

CHAPTER 2.9.4.

CRYPTOSPORIDIOSIS

SUMMARY

Description of the disease: *Cryptosporidiosis is the pathological condition caused by infection with the protozoan Cryptosporidium. Following infection, the Cryptosporidium life cycle, comprising asexual and sexual phases, is completed in a single host, producing sporulated oocysts. There are at least 32 'valid' Cryptosporidium species, some of which cause disease in humans, livestock, poultry and game birds, and companion animals. Cryptosporidium parvum infects mainly the gastrointestinal tract and causes scour in young, unweaned livestock. Mortality is generally low but severe outbreaks may occur occasionally. Weaned and adult animals do not normally exhibit signs of disease, but can excrete oocysts that may contaminate the environment facilitating onward transmission. Cryptosporidium parvum is one of the major causes of zoonotic human cryptosporidiosis. Cryptosporidium andersoni infects the digestive glands of the abomasum of older calves and adult cattle and also bactrian camels. Some infected cows exhibit reduced milk yields and poor weight gain, but do not develop diarrhoea. Cryptosporidium baileyi affects primarily the upper respiratory tract, bursa of Fabricius and cloaca, kidneys and eyes of gallinaceous birds, and has caused outbreaks and mortalities in game and poultry units. Cryptosporidium meleagridis affects primarily the ileum of turkey poults and game birds, and can cause enteritis, diarrhoea and death, and C. galli infects the surface, ductal, and glandular epithelium of the proventriculus of adult hens and some wild birds.*

Identification of the agent: *Laboratory identification is required for diagnosis. Microscopical observation of oocysts, with acid-fast Ziehl–Neelsen, auramine phenol or immunofluorescent stains applied to faecal smears are used commonly. Enzyme immunoassays are also widely used but may have a low specificity. Molecular diagnostic tests are becoming more widely available. The infecting species cannot be identified by oocyst morphology or antibody-based assays, but downstream analysis of DNA amplified by the polymerase chain reaction can be used to determine species. Most cases of cryptosporidiosis in young mammalian livestock are likely to be caused by C. parvum, which is also the most important zoonotic species. There is no standardised subtyping scheme, but sequencing the gp60 gene may be informative in outbreak investigations. Multilocus subtyping schemes are in development but need to be standardised. Oocysts can survive in moist environments for many months, and foodborne and waterborne transmission occurs. However, application of genotyping to the small numbers of oocysts likely to be present in food, water and environmental samples is challenging.*

Requirements for vaccines: *There is no commercially available vaccine for cryptosporidiosis.*

A. INTRODUCTION

Cryptosporidiosis is the pathological condition caused by infection with the protozoan *Cryptosporidium*. Infection of the gastrointestinal tract is most common; the primary symptom is diarrhoea. Respiratory infection occurs in some hosts, especially birds. Other sites may also be infected, again most commonly in birds and immunocompromised patients.

1. Causal pathogen

Cryptosporidiosis is caused by protozoa of the genus *Cryptosporidium*, classified traditionally as phylum Apicomplexa, class Sporozoa, subclass Coccidiasina, order Eucoccidiorida, and family *Cryptosporidiidae*. A revised classification of the Eukaryotes places *Cryptosporidium* in the following descending hierarchical groups: Diaphoretickes; Sar (Stramenopiles, Alveolata and Radiolaria) supergroup; Alveolata; and ultimately Conoidasida wherein *Cryptosporidium* is classified separately from the *Coccidia* and *Gregarinasina* (Adl *et al.*, 2012). At the time of writing (July 2015), there are 26 *Cryptosporidium* species with formally described biological and genetic characteristics (Table 1).

Laboratory detection of the genus is required for a diagnosis of cryptosporidiosis, but only molecular methods will differentiate *Cryptosporidium* species and subtypes as many of the oocyst sizes are similar (Table 1) and there are few species-distinguishing antigens. In the past, many 'species' were described on a generally false premise of host specificity (Fayer, 2010). In livestock, poultry and game birds, *C. parvum*, *C. andersoni*, *C. baileyi* and *C. meleagridis* have been reported to cause morbidity and outbreaks of disease. In humans, *C. parvum*, *C. hominis*, *C. meleagridis* and *C. cuniculus* are considered the main pathogenic species, causing sporadic cases and outbreaks (Table 1). Most cases of cryptosporidiosis in young mammalian livestock are likely to be caused by *C. parvum*, which is also the most significant zoonotic threat for humans. In addition, over 40 *Cryptosporidium* 'genotypes' have been identified in animals on the basis of DNA sequencing but lack sufficient biological data for species status (Fayer, 2010). The *Cryptosporidium* horse, skunk, chipmunk genotype I and the *C. hominis* monkey genotypes have also been described in humans.

A new genus, designated *Piscicryptosporidium*, has been proposed for *C. molnari* and *Cryptosporidium*-like species and genotypes infecting fish but further genetic and biological characterisation is required to determine whether or not *Piscicryptosporidium* is a valid genus.

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

<i>Cryptosporidium</i> species	Mean oocyst dimensions (µm)^a	Major host(s)	Usual site of infection	Infections reported in humans
<i>C. andersoni</i>	7.4 × 5.5	Cattle	Stomach	Yes, but only rarely
<i>C. baileyi</i>	6.2 × 4.6	Poultry	Upper respiratory tract,	No
<i>C. bovis</i> (previously bovine B genotype)	4.9 × 4.6	Cattle	Small intestine	Yes, but only rarely
<i>C. canis</i> (previously dog genotype)	5.0 × 4.7	Dog	Small intestine	Yes, occasionally
<i>C. cichlidis</i> (previously piscine genotype 1 or <i>C. molnari</i> -like)	4.6 × 4.4	Tilapia	Stomach	No
<i>C. cuniculus</i> (previously rabbit genotype)	5.6 × 5.4	Rabbit, humans	Small intestine	Yes, occasionally. One waterborne outbreak
<i>C. ducismarci</i>		Tortoises	Intestine	No
<i>C. erinacei</i>	4.9 × 4.4	Hedgehog	Small intestine	Yes, but only rarely
<i>C. fayeri</i> (previously marsupial genotype I)	4.9 × 4.3	Marsupials	Intestine	Yes, but only rarely
<i>C. felis</i>	4.6 × 4.0	Cat	Small intestine	Yes, occasionally
<i>C. fragile</i>	6.2 × 5.5	Black spined toad	Stomach	No
<i>C. galli</i>	8.3 × 6.3	Chicken	Proventriculus	No
<i>C. hominis</i> (previously referred to as <i>C. parvum</i> human genotype, genotype 1, and genotype H)	4.9 × 5.2	Humans	Small intestine	Yes, commonly. Outbreaks are reported frequently

<i>Cryptosporidium</i> species	Mean oocyst dimensions (µm)^a	Major host(s)	Usual site of infection	Infections reported in humans
<i>C. huwi</i>	4.6 × 4.4	Guppy	Stomach	No
<i>C. macropodum</i> (previously marsupial genotype II)	5.4 × 4.9	Eastern grey kangaroo	Intestine	No
<i>C. meleagridis</i>	5.2 × 4.6	Birds, mammals	Intestine	Yes, frequency depends on setting. One farm-related and one school-related outbreak
<i>C. molnari</i>	4.7 × 4.5	Sea bream	Intestine	No
<i>C. muris</i>	7.0 × 5.0	Rodents	Stomach	Yes, but only rarely
<i>C. parvum</i> (also sometimes previously called bovine genotype, genotype II, and genotype B)	5.0 × 4.5	Humans, pre-weaned mammalian livestock	Small intestine	Yes, commonly and outbreaks are reported frequently
<i>C. proliferans</i>	7.7 × 5.3	Rodents	Stomach	No
<i>C. ryanae</i> (previously deer-like genotype)	3.7 × 3.2	Cattle		No
<i>C. reichenbachklinkei</i> (previously piscine genotype 2)	3.4 × 3.4	Gourami	Stomach	No
<i>C. rubeyi</i>	4.7 × 4.3	Ground squirrels		No
<i>C. scrofarum</i> (previously pig genotype II)	5.2 × 4.8	Pig	Small intestine	Yes, but only rarely
<i>C. serpentis</i>	6.2 × 5.3	Reptiles	Stomach	No
<i>C. suis</i> (previously pig genotype I)	4.6 × 4.2	Pig	Small intestine	Yes, but only rarely
<i>C. tyzzeri</i> (previously mouse genotype I)	4.6 × 4.2	Mice	Small intestine	Yes, but only rarely
<i>C. ubiquitum</i> (previously cervine genotype)	5.0 × 4.7	Various mammals	Small intestine	Yes, occasionally
<i>C. viatorum</i>	5.4 × 4.7	Humans		Yes, occasionally
<i>C. varanii</i> (syn. <i>C. saurophilum</i>)	4.8 × 4.7	Reptiles	Intestine	No
<i>C. wrairi</i>	5.4 × 4.6	Guinea pig	Small intestine	No
<i>C. xiaoi</i> (previously <i>C. bovis</i> -like genotype or <i>C. bovis</i> from sheep or <i>C. agni</i>)	3.9 × 3.4	Sheep, goat		No

^aFrom the original papers describing the species.

2. Description of the disease in animals

Clinical and subclinical infections in animals have been reviewed by Santin (2013).

Cryptosporidium parvum is an important cause of scour in young, unweaned farmed livestock including calves, lambs, goat kids, alpaca and foals. In addition to welfare issues, there are adverse consequences for productivity. Healthy and adult animals can also shed oocysts, often in large numbers, providing a potential reservoir of infection and environmental contamination.

Cryptosporidium parvum infections of cattle are considered endemic globally. Prevalence and severity of disease peak in the second week of life. 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. Affected animals usually recover within 2 weeks of showing signs of illness. Clinical signs can range from a mild to inapparent infection in older animals to severe scouring in young animals, and can cause varying degrees of dehydration, dullness, anorexia, fever and loss of condition. Mortality is generally low unless occurring as a mixed

infection with other enteric pathogens such as *Escherichia coli* or rotavirus, although severe outbreaks of cryptosporidiosis are sometimes reported.

Cryptosporidium parvum infections of small ruminants (sheep, goats) commonly cause neonatal diarrhoea sometimes associated with high morbidity and mortality especially with concurrent infections or deficiencies in nutrition and husbandry. In ewes, a periparturient rise in oocyst shedding has been observed.

Although *C. parvum* appears to be pathogenic to piglets, causing inappetence, depression, vomiting or diarrhoea, *C. suis* and *C. scrophaeum* are the most frequently reported species. However, natural infection does not appear to lead to disease, and clinical signs of cryptosporidiosis in pigs may be related to infection with different *Cryptosporidium* species or genotypes or simultaneous infection with other enteropathogens.

Other *Cryptosporidium* species also infect livestock and companion animals.

Cryptosporidium bovis and *C. ryanae* are generally more common than *C. parvum* in post-weaned calves, but infections with these host-adapted cattle species have not yet been associated with illness and there are no histological or pathological reports. *Cryptosporidium andersoni* colonises the digestive glands of the abomasum of older calves and adult cattle. Infected cattle do not develop diarrhoea, but can excrete oocysts for several months. Some infected beef cattle exhibit reduced weight gain compared with uninfected controls, and one study found that infection may interfere with milk production in dairy cows.

Cryptosporidium ubiquitum and *C. xiaoi* infect lambs and kids. *Cryptosporidium ubiquitum* is prevalent in post-weaned lambs, with one report of involvement with *C. parvum* in outbreaks of diarrhoea, and lambs with *C. ubiquitum* showed higher faecal consistency scores in one study. *Cryptosporidium xiaoi* infection has been associated with outbreaks of neonatal diarrhoea in goats.

Cryptosporidium canis is the most frequently reported species in dogs and although usually asymptomatic, infection has been linked to severe diarrhoea, malabsorption and weight loss especially in younger animals.

Cryptosporidium felis is the most frequently reported species in cats, often in the absence of clinical signs, although infection has been linked to persistent diarrhoea in some cases. Cats with other enteric parasites, or with feline leukaemia virus infection, are more likely to develop cryptosporidiosis and cryptosporidiosis should be included in the differential diagnosis of chronic feline diarrhoea.

Cryptosporidium is a primary pathogen in chickens, turkeys and quail, causing respiratory and/or intestinal disease, leading to morbidity and mortality. Three species infect poultry: *C. baileyi*, *C. meleagridis* and *C. galli*, reviewed by Current (1997) and Ryan (2010).

Cryptosporidium baileyi most commonly infects the upper respiratory tract, although other sites include the renal tract, bursa of Fabricius and cloaca, while the trachea and the conjunctiva are lesser sites of infection. Intestinal infection does not normally result in gross lesions or overt signs of disease, but respiratory cryptosporidiosis of chickens and game birds such as red grouse 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. Cryptosporidiosis in turkeys caused by *Cryptosporidium baileyi* is similar to that observed in chicken. Chicken isolates of *C. baileyi* cause infection in other birds. Respiratory and intestinal cryptosporidiosis has been reported in commercially grown quail caused by *C. baileyi*, with pathological changes similar to those described in chickens. Oral infection of chickens with 100 *C. baileyi* oocysts can result in intestinal cryptosporidiosis (Current, 1997).

Cryptosporidium meleagridis infects turkeys other poults and humans. Villous atrophy, crypt hyperplasia and shortening of microvilli are major pathological changes.

Cryptosporidium galli produces a disease in adult hens and some wild and exotic birds. Unlike the life cycle stages of either *C. meleagridis* or *C. baileyi*, infection with *C. galli* is limited to the epithelial cells of the proventriculus. Clinical signs include puffed plumage with head held under the wing, responsiveness to external stimuli, and failure to thrive. 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 oocysts attached to the surface of glandular epithelial cells.

Outbreaks of disease in game birds (e.g. pheasant, partridge and grouse) suggest cryptosporidiosis should be included among respiratory and enteric diseases routinely tested for in these birds. Both *C. meleagridis* and *C. baileyi* are involved.

The significance of *Cryptosporidium* infections for aquaculture and fish welfare is uncertain, although morbidity and mortality have been reported in hatcheries with high prevalence of infection (Garbor *et al.*, 2011).

3. Human health risk

Human cryptosporidiosis, reviewed by Chalmers & Davies (2010) is usually an acute, self-limiting gastrointestinal disease, characterised by watery diarrhoea, abdominal cramps, vomiting, low-grade fever, and loss of appetite. Symptoms can last for up to 1 month during which time relapse occurs in about one third of cases. Long-term sequelae have been linked to infection with *Cryptosporidium* but require further investigation. Patients with severe immunodeficiency may suffer from chronic, severe and intractable cryptosporidiosis with significant mortality. In malnourished children, infection causes substantial morbidity and mortality (Kotloff *et al.*, 2013). In addition to zoonotic *Cryptosporidium* species (Table 1), *C. hominis* is an important cause of gastrointestinal disease in humans. Although there are a small number of reports of *C. hominis* infections in cattle and sheep, there is no evidence for maintenance of infection in, or transmission between, herds or flocks, or of clinical signs in animals.

4. Transmission

Transmission is by the faecal-oral route and may involve a vehicle such as contaminated food or drinking water. *Cryptosporidium parvum* is highly infectious for young livestock and humans; older livestock can remain infected and excrete oocysts that can be transmitted to other susceptible hosts. Transmission of *C. hominis* is considered to be anthroponotic. Isolates vary in infectivity, and susceptibility is influenced by host-related factors (Borad & Ward 2010; Flores & Okhuysen, 2009; Yang *et al.*, 2010). Dose–response models indicate that there is a high likelihood of human and pre-weaned livestock infection with single numbers of *C. parvum* oocysts, and also that there is a relationship between pre-existing antibodies and protection from infection.

Oocysts can survive for long periods (>6 months) in cool, moist environments, and on fomites such as farm gates, buildings and utensils. Oocysts can be transmitted following direct contact with faeces from an infected individual, or contact with contaminated fomites, or by ingestion of contaminated food or water. Agricultural 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 infected animals. Transmission from clinically normal dams to suckling neonates may occur, but little is known about carriage. The disposal of faeces, farmyard manure or other contaminated waste in land-based dumps, and the spreading of slurry, when followed by periods of heavy rainfall or melting snow can lead to oocyst contamination of water courses and drinking water supplies.

Wild mammals may act as hosts to *Cryptosporidium* spp. (Fayer, 2010; Xiao *et al.*, 2004), but little is known of the importance of their involvement in transmitting infection to, or maintaining infection in, livestock in agricultural environments (Sturdee *et al.*, 1999). Animals normally infected with host-adapted *Cryptosporidium* species may act as vectors of other species including *C. parvum*.

5. Differential diagnosis

The differential diagnosis for *Cryptosporidium* includes other entero-pathogens involved in diarrhoea. Multiple pathogens can be present including other parasites, rotavirus, coronavirus, pathogenic strains of *E. coli* and *Salmonella* spp. Cryptosporidiosis in livestock is confirmed by finding significant numbers of oocysts in diarrhoeic faeces in the absence of other pathogens, and although it has been widely speculated that co-infection may lead to more severe cryptosporidiosis (Lorenz *et al.*, 2011) experimental data to support this are lacking. Gastro-intestinal upset may also have non-infectious causes, for example inflammatory bowel disease in humans.

B. DIAGNOSTIC TECHNIQUES

Table 2. Test methods available for diagnosis of cryptosporidiosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification¹						
Conventional microscopy	n/a	n/a	n/a	+++	++	n/a
FAT	n/a	n/a	n/a	+++	++	n/a
Antigen detection by IC	n/a	n/a	n/a	+	+	n/a
Antigen detection by ELISA	n/a	n/a	n/a	+++	+++	n/a
PCR	n/a	n/a	n/a	+++	+++	n/a
Detection of immune response						
Antibody detection by ELISA	n/a	n/a	n/a	n/a	++	n/a

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable. Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

FAT = fluorescent antibody test; IC = immunochromatography;
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

1. Introduction to tests available

Confirmation of the infection is most commonly by microscopic detection of *Cryptosporidium* oocysts in faeces (Casemore, 1991). Organisms can also be detected in intestinal fluid, tissue samples, or biopsy specimens; antigens in faeces or intestinal fluid; or nucleic acid by polymerase chain reaction (PCR)-based detection in faeces, intestinal fluid, tissue samples, or biopsy specimens. Haematoxylin and eosin stain can be used for histological diagnosis in biopsy material or confirmation of the diagnosis post-mortem.

Species identification is usually by a reference laboratory test, for which the benchmark is sequencing the small subunit (SSU) rRNA gene. Subtyping tools are used in epidemiological investigations of *C. parvum*, *C. hominis*, *C. meleagridis* and *C. ubiquitum* infections. The usual genetic target is the gp60 gene. There is no standardised multilocus subtyping scheme, and although sequencing or fragment size analysis of mini- and microsatellite markers has been described (Xiao, 2010), marker selection, analysis and relationship algorithms need to be harmonised (Widmer & Caccio, 2015).

There are no reproducible *in-vitro* culture techniques available to amplify parasite numbers prior to identification.

Serological tests are not appropriate for diagnosis but can be used for seroepidemiological surveys of exposure.

¹ A combination of agent identification methods applied on the same clinical sample is recommended.

2. Identification of *Cryptosporidium*

2.1. Safety and quality

Cryptosporidium is a laboratory risk and all laboratory procedures that can give rise to infectious aerosols must be conducted in a biosafety cabinet. Specimens may contain other pathogenic organisms and should be processed accordingly. To safeguard the health of laboratory workers, all laboratory manipulations must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

2.2. Collection and submission of samples

Specimens for primary diagnosis should be collected during acute infection. If *Cryptosporidium* only is sought, short-term storage of faeces at 4°C is appropriate as oocyst morphology and antigen structure will be retained. Longer term, –20°C can be used. Alternatively, an equal volume of 5% K₂Cr₂O₇ can be added to facilitate storage at ambient temperature. However, if other parasites and especially trophozoites are sought in differential diagnosis, faeces need to be examined promptly. The deterioration of the morphology of other parasite stages, and overgrowth by other microorganisms particularly yeasts, can be reduced by the addition of preservatives including 10% (v/v) aqueous formalin, merthiolate–iodine–formaldehyde (MIF), sodium acetate–acetic acid–formalin (SAF) and polyvinyl alcohol (PVA). Downstream tests must be considered for preservative compatibility; for example formalin and SAF are generally compatible with enzyme-linked immunosorbent assay (ELISA) and immunochromatographic (IC) kits, but refer to manufacturers' instructions. Preservatives may interfere with PCR-based tests; faecal samples can be preserved in 90% ethyl alcohol for later PCR testing. Faeces in preservative may require concentration by a recognised method before microscopy, but this is not appropriate if ELISA and IC kits are used because soluble antigens may be lost in the process. Some faeces may need additional processing, for example very liquid faeces may be concentrated, high fat faeces may require defatting, mucoid samples may need treatment with KOH or dithiothreitol, high-fibre faeces may need sieving to remove fibres.

Procedures for packaging and shipping of specimens must be as outlined in the International Air Transport Association's Dangerous Goods Regulations (IATA, 2003). These regulations are summarised in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of specimens of animal origin*.

2.3. Microscopy – sample preparation and staining

2.3.1. Preparation of faecal (or appropriate body fluid) smears

Most unpreserved samples can be smeared directly on to microscope slides prior to staining. Include a positive control slide each time this procedure is performed.

2.3.1.1. Test procedure

- i) Wear protective clothing and disposable gloves. Score the reference number of the specimen on a microscope slide with a diamond marker², and use separate microscope slides for each specimen. For formed faeces, place 1 drop of saline (about 50 µl) in the centre of the slide.
- ii) For liquid faeces (or other appropriate body fluid) dispense one drop (about 20 µl) directly on to the slide. For formed faeces, use the tip of a clean applicator stick to remove about 2 mg sample³ and emulsify in the saline by thorough mixing.
- iii) Prepare a medium to thick smear with areas of varying thickness. Ensure that the smear is of the correct transparency⁴.
- iv) Air dry the smear at room temperature.

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

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

4 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.

- v) Fix the smear⁵ in methanol for 3 minutes.
- vi) Stain using modified Ziehl–Neelsen or auramine phenol stains as described below.

2.3.2. Concentration of oocysts from preserved or liquid samples by flotation

2.3.2.1. Preparation of flotation solution

Sucrose, zinc sulphate or sodium chloride solutions may be used to separate oocysts from faecal debris.

2.3.2.1.1. Preparation of sucrose or zinc sulphate solution

Either prepare sucrose solution (specific gravity 1.18) in a glass beaker by adding 256 g of sucrose to 300 ml of deionised water or prepare zinc sulphate solution (specific gravity 1.18) in a glass beaker by adding 100 g of zinc sulphate to 300 ml of deionised water. Gently heat the solution (<60°C) and stir continuously on a hot plate stirrer until the sucrose or zinc sulphate has dissolved completely. Place the solution on ice or in a refrigerator until it cools to 4°C. Pour the cold solution to a 500 ml measuring cylinder and adjust the specific gravity to 1.18 by adding sufficient cold, deionised water (4°C). Pour the solution into a screw-cap glass bottle, labelled, dated, initialled and stored at 4°C until used.

2.3.2.1.2. Preparation of saturated salt solution

Prepare saturated salt solution (specific gravity 1.2) by adding approximately 200 g of sodium chloride to 200 ml of deionised water. Gently heat the solution (<60°C) and stir continuously on a hot plate stirrer. Add further, small amounts of sodium chloride (approximately 10 g) at 10-minute intervals until the solution becomes saturated. Pour the saturated salt solution into a clean glass bottle and either place on ice or in a refrigerator until it has cooled to 4°C. Pour the solution into a 500 ml measuring cylinder and adjust its specific gravity to 1.2 by adding cold, deionised water (4°C). Pour the saturated salt solution into a screw-cap glass bottle, labelled, dated, initialled and stored at 4°C until used.

Alternatively, a cold method may be used; approximately 1.5 kg of sodium chloride is required to saturate 4 litres of deionised water by adding the sodium chloride in small quantities, taking care not to stop the magnetic stirrer and keeping the solution stirring briskly. Continue to add sodium chloride until specific gravity 1.2 is reached. Pour the saturated salt solution into screw-cap glass bottles, labelled, dated, initialled and stored at 4°C until used.

Before use, ensure that the salt solution is mixed by inversion and allowed to settle for 5 minutes.

2.3.2.2. Recovery of *Cryptosporidium* oocysts by centrifugal flotation

2.3.2.2.1. Test procedure

- i) Wear protective clothing and disposable gloves. Transfer approximately 1 to 2 g of faeces⁶ with an applicator stick, or pipette 1 to 2 ml liquid faeces, into 10 ml of flotation solution in a 15 ml centrifuge tube and mix thoroughly.
- ii) Place the centrifuge tube in a bench top centrifuge with swing out buckets, add a balance tube, if necessary, and centrifuge at 1100 *g* for 5 minutes⁷.
- iii) Remove the top 2 ml of fluid (containing the oocysts) from the meniscus, wash 3x in deionised water and finally resuspend in a minimum volume of deionised water.
- iv) Transfer the re-suspended contents on to a microscope slide with a disposable pipette, and air dry.

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

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

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

2.3.3. Concentration of oocysts in preserved or liquid samples by sedimentation

All steps that can generate aerosols (excluding centrifugation) should be performed in an operator protection safety cabinet.

2.3.3.1. Test procedure

- i) Wear protective clothing and disposable gloves. Sample approximately 500 mg to 1 g faeces⁸ with an applicator stick⁹ 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 sieve¹⁰ into a beaker, then pour the filtrate back into the same centrifuge tube.
- iv) Add 3 ml of ethyl acetate¹¹ to the formalinised solution, seal the neck of the tube with a rubber bung 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 slowly.
- v) Centrifuge the tube at 1100 **g** for 2 minutes¹².
- 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 ethyl acetate and formalin, into a marked re-sealable liquid waste container.
- vii) Re-suspend the pellet¹³ by agitation. Transfer the re-suspended contents on to a microscope slide with a disposable pipette, and air dry.

Commercial devices for concentrating helminth ova, larvae and protozoan cysts and oocysts using the formalin-ether method are available.

2.3.4. Staining methods

2.3.4.1. Modified Ziehl–Neelsen (mZN)

- i) 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 (general purpose reagent [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. The concentration of basic fuchsin can vary within the acceptable range of 1 to 3%.
- ii) 1% acid methanol
Carefully add 20 ml concentrated hydrochloric acid to 1980 ml of absolute methanol and mix. Transfer to a stock reagent bottle, and label, date and initial. Commercial supplies are also available.
- iii) 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. Commercial supplies are also available.

8 This is the size of a pea.

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

10 425 µm aperture, 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. Both the sieve and the beaker should be washed thoroughly in running tap water between each sample.

11 Ethyl acetate, although less flammable than diethyl ether, which was used previously, 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.

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

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

2.3.4.1.1. Test procedure

Include a positive control slide each time this procedure is performed.

- i) Wear protective clothing and disposable gloves. Fix the air-dried smear¹⁴ in methanol for 3 minutes.
- ii) Immerse or flood 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 seconds¹⁵.
- 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.
- ix) Examine for the presence of oocysts by scanning the slide systematically using the $\times 40$ objective lens of a bright-field microscope. Confirm the presence of oocysts under the oil immersion objective lens¹⁶.
- x) Measure the size and shape of the red-stained bodies using a calibrated eyepiece graticule.

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.

2.3.4.2. Auramine-phenol

- i) 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. Commercial supplies are also available.

- ii) 3% Acid methanol

Carefully add 60 ml concentrated hydrochloric acid to 1940 ml absolute methanol and mix. Transfer to a stock reagent bottle, and label, date and initial. Commercial supplies are also available.

- iii) 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. Commercial supplies are also available.

2.3.4.2.1. 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 concentrates¹⁷ 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.
- iv) Decolourise with 3% acid alcohol for 5 minutes.

14 Moderately thick smears are recommended for this procedure.

15 Over-destaining must be avoided.

16 The smear can be examined with or without a cover-slip.

17 Moderately thick smears are recommended for this procedure.

- v) Counterstain in 0.1% potassium permanganate for 30 seconds.
- vi) Air dry slide at room temperature¹⁸.
- vii) Examine for the presence of oocysts, using an epifluorescence microscope equipped with fluorescein isothiocyanate (FITC) or UV filters, by scanning the slide systematically under the $\times 20$ objective lens. Confirm the presence of oocysts under the $\times 40$ objective lens.
- viii) Measure the size and shape of the fluorescent bodies using a calibrated eyepiece graticule (see below)¹⁹.

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.

2.3.4.3. Reporting results of microscopic examination

Negative specimens should be reported as '*Cryptosporidium* oocysts NOT 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 1 per field of view
++	=	1 to 10 oocysts per field of view
+++	=	11 or more oocysts per field of view

2.3.5. Immunological methods

Three approaches to the immunological detection of *Cryptosporidium* oocyst antigens have proven useful, immunofluorescence microscopy (IFM), ELISA and IC, and a variety of commercial kits are available. IFM kits are more specific for, and can be more sensitive at, detecting *Cryptosporidium* oocysts in faecal smears than conventional stains (Chalmers & Katzer, 2013). Detection limits of ELISA and IC have been reported in the region of 3×10^5 to 10^6 oocysts per ml (Anusz *et al.*, 1990; Robert *et al.*, 1990; Smith, 2008), which is no more sensitive than conventional microscopical methods, and less sensitive than IFM. However, ELISA in 96-well plate format offers the advantage of streamlined testing of large numbers of samples while IC can be applied outside of the laboratory with less qualified staff. Positive reactions should be confirmed by another method.

2.3.5.1. Direct immunofluorescence microscopy (dIFM)

In dIFM, a FITC-conjugated anti-*Cryptosporidium* MAb (FITC-C-MABs) that recognises surface-exposed epitopes of oocysts is used. It does not distinguish different species of *Cryptosporidium*. Epifluorescence using a FITC filter system 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; it is false economy to dilute), and glycerol-based mounting medium containing a photo-bleaching inhibitor may be included.

Include a positive and negative control slide (usually supplied with the kit) each time this procedure is performed. *Cryptosporidium parvum* oocysts can be purchased from commercial suppliers, diagnostic veterinary laboratories or research facilities.

After staining and mounting slides according to manufacturers' instructions, scan the preparation for oocysts under the $\times 20$ and confirm under the $\times 40$ objective of an epifluorescence microscope equipped with an FITC filter set (maximum excitation wavelength

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

19 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.

490 nm, mean emission wavelength 530 nm). Measure oocysts under the $\times 100$ objective¹⁸. If necessary, slides can be stored at room temperature, in the dark, until read.

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 \times 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. If DAPI is included as counterstain, the nuclei will show blue fluorescence. 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.

Results should be reported as for conventional microscopical methods (above). Numbers can be recorded as identified previously.

2.3.5.2. Detection of *Cryptosporidium* antigens by enzyme linked immunosorbent assay (ELISA)

In the ELISA, the presence of soluble *Cryptosporidium* antigens in faeces (coproantigen) is sought. Depending on the commercial kit, *Cryptosporidium* coproantigens are captured and detected using a mixture of monoclonal and polyclonal antibodies.

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 are also capable of detecting common epitopes from infections with other *Cryptosporidium* species. 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.

Negative reactions should be reported as '*Cryptosporidium* antigen NOT detected'.

Positive reactions should be reported as '*Cryptosporidium* antigen detected'.

It is good practice to confirm positive reactions using a test of equal or better sensitivity and specificity, such as dIFM or PCR.

2.3.5.3. 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 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. The diagnostician should always determine whether any contraindications apply to the use of a commercial test and any fixative used.

IC is a convenient and rapid method for detecting *Cryptosporidium* antigen in stool samples, although false-positive reactions have been reported and positive reactions must be confirmed by a different test. Sensitivity is less than reported for ELISA, dIFM and PCR.

Negative reactions should be reported as '*Cryptosporidium* antigen NOT detected'

Positive reactions should be reported as '*Cryptosporidium* antigen detected' and confirmed using a test of equal or better sensitivity and specificity, such as dIFM or PCR, with the result included in the report.

2.3.6. Nucleic acid recognition methods

PCR offers improved diagnostic sensitivity compared with microscopy and immunological assays for detecting *Cryptosporidium* in faeces (de Waele *et al.*, 2011). The target is the sporozoite DNA within the oocysts. The reported sensitivity of published PCR methods can range between 1 and 10⁶ oocysts, depending on the copy number of the gene target, the oocyst disruption, DNA extraction, amplification and detection reagents, procedures and platforms.

Faecal samples can contain many PCR inhibitors. In addition to bilirubin and bile salts, complex polysaccharides are also significant inhibitors. Boiling faecal samples in 10% polyvinylpyrrolidone (PVPP) before extraction can reduce inhibition, but may not be necessary if abrogation steps are taken during DNA extraction (for example, spin columns) and in the PCR (including bovine serum albumin or appropriate mastermix). Faeces or partially purified oocysts stored in an equal volume of 5% K₂Cr₂O₇ and intended for PCR should be washed in deionised water to remove residual preservative prior to DNA extraction. For oocysts in suspension, a series of three washes each followed by centrifugation (1100 **g** for 10 minutes), removal of the supernatant and resuspension of the pellet in deionised water should minimise PCR inhibition.

No standard method for disrupting oocysts and extracting *Cryptosporidium* sporozoite DNA exists. *Cryptosporidium* DNA can be extracted either following partial purification of oocysts using one of the flotation/sedimentation techniques described above, or directly from oocysts in faeces. If concentration by formol–ethyl acetate sedimentation is the routine laboratory test, oocyst concentrates suitable for lysis and amplification by PCR can be made by washing the pellets by centrifugation in deionised water. Options for oocyst disruption include bead-beating, freeze–thaw cycles, heating or chemical/enzymatic treatments (Elwin *et al.*, 2012). Options for subsequent DNA extraction include commercial spin columns, glassmilk, and chelex resin.

Care is necessary when choosing PCR primers, as some are genus-specific whereas others are species-specific. Validated hydrolysis probe-based real-time PCR assays have been used for detection and identification of *Cryptosporidium parvum* and other selected species (De Waele *et al.*, 2011).

Commercial single and multiplex PCR-based assay kits for the detection of comprehensive panels of gastrointestinal pathogens are becoming available (including for veterinary applications), utilising robotic platforms for DNA extraction, assay set-up and amplicon detection. Pathogen panels should ideally be tailored for the population under investigation. Loci incorporated in such assays include SSU rRNA and *Cryptosporidium* oocyst wall protein (COWP) genes.

2.3.7 Detection of oocysts in drinking water and food

There are standard methods for the detection of *Cryptosporidium* oocysts in drinking water (e.g. International Organization for Standardization [2006], The Environment Agency [2010], Environmental Protection Agency [2012]), based on high volume filtration, elution, concentration, immunomagnetic separation and IFM. A standard method for the detection of *Cryptosporidium* in leafy green vegetables and soft berry fruits is in preparation by the International Standards Organisation.

2.3.8. Typing and subtyping for disease and source tracking

Molecular tools for inter- and intra-species discrimination are usually applied in specialist or reference laboratories for investigating transmission, identifying sources of infection and identifying specific risk factors.

DNA sequence analysis of the *Cryptosporidium* SSU rRNA gene using the “Xiao/Jiang primers” (Xiao & Ryan, 2008) is widely regarded as the benchmark for species identification, as not all *Cryptosporidium* species or genotypes can be identified by procedures such as restriction fragment length polymorphisms (RFLP); however, most of the species that are currently known to be commercially important for livestock can be identified by PCR-RFLP using *Vspl* and *Sspl*

(Xiao & Ryan, 2008). For bovine samples, *DdeI* can be included for differentiation of *C. andersoni* and *C. muris*, and *MboI* for differentiation of *C. parvum* from *C. bovis* or *C. ryanae* (Feng *et al.*, 2007). Sequencing of the amplicon is needed for investigation of water or environmental samples where any species or genotype may be present. Recent work has confirmed the utility of mini- and micro-satellite markers in the study of the population structure of *Cryptosporidium*, but there is a need to harmonise the entire method from marker selection to analytical algorithms (Widmer & Caccio, 2015).

3. Serological tests

Most assays for *Cryptosporidium* antibodies are ELISA based, using various aqueous extracts of native antigens (e.g. Hill *et al.*, 1990) or recombinant proteins (e.g. Priest *et al.*, 2006) derived from *C. parvum* oocysts. They have limited application for epidemiological surveillance, and results should be interpreted with caution as the tests are not fully validated.

C. REQUIREMENTS FOR VACCINES

There is no commercial or rigorously tested vaccine available.

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