

## CRYPTOSPORIDIOSIS

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### SUMMARY

*Cryptosporidiosis is caused by protozoan parasites of the genus Cryptosporidium, in which there are 18 'valid' species. In livestock, C. parvum, C. andersoni, C. baileyi, C. meleagridis and C. galli have been reported to cause morbidity and outbreaks of disease. Laboratory identification is required to confirm diagnosis. Cryptosporidium parvum cryptosporidiosis causes scour in young, unweaned mammalian livestock, however, weaned and adult animals can also become infected. Signs range from a mild inapparent infection to severe scouring, and the young, old or immunocompromised are most susceptible. Mortality is low. Weaned and adult animals normally do not exhibit signs of disease, but will excrete oocysts that may contaminate the environment. Cryptosporidium andersoni cryptosporidiosis affects the digestive glands of the abomasum of older calves and adult cattle. Some infected animals exhibit reduced weight gain, but do not develop diarrhoea. Cryptosporidium baileyi, C. meleagridis and C. galli cryptosporidiosis are diseases of birds. Cryptosporidium baileyi affects primarily the bursa of Fabricius and cloaca of gallinaceous birds, C. meleagridis affects primarily the ileum of turkey poults and C. galli infects the surface, ductal, and glandular epithelium of the proventriculus of adult hens and some wild birds.*

**Identification of the agent:** *There is no prescribed test for cryptosporidium infection. The demonstration of cryptosporidium species oocysts or cryptosporidium antigen in a properly collected and submitted sample is sufficient for a positive diagnosis. Diagnosis is established microscopically, with the acid-fast Ziehl–Neelsen or auramine phenol methods using unconcentrated or concentrated faecal smears. Microscopy-based methods for detecting oocysts and enzyme-linked immunosorbent assays for detecting cryptosporidium antigens are relatively insensitive, but are sufficiently sensitive for detecting clinical cases. Neither tinctorial- nor fluorescence-based stains can determine the species of cryptosporidium present if the oocysts fall within the size range of 4–6 µm. These methods can detect oocysts in clinically ill animals, but sometimes are not sufficiently sensitive to detect infection in clinically normal animals. Nucleic acid detection tests have a greater sensitivity. The polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) and/or sequencing can be used to determine some or all cryptosporidium species/genotypes or subtypes. Those typing and subtyping systems used for veterinary (and human) samples should also be used for environmental samples, to avoid any confusion arising from using different systems during the investigation of disease outbreaks with both veterinary and public health implications. However the sensitivity of subtyping systems will need to be increased so that they can be used with clinical and environmental samples containing small numbers (<10) of oocysts. Current limitations of discriminatory species subtyping systems are that they are only applicable to C. parvum and C. hominis. Discriminatory subtyping systems for non-'parvum' and non-'hominis' pathogens (including the majority of livestock pathogens) have yet to be developed.*

*Specimens for primary diagnosis should be collected during acute infection, and should be processed as soon as possible, ideally, within 24 hours. Transportation to the laboratory should be in accordance with the International Air Transport Association regulations, which are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.*

*Demonstration of Cryptosporidium oocysts or Cryptosporidium-specific antigen in faecal samples is the most appropriate test for most applications. Most infections that cause morbidity and/or mortality in mammalian livestock are likely to be caused by C. parvum cryptosporidiosis. Cryptosporidium bovis is a highly prevalent species that infects primarily post-weaned calves. Cryptosporidium baileyi, C. meleagridis and C. galli cryptosporidiosis cause morbidity and/or mortality in avian livestock. The species of Cryptosporidium responsible can be determined by PCR-RFLP and/or sequencing of cryptosporidium oocyst DNA. There are no international standards for the*

preparation of purified oocysts, antisera, antigens, monoclonal antibodies or hybridomas, although a variety of purified oocysts and coproantigen detection kits using monoclonal antibodies are available commercially.

**Serological tests:** Cryptosporidiosis is often a disease of the newborn and unless there has been exclusion of exposure to infectious oocysts, serological tests do not offer any benefit.

**Requirements for vaccines and diagnostic biologicals:** There is no control programme for cryptosporidiosis, neither is there a rigorously tested and accepted vaccine available.

## A. INTRODUCTION

Originally described in 1907, *Cryptosporidium* spp. were regarded as commensals until their association with diarrhoea in young turkeys (*C. meleagridis*) in the 1950s, and with large outbreaks of diarrhoea in calves (*C. parvum*) in the 1970s. *Cryptosporidium* is an important pathogen of livestock and human beings, and since the 1980s, *C. parvum* cryptosporidiosis has been recognised as a common cause of acute self-limiting gastroenteritis in immunocompetent hosts. Fayer (9) provides a good account of the biology of *Cryptosporidium*.

Cryptosporidiosis is caused by protozoan parasites of the genus *Cryptosporidium* (family Cryptosporidiidae, order Eucoccidiorida, subclass Coccidiasina, class Sporozoasida, phylum Apicomplexa). Although more than 20 'species' of this coccidian parasite have been described on the basis of the animal hosts from which they were isolated, host specificity as a criterion for speciation appears to be ill-founded as some 'species' lack such specificity. Species definition and identification of this genus is constantly changing, with the addition of 'new' species based primarily on molecular criteria. Currently, there are 18 'valid' species (Table 1) namely: *C. hominis* found primarily in humans (previously known as *C. parvum* Type 1), *C. parvum*, found in humans and other mammals (previously known as *C. parvum* Type 2), *C. andersoni* and *C. bovis* in cattle, *C. canis* in dogs, *C. muris* in mice, *C. felis* in cats, *C. wrairi* in guinea-pigs, *C. suis* in pigs, *C. fayeri* in red kangaroo (31), *C. macropodum* in grey kangaroo (28), *C. meleagridis* in turkeys and humans, *C. baileyi* in chickens, *C. galli* in adult hens and some wild birds (26, 27), *C. varanii* in emerald monitor lizards, *C. serpentis* in snakes and lizards, and *C. molnari* in fish (9). In livestock, *C. parvum*, *C. andersoni*, *C. baileyi* and *C. meleagridis* have been reported to cause morbidity and outbreaks of disease.

*Cryptosporidium bovis* has been described recently (10). Previously identified as *Cryptosporidium* genotype Bovine B (GenBank AY120911), *C. bovis* oocysts are morphologically indistinguishable from *C. parvum* oocysts (Table 1). *Cryptosporidium bovis* is a highly prevalent species that infects primarily post-weaned calves (10, 11). *Cryptosporidium bovis* oocysts were not infectious for neonatal BALB/c mice or for two experimentally exposed lambs (<1 week of age), but were infectious for two calves that were previously infected with *C. parvum*. *Cryptosporidium bovis* was detected in calves, 2–7 months of age, none of which had diarrhoea; *C. bovis* has also been detected in a 2-week-old lamb.

In addition to the 18 valid species, there are over 40 *Cryptosporidium* genotypes (9, 48). Some of these are likely to become recognised as species as further research is carried out. The *Cryptosporidium* cervine, skunk, chipmunk genotype I and the *C. hominis* monkey genotypes (Table 2) have been described in humans. Laboratory identification is required to confirm diagnosis.

In human and non-human hosts, molecular methods including the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and DNA sequencing have demonstrated a broader range of cryptosporidium species than previously thought. *Cryptosporidium meleagridis*, *C. canis*, *C. muris*, *C. felis* and *C. suis* has been described in immunocompetent and immunocompromised human patients, as well as *C. hominis* and *C. parvum*. For example, *C. meleagridis* oocysts purified from human faeces are indistinguishable from *C. parvum* by conventional methods (described in this chapter), but show genetic identity to *C. meleagridis* from turkeys at a variety of separate genetic loci. There is some evidence that *C. andersoni* can also infect humans. The most significant zoonotic threat for humans is from *C. parvum* and *C. meleagridis*.

The discovery of sequence-based differences within various genes (ribosomal RNA [rRNA], *Cryptosporidium* heat-shock protein 70, actin, *Cryptosporidium* oocyst wall protein [COWP], *Cryptosporidium* thrombospondin-related adhesive protein-1 & -2) and between individual isolates within a 'previously valid' species has resulted in revision of the taxonomy of the genus. Some of the 40+ *Cryptosporidium* genotypes currently described may represent different species (9, 48). Therefore, as for *Cryptosporidium* species, the current classification of *Cryptosporidium* genotypes will be subject to change.

**Table 1.** Some differences among species within the genus *Cryptosporidium*

Species	Oocyst dimensions ( $\mu\text{m}$ ) <a href="http://www.nmnh.si.edu/msw/">http://www.nmnh.si.edu/msw/</a>	Site of infection	Major host	Infectious to humans
<i>C. hominis</i>	4.5 × 5.5	Small intestine	Humans	Yes
<i>C. parvum</i>	4.5 × 5.5	Small intestine	Neonatal mammalian livestock, humans	Yes
<i>C. suis</i>	5.05 × 4.41	Small intestine	Pigs	Yes
<i>C. felis</i>	4.5 × 5.0	Small intestine	Cats	Yes
<i>C. canis</i>	4.95 × 4.71	Small intestine	Dogs	Yes
<i>C. meleagridis</i>	4.5–4.0 × 4.6–5.2	Intestine	Turkeys	Yes
<i>C. muris</i>	5.5 × 7.4	Stomach	Rodents	Yes
<i>C. andersoni</i>	5.6 × 7.4 (5.0–6.5 × 8.1–6.0)	Stomach	Cattle	No
<i>C. wrairi</i>	4.0–5.0 × 4.8–5.6	Small intestine	Guinea-pigs	No
<i>C. bovis</i>	4.7–5.3 × 4.2–4.8	Small intestine	cattle	No
<i>C. baileyi</i>	4.6 × 6.2	Trachea, bursa of Fabricius, cloaca	Poultry	No
<i>C. fayeri</i>	4.5–5.1 × 3.8–5.0 (mean 4.9 × 4.3)	Intestine	Red kangaroo ( <i>Macropus rufus</i> )	No
<i>C. macropodum</i>			Grey kangaroo ( <i>Macropus giganteus</i> )	No
<i>C. galli</i>	8.0–8.5 × 6.2–6.4	Proventriculus	Finches, chicken	No
<i>C. serpentis</i>	5.6–6.6 × 4.8–5.6	Stomach	Reptiles	No
<i>C. varanii</i>	6.3 × 5.5	Intestine	Emerald monitor lizard ( <i>Varanus prasinus</i> )	No
<i>C. molnari</i>	4.72 × 4.47	Intestine	Fish (gilthead seabream)	No
<i>C. scophthalmi</i>	3.7–5.0 × 3.0–4.7 (mean 4.44 × 3.91)	Intestine, very seldom in the stomach	Fish (turbot)	No

For many of the *Cryptosporidium* species in Table 1, oocyst size and shape are similar. This makes species identification based on oocyst morphometry at the light microscope level difficult if not impossible, due to size overlap.

**Table 2.** Some *Cryptosporidium* genotypes, infection site, and their infection status relative to humans

<i>Cryptosporidium</i> genotypes				
Intestinal				
Bear	Cervine* (×3)	Deer	Deer-like	
Deer mice	Duck	Ferret	Fox (×2)	
Goose (×2)	Horse	Marsupial (×2)	Mongoose	
Monkey*	Mouse	Muskrat (×2)	Opossum (×2)	
Ostrich	Ovine	Pig II	Rabbit	
Raccoon	Seal (×2)	Sheep novel genotype (×2)	Skunk*	
Snake	<i>C. canis</i> Coyote	<i>C. canis</i> Dog	<i>C. canis</i> Fox	

**Table 2 cont.** Some *Cryptosporidium* genotypes, infection site, and their infection status relative to humans

<i>Cryptosporidium</i> genotypes			
Gastric			
Caribou	Lizard	Tortoise	Woodcock
<i>C. galli</i> Finch	<i>C. muris</i> Japanese field mouse genotype		

Key: \* infectious to humans. (Data from references 9, 47, 48).

## 1. Clinical signs

*Cryptosporidium parvum* cryptosporidiosis is a cause of scour in young, unweaned farmed livestock including calves, lambs, goat kids and alpaca. Endogenous stages infect enterocytes of the distal small intestine, caecum and colon. Villous atrophy, shortening of microvilli and sloughing of enterocytes are the major pathological changes associated with disease, and affected animals usually recover within 2 weeks of showing signs of illness. Scouring is most common in young animals but weaned and adult animals can also become infected. Signs can range from a mild to inapparent infection in older animals to severe scouring in young animals. Mortality is low unless occurring as a mixed infection with other enteric pathogens such as rotavirus. Older animals can remain infected and excrete oocysts that can be transmitted to other susceptible hosts.

*Cryptosporidium parvum* infections of cattle can cause varying degrees of dehydration, dullness, anorexia, fever and loss of condition. Mortality may be high. Rarely do they cause the acute dehydration, collapse and high mortality seen with enterotoxigenic *Escherichia coli* or rotavirus, which can occur at a similar time. Oocysts can be detected in clinically normal and clinically ill hosts. Scouring calves and lambs can excrete between  $10^6$  and  $10^8$  oocysts per g of faeces. Infected adult cattle excrete far fewer oocysts, yet subclinical infections of adult cattle can generate similar numbers of oocysts over a 12-month period.

*Cryptosporidium andersoni* colonises the digestive glands of the abomasum of older calves and adult cattle. The microvilli of peptic glands are destroyed by endogenous stages, which may account for the elevated concentrations of plasma pepsinogen detected in infected hosts. Some infected animals exhibit reduced weight gain compared with uninfected controls. Infected cattle do not develop diarrhoea, but can excrete oocysts for several months.

*Cryptosporidium* is a primary pathogen in chicken, turkeys and quail, causing respiratory and/or intestinal disease, leading to morbidity and mortality. Currently, two named species (21) infect chicken and turkeys (*C. baileyi* and *C. meleagridis*), and a third, unnamed species infects quail (*Cryptosporidium* sp.). *Cryptosporidium* spp. are common intestinal infections in broiler chicken in the USA and Japan. *Cryptosporidium baileyi* cryptosporidiosis is a disease of the epithelial lining of the bursa of Fabricius and cloaca of chicken, although the trachea and the conjunctiva are lesser sites of infection. *Cryptosporidium baileyi* intestinal cryptosporidiosis of chicken does not normally result in gross lesions or result in overt signs of disease. Villous atrophy, shortening of microvilli and enterocyte detachment are the major pathological changes associated with disease. *Cryptosporidium baileyi* respiratory cryptosporidiosis of chicken can result in severe morbidity and, on occasion, mortality. Initially, severe disease is accompanied by sneezing and coughing, followed by head extension to facilitate breathing. Epithelial cell deciliation and hyperplasia, mucosal thickening and discharge of mucocellular exudate into the airways are major pathological changes associated with disease in young broilers. Severe signs of respiratory disease can last up to 4 weeks post-infection (7). *Cryptosporidium baileyi* cryptosporidiosis of turkeys is similar to that of chicken. Chicken isolates of *C. baileyi* cause infection in other birds.

*Cryptosporidium meleagridis* cryptosporidiosis is a disease of the ileum of turkey and other poults and human beings. *Cryptosporidium meleagridis* cryptosporidiosis can cause severe diarrhoea in turkey poults. Villous atrophy, crypt hyperplasia and shortening of microvilli are major pathological changes associated with disease (7). Transmission of a turkey isolate of *C. meleagridis* to chicken and domestic ducks has been reported.

*Cryptosporidium galli* cryptosporidiosis is a disease of adult hens and some wild and exotic birds (26, 27, 30). Unlike the life cycle stages of either *C. meleagridis* or *C. baileyi*, the life cycle stages of *C. galli* are limited to the epithelial cells of the proventriculus. Histology reveals *Cryptosporidium* parasites in the proventriculus (surface, ductal, and glandular epithelium) (3, 26, 27, 30). Clinical signs include puffed plumage with head held under the wing, responsiveness to external stimuli, and failure to thrive (30). Histopathology of haematoxylin and eosin stained sections from finches demonstrated necrosis and hyperplasia of proventricular glandular epithelial cells, and a mixed inflammatory cell infiltration into the lamina propria of the proventriculus associated with large numbers of *Cryptosporidium* oocysts attached to the surface of glandular epithelial cells (30). *Cryptosporidium galli* oocysts ( $8.0\text{--}8.5 \times 6.2\text{--}6.4 \mu\text{m}$ ) are larger than those of *C. baileyi*.

Respiratory and intestinal cryptosporidiosis have been reported in commercially grown quail caused by *Cryptosporidium* of an inadequately described species (*Cryptosporidium* sp.) whose oocysts are smaller than those of *C. baileyi* and are not infectious to chicken or turkeys. Pathological changes are similar to those described for *C. baileyi* respiratory and intestinal cryptosporidiosis of chicken (7).

## 2. Infectious dose

The infectious dose for *C. parvum* varies from isolate to isolate and from host species to host species. For mouse (CD-1 strain) neonates the ID<sub>50</sub> (median infectious dose) is between 87 and 60 oocysts (19). Ten oocysts produced infection in two out of two primates tested, and five oocysts produced clinical disease in gnotobiotic lambs. The infectious dose for cattle is not known, but is thought to be small. Whether *C. parvum* isolates vary in their ability to colonise different host species is unknown. In healthy adult human volunteers, the ID<sub>50</sub> is also dependent on both the isolate and the host immune status. In human volunteer infectivity studies, *C. parvum* isolates differ in their ID<sub>50</sub>, their attack rate, and the duration of diarrhoea they induce. The ID<sub>50</sub> of the *C. parvum* UCP isolate (Ungar *Cryptosporidium parvum*, human derived, from Dr B. Ungar, USA) is 1042 oocysts; the *C. parvum* IOWA isolate (bovine derived, from Ames, Iowa, USA) is 132 oocysts; and the *C. parvum* TAMU isolate (equine derived, from Texas A & M University, USA) is 9 oocysts (25). Oral infection with 100 *C. baileyi* oocysts can result in intestinal cryptosporidiosis (7).

## 3. Transmission

Transmission can occur via any route by which material contaminated with viable oocysts excreted by infected individuals can be ingested. Practices likely to enhance the spread of cryptosporidiosis include indoor calving and lambing and the communal feeding and husbandry of neonates, where young susceptible animals are in close contact with each other and the faeces of infected animals. Similarly, the disposal of faeces, farmyard manure or other contaminated waste in land-based dumps, when followed by periods of heavy rainfall or melting snow can lead to *C. parvum* oocyst contamination of water courses. These courses may be used as a source of drinking water for other animals and for potable water for human consumption. Contaminated waste includes both the liquid and solid by-products of animal husbandry.

## 4. Maintenance of infection

A variety of wild mammals act as hosts to *C. parvum* (9, 39, 43, 47), particularly neonates, but little is known of the importance of their involvement in transmitting infection to, or maintaining infection, in domesticated species in farmyard environments. Their role in 'on farm' epidemiology in domesticated species is also uncertain. The methods used for diagnosing infection in small mammals and wildlife are the same as those described for farm animals. Oocysts are environmentally robust and can survive for long time periods (>6 months) in moist, cool microclimates. Evidence exists for transmission of cryptosporidiosis from clinically normal dams to suckling neonates, but, in general, the duration of the carrier state remains unknown. A variety of bird species act as hosts to *C. baileyi*.

# B. DIAGNOSTIC TECHNIQUES

## 1. Identification of the agent

There is no prescribed test for diagnosing *Cryptosporidium* infection. The demonstration of *Cryptosporidium* species oocysts or *Cryptosporidium* antigen in a properly collected and handled sample is sufficient for a positive diagnosis, and the methods of choice for collection of the samples are non-invasive. There are no reproducible *in-vitro* culture techniques available to amplify parasite numbers prior to identification, therefore the detection of the oocyst (the transmissive stage), *Cryptosporidium* antigen and/or DNA from faeces, or other suitable body fluids are the methods of choice. In addition to these tests, haematoxylin and eosin can be used for histological confirmation of the diagnosis on post-mortem. Haematoxylin and eosin histology is useful for confirming diagnosis, is commonplace world-wide, and will not be described in this chapter.

Further analyses including species and/or *C. parvum* subtype identity, can be performed on *Cryptosporidium* DNA using molecular techniques, such as the PCR-RFLP and/or sequencing of products amplified from defined genetic loci. This not only confirms diagnosis, but also provides discrimination beyond that possible with morphology and morphometry using light microscopy.

For *Cryptosporidium* species that infect the gastrointestinal tract (Table 1), primary diagnosis is based on the demonstration of oocysts in faeces by conventional tinctorial stains, fluorescent/immunofluorescent stains or *Cryptosporidium* antigen(s) (copro-antigens) in faeces by enzyme-linked immunosorbent assay (ELISA) or immuno-chromatographic (IC) methods. The majority of diagnostic methods have been developed using *C. parvum* because of its commercial importance and availability. There is anecdotal evidence indicating that in a minority of samples, the methods described below may not detect all isolates. The methods described below are

expected to detect most *C. parvum* infections, but their usefulness for detecting non-*parvum* species from clinical material is less well understood.

Demonstration of *Cryptosporidium* oocysts or *Cryptosporidium*-specific antigen in faecal samples is the most appropriate test for most applications. Most infections that cause morbidity and/or mortality in mammals are likely to be due to *C. parvum* cryptosporidiosis. The species of *Cryptosporidium* responsible can be determined by PCR-RFLP or sequencing of *Cryptosporidium* DNA isolated from oocysts, later. There are no international standards for the preparation of purified oocysts, antisera, antigens, monoclonal antibodies (MAbs) or hybridomas, although a variety of purified oocysts and coproantigen detection kits using MAbs are available commercially.

#### a) Laboratory diagnosis

The diagnostic features of *C. parvum* oocysts viewed in suspension using Nomarski Differential Interference Contrast (DIC) microscopy are as follows. Oocysts are smooth, thick walled, colourless, have spherical or slightly ovoid bodies containing, when fully developed (sporulated), four elongated, naked (i.e. not within a sporocyst[s]) sporozoites and a cytoplasmic residual body. The modal size measurement of *C. parvum* oocysts is  $4.5 \times 5.0 \mu\text{m}$  (range 4–6  $\mu\text{m}$ ).

Diagnosis is normally established by conventional microscopic methods, and the modified Ziehl–Neelsen (mZN) or auramine phenol (AP) methods using unconcentrated faecal smears are frequently used (5, 6, 32, 37). Where low oocyst numbers are expected in samples, or purified oocysts are required for molecular investigations, concentrating oocysts in faecal samples can increase the sensitivity of detection. Sugar (e.g. Sheather), salt, zinc sulphate or formalin-ether (formalin-ethyl acetate) solutions or specific concentration techniques, such as immuno-magnetic separation, are the best options for concentrating oocysts from faeces (32, 33, 37).

#### b) Demonstration in faeces

Stool samples from most clinically ill cases will contain large numbers of thick-walled oocysts and sufficient *Cryptosporidium* antigen, therefore, the use of standard staining and immunological techniques should result in a positive diagnosis. The numbers of clinically normal excretors is not known, and, given the insensitivity of conventional methods, low oocyst excretors may not be diagnosed using conventional techniques, as oocyst numbers may be below the limit of detection of these methods (37, 41, 42). In clinically ill animals, oocysts can normally be demonstrated in unconcentrated stool smears. The use of an oocyst concentration method can enhance the detection rate. Both flotation and sedimentation methods are suitable for concentrating *Cryptosporidium* spp. oocysts, and oocyst antigens can still be sought in faeces following these oocyst concentration procedures.

Oocysts might not be detectable in clinical samples from all cryptosporidiosis cases, and the absence of oocysts in repeated submissions of samples from symptomatic hosts does not necessarily indicate the absence of infection. In these instances, and particularly when clinical suspicion is high, oocyst negative stool samples should be subjected to antigen and/or PCR-based detection, as sufficient *Cryptosporidium* antigen or DNA from asexual life cycle forms should be present in faeces (37). This is a major advantage of copro-antigen detection immunoassays. Nucleic acid detection tests such as the PCR are being used increasingly as they offer both improved sensitivity and species/genotype/subtype identity. For PCR-based methods, nested PCR methods, being more sensitive than direct PCR methods, are likely to have a higher diagnostic index (37).

*Cryptosporidium* spp. positive faecal samples should be available to personnel who are familiarising themselves with staining and concentration techniques, and smears from positive faecal samples should be included each time a test is performed. Stool samples containing *C. parvum* oocysts can be stored at 4°C in either 2.5%  $\text{K}_2\text{Cr}_2\text{O}_7$  or 10% formalin for reference purposes. Similarly, oocyst-positive faecal smears, air dried and fixed in absolute methanol, can be prepared in advance from previous positive samples for use as positive controls. Where bronchio-pulmonary involvement is suspected, similar tests can be performed on bronchial and pleural exudates or lavages.

Note that 2.5%  $\text{K}_2\text{Cr}_2\text{O}_7$  can be inhibitory to PCR. Oocyst positive stool samples or partially purified oocysts stored in 2.5%  $\text{K}_2\text{Cr}_2\text{O}_7$  and intended for nucleic acid amplification by PCR should be washed in deionised water to remove residual 2.5%  $\text{K}_2\text{Cr}_2\text{O}_7$  prior to DNA extraction. A series of three washes each followed by centrifugation (3000 *g* for 10 minutes), removal of the supernatant and resuspension of the pellet in deionised water should minimise PCR inhibition by 2.5%  $\text{K}_2\text{Cr}_2\text{O}_7$ . It should be noted that inhibitory factors can still be present even after long-term (>6 months) storage.

#### c) Laboratory staff and operator safety

*Cryptosporidium* is included in laboratory risk Hazard Group 2 and all laboratory procedures that can give rise to infectious aerosols must be conducted in a biosafety cabinet. Specimens for *Cryptosporidium*

analyses can contain other pathogenic organisms and should be processed accordingly. In order to safeguard the health of laboratory workers, the safety procedures outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities must be followed.

The laboratory should have an internal and external quality assurance programme in place as outlined in Chapter 1.1.3 Quality management in veterinary testing laboratories.

**d) Collections and submission of samples**

Where possible, specimens for primary diagnosis should be collected during acute infection, and should be processed as soon as possible. Ideally, transportation systems should be selected to ensure that specimens arrive at the laboratory within 24 hours. If prompt examination for *Cryptosporidium* cannot be carried out, the deterioration of protozoan morphology and their overgrowth by other microorganisms, particularly yeasts, can be reduced by the addition of 10% aqueous (v/v) formalin, although 10% formalin can interfere with PCR tests. Both oocyst morphology for microscopic identification and sporozoite DNA for PCR testing can usually be preserved for long periods at 4°C without formalinisation. Faeces can be stored frozen for over 2 years without affecting the ability to extract *Cryptosporidium* DNA for molecular analysis. In some instances, this can enhance DNA extraction rates, possibly due to the softening of the outer oocyst wall. Repeated freeze–thawing of frozen faecal samples is not recommended as the released DNA will degrade.

The procedures used for collection and transport of specimens are critically important for successful laboratory analyses. Specimens should be collected in a suitable leak-proof sample container and should be enclosed in secure primary and secondary packaging. Procedures for packaging and shipping of specimens must be as outlined in the International Air Transport Association's Dangerous Goods Regulations (16). These regulations are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

**e) Threshold of detection in faeces**

Most tinctorial and fluorescence methods for detecting oocysts, and ELISA and IC for detecting *Cryptosporidium* antigens, are relatively insensitive. These methods can detect oocysts in clinically ill animals, but may not be sufficiently sensitive to detect infection in clinically normal animals. Anusz *et al.* (2) reported a detection limit of  $10^6$  oocysts per ml of faeces using the Kinyoun modification of mZN on unconcentrated faecal smears. Concentrating oocysts in the sample can increase the sensitivity of detection. In oocyst-positive human stool samples, between  $1 \times 10^4$  and  $5 \times 10^4$  oocysts per g of unconcentrated stool are necessary to obtain a 100% detection efficiency using the Kinyoun mZN staining method (41). Variations in faecal consistency influence the ease of detection, with oocysts being more easily detected in concentrates made from watery, diarrhoeal specimens than from formed stool specimens (41). In addition to microscopic techniques, a number of antigen-capture ELISAs and ICs have been reported in the literature with detection limits in the region of  $3 \times 10^5$ – $10^6$  oocysts per ml (2, 29, 37), which indicates that they do not appear to offer increased sensitivity over microscopical methods.

In bovine faecal samples, oocysts were not detected in samples seeded with 10,000 *C. parvum* oocysts per g following formol–ether sedimentation and examination using AP or immunofluorescence (IF) staining. When oocysts were concentrated using sucrose flotation, the threshold of detection was 4000 oocysts per g for both staining methods. After salt flotation, 4000 oocysts per g could be reliably detected by AP staining, but the detection limit was increased to 6000 oocysts per g using IF staining (41). Webster *et al.* (42) also compared microscopy with PCR and found that PCR coupled with immunomagnetic particle separation (IMS) of oocysts from faecal samples detected five oocysts per ml of diluted faeces, which corresponds to 80–90 oocysts per g. Even allowing for the dilution of formed faecal samples required for IMS, this represented an increase in sensitivity of several orders of magnitude over the conventional coprodiagnostic methods. Currently, a variety of sensitive, PCR-based tests are available (see Section B.1 Nucleic acid recognition methods).

**o Preparation of unconcentrated faecal (or appropriate body fluid) smears (include a positive control slide each time this procedure is performed)**

**o Test procedure**

- i) Wear protective clothing and disposable gloves. Score the reference number of the specimen on a microscope slide with a diamond marker<sup>1</sup>, and use separate microscope slides for each specimen. Place 1 drop of saline (about 50 µl) in the centre of the slide.
- ii) Remove a small sample of faeces (about 2 mg) with the tip of a clean applicator stick<sup>2</sup> (or pipette after mixing thoroughly, if liquid) and emulsify the sample in saline by thorough mixing. For liquid stools (or other appropriate body fluid) dispense one drop directly on to the slide. In liquid stools, mucus strands

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1 Alternatively, a lead pencil can be used to mark the etched (frosted) portion of a frosted glass microscope slide.

2 For formed stools, the sample should include portions from the surface and from within the stool.

and exudates or pus can be mixed with saline on the microscope slide. Liquid stools can be diluted with a drop of 150 mM saline solution.

- iii) Prepare a medium to thick smear with areas of varying thickness. Ensure that the smear is of the correct transparency<sup>3</sup>.
  - iv) Air dry the smear at room temperature.
  - v) Fix the smear<sup>4</sup> in methanol for 3 minutes.
- f) **Preparation of faecal (or appropriate body fluid) smears following concentration by flotation or sedimentation**

No flotation or sedimentation method is specific for *Cryptosporidium* spp. oocysts. Flotation fluids are denser than the parasites to be concentrated and are formulated to a defined specific gravity using a suitable hydrometer available from most large laboratory suppliers. Parasites concentrated by flotation or sedimentation methods can be identified by all the methods described in this chapter. Flotation/sedimentation fluids can sometimes interfere with diagnostic tests. Excess sucrose can reduce both oocyst attachment to glass slides and subsequent antibody binding, prolonged exposure to NaCl can distort morphology and morphometry, and formalin can reduce the sensitivity of PCR reactions. When oocysts are concentrated, excess flotation/sedimentation fluid can be removed by washing the concentrate in water and re-centrifuging. The supernatant is then aspirated and discarded, care being taken not to disturb the pellet. These concentration methods are suitable for any appropriate body fluid that could contain oocysts.

#### 1. Flotation

The flotation principle uses a liquid suspending medium, which is denser than the oocysts to be concentrated. Therefore, when mixed with flotation fluid, the oocysts rise to the surface and can be skimmed out of the surface film and detected using the chosen method. For a flotation fluid to be useful in diagnostics, when morphology and morphometry are the critical factors, the suspending medium must not only be heavier than the object to be floated but must not produce shrinkage sufficient to render the object undiagnosable (32). Sucrose flotation, zinc sulphate flotation and saturated salt flotation methods are all suitable for concentrating cryptosporidium oocysts. The following is a description of the methods used to prepare flotation solutions and to concentrate oocysts.

##### o Sucrose flotation

Sucrose solution (specific gravity 1.18) is prepared in a glass beaker by adding 256 g of sucrose to 300 ml of deionised water. The solution is gently heated (<60°C) and continuously stirred with the aid of a magnetic stirrer on a hot plate stirrer, until the sucrose has dissolved completely. The sucrose solution is either placed on ice or in a refrigerator until its temperature has adjusted to 4°C. The cold sucrose solution is transferred to a 500 ml measuring cylinder and its specific gravity is adjusted to 1.18 by adding sufficient cold, deionised water (4°C). The sucrose solution is transferred to a screw-cap glass bottle, labelled, dated, initialled and stored at 4°C until used.

##### o Zinc sulphate flotation

Zinc sulphate solution (specific gravity 1.18) is prepared in a glass beaker by adding 100 g of zinc sulphate to 300 ml of deionised water. The solution is gently heated (<60°C) and continuously stirred with the aid of a magnetic stirrer on a hot plate stirrer, until the zinc sulphate had dissolved completely. The zinc sulphate solution is transferred to a 500 ml measuring cylinder and its specific gravity is adjusted to 1.18 by adding sufficient cold, deionised water (4°C). The zinc sulphate solution is transferred to a screw-cap glass bottle, labelled, dated, initialled and stored at 4°C until used.

##### o Salt flotation

Saturated salt solution (specific gravity 1.2) is prepared by adding approximately 200 g of sodium chloride to 200 ml of deionised water. The solution is gently heated (<60°C) and continuously stirred with the aid of a magnetic stirrer on a hot plate stirrer. Small amounts of sodium chloride (approximately 10 g) are added at 10-minute intervals until the solution becomes saturated. The saturated salt solution is then decanted into a clean glass bottle and either placed on ice or in a refrigerator until its temperature had adjusted to 4°C. The cold saturated salt solution is then transferred into a 500 ml measuring cylinder and its specific gravity is adjusted to 1.2 by adding cold, deionised water (4°C). The

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- 3 Moderately thick smears are recommended for this procedure. If the smear is too thin or thick, oocysts will be missed. An acceptable thickness can be achieved when either the hands of your watch or the print on this page can just be read when viewed through the preparation.
  - 4 Air-dried, methanol-fixed smears can be kept at room temperature for >6 months before staining.

saturated salt solution is transferred to a screw-cap glass bottle and labelled, dated, initialled and stored at 4°C until used.

Brine is a concentrated aqueous NaCl solution, which has a specific gravity between 1.120 and 1.200 depending on the impurity of the salt used. While suitable for concentrating *Cryptosporidium* spp. oocysts, some protozoan cysts can become badly shrivelled or open up in this flotation fluid. The optimal time to examine specimens obtained from brine flotation is between 5 and 20 minutes after their recovery following flotation.

Centrifugal flotation has also been used to recover *Cryptosporidium* oocysts (and a various other parasite cysts and ova) from faeces. Most centrifugal flotation methods are based on modifications of the Clayton-Lane technique, whereby oocysts are concentrated by flotation and collected as a hanging drop on the underside of a glass cover-slip placed onto the positive meniscus of the flotation fluid. Centrifugation is used to separate particles which are denser than the flotation fluid from oocysts and particulates which will float on the surface of the flotation fluid. The inclusion of a centrifugation step speeds up separation of oocysts from other particulates (and hence, the concentration of oocysts) and minimises the risk of the flotation fluid adversely affecting the morphometry or morphology of the oocysts. The operator should note the health and safety issues, including lacerations and puncture wounds, associated with handling cover-slips.

#### Concentration of *Cryptosporidium* spp. oocysts by flotation

##### o Test procedure

- i) Wear protective clothing and disposable gloves. Transfer approximately 1–2 g of faeces<sup>5</sup> with an applicator stick to 3 ml of flotation fluid in a 12 ml test tube and mix thoroughly. If the stool is liquid, mix thoroughly, and dispense approx. 1–2 ml fluid into the test tube.
- ii) Add, with gentle stirring, sufficient flotation fluid to form a positive meniscus at the rim of the test tube. Remove any large particles from the surface and if necessary, add more flotation fluid to maintain this positive meniscus.
- iii) Leave for 20 minutes, then, taking great care not to disturb the positive meniscus gently remove the meniscus with a disposable pipette and dispense gently on to a microscope slide<sup>6</sup>.
- iv) Air dry the smear at room temperature.
- v) Fix the smear<sup>7</sup> in methanol for 3 minutes.

#### Concentration of *Cryptosporidium* spp. oocysts by centrifugal flotation

##### o Test procedure

- i) Wear protective clothing and disposable gloves. Transfer approximately 1–2 g of faeces<sup>8</sup> with an applicator stick to 3 ml of flotation fluid in a 12 ml centrifuge tube and mix thoroughly. If the stool is liquid, mix thoroughly, and dispense approx. 1–2 ml fluid into the centrifuge tube.
- ii) Add, with gentle stirring, sufficient flotation fluid to form a positive meniscus at the rim of the centrifuge tube. Remove any large particles from the surface and if necessary, add more flotation fluid to maintain this positive meniscus.
- iii) Place the centrifuge tube in a bench top centrifuge with swing out buckets, and place a 22 mm × 22 mm glass cover-slip onto the rim of the centrifuge tube, so that it flattens the positive meniscus. Add a balance tube if necessary, and centrifuge at 1100 **g**<sup>9</sup> for 5 minutes.
- iv) Once the centrifuge stops, pick up the glass cover-slip between index finger and thumb at opposing corners of the cover-slip. A hanging drop will be present on the underside of the cover-slip. Carefully place the cover-slip, with the hanging drop lowermost, onto a glass microscope slide.

## 2. Centrifugal sedimentation

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5 For formed stools, the sample should include portions from the surface and from within the stool.

6 Moderately thick smears are recommended for this procedure. If the smear is too thin or thick, oocysts will be missed. An acceptable thickness can be achieved when either the hands of your watch or the print on this page can just be read when viewed through the preparation. Score the reference number of the specimen on a microscope slide with a diamond marker, and use separate microscope slides for each specimen. Alternatively, a lead pencil can be used to mark the etched (frosted) portion of a frosted glass microscope slide.

7 Air-dried, methanol-fixed smears can be kept at room temperature for >6 months before staining.

8 For formed stools, the sample should include portions from the surface and from within the stool.

9 Centrifugation at speeds higher than 1100 **g** for longer (>5 minutes) periods of time is not advised as some parasites may deform and/or rupture and collapse.

Parasites will settle more rapidly if the stool suspension is subjected to centrifugation, however, food particles will also sediment more rapidly and can mask the presence of parasites in the film examined. To overcome this potential problem, larger food particles can be removed prior to centrifugation by filtering the emulsified stool through a sieve with an aperture size large enough for parasites to pass through, but which retains the larger food particles. As this process is more efficient than sedimentation by gravity, a smaller faecal sample (500 mg–1 g: the size of a pea) is sufficient for examination. Although centrifugation concentrates the material more quickly, faecal debris, which can obscure parasites, remains present. The efficiency of detection is increased by adding formalin for fixation and preservation of parasites, and ether to remove fats and oils. Both 10% formalin and ether are bactericidal. After centrifugation, a fatty plug, which may adhere to the inner walls of the tube, can be seen at the interface of the two liquids. The ether layer, the fatty plug and the formalin below it are discarded and the whole pellet is retained for examination.

Many modifications to this procedure have been advocated, and the following protocol is typical of the method used in diagnostic laboratories. Less distortion of protozoan cysts occurs with this method than with zinc sulphate flotation. This method achieves a concentration of 15–50-fold, dependent on the parasite sought, and provides a good concentrate of protozoan cysts and helminth eggs, which are diagnostically satisfactory. All steps that can generate aerosols (excluding centrifugation) should be performed in an operator protection safety cabinet.

#### Concentration of *Cryptosporidium* spp. oocysts by (formol/ether) centrifugal sedimentation

##### o Test procedure

- i) Wear protective clothing and disposable gloves. Sample approximately 500 mg–1 g faeces<sup>10</sup> with an applicator stick<sup>11</sup> and place in a clean 12–15 ml centrifuge tube containing 7 ml of 10% formalin. If the stool is liquid, dispense about 750 µl into the centrifuge tube.
- ii) Break up the sample thoroughly and emulsify using the applicator stick.
- iii) Filter the resulting suspension through a sieve into a beaker, then pour the filtrate back into the same centrifuge tube<sup>12</sup>.
- iv) Add 3 ml of diethyl ether (or ethyl acetate<sup>13</sup>) to the formalinised solution, seal the neck of the tube with a rubber bung (or a gloved thumb over the top of the tube) and shake the mixture vigorously for 30 seconds. Invert the tube a few times during this procedure and release the pressure developed gently by removing the rubber bung (or your thumb) slowly.
- v) Centrifuge the tube at 1100 *g*<sup>14</sup> for 2 minutes.
- vi) Loosen the fatty plug with a wooden stick by passing the stick between the inner wall of the tube and the plug. Discard the plug and the fluid both above and below it by inverting the tube, allowing only the last one or two drops to fall back into the tube. Discard this fluid, containing diethyl ether and formalin, into a marked re-sealable liquid waste container.
- vii) Re-suspend the pellet<sup>15</sup> by agitation. Pour the whole, or the majority of the re-suspended pellet on to a microscope slide, or transfer the re-suspended contents on to a microscope slide with a disposable pipette, and air dry.

A commercial device for concentrating helminth ova, larvae and protozoan cysts and oocysts using the formalin-ether method is available. Sold as the Fecal Parasite Concentrator<sup>16</sup> it is an enclosed system, and consists of two polypropylene tubes, a flat-bottomed tube used for emulsifying the stool, and a conical tube used for centrifugation, with an interconnecting sieve. The comprehensive method states that both fresh and preserved (10% formalin, merthiolate-iodine-formalin, polyvinyl alcohol, and sodium acetate-formalin) stool specimens may be used.

10 This is the size of a pea.

11 The sample should include portions from the surface and from within a formed stool.

12 425 µm aperture size, 38 mm diameter is equivalent to 36 mesh British Standard (BS 410-86) or 40 mesh American Standard (ASTM E11-81). The skirt of the sieve should fit neatly into the rim of the beaker. Debris trapped on the sieve is discarded by inverting the sieve and passing a stream of tap water through the mesh. Both the sieve and the beaker should be washed thoroughly in running tap water between each sample.

13 Ethyl acetate, although less flammable than diethyl ether is nevertheless flammable, therefore the procedure should be performed in well ventilated areas, ensuring that they contain no naked flames. Avoid prolonged breathing or skin contact.

14 Centrifugation at speeds higher than 1100 *g* for longer (>5 minutes) periods of time is not advised as some parasites may deform and/or rupture and collapse.

15 Too large a pellet is indicative of one or more of the following: centrifuging above the recommended speed and/or time, insufficient shaking (step iv), taking too large a faecal sample.

16 FPC, Evergreen Scientific, 2300 East 49th Street, P.O. Box 58248, Los Angeles, California 90058, USA; <http://www.evergreensci.com/micro/hfpc.htm>

## g) Conventional staining methods

Both mZN and AP are effective for detecting *Cryptosporidium* oocysts in faeces (5, 6, 32, 37). mZN-stained slides should be screened under the  $\times 40$  objective lens and putative oocysts confirmed and measured under the  $\times 100$  objective lens (morphology and morphometry) using a bright-field microscope with a  $\times 10$  eyepiece. AP-stained slides require to be read using an epifluorescence microscope equipped with a fluorescein isothiocyanate (FITC) filter set (excitation 490 nm; emission 510 nm). A UV filter set (excitation 355 nm, emission 450 nm) can assist in visualising AP-stained sporozoites. AP-stained slides can be screened under the  $\times 20$  objective lens and oocysts with typical morphology can be confirmed under the  $\times 40$  objective lens. The  $\times 100$  objective lens must be used for all morphometric (size) measurements. AP-stained oocysts visualised under either the FITC or UV filters can be measured by slowly increasing the voltage (light intensity) of the bright-field light source so that both fluorescent and bright-field images can be seen concurrently. The object can then be measured with the eye-piece graticule.

## o Calibration of size using the eye-piece and stage micrometers

Diagnosis of intact organisms often necessitates the measurement of the size and shape (morphometry) of the organism in question, in order to ensure that it falls within the accepted range of standard parameters (e.g. size and shape) for the species in question. At the light microscope level, measurement of objects  $< 1$  mm is achieved by means of a stage micrometer used in conjunction with an eye-piece micrometer. Objects are measured in Systeme International (SI) units, and the standard unit of measurement for conventional microscopy is the micron ( $\mu = 0.001$  mm).

The stage micrometer consists of a  $76 \times 26$  mm glass slide that has a millimetre scale, graduated in microns permanently mounted on it. The eye-piece micrometer is a disc of transparent glass or plastic bearing a graduated scale, which is placed in one of the eye-pieces of a binocular microscope. The scale is usually 1 cm in length and is subdivided into millimetre intervals. When the microscope is focused on the object to be measured, both the scale on the eye-piece micrometer and the image of the object are seen simultaneously in focus. The standard scale on the stage micrometer is usually 1 or 2 mm.

When measurements are to be made, the appropriate objective lens, which is dependent on the magnification required, is chosen, and the number of divisions corresponding to the length or breadth of the image of the object is read on the scale of the eye-piece micrometer. The observed measurement is translated into *real length* (which corresponds to the number of eye-piece micrometer divisions representing the chosen parameter to be measured) by substituting the stage micrometer for the object and determining the number of divisions on the eye-piece micrometer corresponding to a definite number of divisions of the millimetre scale on the stage micrometer, *under the same magnification*.

Remember that your calculation, in *real length*, of the value of the division on the eye-piece micrometer scale will only be valid for the magnification of the objective chosen. You will have to recalculate the value of a division on the eye-piece micrometer for each objective of differing magnification on the microscope.

Because morphometry is a significant component of diagnostic parasitology, repetitive measurements of similar objects present in a single sample, or of various objects of varying sizes in sequential samples, necessitating the use of a variety of magnifications, will have to be undertaken. By determining the *micrometer value* of the eye-piece scale for *each objective used*, the constant interchange of objects and stage micrometer can be overcome. This enables rapid calculation of morphometry, in millimetres, or fractions thereof, to be undertaken with any of the objective lenses available.

The graticule is placed in the eye-piece by unscrewing the lower component of the eye-piece and placing it into the open tube. It must be seated correctly before the lower component is screwed back on to the eye-piece. Ensure that the diameter of the graticule is similar to the internal diameter of the lower lens tube. Do not touch the surface of the graticule – hold it by its edges. Make sure it is dust and grease free. The eye lens is focused on to the graticule by adjusting it until the scale on the graticule is critically sharp.

The determination is carried out as follows:

- i) Insert the eye-piece micrometer, with its scale already in focus, into the microscope, making sure that the graticule scale is the right way up.
- ii) Select the lowest power objective lens (e.g.  $\times 10$  objective) and focus the microscope on the stage micrometer, rotating the eye-piece and positioning the stage micrometer until the scales of the eye-piece micrometer and the stage micrometer lie parallel and close to or overlapping each other.
- iii) Count the number of intervals on the stage micrometer that correspond exactly to a whole number of divisions on the eye-piece micrometer.

- iv) Divide the value observed on the stage micrometer by the number of divisions counted on the eye-piece micrometer scale to determine the value of each division on the eye-piece micrometer scale.
- v) Repeat the above for each objective lens<sup>17</sup>.
- vi) Keep a permanent record of the calculation of the value for each of the divisions on the eye-piece micrometer for each objective lens in close proximity to or attached on to the body of the microscope (e.g. a piece of cardboard stuck to the front of the microscope).

o **Example**

For each objective lens:

x eye-piece divisions = y  $\mu$ m (on the stage micrometer)

1 eye-piece division = y/x  $\mu$ m

o **Microscopical examination of a sample**

The sample must be examined in a systematic manner. Observation should commence using the lowest suitable objective, ensuring that the entire sample is viewed. A suggested scheme is as follows: commence in the upper left-hand corner of the sample, working across the slide from left to right, one field width at a time, until the upper right-hand edge of the sample is reached. Move down one field height and continue working across the slide from right to left, field by field, until the left-hand edge of the sample is reached. Continue in this manner until the end of the sample (lower right-hand corner) is reached. During this period of observation, the fine focus should be adjusted continuously so that the depth of the sample is also scanned. When a suspicious object is located, it is inspected under high power magnification and either verified or disregarded. If the magnification of the image of the object is insufficient to be able to visualise definitive morphological characteristics under the high (dry) objective, the immersion lens ( $\times 100$ ) must be used. Wet mounts can be sealed with nail varnish or a proprietary permanent sealant.

Neither tinctorial- nor fluorescence-based stains can determine the species of cryptosporidium present if the oocysts fall within the size range of 4–6  $\mu$ m (see Table 1). For mammalian livestock, the consensus opinion is that the majority of infections are likely to be due to *C. parvum*, therefore, a preliminary diagnosis of *C. parvum* cryptosporidiosis can be made. However, the presence of oocysts of the size range 4–6  $\mu$ m does not necessarily indicate that the infectious species is *C. parvum*. Similarly, for birds, a preliminary diagnosis of *C. baileyi*, *C. meleagridis* or *C. galli* cryptosporidiosis can be made depending on infection site and oocyst size. Molecular identification of species/genotype/subtype can be performed later.

o **Reporting results of microscopical examination**

Negative specimens should be reported as 'NO *Cryptosporidium* oocysts seen'.

Positive specimens should be reported as '*Cryptosporidium* oocysts seen'.

A scoring system for positive samples can be used, based on the number of oocysts observed under the  $\times 40$  objective lens. However, microscopic examination cannot be considered as a quantitative determination as oocyst numbers vary considerably during the course of infection.

+ = less than 5 oocysts per slide  
 ++ = 1 to 10 oocysts per field of view  
 +++ = 11 or more oocysts per field of view

o **Modified Ziehl-Neelsen (mZN)**

**Strong carbol fuchsin:** Dissolve 20 g basic fuchsin in 200 ml absolute methanol and mix on a magnetic stirrer until dissolved. Add 125 ml liquid phenol (GPR [80% w/w in distilled water]) carefully until well mixed, and make up to the final volume with 1675 ml deionised water. Mix thoroughly. Filter before use through Whatman No.1 filter paper to remove debris and store in a stock reagent bottle. Label, date and initial. Store the stock reagent in a dark cupboard at room temperature.

Commercial supplies are also available. Often the concentration of basic fuchsin can vary within the acceptable range of 1–3%.

**1% acid methanol:** Carefully add 20 ml hydrochloric acid (GPR/SLR) to 1980 ml of absolute methanol and mix. Transfer to a stock reagent bottle, and label, date and initial.

17 Notice that the value calculated in millimetres for each of the divisions on the eye-piece micrometer will be different for objectives of different magnifications, with values calculated for real length being smaller for each of the divisions of the eye-piece micrometer with increasing magnification.

**0.4% malachite green:** Add 2 g malachite green to 480 ml deionised water and mix on a magnetic stirrer. Filter through Whatman No.1 filter paper into a stock reagent bottle, label, date and initial.

o **Test procedure**

Include a positive control slide each time you perform this procedure.

- i) Wear protective clothing and disposable gloves. Fix the air-dried smear or concentrate<sup>18</sup> in methanol for 3 minutes.
- ii) Immerse the slide in cold strong carbol-fuchsin and stain for 15 minutes.
- iii) Rinse the slide thoroughly in tap water.
- iv) Decolourise in 1% acid methanol for 10–15 seconds<sup>19</sup>.
- v) Rinse the slide in tap water.
- vi) Counterstain with 0.4% malachite green for 30 seconds.
- vii) Rinse the slide in tap water.
- viii) Air-dry the slide. (The smear can be examined with or without a cover-slip. A little immersion oil is spread over the smear which is then viewed with either dry or oil immersion lenses, without the addition of a cover-slip. An alternative method is to add a cover-slip and mounting medium and then examine the smear.)
- ix) Examine for the presence of oocysts by scanning the slide using the ×40 objective lens of a bright-field microscope. Confirm the presence of oocysts under the oil immersion objective lens.
- x) Measure the size and shape of the red-stained bodies<sup>20</sup>.

o **Diagnostic features of *Cryptosporidium* spp. oocysts stained with mZN**

*Cryptosporidium* spp. oocysts stain red on a pale green background. The degree and proportion of staining varies with individual oocysts. In addition, the internal structures take up the stain to varying degrees. Some may appear amorphous while others may contain the characteristic crescentic forms of the sporozoites. *Cryptosporidium parvum* oocysts appear as discs, 4–6 µm in diameter. Yeasts and faecal debris stain a dull red. Some bacterial spores may also stain red, but these are too small to cause confusion.

o **Auramine-phenol**

**Auramine phenol (AP):** Dissolve 3 g phenol in 100 ml deionised water and slowly add 0.3 g Auramine O. Filter through Whatman No. 1 filter paper into a stock reagent bottle. Label, date and initial the stock reagent. Store at room temperature in a light-proof glass bottle with an airtight stopper. Commercially available stains, such as Lempert's reagent, are also acceptable.

**3% Acid methanol:** Carefully add 60 ml hydrochloric acid (GPR/SLR) to 1940 ml absolute methanol and mix. Transfer to a stock reagent bottle, and label, date and initial.

**0.1% potassium permanganate:** Add 0.5 g potassium permanganate to the 499.5 ml deionised water and mix using a magnetic stirrer. Filter through Whatman No. 1 filter paper into a stock reagent bottle, and label, date and initial.

o **Test procedure**

Include a positive control slide each time you perform this procedure.

- i) Wear protective clothing and disposable gloves. Fix air-dried smears or concentrate<sup>21</sup> in absolute methanol for 3 minutes.
- ii) Immerse the slides in AP stain for 10 minutes.
- iii) Rinse in tap water to remove excess stain.

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18 Moderately thick smears are recommended for this procedure.

19 Over-destaining must be avoided.

20 *Isospora* spp. oocysts stain red and appear as large elongated ovoid bodies, tapered at the end and containing either a granular zygote or two sporoblasts. *Cyclospora* spp. oocysts stain pinkish red and appear as circular discs (8–10 µm in diameter) containing a central morula. The degree and proportion of staining varies with individual oocysts. The unsporulated oocyst is seen generally in stool samples.

21 Moderately thick smears are recommended for this procedure.

- iv) Decolourise with 3% acid alcohol for 5 minutes.
- v) Counterstain in 0.1% potassium permanganate for 30 seconds.
- vi) Air dry slide at room temperature<sup>22</sup> (see Modified Ziehl–Neelsen (mZN) step viii, above).
- vii) Examine for the presence of oocysts, using an epifluorescence microscope equipped with FITC filters, by scanning the slide under the ×20 objective lens. Confirm the presence of oocysts under the ×40 objective lens.
- viii) Measure the size and shape of the fluorescent bodies<sup>23</sup>.

o **Diagnostic features of *Cryptosporidium* spp. oocysts stained with AP**

*Cryptosporidium* spp. oocysts appear ring or ovoid shaped and exhibit a characteristically bright apple-green fluorescence against a dark background. *Cryptosporidium parvum* oocysts are ring or doughnut shaped, measuring 4–6 µm in diameter. If available, view the preparation under a UV filter (excitation 355 nm, emission 450 nm), as sporozoites are more readily seen under the UV rather than the FITC filter set. Under the UV filter, oocysts appear light green and sporozoites appear yellow green.

o **Culture**

There is no reproducible method for culturing cryptosporidium from body fluids. *In-vitro* cell culture systems have been described for semi-purified, infectious oocysts, but they have not been tested in sufficient depth with enough isolates or inhibitory materials to be recommended for routine purposes.

o **Immunological methods**

Three approaches to the immunological detection of *Cryptosporidium* oocysts have proven useful, and a variety of commercial kits are available. Each has a similar level of sensitivity, and either unconcentrated or concentrated stool samples can be used depending on the likely number of oocysts in the sample. Immunofluorescence-based kits, using a fluorescein isothiocyanate-conjugated anti-*Cryptosporidium* MAb that recognises surface exposed epitopes of oocysts (FITC-C-MAbs) are more specific for, and can be more sensitive at, detecting *Cryptosporidium* oocysts in faecal smears than conventional tinctorial stains. Compared with conventional tinctorial stains, antibody-based detection kits (immunofluorescence, ELISA and immunochromatography) appear to be expensive, considering that many report a similar detection threshold.

a) **Direct immunofluorescence**

In direct immunofluorescence, a FITC-labelled MAb reactive with genus-specific, surface-exposed epitopes on cryptosporidium oocysts binds to oocysts present in the sample. UV excitation using a FITC filter system (maximum excitation wavelength 490 nm, mean emission wavelength 530 nm) causes the labelled oocysts to exhibit a bright apple-green fluorescence. Materials provided with commercial kits vary but *C. parvum* oocyst positive and negative controls, FITC-labelled anti-cryptosporidium MAb (provided at the working dilution), and glycerol-based mounting medium containing a photo-bleaching inhibitor are normally included. Known negative and positive samples must always be included in each test.

Air-dried faecal smears or faecal concentrates are fixed in absolute methanol (or acetone, depending on the manufacturers' instructions) and air dried. Often the slides provided in the kit are well slides into which both sample and kit reagents are dispensed and retained. The manufacturers' instructions must be followed. It is false economy to dilute kit reagents to increase the testing volume. The FITC-labelled anti-*Cryptosporidium* genus-specific MAb (*Cryptosporidium* MAb), at the predetermined working dilution, is applied over the fixed, air-dried specimen(s) and the slide(s) are incubated horizontally in the dark in a humidified chamber. Excess antibody is removed by gentle rinsing, and the excess moisture drained. Mounting medium is placed over the specimen and a cover-slip is applied to the sample, ensuring that no air bubbles are trapped over the specimen. If mounting medium is not supplied, a mixture of 50% non-fluorescent glycerol: 50% phosphate buffered saline (PBS) (v/v) is suitable. Samples are scanned using ×20 objective lens, oocysts are confirmed using the ×40 objective lens, and the number of oocysts present is determined. Numbers can be recorded as identified previously. In the absence of a manufacturer's method, the following method will produce satisfactory results.

The nuclear fluorogen, 4'6-diamidino-2-phenyl indole (DAPI; [C<sub>16</sub>H<sub>15</sub>N<sub>5</sub>·2HCl, FW 350.2]), can be used to highlight sporozoite nuclei within fluorescent oocysts providing further confirmatory morphological information (13, 34). DAPI is a non-specific DNA intercalator, therefore DNA of other cellular interferents including

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22 Do not blot slides dry, as some blotting papers contain fluorescent fibres.

23 Putative oocysts are measured by slowly increasing the voltage (light intensity) of the bright-field light source so that both fluorescent and bright-field images can be seen concurrently. Objects can then be measured with the eye-piece graticule.

bacteria and yeasts will also be stained. DAPI at a working strength of 0.4 µg/ml is particularly useful when oocysts are sought in non-faecal samples (e.g. water and foodstuffs). DAPI intercalates with the nuclei of the sporozoites within viable or non-viable oocysts and causes them to fluoresce sky blue.

A blue filter block (excitation 490 nm; emission 510 nm) is used to visualise FITC-C-mAb localisation and an ultra-violet (UV) excitation (excitation 355 nm, emission 450 nm) is used to determine the presence of DAPI stained sporozoite nuclei.

#### o Test procedure

Include a positive and negative control slide each time this procedure is performed. Positive and negative slides are supplied with the majority of immunofluorescence kits, and *C. parvum* oocysts can be purchased from commercial suppliers<sup>24</sup>.

Sources other than *C. parvum* may be available from diagnostic veterinary laboratories or research institutes or facilities. Local distributors may also supply oocysts obtained from commercial sources.

- i) Wear protective clothing and disposable gloves. Fix air-dried faecal smears or faecal concentrates in absolute methanol for 5 minutes<sup>25</sup>.
- ii) Dispense 50 µl of anti-cryptosporidium MAb at its working dilution on to the well of each slide. Ensure complete coverage of the well.<sup>26</sup>
- iii) Place the prepared slide(s) in a humidity chamber with the slide(s) elevated above the absorbent material used to generate humidity. Ensure that the absorbent material is moist.
- iv) Place the humidity chamber in an incubator at approximately 37°C for the period of time prescribed by the manufacturer (normally 30–60 minutes).<sup>22</sup>
- v) Using a Büchner-type aspirator gently aspirate excess MAb from each well. Tilt the slide to an angle of about 45° from the horizontal towards the operator, and aspirate the fluid that collects at the bottom of the well(s) by placing the tip of an aspirator close to, but not touching, the fluid. The suction at the aspirator tip will draw the fluid to waste. Repeat this procedure for each slide well containing a sample.
- vi) Dispense 50 µl of PBS to each well and allow to stand for 2 minutes at room temperature.<sup>22</sup>
- vii) Gently aspirate the PBS from each well as described in step v. Apply a further 50 µl of PBS to each well and allow to stand for a further 2 minutes, before gently aspirating the PBS as described.<sup>22</sup>
- viii) Apply 50 µl of a 1/5000 DAPI in PBS solution to each well and allow to stand for 2 minutes at room temperature. The working solution of DAPI is prepared by diluting a 2 mg/ml DAPI stock solution by 1/5000 in PBS (150 mM, pH 7.2). The working solution should be prepared for each day it is required. A stock solution of DAPI (2 mg/ml in methanol) can be stored at 4°C in the dark, indefinitely.
- ix) Gently aspirate off the DAPI solution from each well as described in step v.
- x) Apply 50 µl of deionised water to each well and leave to stand for 1–3 seconds at room temperature, then gently aspirate the deionised water from each well as described in step v.
- xi) Dispense 50 µl of mounting medium to the centre of each well of each slide, then gently apply a cover-slip on to the microscope slide.<sup>22</sup>
- xii) Allow the cover-slip to settle into place before scanning the slide<sup>27</sup>.
- xiii) Scan the preparation for oocysts under the ×20 and confirm under the ×40 objective of an epifluorescence microscope equipped with an FITC filter set. Measure oocysts under the ×100 objective<sup>28</sup>. If necessary, slides can be stored at room temperature, in the dark, until read.

24 For example from Kate Miller, Sterling Parasitology Laboratory, University of Arizona, Dept of Veterinary Science and Microbiology, Building 90, Room 308, Tucson, Arizona, 85721, USA; [millerk@email.arizona.edu](mailto:millerk@email.arizona.edu) or Geoff and Sue Pritchard, Bunch Grass Farm, 1301 Drury Road, Deary, Idaho, 83823, USA. [pritchard@turbonet.com](mailto:pritchard@turbonet.com)

25 Alternatively, allow the methanol to evaporate to dryness at room temperature.

26 Volumes, times, etc. can vary according to manufacturers' instructions. Always follow the manufacturers' instructions.

27 These temporary mounts can be made semi-permanent by sealing around the edges of the cover-slip with clear nail varnish. Allow the cover-slip to settle into place for approximately 30 minutes to 1 hour before sealing with nail varnish. Using the brush supplied with the nail varnish, carefully apply the nail varnish around the perimeter of the settled cover-slip, using the width of the brush as the guide to the width of nail varnish applied. Ensure even coverage around the cover-slip perimeter. Do not leave any gaps. Allow the nail varnish to dry at room temperature before labelling the slide(s) appropriately with their unique identifier number. If required, use a scalpel to carefully shave/scrape any excess dried and hardened nail varnish.

28 Putative oocysts are measured by slowly increasing the voltage (light intensity) of the bright-field light source so that both

- o **Diagnostic features of *Cryptosporidium* spp. oocysts stained by FITC-labelled anti-cryptosporidium MAb**

*Cryptosporidium* spp. oocysts are round or slightly ovoid objects that exhibit a bright apple-green fluorescence under the FITC filter set. Their measurements (measured length × breadth) are presented in Table 1. Often the fluorescence has an increased intensity around the entire circumference of the oocyst, with no visible breaks in oocyst wall staining. If Evans' blue, which reduces nonspecific fluorescence, is included in the kit, the background fluorescence will be red. Nonspecific fluorescence is yellow. Always refer to the positive control to ensure that the size, shape and colour of the putative oocyst is consistent with those of the positive control. DAPI intercalates with the nuclei of the sporozoites within viable or non-viable oocysts and causes them to fluoresce sky blue. Under the ×100 oil immersion lens, a sporozoite nucleus is spherical to subspherical, measuring approximately 1 micron in diameter. In the event of an oocyst being distorted, the demonstration of up to four fluorescent nuclei in an object of a comparable size to an oocyst will assist in its identification (13, 34).

- b) **Detection of *Cryptosporidium* antigens by enzyme linked immunosorbent assay**

In the ELISA, the presence of *Cryptosporidium* antigens in faeces (coproantigen) is sought. Depending on the commercial kit, *Cryptosporidium* coproantigens are captured and developed using a mixture of monoclonal and polyclonal antibodies. With the exception of increased throughput and automation, coproantigen detection kits do not offer increased sensitivity beyond the methods described.

Commercially available sandwich ELISA antigen detection kits contain anti-*Cryptosporidium*-coated well strips for capturing *Cryptosporidium* coproantigens, anti-*Cryptosporidium* antibodies for developing the reaction that is conjugated to an enzyme (frequently horseradish peroxidase), substrate, chromogen/substrate development system and stopping solution (which inhibits further enzyme catalysis when added to the reaction mixture). These have been developed to detect *C. parvum* antigens in stool samples, but they may also be capable of detecting common epitopes from non-*C. parvum* infections. Known negative and positive samples are included in commercial kits. Commercial kits normally contain all the necessary reagents to perform the analysis and the manufacturers' instructions must be followed. It is false economy to dilute kit reagents to increase testing capacity. A comprehensive method and a formula for calculating the cut-off value and assigning positive or negative status to samples are usually included. Kit reagents are normally stored at 4°C when not in use. All reagents should reach room temperature before being used. The diagnostician should always determine whether any contraindications apply to the use of a commercial test and any stool/sample fixative used. Because of the variation in the methods described for different commercial kits, no method for ELISA or IC coproantigen detection is included in this chapter.

- c) **Detection of *Cryptosporidium* antigens by immunochromatography**

Rather than relying on molecular diffusion to dictate the rate of antigen binding by the capture antibody as in the ELISA format, which normally takes about an hour per reaction, in lateral flow immunochromatography (IC), the speed of antigen binding to the solid phase-bound capture antibody is increased by a wicking action. This draws all fluids rapidly through a membrane enclosed in the immunochromatography cassette and reduces the time required for analysis from hours to minutes or seconds. Soluble *Cryptosporidium* antigens in the test sample are drawn through the membrane and come into contact with, and bind to, immobilised antibodies raised against *Cryptosporidium* antigens, which dramatically increases the speed of antigen-antibody interaction. Positive reactions are qualitative and are seen as a band of colour at a specific location on the membrane, normally identified by a line on the cassette. The assay format can vary between commercial kits. As for antigen detection by ELISA, the diagnostician should always determine whether any contraindications apply to the use of a commercial test and any fixative used.

IC is a convenient alternative method for detecting *Cryptosporidium* antigen in stool samples and specificity is reported to be high (98–100%). Debate continues as to whether IC (or indeed ELISA) has reduced, equal or better sensitivity than oocyst-staining methods. As with ELISA, IC assays can be invaluable in cases of infection in the absence of detectable oocysts. Because of the variation in the methods described for different commercial kits, no method for IC coproantigen detection is included in this chapter.

- o **Nucleic acid recognition methods**

PCR is more sensitive than conventional and immunological assays for detecting oocysts in faeces, although the sensitivity of published methods can range between 1 and 10<sup>6</sup> oocysts. These techniques are often restricted to specialist laboratories. Care is necessary when choosing primers, as some primers amplify genus-specific DNA whereas others amplify species-specific DNA. Prior to routine adoption in clinical laboratories, both the variability

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fluorescent and bright-field images can be seen concurrently. Objects can then be measured with the eye-piece graticule.

between methods and the recognised difficulties in amplifying nucleic acids from faecal specimens by PCR must be overcome.

Faecal samples can contain many PCR inhibitors. In addition to bilirubin and bile salts, complex polysaccharides are also significant inhibitors. For *Cryptosporidium*, boiling faecal samples in 10% polyvinylpyrrolidone (PVPP) before extraction can reduce inhibition.

The most robust information regarding species/genotype/subtype information has been derived from the study of three genetic loci (two 18S rRNA [18, 24, 45, 46] and the *Cryptosporidium* Oocyst Wall Protein [COWP] [15, 38]) gene fragments by PCR-RFLP and/or sequencing amplicons.

PCR amplification of *Cryptosporidium* DNA using the 18S rRNA primers (CPB-DIAGF/R) of Johnston *et al.* (18) yields products that vary in length from 428 bp to 455 bp. The Johnson *et al.* primers (18) are included because they have been evaluated for cross reactions against a total of 23 microorganisms and the primers have been shown to work in a variety of matrices. The Ward *et al.* (40) modification of the Johnson *et al.* (18) reverse primer (substitution of CPB-DIAGR with PW99R [TAA-GGA-ACA-ACC-TCC-AAT-CTC], which produces an amplicon of approximately 420 bp) was shown by the authors to be more sensitive than CPB-DIAGF/R (18) in both direct and nested PCR assays. Further corroboration is required in different matrices before PW99R (40) can be fully recommended as a replacement for CPB-DIAGR. The nested (Nichols–Johnson; 24) 18S rRNA assay has also been shown to be sensitive. A multi-locus approach to characterising *Cryptosporidium* isolates is essential. A multiplex allele-specific PCR (MAS-PCR) based on the dihydrofolate reductase gene sequence differentiates *C. hominis* (357 bp) from *C. parvum* (190 bp) in a one-step reaction, which can be distinguished on agarose gel, without the requirement for endonuclease digestion and RFLP analysis, thus reducing assay time considerably (12). MAS-PCR is as sensitive as other diagnostically used assays targeting the 18S rRNA gene for *C. hominis* and *C. parvum* detection, and can detect more mixed (*C. hominis* and *C. parvum*) species infections than 18S rRNA gene assays (12). However, MAS-PCR failed to detect the DNA of *C. felis*, *C. canis*, *C. muris* and *Cryptosporidium* pig genotype I in human samples (17) and its usefulness for determining the range of pathogenic species found in livestock samples remains to be determined.

Currently, there is no 'standard' genetic locus recommended for species identity, but RFLP or sequencing of 18S rRNA gene loci are widely used (44) and provide information about more species than the COWP gene locus. For detecting small numbers of oocysts (<100) consistently, a nested PCR is required. Two 18s rRNA PCRs have been tested extensively in the author's laboratory (24, 45, 46) and the Nichols–Johnson primers (24) appear to be more sensitive, particularly with small numbers ( $\leq 10$ ) of *C. parvum*, *C. hominis*, *C. felis*, and *C. muris* oocysts (24). Although less sensitive, the Xiao *et al.* assay (45, 46) has the benefit of being able to detect all species by RFLP, particularly *C. parvum* and *C. hominis*, which the Nichols–Johnson assay (24) cannot.

Table 3 contains the *Cryptosporidium* spp. and genotypes determined by RFLP of the amplicon defined by the CPB-DIAGR/F primers following simultaneous digestion with restriction enzymes *Vspl/DraI* (24), which can identify five major species. Table 4 contains the RFLP result of the same amplicons separately digested with the restriction enzymes *Vspl/Asel*, *Sspl* and *Ddel* according to available GenBank complete/partial sequences of the 18S rRNA gene (ref. 24 and unpublished data). Table 5 contains the *Cryptosporidium* spp. and genotypes determined by RFLP of the amplicon defined by the XR2/XF2 primers following digestion with enzymes *Asel* and *Sspl* (45).

Not all *Cryptosporidium* species/genotypes can be identified by PCR-RFLP of the 18S rRNA loci; however most of the species that are currently known to be commercially important for livestock can be identified by PCR-RFLP. Sequencing has been used to distinguish *C. parvum* from *C. bovis* or the *Cryptosporidium* deer-like genotype, but they can also be readily differentiated by digestion with *MbolI* (11). Sequencing of the amplicon can offer better information than PCR-RFLP, but sequencing is more expensive and takes longer than PCR-RFLP. Currently, sequencing availability varies in different parts of the world and, for the more common species that infect livestock, PCR-RFLP has an important role to play.

A single-tube nested PCR-RFLP assay (15) amplifying a fragment of the gene coding for COWP distinguishes between *C. hominis* and *C. parvum*. This assay is recommended over and above that in reference 38 as it is more sensitive and offers a solution to the contamination frequently experienced in nested PCRs due to re-amplification of PCR products. In this single-tube nested PCR, the inner and outer primers are added to the initial reaction mixture. Optimisation of primer set concentrations and annealing temperatures result in the preferential amplification of one product size only, defined by the inner primers.

No recommended method for extracting *Cryptosporidium* DNA from oocysts exists, and the sensitivity of most methods described has not been addressed fully. *Cryptosporidium* DNA can be extracted either following partial purification of oocysts using one of the flotation/sedimentation techniques described above, or from oocysts in faeces following zirconia bead extraction (20). If concentration by formal–ether sedimentation is the routine laboratory test, oocyst concentrates suitable for lysis and amplification by PCR can be made by substituting deionised water for the 10% formalin used in the method described. DNA loss can be a consequence of

subsequent DNA purification using commercial purification columns, but normally there should be an adequate number of oocysts present in the sample to extract sufficient *Cryptosporidium* DNA for PCR-RFLP/sequencing analysis. The selection of a suitable DNA extraction technique is the most important step in determining the final sensitivity of oocyst DNA detection.

**Table 3.** Structural analysis of the 18S rRNA gene defined by CPB-DIAGR primers after simultaneous digestion with the restriction enzymes *VspI* or *AseI* and *DraI* (reproduced from ref. 24)

<i>Cryptosporidium</i> species (amplicon length in bp)	Number of <i>VspI/AseI</i> sites (AT↓TAAT)	Number of <i>DraI</i> sites (TTT↓AAA)	Fragments' length in bp
<i>C. hominis</i> (438)	2	None	222; 112; 104
<i>C. parvum</i> (435)	2	None	219; 112; 104
<i>C. muris</i> (431 or 432)	1	None	319; 112
<i>C. felis</i> (455)	2	1	189; 112; 104; 50
<i>C. baileyi</i> (428)	2	1	128; 112; 104; 84
<i>C. meleagridis</i> (434)	3	None	171; 112; 104; 47

**Table 4.** *Cryptosporidium* spp. and genotypes determined by RFLP of the amplicons defined by the CPB-DIAG primers following digestion with enzymes *VspI* or *AseI*, *SspI* and *DdeI*, according to available GenBank complete/partial sequences of the 18S rRNA gene

<i>Cryptosporidium</i> species (amplicon length in bp)	<i>VspI / AseI</i>	<i>SspI</i>	<i>DdeI</i>	GenBank Accession No.
<i>C. hominis</i> (438)	222, 104,112	264,111, 40,12, 11	204, 166,68	L16997
<i>C. parvum</i> (435)	219, 104,112	264, 108, 40,12, 11	201, 166, 68	L16996
<i>C. muris</i> (432)	320, 112	395, 37	224,166, 42	AF093498
<i>C. andersoni</i> (431)	319, 112	394, 37	265, 166	L19069
<i>C. felis</i> (455)	239, 104,112	401, 40,14	221, 166, 68	AF087577
<i>C. baileyi</i> (428)	212, 104,112	264, 164	262, 166	L19068
<i>C. meleagridis</i> (434)	171, 104,112, 47	264, 119, 40,11	200, 166, 68	AF112574
<i>C. serpentis</i> (430)	318, 112	380, 36,14	264, 166	AF093502
<i>C. wrairi</i> (435)	219, 104,112	264, 109, 40,11,11	201, 166, 68	AF115378
<i>Cryptosporidium</i> pig (435)	219, 104,112	375, 40, 11, 9	201, 166, 68	AF108861
<i>C. saurophilum</i> (432)	216, 108,112	264, 109, 40,19	198, 166, 68	AF112573
<i>Cryptosporidium</i> mouse (439)	175, 104,112,48	264, 112, 40,12,11	205, 166, 68	AF108863
<i>Cryptosporidium</i> ferret (438)	174, 103,113, 48	264, 111, 40,23	204, 166, 68	AF112572
<i>Cryptosporidium</i> dog (429)	213, 104,112	264, 105, 40,20	195, 166, 68	AF112576
<i>Cryptosporidium</i> koala (436)	220, 104,112	264, 109, 63	202, 166, 68	AF108860

<i>Cryptosporidium</i> kangaroo (436)	220, 104, 112	373, 63	202, 166, 68	AF112570
<i>Cryptosporidium</i> monkey (436)	220, 104, 112	264, 109, 52, 11	202, 166, 68	AF112569

**Table 5.** *Cryptosporidium* spp. and genotypes determined by RFLP of the amplicons defined by the XR2/XF2 primers following digestion with enzymes AseI and SspI (Reproduced from ref. 45)

<i>Cryptosporidium</i> species	Vspl	Sspl
<i>C. hominis</i>	561, 104, 102, 70	449, 254, 111, 12, 11
<i>C. parvum</i> gene type A	628, 104, 102	449, 254, 108, 12, 11
<i>C. parvum</i> gene type B	625, 104, 102	449, 254, 119, 9
<i>C. muris</i>	731, 102	448, 385
<i>C. andersoni</i>	731, 102	448, 385
<i>C. felis</i>	476, 182, 104, 102	426, 390, 33, 15
<i>C. baileyi</i>	620, 104, 102	572, 254
<i>C. meleagridis</i>	456, 171, 104, 102	449, 254, 108, 11, 11
<i>C. serpentis</i>	729, 102	414, 370, 33, 14
<i>C. wrairi</i>	628, 104, 102	449, 254, 109, 11, 11
<i>Cryptosporidium</i> pig	632, 104, 102	453, 365, 11, 9
<i>C. saurophilum</i>	628, 104, 102	418, 255, 109, 33, 19
<i>Cryptosporidium</i> mouse	457, 175, 104, 102	449, 254, 112, 12, 11
<i>Cryptosporidium</i> ferret	457, 174, 104, 102	449, 254, 111, 12, 11
<i>Cryptosporidium</i> kangaroo, koala	631, 104, 102	441, 254, 109, 33
<i>Cryptosporidium</i> dog	633, 102, 94	417, 254, 105, 33, 20
<i>Cryptosporidium</i> monkey	559, 104, 102, 70	461, 254, 109, 11

The following method is effective in extracting DNA from small numbers (~10+) of partially purified oocysts, and is used in the author's laboratory (22–24). Partially purified oocysts are suspended in 100 µl of lysis buffer (50 mM Tris/HCl, pH 8.5, 1 mM ethylene diamine tetra-acetic acid, pH 8, 0.5% sodium dodecyl sulphate [SDS], Sigma-Aldrich) and subjected to 15 freeze–thaw cycles (1 minute in liquid nitrogen; 1 minute 65°C). Samples are then transferred to a 55°C water bath, proteinase K (at a final concentration of 200 µg/ml) is added, and the samples are incubated for 3 hours. Proteinase K is heat denatured (90°C, 20 minutes), samples are chilled on ice for 1 minute, centrifuged (16,000 g, 5 minutes) then 70 µl of supernatant is removed for PCR amplification. SDS is inhibitory to *Taq* polymerase at concentrations as low as 0.01%, therefore, neutralisation of SDS in the extracted DNA is necessary. The addition of 2% Tween 20 will neutralise up to 0.05% SDS.

Reagents for PCR reactions are dispensed in 0.5 ml thin-walled tubes. Each tube contains 90 µl of pre-mixed reagents (200 µM of each of the four dNTPs, 200 nM each of primers CPB-DIAGR and CPB-DIAGF, bovine serum albumin at a final concentration of 400 µg/ml, MgCl<sub>2</sub> at 3.5 mM, 2.5 U of *Taq* polymerase in PCR buffer and Tween 20 at a final concentration of 2% to inactivate 0.05% SDS). Finally, 10 µl of DNA template is introduced below approximately 40 µl of mineral oil. Samples are subjected to 39 amplification cycles, and products are visualised following ethidium bromide staining of 1.4% agarose gels (24).

The primers and step cycle protocols for amplifying either 18S rRNA gene fragment (24, 45, 46) or the COWP gene fragment (15) are given in Table 6.

#### o Reporting results of PCR-RFLP/sequencing examination

Negative specimens should be reported as 'NO *Cryptosporidium* DNA detected'.

Positive specimens should be reported as '*Cryptosporidium* DNA detected' inserting the species/genotype(s)/subtype(s) identified (see Tables 3–5) after identifying the respective species from the RFLP profiles presented in Table 2.

**Table 6.** Step cycle PCR protocols for 18S rRNA (refs 24, 45 & 46) and single-tube nested COWP (ref. 15) PCR

Primers	Step cycle protocol		Ref.
CPB-DIAGF AAG-CTC-GTA-GTT-GGA-TTT-CTG CPB-DIAGR TAA-GGT-GCT-GAA-GGA-GTA-AGG	80°C, 5 minutes; 98°C, 30 seconds 55°C, 30 seconds; 72°C, 1 minute; 94°C, 30 seconds 72°C, 10 minutes 4°C	1 cycle 39 cycles 1 cycle soak	24
XF1 (outer) TTC-TAG-AGC-TAA-TAC-ATG-CG XR1 (outer) CCC-ATT-TCC-TTC-GAA-ACA-GGA XF2 (inner) GGA-AGG-GTT-GTA-TTT-ATT-AGA-TAA-AG XR2 (inner) AAG-GAG-TAA-GGA-ACA-ACC-TCC-A	94°C, 3 minutes 94°C, 35 seconds; 55°C, 45 seconds; 72°C, 1 minute 72°C, 7 minutes 4°C	1 cycle 35cycles 1 cycle soak	45, 46
oocry3 (outer) AGA-TTA-ACA-GAA-TGC-CCA-CCA-GGT-A oocry4 (outer) CCA-TGA-TGA-TGT-CCT-GGA-TTT-TGT-A oocry1 (inner) CCT-GGA-TAT-CTC-GAC-AAT Oocry2 (inner) GCG-AAC-TAA-TCG-ATC-TCT-CT	94°C, 5 minutes 94°C, 1 minute; 67°C, 1 minute; 72°C, 1 minute 94°C, 1 minute; 54°C, 1 minute; 72°C, 1 minute 72°C, 10 minutes 4°C	1 cycle 20cycles 35 cycles 1 cycle soak	15

**a) Quantitative real-time PCR**

Quantitative real-time PCR methods use patented technologies (e.g. TaqMan™, Applied Biosystems, LightCycler™, Roche Molecular Biochemicals). For both technologies, fluorogenic dyes are incorporated into the amplicon during PCR, thus amplicon fluorescence increases as more PCR product is generated. Both systems can detect PCR products during the initial cycles of the PCR reaction when amplification is exponential, thus enabling quantitative analysis of fluorescent product. Quantitative real-time PCR methods have been used to identify different *Cryptosporidium* species by exploiting the genetic polymorphism of the 18S rRNA gene for devising probes with differing melting temperatures, and for the quantitative detection of cryptosporidium oocysts in environmental water samples and sewage. The increased sensitivity of real-time PCR guarantees increased speed of detection and qualitative diagnosis while the quantitative nature of the assay will be invaluable in estimating levels of contamination. The 'closed-tube' assay format reduces the danger of contamination from 'carry over' (36). Quantitative real-time PCR should be a useful tool for the future, once issues surrounding matrix inhibition are overcome. Currently, there are no standardised quantitative real-time PCR methods.

**b) Typing and subtyping for disease and source tracking**

The value of characterising the genetic diversity of *Cryptosporidium* at different levels of specificity and the importance of appropriate nucleic acid analysis cannot be over-emphasised. Molecular tools for inter- and intra-species discrimination differ (4) and the publication of complete genomic sequences of *C. parvum* (1) and *C. hominis* (49) has assisted in developing appropriate typing and subtyping tools (4). Genetic markers differ in their information content and the nature of the DNA fragment selected for detecting and characterising cryptosporidium and *Giardia* should be considered carefully (see Table 4 below). In order to detect species, analysis of highly or moderately conserved coding regions (e.g. 18S ribosomal DNA, structural and house-keeping genes) is necessary, whereas investigations into the transmission of genotypes and subtypes, identifying sources of infection and risk factors, requires more discriminatory fingerprinting techniques, which can identify individual isolates or clonal lineages (4, 35).

Typing and subtyping systems used for veterinary (and human) samples should also be used for environmental samples, particularly for source and disease tracking in order to avoid any confusion arising from using different systems for human, non-human hosts and environmental samples for veterinary and public health investigation of disease outbreaks (4, 35). Species determination should be based on the analysis of at least two loci since this provides more robust information. At least one locus should be 18S rRNA, and, where possible, another should be suitable for both species identification and further subtyping analysis. For *Cryptosporidium*, mini- and micro-satellite typing, GP60 sequencing and analysis of a double stranded RNA element have been used to subtype *C. parvum* and *C. hominis*, and may offer sufficient sub-species discrimination to address veterinary and public health investigations, either separately or in combination (reviewed in references 4 and 35). Further development of these subtyping systems is required e.g. gp60 sequencing shows variability within *C. parvum* populations but not *C. hominis*. The goal would be

to find common isolate identifiers. There is also evidence that mixed species infections occur in both the human and animal populations. It is therefore important that species and subtyping systems be capable of identifying mixed infections to give accurate diagnosis and information. Recent work has confirmed the utility of mini- and micro-satellite markers in the study of the population structure of *Cryptosporidium*, and in understanding transmission dynamics of infection (reviewed in references 4, 8 and 35). The most commonly used PCR assays for detecting and typing *Cryptosporidium* are listed in Table 7.

**Table 7.** List of targets, type of assay and main use of amplification-based techniques for *Cryptosporidium* (refs 4 & 35)

Amplification target	Assay type	Main application
18S rDNA	PCR, nested PCR, sequencing, PCR-RFLP, Real-time PCR, microarray	Species & genotype identification
Hsp70	PCR, nested PCR, sequencing, Real-time PCR, microarray	Species & genotype identification
COWP	PCR, nested PCR, sequencing, PCR-RFLP, microarray	Species & genotype identification
Actin	PCR, nested PCR, sequencing,	Species & genotype identification
$\beta$ -tubulin	PCR, nested PCR, sequencing, PCR-RFLP	Species & genotype identification
GP60	PCR, nested PCR, sequencing,	Subgenotype identification
Microsatellites	PCR, nested PCR, sequencing, fragment typing	Subgenotype identification
Minisatellites	PCR, nested PCR, sequencing, fragment typing	Subgenotype identification
Extra-chromosomal double stranded RNA	Reverse transcriptase, PCR, sequencing, heteroduplex mobility assays	Subgenotype identification

**Key:** RFLP = restriction fragment length polymorphism; Hsp70 = heat shock protein 70; COWP = *Cryptosporidium* oocyst wall protein; GP60 = glycoprotein 60.

## 2. Serological tests (and/or tests for cellular immunity where relevant)

Cryptosporidiosis is often a disease of the newborn and unless there is sufficient evidence to exclude exposure to infectious oocysts, serological tests do not offer any benefit. Serological tests can be used for seroepidemiological surveys of exposure: most are ELISA based, using various aqueous extracts of *C. parvum* oocysts (e.g. ref. 14). Tests for cellular immunity do not appear to offer specific benefit, and are not available.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There is no control programme for cryptosporidiosis, neither is there a rigorously tested and accepted vaccine available.

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