CHAPTER 9

# Waterborne Parasites and Diagnostic Tools

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## 9.1 PREFACE

The purpose of parasite detection is not limited to curing disease in an infected individual but is crucial in the prevention and spread of disease. For these reasons, detection methods must be held at the highest standard of both sensitivity and specificity so that false-negative and false-positive results are avoided. Achieving these two requirements has been challenging, despite the development of various detection methodologies over the last several years. For parasitic organisms, those spread by food and water alike, microscopic based detection and diagnostic techniques are still revered as the gold standard. However, the advent and employment of molecular methodologies has proven to surpass microscopy in three major aspects: sensitivity, specificity, and the ability to speciate.

The choice and application of the appropriate detection or diagnostic technique begins with asking questions surrounding the target organism itself and the analytical objective. This task can be reduced to three essential questions. First, what is the matrix type to be assayed for the presence of the parasite; is it food (berries, lettuce, meat/tissue); is it from an infected individual (blood, fecal, or tissue); or is it environmental (water, wastewater, soil, etc.)? Second, what is the form of the parasite? Is it the trophozoite or cyst, as in the case of *Giardia lamblia*, the oocyst as for *Toxoplasma gondii*, or the nurse-cell for *Trichinella spiralis*? Finally, what is the purpose for detecting the organism's presence? Is it for patient diagnosis so that disease may be treated; is it source tracking for watershed management; or possibly epidemiological fingerprinting? Answering these questions is essential so that appropriate recovery and diagnostic techniques are used to confidently and consistently ascertain the answer. Once these questions have been answered, then the molecular technique or combination of techniques can be selected. Depending on the technique(s) selected, different analytes are targeted for recovery and purification from the sample matrix. These analytes can be divided into three groupings, the whole organism, nucleic acids (DNA and RNA), and proteins (including enzymes).

The parasites *Cryptosporidium parvum* and *G. lamblia* (syn. *G. intestinalis*, *G. lamblia*) are proven to (1) be endemic throughout the world, causing infections in both developed and developing countries; (2) be capable of causing outbreaks with significant illness and economic effects; (3) have a low infectious dose; and (4) utilize multiple transmission routes including ingestion of contaminated water and food, as well as direct fecal-oral transmission. In addition, *C. parvum* also poses special challenges to the water industry. It is small in size ($\sim$5 μm) making it difficult to remove via filtration; resistant to common disinfection processes, including chlorination; and environmentally hardy, remaining infectious for months. For these reasons, this chapter briefly discusses transmission routes and reported incidences

of these two parasites while largely focusing on the multiple diagnostic techniques that have been developed for their detection. Other parasites will also be alluded to, as many life cycle particulars are shared between the different genera. For example, parasites that use contaminated water as a direct conduit for transmission or are foodborne include *Ascaris lumbricoides*, *A. suum*, *Balantidium coli*, *Blastocystis hominis*, *Cyclospora cayetanensis*, *Fasciola hepatica*, *Fasciolopsis buski*, the Microspora group, and *T. gondii*. In addition, it is noteworthy that many of the methods used for detecting parasites (helminth and protozoa alike) in environmental samples have derived from protocols developed in the clinical setting. Therefore, the molecular techniques discussed in this chapter, with modification to isolation procedures, are used in the detection of the foodborne parasites as well. Examples of these parasites include *C. parvum*, *G. lamblia*, *Diphyllobothrium latum*, *Paragonimus westermani*, *Taenia saginata* and *T. soilium*, *Trichinella spiralis*, and *T. gondii*.

Two important notes should be made at this time. First, it should be stated that molecular techniques are powerful tools and have different levels of complexities within the answers they provide. When used properly, the appropriate technique, even those that are less sophisticated, will give the information needed to answer the proposed question. However, sometimes a novel technique is applied to a question where the need for more complex information is not necessary (i.e., more information than needed is collected). And second, since the advent of the polymerase chain reaction (PCR) in 1985, and the multitude of subsequent derivative techniques, parasite detection methodologies have evolved more rapidly than the necessary and required quality assurance (QA) and quality control (QC) procedures and strategies. Proper QA/QC evaluation of these new techniques is discussed in the last section of this chapter.

## 9.2 PARASITES

The biology of many of the parasites are discussed elsewhere in other chapters. The purpose of these brief paragraphs is to outline important biological factors that need to be considered when developing a detection technique.

*Cryptosporidium* spp.: Taxonomy places the obligate intracellular parasite *Cryptosporidium* in the phylum Apicomplexa and class Sporozoa, which includes many medically important human and veterinary parasites such as *Cyclospora cayetanensis*, *Eimeria* spp., *Isospora* spp., *Plasmodium* spp., *Sarcocystis* spp., and *T. gondii* (Levine, 1984; Morgan *et al.*, 1999). The species *C. parvum* was originally described in 1912 (Tyzzer, 1912), however it wasn't until 1971 that the parasite was perceived important due to the economic impact as a result of bovine diarrhea (Panciere *et al.*, 1971). Later, cryptosporidiosis gained worldwide interest in 1982 with the emergence of the human immunodeficiency virus (HIV) and AIDS (Anonymous, 1982). Currently, 14 *Cryptosporidium* species are recognized based on vertebrate host infectivity, morphology and DNA classification. Of these, two species, *C. hominis* and *C. parvum*, are the major cause of human infection and outbreaks including ingestion of contaminated food and water (Table 9.1). These two species are morphologically similar and therefore proper classification relies upon

**Table 9.1.** Cryptosporidiosis outbreaks due to contaminated drinking water.[a]

| *Year* | *Location* | *Number of cases* | *Suspected cause* | *Reference* |
|---|---|---|---|---|
| 1984 | Braun Station, TX | 2006 | Contaminated well water | (D'Antonio *et al.*, 1985) |
| 1987 | Carrollton, GA | 12,960 | Treatment deficiencies | (Hayes *et al.*, 1989) |
| 1989 | Ayrshire, UK | 27 | Treatment deficiencies | (Smith *et al.*, 1989) |
| 1990–1991 | Isle of Thanet, UK | 47 | Treatment deficiencies | (Joseph *et al.*, 1991) |
| 1991 | Berks County, PA | 551 | Treatment deficiencies | (Moore *et al.*, 1993) |
| 1992 | York Shire, UK | 125 | Contaminated tap water | (Furtado *et al.*, 1998) |
| 1992 | Jackson County, OR | 15,000 | Treatment deficiencies | (Frost *et al.*, 1998; Moore *et al.*, 1993) |
| 1993 | Milwaukee, WI | 403,000 | Treatment deficiencies | (MacKenzie *et al.*, 1994) |
| 1993 | Waterloo, Canada | >1000 | Contaminated tap water | (Rose *et al.*, 1997) |
| 1993 | Las Vegas, NV | 103 | Unknown | (Goldstein *et al.*, 1996) |
| 1993 | Wessex, UK | 27 | Contaminated tap water | (Furtado *et al.*, 1998) |
| 1994 | Kanagawa, Japan | 461 | Contaminated drinking water | (Kuroki *et al.*, 1996) |
| 1994 | Walla Walla, WA | 104 | Sewage contaminated well | (Dworkin *et al.*, 1996) |
| 1995 | Northern Italy | 294 | Contaminated water tanks | (Frost *et al.*, 2000; Pozio *et al.*, 1997) |
| 1995 | Gainesville, FL | 77 | Contaminated tap water | (Anonymous, 1996) |
| 1996 | Ogose, Japan | >9000 | Unfiltered ground water | (Yamazaki *et al.*, 1997) |
| 1996 | New York, NY | >30 | Contaminated apple cider | (Anonymous, 1997b) |
| 1997 | Shoal Lake, Ontario | 100 | Unfiltered lake water | (Anonymous, 1997a) |
| 1998 | Brushy Creek, TX | 32 | Sewage contaminated well | (Anonymous, 1998) |
| 1999 | Northwest England, UK | 360 | Unfiltered surface water | (Anonymous, 1999) |
| 2000 | Belfast, Northern Ireland | 129 | Contaminated drinking water | (Glaberman *et al.*, 2002) |
| 2000 | Lancashire, England | 58 | Contaminated drinking water | (Howe *et al.*, 2002) |
| 2001 | Belfast, Northern Ireland | 230 | Contaminated drinking water | (Glaberman *et al.*, 2002) |

[a] Table adapted from Fayer *et al.*, 2000.

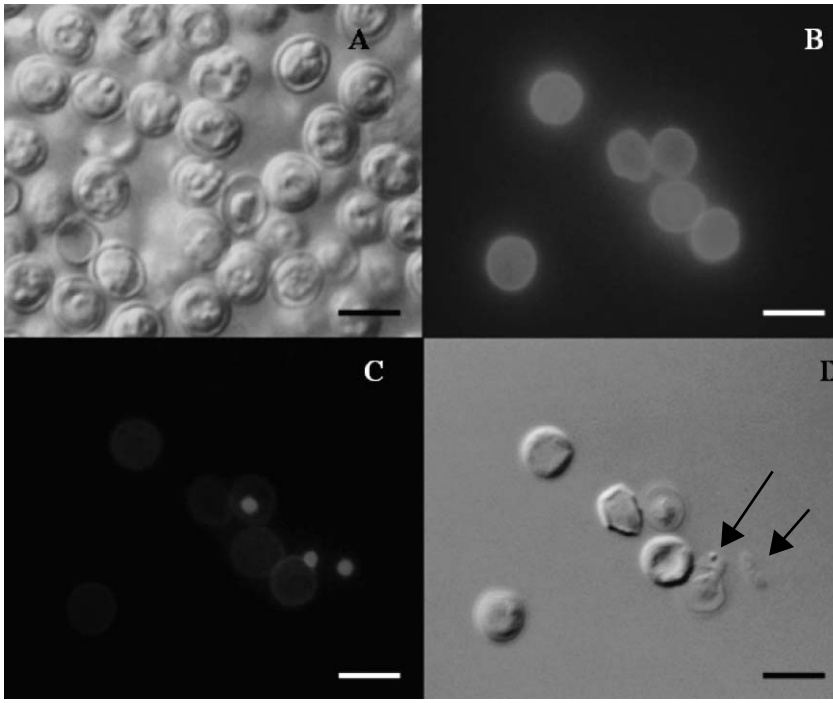


**Figure 9.1.** *Cryptosporidium parvum* photomicrographs at 1000X magnification. Panel A: Purified oocysts, differential interference contrast (DIC) microscopy; Panel B: Purified oocysts, immunofluorescent microscopy; Panel C: Purified oocysts stained with DAPI highlighting excysted sporozoite nuclei; Panel D: Purified oocysts, differential interference contrast (DIC) microscopy highlighting excysted sporozoite (arrowhead). Bar represents 5 μm.

molecular methods. Confounding matters, *C. hominis* has evolved to preferentially infect humans (Xiao *et al.*, 2002), making laboratory infectivity and disinfection studies with *C. hominis* nearly impossible to perform due to a lack of a consistent and standardized supply of oocysts. The characteristics of *C. parvum* (and by association *C. hominis*) complicates matters with two major aspects having a critical role for proper identification. First, the small size of *C. hominis* and *C. parvum* oocysts (4–6 μm) confound microscopic identification in fecal and environmental matrices since algae and yeast spores appear as the same size and shape and may cross-react with FITC labeled anti-*Cryptosporidium* monoclonal antibodies. In addition, as the oocysts for both species are similar in size and shape, speciation relies upon molecular methods. Second, oocyst concentrations in environmental samples are low thus making collection, concentration, and recovery techniques critical first steps for the application of molecular methods. In separate studies, a geometric mean of 2.7 oocysts/L (range: <0.007 to 484 oocysts/L, n = 66) was documented in source waters (LeChevallier *et al.*, 1991) and <1 oocysts/L (range: 0.001 to 0.48 oocysts/L, n = 158) is reported in drinking waters (Rose *et al.*, 1997). These combined aspects

make proper detection of *Cryptosporidium* spp. vital for the protection of public health and are technically very challenging.

*Giardia lamblia*: Of the six recognized *Giardia* species, *G. lamblia* (syn *G. intestinalis*, *G. duodenalis*) is the only species infectious to humans (Thompson, 2000). This primitive flagellate protozoan also infects a wide range of vertebrate hosts including pets, livestock, and wild animals, thus increasing the potential for zoonotic transmission (Thompson, 2000). The cysts of *G. lamblia* isolates are morphologically similar, however molecular characterization has determined seven different genotypes or assemblages, A through G (Monis *et al.*, 1999). Of these, humans are only infected with Assemblages A and B (Homan *et al.*, 1992; Maryhofer *et al.*, 1995; Nash and Mowatt, 1992). Assemblages C and D are dog specific (Hopkins *et al.*, 1997; Monis *et al.*, 1998). Assemblage E is found in hoofed livestock (cattle, goats, pigs, and sheep) (Ey *et al.*, 1997). Finally, Assemblages F and G are infectious for cats and rats, respectively (Monis *et al.*, 1999). As with *Cryptosporidium* spp., objects of similar size and shape (e.g., algal cells) confound proper identification of *Giardia* cysts in fecal and environmental samples due to the use of non-specific dyes and cross-reacting FITC labeled monoclonal antibodies. Again, it is observed that molecular techniques are required for epidemiological and source tracking studies.

## 9.3 CONCENTRATION AND ISOLATION TECHNIQUES

Whether a microscopic or molecular technique is employed, a general first step is to execute an isolation protocol that both concentrates and purifies the parasite from the matrix (berries, meat, water, etc.), while reducing potential cross reactors or inhibiting substances. In general, the majority of the isolation techniques target the environmentally resistant stage of a parasite's life cycle (e.g., cyst, oocyst, and ova) based on the inherent robustness of this form rather than the active trophozoite and larval stages (cercariae and other infective larvae). The robustness of this resistant stage allows for exposure to chemicals such as surfactants or alcohols, as well as the separation steps such as centrifugal flotation or immunomagnetic separation (IMS) while having limited physical effect on the organism and subsequent microscopic or molecular detection. However, for some strictly foodborne parasites such as *Trichinella spiralis*, the direct carnivorous nature of the transmission route leaves detection protocols relying upon tissue sectioning for histology or tissue mincing for molecular protocols (such as ELISA or PCR).

The majority of collection/concentration techniques employed today are adopted and modified from isolation protocols developed within the clinical setting. For fecal samples, different protocols include an initial treatment step dilution with phosphate buffered saline (PBS) or a preservative followed by sieving through metal mesh sieves or cheesecloth to remove large debris. This semi-purified sample would then be subjected to an organic solvent such as phenol or diethyl ether in order to do a primary removal of lipids. Purification and concentration steps would include selective centrifugation such as zinc sulphate (Ryley *et al.*, 1976), diethyl-ether/Percoll (Garcia *et al.*, 1979; Garcia and Shimizu, 1981; Horen, 1983), Percoll density gradients (Waldman *et al.*, 1986), sequential discontinuous Percoll®/Sucrose (Arrowood

and Sterling, 1987), Sheather's sugar flotation (Current *et al.*, 1983; Sheather, 1923), or for highly purified organisms, sucrose/cesium-chloride gradient (Arrowood and Donaldson, 1996). While none of these flotation techniques provide debris free parasite recovery, all assist in concentrating target organisms.

For inherently low parasite concentrations found in environmental samples, the goal is to concentrate and purify at the same time. Current detection protocols for *Cryptosporidium* spp., and *T. gondii*, as well as other parasites, have all evolved from techniques developed for *Giardia*. An outbreak of giardiasis in Aspen, CO, in 1965 (Craun, 1986) initiated the development of a microscopic based method that combined water filtration to concentrate *Giardia* cysts, followed by zinc sulfate floatation for separation and iodine staining for microscopic identification (Quinones *et al.*, 1988; Reference-Method, 1992). This basic detection strategy of concentration, separation/isolation, and microscopic detection is generally followed currently (oo)cyst detection in water samples, but with modern improvements. Filtration of water samples is used to concentrate water particulates, including (oo)cysts. Types of samples include treated drinking water, ground water, surface waters (lakes/reservoirs, irrigation ditches, rivers, etc.), storm water, and sewage treatment effluent. Various approaches to large volume water sample concentration have been used. Adapted from the *Giardia* recovery methods, a 10-inch polypropylene cartridge filter with a 1 μm nominal porosity was the filter of choice of the US Environmental Protection Agency (USEPA)-mandated Information Collection Rule (ICR) (Musial *et al.*, 1987; USEPA, 1996). However, this filter was later demonstrated to have low recovery efficiencies (LeChevallier *et al.*, 1995; Shepherd and Wyn-Jones, 1996) and therefore, other filtration/concentration techniques were developed, including membrane filtration (Ongerth and Stibbs, 1987), calcium carbonate flocculation (for small volumes) (Vesey *et al.*, 1993), continuous flow centrifugation (Borchardt and Spencer, 2002; Renoth *et al.*, 1996; Zuckerman *et al.*, 1999), flow cytometry (Vesey *et al.*, 1994a, 1994b), wound fiberglass cartridge filters (Kaucner and Stinear, 1998), foam filters (McCuin and Clancy, 2003), and capsule filters (Matheson *et al.*, 1998). The current USEPA Method 1623 for detection of *Cryptosporidium* and *Giardia* in water has approved various filter systems for (oo)cyst recovery, typically from 10 to 50 L surface water samples (USEPA, 2003). For example, the Envirochek™ High Volume (HV) Sampling Capsule (Pall Gelman Laboratory, Ann Arbor, MI), a self-contained 1 μm absolute porosity pleated membrane capsule filter, has a reported recovery rate of $>70\%$ (Matheson *et al.*, 1998). The other approved filter system is based on compression of foam disks to achieve the appropriate porosity to collect (oo)cysts on the surface of the filter. For example, the Filta-Max® (IDEXX Laboratories, Inc., Westbrook, Maine) recovery efficiency in tap water was $48.4\% \pm 11.8\%$, and in raw source waters, the range was 19.5 to 54.5% (McCuin and Clancy, 2003).

Once the water sample has been filtered and the water particulates, including (oo)cysts, have been captured on the surface of the filter, a surfactant is applied to release the particulates back into solution as individual particles for downstream purification protocols. Different surfactants have been used, including sodium dodecyl sulfate (SDS), Laureth 12, Tween 20, and Tween 80. Generally, these surfactants are mixed solely or in combinations with a buffering solution such as EDTA,

PBS, and/or Tris. For use while sampling finished drinking waters, during which scaling (residue accumulation) can occur on the surface of a membrane filter and potentially trap target organisms (e.g., *C. parvum* oocysts), the addition of sodium hexametaphosphate to the elution solution has been proposed to breakdown/dissolve scaling to release any trapped organisms (Fireman and Reitemeier, 1944; Kasper, 1993). Once the (oo)cysts have been resuspended, a concentration step is performed solely or in conjunction with a separation step to further remove sample debris as well as concentrate the (oo)cysts. Two techniques that are performed without a separation step are calcium carbonate flocculation and continuous flow centrifugation. The recovery efficiencies for calcium carbonate flocculation and continuous flow centrifugation have been reported as $> 70\%$ (Shepherd and Wyn-Jones, 1996) and $>90\%$ (Borchardt and Spencer, 2002), respectively. However time constraints ($>4$ and $>2$ h required, respectively) restrict the use of these techniques for day-to-day applications. The selective centrifugation techniques developed for fecal material combine concentration and separation in one step and were directly transferred to use with environmental samples. However, these selective centrifugation techniques have been shown to have moderate recovery efficiencies and while appropriate for use with fecal samples in which (oo)cyst concentrations are generally high, the low (oo)cyst concentrations in environmental samples prove to be an issue. The most common techniques used for (oo)cyst separation from environmental samples include Percoll/sucrose flotation (Arrowood and Sterling, 1987; LeChevallier *et al.*, 1995), Percoll/Percoll step gradient (Nieminski *et al.*, 1995), flow cytometry (Vesey *et al.*, 1994a), and immunomagnetic separation (IMS) (Johnson *et al.*, 1995). Of these, IMS has gained the most notoriety and is currently the separation method of choice for the USEPA and United Kingdom Drinking Water Inspectorate (UKDWI).

## 9.4 DETECTION METHODOLOGIES

### 9.4.1 Microscopic Techniques

#### 9.4.1.1 Strengths and Weaknesses

Microscopic identification has several positive attributes that preserve its status as the "Gold" standard even in light of flourishing molecular techniques. Under the qualified eye of a technician, the physical observance of a life cycle stage is convincing proof that the patient or environmental sample has that particular parasite present. To assist in detection and identification, several methods can be used including various stains, fluorescence, and/or Nomarski differential interference contrast microscopy (DIC). These different methods highlight and allow the microscopist to visualize defining characteristics such as internal structures (i.e., hooks, median bodies, nuclei, etc.) or abnormal characteristics such as algal chloroplasts. However, two qualities make microscopy a less than optimal detection technique. First, as stated above, demands on the technician are not limited to knowing all the different parasites and the respective different life cycle stages but also all the potential cross-reactors that might be present in any given sample. Months, if not

years, are required for a technician to become proficient in properly identifying all the different parasites. Second, microscopy lacks the ability to speciate, even with the best-qualified technicians. As in the case of *Taenia* spp., the eggs of *T. saginata* and *T. soilium* are identical via microscopy, however, the corresponding diseases are drastically different and type of treatment depends on proper identification of the species.

9.4.1.2 Dye Stains

A multitude of dye stains for brightfield, negative, and fluorochrome microscopy have been used to detect various parasite ova and (oo)cysts in stool specimens (Fig. 9.1). These techniques have also been used for detection in environmental samples with limited success. Examples for bright field staining for *C. parvum* detection include safranin methylene blue stain (Baxby *et al.*, 1984), Ziehl-Neelsen modified acid-fast (Henricksen and Pohlenz, 1981), and DMSO-carbolfuchsin (Pohjola *et al.*, 1985). Parasite staining with these techniques is not 100% uniform and the discernment from fecal debris is difficult and require's experienced microscopists. The advantage of using bright field stains, for example the employment of the Ziehl-Neelsen modified acid-fast to detect *C. parvum* oocysts, is that other organisms such as *Cyclospora cayetanensis* and *Isospora* spp. are also stained and thus have a greater potential of being detected. Other techniques include negative staining with nigrosin (Pohjola, 1984) and malachite green (Elliot *et al.*, 1999) where the background is stained.

As observed with the non-specific bright field stains, fluorescent dye stains such as acridine orange (Ma and Soave, 1983), propidium iodide (PI) and 4,6 diamidino-2′-phenylindole (DAPI) (Kawamoto *et al.*, 1987) have a higher propensity for false-positive results and have variable staining characteristics depending on the organism's viability status. However, the use of these dyes, especially DAPI, can greatly assist in identification when used concurrently with other microscopic techniques such as fluorescence and DIC (see below).

9.4.1.3 Fluorescent Labeling

The detection of *Cryptosporidium* spp. in stool specimens was greatly enhanced with the introduction of immunofluorescent assays in 1985 and 1986 (Fig. 9.1) (Casemore *et al.*, 1985; Garcia *et al.*, 1987; McLauchlin *et al.*, 1987; Sterling and Arrowood, 1986; Stibbs and Ongerth, 1986). Both sensitivity and specificity were shown to outperform conventional staining techniques (mentioned above) (Arrowood and Sterling, 1989; Garcia *et al.*, 1992; Grigoriew *et al.*, 1994). Immunofluorescent assays for the detection of *Giardia* spp. were introduced at approximately the same time and again were shown to have high sensitivity and specificity (Alles *et al.*, 1995; Grigoriew *et al.*, 1994; Nieminski *et al.*, 1995; Riggs *et al.*, 1983; Rose *et al.*, 1989; Sauch, 1985; Winiecka-Krusnell and Linder, 1995). In addition, one study found that *G. muris* and *G. lamblia* cysts subjected to freezing and thawing remained detectable via immunofluorescent microscopy, whereas the cyst walls often became distorted and therefore were not detected by bright field microscopy (Erlandsen *et al.*, 1990).

9.4.1.4 USEPA Approved Microscopic Methods

Among the diverse examples of concentration, separation, and detection methodologies, one combination has been adopted by the USEPA for the detection of *Cryptosporidium* spp. and *Giardia* spp. in surface water (USEPA, 2003). Method 1623 combines filtration (membrane or foam) followed by elution, concentration by centrifugation, and oocyst separation via IMS. Detection and identification uses the triple combination of immunofluorescence, DAPI staining, and DIC microscopy. Reported recovery efficiencies range from 36 to 75% (DiGiorgio *et al.*, 2002; LeChevallier *et al.*, 2003; McCuin and Clancy, 2003). (Oo)cysts in environmental samples are identified based on three general (oo)cyst morphological characteristics. First, (oo)cysts are detected at 200X magnification via immunofluorescence labeling of the (oo)cyst wall. Fluorescence intensity, (oo)cyst wall thickness, and characteristic folds in the wall are observed and assessed. Second, 4,6 diamidino-2′-phenylindole (DAPI) staining assists in identification, location, and number of nuclei, if present. Finally, DIC microscopy facilitates (oo)cyst internal morphology including identification of sporozoites and nuclei for *Cryptosporidium*, and nuclei, median bodies and axonemes for *Giardia*. The triple combination of fluorescence, DAPI staining and internal structure assessment assists the microscopist to decide if the object is an (oo)cyst or not. Again, it is important to note that this methodology is not 100% specific and cross-reacting algae and other debris can interfere, thus making the identification process very subjective. Table 9.2 lists a summary of advantages and disadvantages of the various detection methods.

## 9.4.2 Nucleic Acid Techniques

9.4.2.1 Strengths and Weaknesses

The greatest strengths of nucleic acid techniques over microscopic techniques are specificity and the ability to determine the species and potentially subspecies/genotype of the parasite. This feature is notably important when considering the different species of *Cryptosporidium*, *Entamoeba*, *Giardia*, and *Taenia*. With each of these parasites, the transmissible form is microscopically indistinguishable between the different species within its genera. As stated previously, correct identification of species has implications for pathogenesis, physician treatment, epidemiological studies, and source tracking, and therefore this advantage of molecular techniques is highly desirable.

Another strength of many molecular-based protocols is that many have improved over the years to be less labor intensive and at the same time shown to be as sensitive, if not more, as microscopic protocols. Examples of specific recovery efficiencies and lower limits of detection (LLD) are reviewed later in this chapter. In addition, the subjective nature of microscopy is eliminated with molecular based protocols. Not to be confused with specificity (discussed in the following paragraph), molecular based detection techniques give a yes or no answer. With microscopy, detection is reliant on the technician's experience as well as the ability to stay focused while analyzing the sample.

However, while certain molecular techniques are showing promise, each is not without its limitations. Limitations of most include concerns over specificity, risk

**Table 9.2.** Advantages and disadvantages of detection methods for parasites.

| *Method* | *Target* | *Advantage(s)* | *Disadvantage(s)* | *Required time* | *Speciation* | *Viability* | *Setup cost* ($)[a] | *Cost per sample* ($) |
|---|---|---|---|---|---|---|---|---|
| Microscopic detection | Whole organism | Specific; Quantitative | Microscopist dependent; Subjective | 1–4 days | No | No | M | 35–100 |
| IFA Microscopy | Whole organism | Specific; Quantitative | Microscopist dependent; Subjective | 1–4 days | No | No | M | 35–300 |
| Culture and cell culture | Whole organism | Viability assessment; Semi-quantitative | Subject to inhibitors; Many organisms are not cultivable | 1–7 days | No | Yes | H | 25–50 |
| PCR | DNA | Simple; Specific | Single target/organism reactions; Subject to inhibitors; Non-quantitative | 1–2 days | Yes, via DNA seq. and/or RFLP | No | L | <10 |
| Nested PCR | DNA | Increased sensitivity; Specific | Higher chance for contamination; Time consuming; Single target/organism reactions; Subject to inhibitors; Non-quantitative | 2–3 days | Yes, via DNA seq. and/or RFLP | No | L | <20 |
| Multiplex PCR | DNA | Multiple targets/ organisms | Difficult to optimize; Subject to inhibitors; Non-quantitative | 1–2 days | Possible but difficult | No | L | <20 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Real-Time PCR | DNA/RNA | Multiple targets/ organisms; Quantification assessment | Difficulty working with RNA; Difficult to optimize; Subject to inhibitors | <1 day | Yes, via multiple probes | No | M | 10–25 |
| RT-PCR[b] | RNA | Viability assessment | Single target/organism reactions; Difficulty working with RNA; Difficult to optimize; Subject to inhibitors | 1–2 days | Yes, via DNA seq. and/or RFLP | Yes | M | 10-25 |
| CC-PCR | DNA | Viability assessment | Single target/organism reactions; Difficulty working with environmental samples; Subject to inhibitors | 3–7 days | Yes, via DNA seq. and/or RFLP | Yes | H | 25–50 |
| DNA Array | DNA/RNA | Multiple targets/ organisms per reaction | Difficulty working with environmental samples; Subject to inhibitors | 2–3 days | Yes | Yes | H | 25–50 |

[a] Setup cost for laboratory equipment (US$): (L) Low, <10,000; (M) Moderate, 10,000–35,000; (H) High, >35,000
[b] RT-PCR, Reverse Transcription PCR

of laboratory contamination, and sensitivity. First, specificity is of concern because of the "yes or no" answer and the response for any test is based on that simple answer. Therefore, techniques that have not been properly evaluated for specificity are of great concern and lead to confounded results. The general trend for specificity testing is to challenge the new technique with organisms that are readily available. These would include a suite of fecal bacteria including *E. coli* and other organisms that are closely related to the target organisms. This list should also be extended to distantly related organisms that might also be present in any given fecal or environmental sample. It is also recommended that the developed method be amenable to confirmatory tests, such as oligonucleotide probe hybridization and sequence analysis of PCR amplicons. Second, contamination and false-positive results are of concern with any detection method but with molecular techniques, especially those that exponentially amplify a specific nucleic acid target, there is an increased risk due to potential laboratory contamination with amplified product. Proper quality assurance (QA) and quality control (QC) measures must be established and practiced with vigilance in order to reduce the risk of contamination. Third, while nucleic acid based detection methods continue to gain popularity in the research and commercial laboratory settings, methods for liberation and recovery of high quality nucleic acid are still in need of improvement.

9.4.2.2 Nucleic Acid Recovery Techniques

As observed with the microscopic based protocols, a limitation in parasite detection, notably within environmental samples, is the low concentration of ova and (oo)cysts. Therefore, molecular detection techniques are also limited by current concentration and isolation protocols that target the environmentally stable form of the parasite. The same concentration and isolation techniques outlined earlier are also used as precursor steps followed by a molecular detection technique. Following Method 1623 as an example, the combination of filtration for concentration and IMS for isolation has become the norm for detecting *Cryptosporidium* spp. and *Giardia* spp. in water samples with a molecular detection technique. For example, in combination with capsule filtration followed by IMS, nested PCR was able to detect eight *C. parvum* oocysts seeded into treated waters (Monis and Saint, 2001); real-time PCR had a detection limit of five *C. parvum* oocysts seeded into purified water and eight oocysts seeded into raw water sample (Fontaine and Guillot, 2003a); and reverse transcription-PCR was able to detect ten oocysts seeded into tap water (Hallier-Soulier and Guillot, 2003). In 2003, an IMS-PCR system was introduced to detect *Enterocytozoon bieneusi* (Sorel *et al.*, 2003). Development of a filtration/IMS recovery system for *T. gondii* has also been outlined (Dumetre and Darde, 2003).

The second challenge is liberating, capturing, and purifying the nucleic acid from the organisms and the matrix in which the parasite is suspended. The ova and (oo)cysts serve as perfect package delivery systems that contain and protect the nucleic acid from the surrounding matrix. However, the problem arises when trying to break open these environmentally resistant stages and gain access to their nucleic acids for detection. A spectrum of different approaches has been used, from simple boiling to complex automated systems. The most common nucleic acid liberating schemes include: (1) simple boiling; (2) freeze/thawing (Johnson *et al.*, 1995; Sluter

*et al.*, 1997) in the presence of an inhibitor removing substance in conjunction with an inhibitor remover such as InstaGene™ Chelex resin (BioRad) (Higgins *et al.*, 2001b; Sturbaum *et al.*, 1998), Maximator® (Connex), and GeneReleaser® (Bioventures) (Kramer *et al.*, 2002); (3) exposure to guanidinium thiocyanate in the presence of silica (Boom *et al.*, 1990); (4) exposure to digestive enzymes such as proteinase-K (Abbaszadegan *et al.*, 1991; Gross *et al.*, 1992); microwave heating (Goodwin and Lee, 1993); a variety of commercially available spin columns are available such as the QIAamp® DNA Mini Kit (QIAGEN Inc., Valencia, CA); (6) novel approaches such as the FTA filter paper (McOrist *et al.*, 2002; Orlandi and Lampel, 2000; Subrungruang *et al.*, 2004); and (7) automated silica-based membrane capture systems such as the MagNA Pure LC System (Roche Diagnostics Corp.) (Knepp *et al.*, 2003; Wolk *et al.*, 2002).

Yet, even though different combinations of these listed techniques are promising, limitations remain due to the fact that molecular based techniques are subject to inhibition by substances that are introduced during the preparation process, or naturally occur and are coextracted with the nucleic acid (Johnson *et al.*, 1995; Sluter *et al.*, 1997). Naturally occurring substances such as humic acids (Ijzerman *et al.*, 1997; Kreader, 1996), chemicals such as SDS commonly used in elution and lysis buffers, and residual alcohol used during the nucleic acid purification have been shown to inhibit and reduce the activity of the enzymes used in various detection methods. One approach to guard against false-negative results due to inhibitory substances is the introduction of an internal positive control (IPC) (Kaucner and Stinear, 1998; Stinear *et al.*, 1996).

Currently, a consensus method has not been agreed upon for the best combination of parasite recovery and nucleic acid liberation/purification method for any one given parasite, let alone a consensus method that potentially can be used for multiple parasites. The latter is a lofty goal that will be difficult to achieve due to the multiple characteristics of each parasite and the different matrixes to which the detection protocol will need to be applied.

#### 9.4.2.3 DNA Based Protocols

##### *9.4.2.3.1 Polymerase Chain Reaction (PCR)*

PCR is the selective exponential amplification of a nucleic acid locus and countless numbers of studies employing PCR as a detection tool for parasites in clinical and environmental matrixes have been published since the technique's introduction in 1985 (Mullis *et al.*, 1986; Saiki *et al.*, 1985). The nucleic acid locus is targeted by oligonucleotide primers that flank the region to be amplified. Amplification is achieved via a heat stable DNA polymerase that synthesizes a new strand of DNA determined by the primer locations during thermal cycling, typically between three temperatures for denaturation, primer annealing, and elongation. The choice of the targeted locus is of critical concern and issues such as single versus multicopy targets for sensitivity, level of genetic relatedness for specificity, and amount of observed genetic variability need to be considered.

PCR-based protocols for *Cryptosporidium* spp. include targeting the 18S rRNA (Johnson *et al.*, 1995; Leng *et al.*, 1996; Lowery *et al.*, 2000; Sturbaum *et al.*, 2002; Xiao *et al.*, 1999), *hsp*70 gene (Gobet and Toze, 2001; Kaucner and Stinear, 1998;

Monis and Saint, 2001), beta tubulin (Perz and Le Blancq, 2001), an unknown locus (Laxer *et al.*, 1991) as well as multiple other protocols (Awad-el-Kariem *et al.*, 1994; Gibbons and Awad-El-Kariem, 1999; Gile *et al.*, 2002; Hallier-Soulier and Guillot, 2000; Morgan *et al.*, 1998; Scorza *et al.*, 2003; Xiao *et al.*, 2001). Various studies have determined that the lower limit of detection (LLOD) of *C. parvum* with PCR in different matrixes is as low as a single oocyst (Table 9.3). It is necessary to keep in mind that the sensitivity of these different protocols is only as good as the method that is used to determine the actual number of oocysts seeded into the matrix. This is discussed in the final section of this chapter. A choice of *G. lamblia* PCR detection protocols is also noted in the literature. Examples include PCR protocols targeting the rRNA genes (Hopkins *et al.*, 1999; Hopkins *et al.*, 1997; van Keulen *et al.*, 1995), two genes encoding cysteine-rich trophozoite surface proteins (Ey *et al.*, 1993), and the triose phosphate isomerase gene (*tim*) (Lu *et al.*, 1998). The LLOD with PCR detection is also as low as a single cyst in purified water and five cysts in environmental water (Caccio, 2003; Rochelle *et al.*, 1997a). While many PCR protocols are available to detect *T. gondii* in clinical specimens (Dupon *et al.*, 1995; Dupouy-Camet *et al.*, 1993; Lamoril *et al.*, 1996; Savva *et al.*, 1990; Schoondermark-van de Ven *et al.*, 1993; Weiss *et al.*, 1991), only recently has interest been shown for *T. gondii* detection in environmental samples (Dumetre and Darde, 2003; Ellis, 1998; Kourenti and Karanis, 2004). A few PCR protocols have been described detecting *Encephalitozoon intestinalis* and *Enterocytozoon bieneusi* spores in formalinized fecal samples and environmental water samples (Dowd *et al.*, 1998; Sorel *et al.*, 2003; Thurston-Enriquez *et al.*, 2002). One cestode PCR protocol allows for diagnostic differential detection of *Taenia saginata* and *Taenia solium* (Gonzalez *et al.*, 2000).

*9.4.2.3.2 Nested PCR*

A second round PCR amplification of a DNA segment internal to the priming sites of a first round PCR product (amplicon) is referred to as "nested PCR." The nested PCR amplicon is generated by performing a second round of PCR using the first round PCR amplicons as the template DNA and primers designed to "nest" within the DNA template extremities. The use of one of the first round PCR primers and one new second round primer is referred to as "semi-nested PCR." The major strength of nested PCR is increased sensitivity, while the major weakness is the greatly increased risk of contamination and false-positives. Several nested PCR protocols have been published for use with environmental samples. For example, in combination with IMS, nested PCR targeting the 18S rRNA gene was able to detect eight *C. parvum* oocysts seeded into treated waters (Monis and Saint, 2001). A sensitivity study carried out with micromanipulated *C. parvum* oocysts determined that a single oocyst was detected using nested PCR 38% of the time (19 out of 50 replicates) and 10 micromanipulated oocysts were detected in all 50 replicates (Sturbaum *et al.*, 2001). The same nested PCR protocol, in conjunction with IMS, was then evaluated at the five, ten, and fifteen oocysts levels seeded into two different environmental source waters (Sturbaum *et al.*, 2002). The seeded oocysts were detected in both source waters at all three seeding levels (Sturbaum *et al.*, 2002). However, false positive results were noted as naturally occurring *C. muris*, a nontarget species, was

**Table 9.3.** *Cryptosporidium parvum* detection methodologies and associated lower limits of detection (LLOD).[a]

| *Detection* | *Oocyst concentrations* | *Determined by* | *LLOD*[a] | *Reference* |
|---|---|---|---|---|
| Oocysts/PCR/18S rRNA[b] | 900, 90, 9, 1 | Serial Dilution | 90 oocysts | (Johnson *et al.*, 1995) |
| Oocysts/RT-PCR/*hsp70*[c] | 1000, 100,50,25,12,1 | Serial Dilution | 1 oocyst | (Stinear *et al.*, 1996) |
| Oocysts/PCR/18S rRNA | 5000, 1000,500, 50, 5 | Serial Dilution | 50 oocysts | (Rochelle *et al.*, 1997a) |
| PCR/Unknown region | 5000, 1000,500, 50, 5 | Serial Dilution | 50 oocysts | (Rochelle *et al.*, 1997a) |
| PCR/Unknown region | 5000, 1000,500, 50, 5 | Serial Dilution | 5 oocysts | (Rochelle *et al.*, 1997a) |
| Oocysts/IMS[d]/IFA[e] | 100 | Flow Cytometry | 100 oocysts | (Reynolds *et al.*, 1999) |
| PCR/Unknown region | 100, 50, 10, 1 | Serial Dilution | 1 oocyst[f] | (Wu *et al.*, 2000) |
| Oocysts/membrane dissolution/IFA | $4 \times 10^4$, 100 | Serial Dilution | <100 oocysts | (McCuin *et al.*, 2000) |
| Oocysts/PCR-18s rRNA | 10, 7, 5, 4, 3, 2, 1 | Micromanipulation | 1 oocyst | (Sturbaum *et al.*, 2001) |
| Oocysts/IMS/PCR-18s rRNA | 1000, 500, 250, 100, 10, 1 | Serial Dilution[1] | 10 oocysts | (Lowery *et al.*, 2000) |
| Oocysts/IMS/IFA | 10, 5 | Serial Dilution | 5 oocysts | (McCuin *et al.*, 2001) |
| Oocysts/IMS/RT-PCR/*hsp70* | 10000, 1000, 100, 8 | Serial Dilution | 8 oocysts | (Monis and Saint, 2001) |
| Oocysts/IMS/PCR-18s rRNA | 15, 10, 5 | Flow Cytometry | 5 oocysts | (Sturbaum *et al.*, 2002) |
| Hollow fiber ultrafilter/IFA | 10000, 1000, 600 | Serial Dilution | ND[g] | (Kuhn and Oshima, 2001) |
| Oocysts/Flocculation/IFA | $1 \times 10^f$, 1000, 10, 5, 1 | Serial Dilution | 10 oocysts | (Karanis and Kimura, 2002) |
| Oocysts/IMS/Real-Time PCR | 10000, 1000, 100, 10, 5 | Serial Dilution | 10 oocysts | (Hallier-Soulier and Guillot, 2003) |
| DNA/Real-Time PCR | 1000, 100, 10, 1 | Serial Dilution | 1 oocyst[f] | (Guy *et al.*, 2003) |
| Oocysts/Real-Time PCR | 775, 75 | Serial Dilution | 75 oocysts | (Fontaine and Guillot, 2003a) |

[a] lower limits of detection (LLOD)
[b] 18S rRNA, Small Subunit ribosomal RNA
[c] *hsp70*, 70 KDa heat shock protein
[d] IMS, Immunomagnetic Separation
[e] IFA, Immunofluorescent microscopy
[f] as determined by serial dilution of *C. parvum* previously isolated DNA
[g] ND, not determined

detected in one source water, and DNA from an algal species, *Gymnodinium fuscum*, was amplified from the other source water (Sturbaum *et al.*, 2002). These results only emphasize the importance of primer design and specificity testing.

Multiple other protocols using nested or hemi-nested PCR have been outlined for detection of *G. lamblia*, *C. parvum*, and *T. gondii* in both clinical and environmental samples (Amar *et al.*, 2002; Balatbat *et al.*, 1996; Deng *et al.*, 1997; Fischer *et al.*, 1998; Gibbons and Awad-El-Kariem, 1999; Jones *et al.*, 2000; Kato *et al.*, 2003; Kostrzynska *et al.*, 1999; Mayer and Palmer, 1996; Nichols *et al.*, 2003; Ostergaard *et al.*, 1993; Ward *et al.*, 2002; Zhu *et al.*, 1998).

*9.4.2.3.3 Real-time PCR*

When compared with PCR protocols that require 6–8 h to conduct and nested PCR protocols that require 8–12 h to complete, real-time PCR is superior in that results can be produced in less than 2 h. This time frame is achieved with real-time PCR via: (1) the rapid heating and cooling processes unique to each platform; (2) the relatively small amplicon size (<130 base pairs); and (3) measurable signal (or lack thereof) is generated via fluorescence during the actual temperature cycling. For an excellent review of the different real-time PCR platforms and fluorescent probe variations see Wolk, 2001 (Wolk *et al.*, 2001). In addition, multiple probes, each with its own fluorescent signal, can be used in the same reaction vial, which allows for the detection of multiple organisms or genotypes at the same time. Finally, due to the nature of signal amplification and measurement during temperature cycling, real-time PCR is being used for estimating starting DNA template concentration, which can then be used to estimate the number of organisms present in any given sample.

TaqMan probe chemistry is commonly used for real-time quantitative PCR. TaqMan quantitative PCR requires the use of primers similar to those used in conventional PCR; however, unlike conventional PCR, TaqMan quantitative PCR also requires an oligonucleotide probe labeled with 5′ reporter and 3′ quencher fluorescent dyes and a thermal cycler equipped with a fluorometer (Heid *et al.*, 1996). During each cycle of the PCR, if the target of interest is present, the probe specifically anneals to the target amplicon between the forward and reverse primer sites. Due to 5′ nuclease activity of *Taq* DNA polymerase, the probe is cleaved during the polymerization step (PCR product formation) of the PCR resulting in an increase in reporter fluorescence detected by the instrument. Target signal increases in direct proportion to the concentration of the PCR product being formed. The threshold cycle ($C_T$) is the fractional PCR cycle number at which a significant increase in target signal fluorescence above baseline is first detected for a sample. By using amplification standards consisting of known quantities of target nucleic acid or organisms to generate a standard curve, the starting copy number of nucleic acid targets or target organisms for each sample can be estimated.

Real-time PCR protocols developed for *Cryptosporidium* spp. and *G. lamblia* are demonstrating sensitivity and are able to distinguish different species and genotypes (Amar *et al.*, 2003, 2004; Fontaine and Guillot, 2003a, b; Guy *et al.*, 2003; Higgins *et al.*, 2001a; Limor *et al.*, 2002; MacDonald *et al.*, 2002; Tanriverdi *et al.*, 2003; Tanriverdi *et al.*, 2002). Protocols for *T. gondii* (Buchbinder *et al.*, 2003; Jauregui *et al.*, 2001; Reischl *et al.*, 2003) *Encephalitozoon intestinalis* (Wolk *et al.*,

2002) and *Entamoeba histolytica* and *Entamoeba dispar* (Blessmann *et al.*, 2002) detection have also been described. One study outlines the simultaneous detection of *Entamoeba histolytica*, *G. lamblia*, and *C. parvum* in stool samples at the same time (Verweij *et al.*, 2004).

*9.4.2.3.4 Multiplex PCR*

The incorporation of multiple oligonucleotide primer sets into the same PCR reaction tube permits amplification of multiple loci at the same time. As with real-time PCR, different organisms, species, and/or genotypes can be detected within a single sample. Limitations to be considered are the nucleotide ratios (guanine, cytosine, adenine, and thymine; GC:AT) of the different parasite genomes and the biased amplification nature of PCR. First, the GC:AT ratio, which is unique to each organism, has a direct affect on annealing temperatures of PCR primers. Second, even if the multiple targets are present, there is no guarantee that PCR will amplify all the targets consistently, equally or at all (Reed *et al.*, 2002), especially when there are large differences in concentrations of different targets.

Considering these limitations, multiplex PCR has been applied with success for detecting *C. parvum*, *Eimeria spp*., *E. histolytica*, *E. dispar*, *G. lamblia*, *T. gondii*, and *Taenia* spp. in clinical as well as environmental samples (Abe *et al.*, 2002; Evangelopoulos *et al.*, 2000; Fernandez *et al.*, 2003; Gomez-Couso *et al.*, 2004; Guay *et al.*, 1993; Lindergard *et al.*, 2003; Patel *et al.*, 1999; Rochelle *et al.*, 1997a; Yamasaki *et al.*, 2004).

*9.4.2.3.5 DNA Arrays*

The unique feature that DNA microarray technology provides is that literally thousand of probes representing entire genomes or a mixture of specific target genes from a variety of organisms can be immobilized on a chip. Therefore multiple target loci from multiple target organisms can be screened for their presence at the same time from the same sample. Two formats of DNA microarray technology are currently available. The first format, called DNA microarray, immobilizes cDNA probes (generally 500 to 5000 nucleotide base pairs in length) that are placed or "spotted" directly onto a solid surface, generally glass and sometimes nylon substrates, via robotics (Ekins and Chu, 1999). The second format, DNA chips (Affymetrix, Inc.) differs in that probes of 20 to 80 base pairs in length are synthesized *in vitro* directly onto the chip or spotted onto the chip via robotics. With both formats, the chip is then probed with previously labeled target RNA, cDNA, or DNA either separately or in a mixture and signal from hybridization between the immobilized probe and the target is measured and recorded.

While, this technology is promising, however, it is rather sophisticated to perform and can be costly with designed chips costing as much as $500 to $5000. As with other detection methodologies, DNA array detection is only as good as the upstream sample processing protocol for the concentration and purification of the target organism from the matrix, whether clinical or environmental.

DNA array technology has been adapted to detect and discriminate between different *Cryptosporidium* species and genotypes (Straub *et al.*, 2002) as well as detecting genotypes of *E. histolytica*, *E. dispar*, *G. lamblia*, and *C. parvum* on the same chip (Wang *et al.*, 2004).

9.4.2.4 RNA

*9.4.2.4.1 Reverse Transcription PCR (RT-PCR)*

Employing messenger RNA (mRNA) as the target template, reverse transcription PCR (RT-PCR) has the advantages of lower inhibitory substance contamination issues as well as the ability of addressing viability questions. Reverse transcriptase is the enzyme that is able to make a copy of complementary DNA (cDNA) from mRNA. This newly synthesized strand of cDNA is then used in a PCR protocol as template. Due to continual degradation of mRNA, this molecule is suitable for viability assessment, as only viable organisms are able to synthesize mRNA.

This technique has been used solely or in combination with water filtration and IMS recovery for the detection of *C. parvum* and other *Cryptosporidium* species in clinical and environmental samples (Hallier-Soulier and Guillot, 2003; Rochelle *et al.*, 1999; Rochelle *et al.*, 1997b).

*9.4.2.4.2 NASBA*

A second RNA based technique is nucleic acid sequence-based amplification (NASBA) (Cook, 2003; Romano *et al.*, 1997). NASBA is an isothermal amplification method, which, like reverse transcriptase PCR, uses RNA as the target molecule (DNA can also be used with modifications to the protocol) (Wolk *et al.*, 2001). RNA is reverse transcribed into cDNA that is then used as a template to produce more RNA transcripts via RNA polymerase. NASBA, in conjunction with other novel detection protocols, is currently being used to detect *C. parvum* in water samples (Baeumner *et al.*, 2001; Esch *et al.*, 2001a; Esch *et al.*, 2001b) and is predicted to gain importance for detection of other food-borne and waterborne parasites (Caccio, 2003; Cook, 2003).

9.4.2.5 Innovative Techniques

Innovative techniques such as fiber optics and biosensors (Snowden and Anslyn, 1999; Wang, 2000), transcription mediated amplification (TMA) (Walker *et al.*, 1992), Invader® technology (Third Wave Technologies, Inc.) (Kwiatkowski *et al.*, 1999), Q-beta replicase (Gene-Trak Systems) (Cahill *et al.*, 1991; Pritchard and Stefano, 1990), the Ligase Chain Reaction (Barany, 1991), and Laboratory Multi-Analyte Profiling with suspension arrays (Luminex, Inc.) are demonstrating insightful thought and promise as new detection methodologies. A review for several of these different approaches is given by Wolk, 2001 (Wolk *et al.*, 2001).

## 9.4.3 Immunological-based Techniques

9.4.3.1 Strengths and Weaknesses

Non-microscopic based detection methods include immunological and molecular based techniques. Examples of immunological-based techniques include enzyme-linked immunosorbent assays (ELISA) (Anusz *et al.*, 1990; Chapman *et al.*, 1990; Knowles and Gorham, 1993; Ungar, 1990), reverse passive haemagglutination (Farrington *et al.*, 1994), and solid-phase qualitative immunochromatographic assays (Garcia and Shimizu, 2000). Used primarily for clinical diagnosis, numerous antigen detection assays have been developed for parasites in stools including

*C. parvum* (Garcia and Shimizu, 1997; Newman *et al.*, 1993; Rosenblatt and Sloan, 1993), *G. lamblia* (Boone *et al.*, 1999; Ungar *et al.*, 1984; Vinayak *et al.*, 1991), and *E. histolytica*/*E. dispar* (Gonzalez-Ruiz *et al.*, 1994; Haque *et al.*, 1993; Jelinek *et al.*, 1996; Schunk *et al.*, 2001). A single enzyme immunoassay (EIA), the Triage Parasite Panel (Biosite Diagnostics, Inc., San Diego, CA), has been developed to simultaneously detect *C. parvum*, *G. lamblia*, and *E. histolytica*/*E. dispar* (Garcia *et al.*, 2000; Sharp *et al.*, 2001). The test immobilizes organism specific proteins that are present in a stool specimen onto a membrane via antibodies. Incubation with an antibody-enzyme conjugate followed by the addition of the substrate results in a presence/absence marker that is detected visually. These various immunological-based techniques have proven effective with reported sensitivity and specificity results at >90% when compared to O & P examination (Garcia and Shimizu, 1997, 2000; Garcia *et al.*, 2000; Jelinek *et al.*, 1996), however specific reports do cite false-positive and false-negative results (Doing *et al.*, 1999; Sharp *et al.*, 2001). Taken together, questionable results from an immunological-based technique should be confirmed via a second assay.

#### 9.4.3.2 Speciation and Genotyping Techniques

##### *9.4.3.2.1 Strengths and Weaknesses*

While some of the above listed molecular techniques have speciation and genotyping capabilities inherent in the methodology (such as real-time PCR and the use of multiple probes in the same reaction tube), the majority of the techniques require an additional step for further molecular characterization. The goal of these various techniques is to detect variations in the genetic code as low as single nucleotide polymorphisms (SNPs). However, it should be noted that detection of a SNP does not necessarily depict a new genotype or species. Classification schemes for new parasite genotypes and species need to be based on morphology, host specificity, and multiple nucleic acid differences identified within coding regions of the genome.

##### *9.4.3.2.2 Techniques*

Examples of speciation and genotyping techniques include restriction fragment length polymorphism (RFLP), random amplified DNA polymorphism (RAPD), single strand confirmation polymorphism (SSCP), direct DNA sequencing, amplified fragment length polymorphism (AFLP), denaturing gradient gel electrophoresis (DGG), real-time PCR, and microsatellite analysis, as well as many others. While each of these techniques is able to successfully detect variation and SNPs, two are used with high fidelity, RFLP, and direct DNA sequencing. This claim is based on the number of variables that can be introduced for any of these given techniques. For RFLP and direct DNA sequencing, with proper QA and QC checks such as the use of proof-reading Taq polymerase, only one variable is introduced which is the PCR amplicon itself. If one suspects that multiple alleles are present, the PCR amplicon can be subject to cloning followed by direct DNA sequencing. With the financial cost being <$30 and the potential for valuable information to be gained, direct DNA sequencing is considered the gold standard when detecting variation in nucleotide sequences.

It should be noted that techniques that analyze the entire genome, such as RAPD or AFLP analysis, are designed for fingerprinting pure cultures and not fecal or environmental samples. Current separation and purification schemes do not isolate the target organism with 100% fidelity and extraneous microbes (e.g., bacteria) will compromise the analysis.

## 9.4.4 Viability Techniques

9.4.4.1 Strengths and Weaknesses

Several *in vitro* surrogate methods have been proposed as convenient, user-friendly alternatives to animal infectivity assays for determining the viability and/or infectivity for a number of parasites. Of the listed detection techniques, only one has the ability to assess viability and none are able to assess infectivity. To be clear, viability differs from infectivity. Viability requires that the organism be intact and capable of metabolic activity. Infectivity includes the definition of viability and extends this capacity to the ability to cause disease in animal models, or invade and multiply for *in vitro* cell culture models. The need for establishing viability and/or infectivity is two-fold. First, methodologies such as drug therapy for treatment and UV exposure or radiation for disinfection need to be accurately verified. To install confidence in a treatment or disinfection methodology, the viability and/or infectivity validation techniques need to be accurate and absolute. Second, organisms, such as *C. parvum* oocysts, detected during routine monitoring or in an outbreak situation should be evaluated for viability/infectivity to assess the public health risk. The ideal detection method would not only have high sensitivity and specificity, but would also be able to evaluate the organism's viability/infectivity status.

9.4.4.2 Dye Permeability Assays

Dye permeability assays (inclusion or exclusion) have been used for years to assess viability in a number of parasites including *C. parvum* (Arrowood *et al.*, 1991; Campbell *et al.*, 1992; McCuin *et al.*, 2000), *Entamoeba* cysts (Kawamoto *et al.*, 1987) and trophozoites (Cano-Mancera and Lopez-Revilla, 1988), *Giardia* spp. (deRegnier *et al.*, 1989; Labatiuk *et al.*, 1991; Sauch *et al.*, 1991; Schupp and Erlandsen, 1987a, b; Smith and Smith, 1989), *Schistosoma mansoni* schistosomula (Van der Linden and Deelder, 1984), and *Taenia saginata* (Owen, 1985). The most commonly used dyes include fluorescein diacetate (FDA), propidium iodide (PI), 4'-6-diamidino-2-phenylindole (DAPI), Hoechst 33258, acridine orange, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT), and trypan blue (Korich *et al.*, 1993). While the fluorogenic dyes, FDA, DAPI, and PI, were shown to accurately assess *Giardia* cyst (Sauch *et al.*, 1991; Schupp and Erlandsen, 1987a, b) and *C. parvum* oocyst (Campbell *et al.*, 1992; Jenkins *et al.*, 1997) viability when compared with *in vitro* excystation, DAPI and PI were shown to overestimate *C. parvum* oocyst viability when compared with the neonatal mouse infectivity model (Black *et al.*, 1996; Korich *et al.*, 1993). However, separate studies demonstrated that viable *C. parvum* oocysts, which excluded the fluorogenic dyes SYTO-9 and SYTO-59 (while non-viable oocysts were permeable and fluoresced), were infectious in neonatal CD-1 mice (Belosevic *et al.*, 1997; Neumann *et al.*, 2000b).

9.4.4.3 *In vitro* excystation

*In vitro* excystation for *Cryptosporidium* spp. (Fayer and Leek, 1984; Robertson *et al.*, 1993; Sundermann *et al.*, 1987; Woodmansee, 1987) and *Giardia* spp.(Bingham *et al.*, 1979; Bingham and Meyer, 1979; Isaac-Renton *et al.*, 1992; Rice and Schaefer, 1981; Schaefer *et al.*, 1984; Smith and Smith, 1989), and sporulation for *C. cayetanensis* (Ortega *et al.*, 1993; Siripanth *et al.*, 2002) and *T. gondii* (Lindsay *et al.*, 2002; Lindsay *et al.*, 2003), are principle methods to assess viability. However, while excystation has been shown to be an effective measure of viability for *Giardia* spp. (Bingham *et al.*, 1979; Hoff *et al.*, 1985), it is not a reliable measure for *C. parvum* oocyst viability (Black *et al.*, 1996), and for both organisms it should not be used as a surrogate for infectivity (Black *et al.*, 1996; Bukhari *et al.*, 2000; Labatiuk *et al.*, 1991; Neumann *et al.*, 2000a). Finally, environmental triggers that influence *C. cayetanensis* and *T. gondii* sporulation have yet to be characterized (Lindsay *et al.*, 2002; Ortega *et al.*, 1993; Siripanth *et al.*, 2002), and therefore, sporulation as a measurement of oocyst viability should remain questionable.

9.4.4.4 FISH

Utilizing fluorescently labeled oligonucleotide or peptide nucleic acid probes, fluorescence *in situ* hybridization (FISH) (Nath and Johnson, 1998) has been used in detection assays as a measure of viability for both *C. parvum* and *G. lamblia* (Deere *et al.*, 1998a; Deere *et al.*, 1998b; Dorsch and Veal, 2001; Jenkins *et al.*, 2003; Smith *et al.*, 2004; Vesey *et al.*, 1998). The ability of FISH to determine viability is based on the presence of the target nucleic acid, which in certain reports is rRNA (Smith *et al.*, 2004; Vesey *et al.*, 1998), and is present only in viable organisms. For example, after loss of viability, rRNA synthesis will cease and be subject to degradation and therefore the FISH probe will not have the rRNA target to hybridize with. In a separate study, *C. parvum* oocyst viability was assessed by comparing FISH to cell culture and mouse infection (Jenkins *et al.*, 2003). The results showed that oocyst viability decreased proportionately over a 9-month period according to all methods, however, cell culture and mouse infection had the best agreement (Jenkins *et al.*, 2003). A unique advantage of FISH is that it may be combined with immunofluorescent microscopy or flow cytometry.

9.4.4.5 Reverse Transcription PCR

A final molecular technique for viability assessment is reverse transcription PCR (RT-PCR). Viability can be measured with RT-PCR because the target, mRNA, is degraded and thus constantly requires de novo synthesis. This results in nonviable organisms being undetected. Confirmation that residual RNA has been degraded should be included with all detection and/or viability protocols. Several studies report the reproducible RT-PCR detection of *C. parvum* and *G. lamblia* in reagent grade as well as environmental waters (Fontaine and Guillot, 2003b; Hallier-Soulier and Guillot, 2003; Jenkins *et al.*, 2003; Jenkins *et al.*, 2000; Kaucner and Stinear, 1998; Stinear *et al.*, 1996; Widmer *et al.*, 1999). However, in a single report directly comparing mouse infectivity, cell culture and RT-PCR targeting *C. parvum* amyloglucosidase mRNA, the RT-PCR assay underestimated oocyst viability (Jenkins *et al.*, 2003). Therefore, while in theory, RT-PCR is able to assess viability, additional research is needed to support this claim.

9.4.4.6 Cell Culture and Animal Infectivity Models

Animal infectivity models remain the current gold standard to assess viability and infectivity and all surrogate methods should be compared to animal infectivity. However, animal infectivity models are expensive, time consuming, and require experienced personnel and facilities (Rochelle *et al.*, 2002). In addition, not all parasites have been adapted to an animal model. For example, multiple animal models have been tested without success for *C. cayetanensis* (Eberhard *et al.*, 1999, 2000) infection and only gnotobiotic piglets have been shown to be susceptible to *C. hominis* infection (Widmer *et al.*, 2000a). While many of the surrogate techniques are being evaluated with *C. parvum* and *G. lamblia*, animal infectivity remains the technique of choice when evaluating viability/infectivity of *T. gondii* oocysts (Jauregui *et al.*, 2001; Lindsay *et al.*, 2002; Lindsay *et al.*, 2003).

The best surrogate for animal testing is *in vitro* cell culture. Cell culture (often abbreviated as "CC" when included in method acronyms) is well established with multiple food-borne and waterborne parasites. Complete life cycle development for *Encephalitozoon intestinalis* and other microsporidia spp.(Huffman *et al.*, 2002; Li *et al.*, 2003; Visvesvara, 2002; Wolk *et al.*, 2000) as well as *T. gondii* (Hoff *et al.*, 1977; Kniel *et al.*, 2002; Lindsay *et al.*, 1991) is documented and specifically for *E intestinalis*, cell culture is used to generate large numbers of spores (Wolk *et al.*, 2000). For *Cryptosporidium* spp., several cell culture methods have been described and even though final development of oocysts, the end product of the complete *Cryptosporidium* life cycle, is not robust; cell culture in combination with PCR or other molecular techniques has proven to be very reliable and a good measure of viability (Di Giovanni *et al.*, 1999; Gennaccaro *et al.*, 2003; Hijjawi *et al.*, 2001; Hijjawi *et al.*, 2002; Joachim *et al.*, 2003; Keegan *et al.*, 2003; LeChevallier *et al.*, 2003; Rochelle *et al.*, 1996, 1997b; Slifko *et al.*, 1997; Upton *et al.*, 1994, 1995; Widmer *et al.*, 2000b). For a sample to be considered positive, all that is needed is initial life cycle stages to begin development, and these stages can then be targeted by molecular methods. *C. parvum* oocysts recovered by IMS have been detected and determined viable by both PCR (Di Giovanni *et al.*, 1999; Jenkins *et al.*, 2003; LeChevallier *et al.*, 2003; Rochelle *et al.*, 1996) and RT-PCR (Rochelle *et al.*, 1999; Rochelle *et al.*, 1997b; Rochelle *et al.*, 2002). Cell culture has been shown to be equivalent to the "gold standard" mouse infectivity for disinfection studies (Rochelle *et al.*, 2002). Recently, CC-PCR has been used to determine the risk posed by infectious *C. parvum* and *C. hominis* in finished drinking water (Aboytes *et al.*, 2004). Another *Cryptosporidium* cell culture technique relies upon the immunofluorescent assay detection of foci of infection, referred to as the focus detection method (FDM) (Slifko *et al.*, 1997). The FDM method has been used for disinfection trials (Slifko *et al.*, 2002; Slifko *et al.*, 2000) and the detection of naturally occurring infectious *Cryptosporidium* in wastewater (Gennaccaro *et al.*, 2003; Quintero-Betancourt *et al.*, 2003). Despite the significant progress made in the area of *Cryptosporidium* cell culture and detection methods, optimization of culture conditions, pretreatment of oocysts for cell culture and further evaluation of detection methods is needed (Di Giovanni and Aboytes, 2003) and are the focus of current research efforts.

## 9.5 PROPER EVALUATION (QA/QC)

In addition to Good Laboratory Practices (GLP) and QA and QC checks, certain concerns need to be addressed with any detection method. These include specificity issues and proper determination of the lower limit of detection (LLOD).

Specificity evaluation of new methods is critical, as methods will be challenged with multiple unknown organisms in clinical and especially environmental samples. As discussed above, the majority of the concentration and isolation techniques are nonspecific and even those that incorporate specificity measures, such as IMS, may allow nonspecific organisms to be carried over to subsequent detection methods. Moreover, detection techniques that circumvent isolation protocols altogether, such as direct PCR on fecal material, are subject to more potential cross-contaminating organisms.

Understanding that the evaluation of a detection method cannot include every possible organism, certain criteria should be considered when selecting the organisms that are used for specificity testing. These include physical shape (for microscopic based techniques), genetic relatedness (for microscopic and molecular based techniques) and potential co-occurrence or presence in the sample matrix. However, in many cases, detection methods are only evaluated with organisms that are easily accessible without taking these criteria into consideration.

For example, in consideration of the potential co-occurrence or presence in the sample matrix, those techniques that are used with fecal samples should be evaluated with a higher proportion of coliform bacteria; while those techniques used with source waters should include microbiota such as algae. If a technique is to be used with sewage effluent or run-off water, then both groups of organisms should be included in the specificity evaluation analysis.

For physical shape, two criteria should be considered. First, genetically unrelated organisms, which are similar in same size and shape of the target organism, should be evaluated. Examples of such organisms include the algal species *Oocystis* spp. for *Giardia* and *Chlorella* spp. for *C. parvum*. Second, genetically related species, especially those within the same genus, need to be evaluated such as the 14 now recognized *Cryptosporidium* spp. For example, in the case of a new *Cryptosporidium* microscopic technique, all 14 different species should be evaluated for cross-reaction and the ability to distinguish the oocysts.

For genetic relatedness, criteria that should be considered include multiple species within the targeted genus, related organisms outside the genus, and homologous genetic loci. Methods for those genera that have multiple confounding species (*Cryptosporidium* spp., *Giardia* spp., the microsporidia group, *Taenia* spp.) should be thoroughly evaluated, especially if they will be applied to environmental samples. For example, in one study, storm waters contained four different *Cryptosporidium* spp. and 12 different genotypes based on detection of rRNA gene and subsequent DNA sequencing (Xiao *et al.*, 2000). Further, distantly related organisms to the target organism have largely not been included in any specificity trials. Again, using *C. parvum* as an example, the genus *Cryptosporidium* is genetically placed in the classification group, the Alveolata. Based on rRNA, the Alveolata is a robust,

monophyletic taxon consisting of three phyla: Apicomplexa (e.g., *Cryptosporidium*, *Toxoplasma*), Ciliophora (ciliates) and Dinozoa (dinoflagellates) (Cavalier-Smith, 1993). Using a nested PCR primer set targeting the 18S rRNA and previously tested for *Cryptosporidium* spp. specificity, DNA from a common fresh water dinoflagellate, *Gymnodinium* spp., was nonspecifically amplified and detected via this *C. parvum* detection technique (Sturbaum *et al.*, 2002). This example further exemplifies the final point of DNA target selection. When choosing a locus for molecular detection, consideration must be given to the conserved nature of the locus, as well as the copy number per genome. In the case of the cross-reacting dinoflagellate, the 18S rRNA gene is highly conserved, and therefore it was not surprising that a related, nontarget organism was amplified, especially using a sensitive nested PCR primer set with environmental samples. As a second example, the Apicomplexa members *Cryptosporidium*, *Eimeria*, and *Toxoplasma* all produce oocysts that are present in the environment ubiquitously and concentration techniques will nonspecifically collect all three genera. Of the possible molecular loci as targets for detecting *C. parvum* in the environment, the thrombospondin-related anonymous protein (TRAP) gene has emerged as a potential candidate (Spano *et al.*, 1998a). However, the TRAP genes are conserved not only within the *Cryptosporidium* genus but also between the different members of the Apicomplexa (Kappe *et al.*, 1999; Spano *et al.*, 1998a; Spano *et al.*, 1998b; Sulaiman *et al.*, 1998). Therefore, when using a TRAP gene as the target loci in an environmental detection protocol, the protocol needs to incorporate a subsequent confirmation step to verify the amplicon came from the target organism.

Proper determination of the LLOD is the second major aspect of method development that is generally not evaluated thoroughly. First, to determine the LLOD of any detection system, accurate numbers of organisms need to be used. Historically for many parasites, the combination of hemocytometer counting followed by serial dilution was used to generate low numbers of target organisms. This technique, which is based on an estimated value for the initial count, introduces a standard error with every dilution introduced. Therefore the final estimated number of target organisms is always $\pm$ an accumulated standard error. While this procedure is acceptable for estimating high levels of organisms ($>100$), it should not be used to generate suspensions with low numbers of organisms.

To generate low numbers of accurately counted organisms, micromanipulation and flow cytometric enumeration and sorting (flow cytometry) have emerged (Reynolds *et al.*, 1999; Sturbaum *et al.*, 2001; Tanriverdi *et al.*, 2002). Micromanipulation, while determined to be accurate, is time consuming and labor intensive and therefore is recommended for use with small "in-house" research projects in which large numbers of replicates are not needed (Sturbaum *et al.*, 2002). Flow cytometry triumphs in cases where high numbers of replicates with a low standard deviation ($\pm 1$ organism) are needed (Chesnot *et al.*, 2002; Reynolds *et al.*, 1999). Flow cytometry has been used to enumerate and sort oocysts of *C. parvum*, *G. lamblia* cysts (Ferrari and Veal, 2003; Vesey *et al.*, 1994a), *E. intestinalis* spores (Hoffman *et al.*, 2003), and *T. gondii* oocysts (Everson *et al.*, 2002) from both reagent grade water and environmental sources. In addition to the small standard deviation, flow

cytometry can be used to directly sort (oo)cysts into the test vial of interest, and thus eliminating any additional standard errors that occur during a dispensing step. For instance, (oo)cysts can be directly sorted into a thin-walled PCR tube or the Leighton tube that is used in EPA Method 1623.

Currently, the EPA has approved flow cytometry for establishing low numbers of *C. parvum* oocysts and *G. lamblia* cysts that are used in QC recovery trials for Method 1623. The method requires that spiking suspensions contain between 100 and 500 (oo)cysts. Currently, commercial sources of flow cytometer-counted (oo)cysts are the Wisconsin State Laboratory of Hygiene Flow Cytometry Unit (WSLH) and BioTechnology Frontiers, (BTF). WSLH prepares and distributes (oo)cysts that are live and infectious while BTF prepares and distributes (oo)cysts that have been inactivated via irradiation. BTF also prepares and distributes oocysts that have been permanently stained with a fluorochrome similar to Texas Red. The utility of this product is an immunofluorescent assay internal positive control that can be used to measure step-by-step losses that occur throughout processing as well as cross-contamination.

The final aspect of proper LLOD determination is directed at molecular based methods. The majority of manuscripts reporting recovery efficiencies and the LLOD use serial dilutions of whole organisms or previously extracted DNA. Again, serial dilutions of whole organisms incorporate a standard error from which the actual number of organisms being seeded and tested for detection/recovery is not known. Second, working with previously extracted DNA does not incorporate the difficulty of liberating DNA from the organism or unintentional co-extraction of PCR inhibitors. In general, nucleic acid is mass extracted from large quantities of organisms ($>10^5$) and subsequently diluted to achieve a low number equivalent ($<10^2$) or even a single organism. This DNA "equivalent" is then used in a recovery assay to determine the LLOD. Following this protocol completely avoids the difficulty of liberating DNA from the low numbers of organisms typically present in purified environmental sample matrices. The best way to determine the LLOD of a given molecular detection method is to use whole organisms (ideally flow cytometry-enumerated) and evaluate the entire method, including concentration, purification, and nucleic acid liberation and recovery with a variety of different sample matrices.

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