using commercial DNA isolation kits and an automated MagNA Pure LC instrument could identify microsporidia. The sensitivity of the PCR was 100–10,000 spores/ml of feces (145). PCR followed by sequencing of the ITS fragment of various Microsporidia isolates from human and animal origin has demonstrated high variability among isolates, and that few of the isolates from animal origin may be of public health relevance (122).

HELMINTH INFECTIONS

There are three groups of helminthic parasites and each group is significantly relevant to public health. Cestodes or flatworms associated with foodborne outbreaks include *Taenia solium* and *T. saginata, Diphylobrotrium latum, Echinococcus granulosus,* and *E. multilocularis*. Identification of the parasites can be done by observation of the larval (or cystic) stages of the parasite in meat. Nematodes or round worms have also been identified in meats as larval forms (*Trichinella spiralis, Anisakis*) and the eggs can be present in fresh produce or in contaminated water (*Ascaris lumbricoides, Toxocara canis, Capillaria, Gnatostoma,* and *Angiostrongilus*). Flukes or trematodes can also be acquired by ingestion of the cystic forms in fish, crabs, or shellfish (*Paragonimus westermani, Heterophyes,* and *Nanophyetus*). In most instances, these parasites are ingested when foods are eaten fresh or raw (not frozen). Another fluke (*Fasciola* or *Fasciolopsis*) can be acquired by ingestion of raw vegetables containing the metacercaria stages. These parasites can be isolated from meats and produce (39, 75, 112, 129, 144). Molecular assays for detection of these parasites are currently not done as routine procedure; however, laboratories that do molecular epidemiology have developed assays to describe the genotypes most commonly isolated in certain animal species. Most of these parasites have been reported in developing areas and countries, where Good Agricultural Practices (GAPs) are not established. Some of these parasites can be acquired by ingestion of game meats, raw fish, or shellfish. Most of these parasites can be inactivated when frozen.

VIABILITY ASSAYS

The viability of cysts or oocysts has been examined by using vital dyes. *In vitro* cultivation has been successful for some genotypes and species of *Cryptosporidium* and *Toxoplasma*. Most of the microsporidian spores can be propagated *in vitro* except for *E. bieneusi,* which is the most commonly identified microsporidia in humans and associated with gastrointestinal illness (25, 27, 28). To date, there is no effective *in vitro* cultivation assay for *Cyclospora. Giardia* can be excysted and grown in TYI-S-33 media; however, a large number of cysts are required for successful propagation. To date, some *Giardia* assemblages or genotypes cannot be propagated *in vitro*. Animal models that could be used to determine infectivity and viability are limited to *Cryptosporidium* and *Toxoplasma* (108). *Cryptosporidium* can be propagated using neonate calves or mice (110). However, *C. hominis*, which is anthroponotic, does not infect these animals. Gnotobiotic pigs have been used to propagate *C. hominis*, but they are not a practical animal model for inactivation and viability studies. No animal model is currently available for *Cyclospora* (31), nor has the disease been reproduced with this agent in healthy, human volunteers. *Toxoplasma* tachyzoites can be propagated using the MRC-5 cell line and most other fibroblast cell lines. It can infect cats, mice, and chickens. Whether this infectivity is selective to certain genotypes is currently not known.

As evident from this discussion, there are several viability assays available for some but not all foodborne parasites. However, how practical and cost-effective are these assays in assessing effectiveness of certain processes for eliminating these pathogens, or reducing their load in food? Obviously, there is a need for developing methods to discern the effectiveness of certain food processes for eradicating or reducing these parasites. Reverse transcriptase PCR developed as "real-time" detection of these foodborne parasites might prove to be an important tool, in the future, for this endeavor.

CONCLUSIONS

Parasites and the intestinal diseases they cause are more frequently being associated with consumption of raw vegetables, fruits, and nonfiltered water. These outbreaks are most often associated with produce imported from areas, where these parasites are endemic. Importation of foods is now necessary in order to satisfy the consumer demands for certain commodities, especially fresh fruits and vegetables. Incentives for importation of foods include the cost of production for particular crops throughout the year. The fast transportation of fresh produce from the farm to the consumers has favored the survival of these pathogens on these commodities. Due to advances in medicine, the U.S. demography has also changed. The elderly, immunocompromised individuals and children are at higher risks of acquiring and having a more severe illness. Most foodborne outbreaks are considered to be bacterial or viral. To determine the etiology of an outbreak may take many days, by which time samples of the

implicated product may not be available for investigation. Because of this factor, food scientists and the medical community need to be aware that parasites are significant agents for foodborne outbreaks and routine examination of clinical samples may miss the identification of parasites, particularly with *Cyclospora* and *Cryptosporidium*. Molecular assays have been very helpful in foodborne outbreak investigations, but they have also demonstrated the current limitations in food parasitology. Parasite isolation and recovery procedures are crucial to have a very accurate and specific diagnosis. This is particularly important, since parasites are generally inert in the environment and enrichment procedures necessary for bacterial contaminants are not an option in the detection of these pathogens in foods. With further development and refinement, PCR will prove to be an important tool in surveillance of foods for these emerging human parasites.

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Table 7.1. Primers used for the identification of protozoan parasites.

Organism	*Target Gene*	*Sequence*	*Expected Size (bp)*	*Reference*
Cryptosporidium				
	18S rRNA	F:AGCTCGTAGTTGGATTTCTG	435	92
		R:TAAGGTGCTGAAGGAGTAAGG		
	SSU-rRNA	F:TTCTAGAGCTAATACATGCG[1]	1,325	146
		R:CCCATTTCCTTCGAAACAGGA[1]		
		F:GGAAGGGTTGTATTTATTAGATAAAG[2]	826–864	
		R:AAGGAGTAAGGAACAACCTCCA[2]		
	chromosomal fragment	F:CCGAGTTTGATCCAAAAAAGTTACGAA[1]	402	48
		R:TAGCTCCTCATATGCCTTATTGAGTA[1]		
		F:GCGAAGATGACCTTTTGATTTG[2]	194	
		R:AGGATTTCTTCTTCTGAGGTTCC[2]		
Cyclospora cayetanensis	18S rRNA	F:TACCCAATGAAAACAGTTT[1]	636	92
		R:CAGGAGAAGCCAAGGTAGG[1]		
		F:CCTTCCGCGCTTCGCTGCGT[2]	294	
		R:CGTCTTCAAACCCCCTACTG[2]		
Encephalitozoon intestinalis	18S rRNA	F:TTTCGAGTGTAAAGGAGTCGA	520	92
		R:CCGTCCTCGTTCTCCTGCCCG		
Microsporidia	18S rRNA	F:CACCAGGTTGATTCTGCCTGA[3]	1,300	
		R:TAATGATCCTGCTAATGGTTCTCCAAC[3]		
Enterocytozoon bieneusi	18S rRNA	F:GAAACTTGTCCACTCCTTACG[3]	607	92
		R:CAATGCACCACTCCTGCCATT[3]		
Encephalitozoon cuniculi	18S rRNA	F:ATGAGAAGTGATGTGTGTGCG[3]	549	92
		R:TGCCATGCACTCACAGGCATC[3]		
Encephalitozoon hellem	18S rRNA	F:TGAGAAGTAAGATGTTTAGCA[3]	547	92

		R:GTAAAAACACTCTCACACTCA[3]		
Giardia	18S rRNA	F:GCGCACCAGGAATGTCTTGT	183	130
		R:TCACCTACGGATACCTTGTT		
Toxoplasma gondii	SSU rRNA	F:CCGGTGGTCCTCAGGTGAT	120	8
		R:TGCCACGGTAGTCCAATACAGTA		
		FAM-ATCGCGTTGACTTCGGTCTGCGC-TAMRA[4]		
	B1	F:TCGAAGCTGAGATGCTCAAAGTC	129	8
		R:AATCCACGTCTGGGAAGAACTC		
		FAM-ACCGCGAGATGCACCCGCA-TAMRA[4]		
	B1	F:Biotin-GGAACTGCATCCGTTCATGAG[5]	193	114
		R:TCTTTAAAGCGTTCGTGGTC[5]		
		FITC-GGCGACCAATCTGCGAATACACC[5]		
		FITC-TCGTCAGTGACTGCAACCTATGC[5]		

[1]Nested PCR, external primers
[2]Nested PCR, internal primers
[3]Multiplex PCR primers
[4]TaqMan primer. FAM: 6-carboxyfluorescein, TAMRA, 6-carboxytetramethylrhodamine.
[4]PCR-ELISA. See text or reference 114 for details.