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Cryptosporidiosis in Animals and Man: 2. Diagnosis

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ABSTRACT

Different diagnostic methods including clinical examination, detection of endogenous developmental stages by histopathology and electron microscopy, detection of oocysts by concentration and purification of *Cryptosporidium* oocysts and staining techniques, immunological assays and molecular identification techniques are described. Clinical examination revealed that diarrhoea begins in infected neonates at 3-5 days post-infection and last for 4-17 days. High mortality due to cryptosporidiosis has been reported, even in the absence of other enteropathogens. *Cryptosporidium* infections can be diagnosed by histological examination of autopsy or biopsy material from the intestinal mucosa. Endogenous developmental stages can be recognized associated with villous atrophy, villous fusion and changes in the surface epithelium. Scanning and transmission electron microscopy have confirmed the intracellular but extra cytoplasmic location within parasitophorus vacuoles formed by a continuous covering of microvillous membranes. Many specialized staining procedures have been described to stain the wall and/or contents of mature oocysts. The stain of choice for many diagnostic laboratories has been acid-fast staining and safranine-methylene blue. Oocysts stained bright red whereas yeast, bacteria and other fecal debris only take up the counter stain (green or blue). More recently, several immunolabelling techniques using polyclonal or monoclonal antibodies have been developed to detect oocysts.

Key words: Cryptosporidiosis, diagnosis, animals, humans

INTRODUCTION

The intracellular parasites of the genus Cryptosporidium infect mammals, birds, reptiles and amphibians (Santin, 2013).Cryptosporidium spp. cause significant diarrheal disease in humans and animals worldwide (Bouzid *et al*., 2013). Clinical manifestations may include acute, persistent or chronic diarrhea, biliary and pulmonary disease. Disease severity ranges from asymptomatic or mild to severe and intractable diarrhea with wasting depending on immune status, nutrition and age (Shaapan and Khalil, 2014).

Several studies have been published to evaluate and compare various methods used for diagnosis of cryptosporidiosis. Diagnosis is based on clinical signs, intestinal biopsy and detection of *Cryptosporidium* spp. oocysts or antigens in fecal samples by microscopical examination, immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA) and immune chromatographic assays (Geurden *et al*., 2008). However, most diagnostic methods rely on the detection of oocysts in feces by microscopy with or without prior concentration of oocysts in the specimen. Microscopic examinations are improved by various staining methods including negative,

acid-fast and fluorescent stains. At post-mortem, infection can be detected by histological examination of sections of the intestine (Quilez *et al*., 1996a). Polymerase Chain Reaction (PCR) is becoming increasingly popular as a tool to detect *Cryptosporidium* DNA in faeces. In recent years, many researchers have developed several PCR-based techniques for differentiation between species and genotypes of *Cryptosporidia*. PCR methods have proved to be more sensitive and more specific than the traditional microscopic techniques for the detection of *Cryptosporidium* spp. in clinic and environmental samples. PCR assays allow a rapid and accurate diagnosis in outbreak situations (Fayer *et al*., 2000; Zhou *et al*., 2007; Shaapan *et al*., 2011).

Therefore, the objective of the current review article was to highlight and evaluate the new trends for diagnosis of cryptosporidiosis in animals and humans.

Diagnosis

Clinical signs: Calves usually become infected with *Cryptosporidium* between 1 and 4 weeks of age and the duration of infection is short, lasting around 2 weeks. Calves begin shedding oocysts as early as 3 days of age with peak shedding occurring at 14 days of age (Olson *et al*., 2004). The kinetics of oocyst shedding of experimentally *C. parvum* infected neonatal calves revealed a pre-patent and patent period ranging from 3-6 and 4-13 days, respectively (Fayer *et al*., 1998). Calves raised in isolation from *Cryptosporidium* remain susceptible to infection at older age but clinical signs become less severe (Harp *et al*., 1990). In most calves, diarrhea has already begun 3-5 days post-infection and lasted 4-17 days (Fayer *et al*., 1998). Cryptosporidium diarrhea is associated with the excretion of tremendous numbers of oocysts. High mortality due to cryptosporidiosis has been reported, even in the absence of other entero-pathogens (De Graaf *et al*., 1999). The pathogenesis of *Cryptosporidium* diarrhea is believed to result from parasite invasion and epithelial destruction, resulting in mild to moderate villous atrophy and microvillous shortening and destruction (Olson *et al*., 2004). This leads to impaired nutrient digestion and absorption. The main clinical manifestations of *C. parvum* in cattle are diarrhea, depression, anorexia and abdominal pain (Fayer *et al*., 2000; Ralston *et al*., 2003). The severity and duration of clinical cryptosporidiosis are highly variable among calves. The diarrhea, which is pale yellow with mucus can be mild to severe and can last for up to 2 weeks. Calves are usually lethargic, anorexic and dehydrated. In severe cases, calves die from dehydration and cardiovascular collapse. Other enteric viral, bacterial and parasitic pathogens such as rotavirus, *Escherichia coli* and *Giardia* could also be observed in calves during the first 4 weeks of life that could contribute the severity of cryptosporidiosis (O'Handley *et al*., 1999; Joachim *et al*., 2003). Calves with severe cryptosporidiosis can take 4-6 weeks to recover fully and there could be an initial negative impact on production due to weight loss or impaired weight gain.

Unlike young ruminants, infections in swine are typically asymptomatic, even in young animals. However, serious naturally occurring problematic cryptosporidium infections in nursing piglets (<3 weeks of age) have been reported in Europe (Rotkiewicz *et al*., 2001). Experimental infections of piglets resulted only in diarrheic problems when the animals were inoculated before 2 weeks of age. In older piglets, experimental *Cryptosporidium* infection did not cause clinical manifestations (Ramirez *et al*., 2004). The prevalence of cryptosporidiosis was reported to be very low in nursing piglets. Several studies demonstrated that naturally occurring cryptosporidiosis is delayed until after weaning (Quilez *et al*., 1996a). Previous studies have confirmed that diarrhoea in suckling and weaned piglets is usually a multifactorial problem, where mixed infections between *C. parvum* and *E. coli* or rotavirus are frequent (De Graaf *et al*., 1999).

Several episodes of swine infections have been reported in a variety of geographical localities globally and have been connected with diarrhoea and morbidity. In Southern California, a prevalence of 5% was reported in feeder pigs and butcher hogs. Oocyst shedding was reported in both clinically healthy pigs and those with diarrhoea (Ramirez *et al*., 2004). Higher prevalence of *Cryptosporidium* infection in pigs has been reported in Spain (21.9%) and Trinidad (19.6%) with asymptomatic infections in most of the pigs and higher rates in 1-2 month old pigs (Kaminjolo *et al*., 1993; Quilez *et al*., 1996a).

Increasingly, outbreaks of foal diarrhea have been attributed to *C. parvum* infection, which has been recently described as one of the most prominent nonbacterial causes of diarrhoea in foals (Cole *et al*., 1998). Several studies indicate that the infection is common in foals and adult horses with a prevalence of 6.4% in the UK, 9.4% in Poland, 17% in Canada, 15-31% in Ohio and Kentucky and 100% in Louisiana (Xiao and Herd, 1994; Olson *et al*., 1997; Majewska *et al*., 1999; Sturdee *et al*., 2003). In Egypt (Ghazy *et al*., 2004) revealed a prevalence level of 46.3% in foals suffering from emaciation, anemia, recurrent colic and chronic intermittent diarrhea.

Detection of endogenous developmental stages by histopathology and electron microscopy: *Cryptosporidium* infections were first diagnosed in animals and man by the histological examination of autopsy or biopsy material from the intestinal mucosa. *Cryptosporidium* infections are mainly concentrated in the distal small intestine but lesions were also found in the caecum and colon and occasionally in the duodenum (De Graaf *et al*., 1999). The pathological findings associated with *Cryptosporidium* are a mild villous atrophy, villous fusion and changes in the surface epithelium. In histological sections, most endogenous developmental stages appear as small basophilic bodies by (H and E or Giemsa stains) apparently attached to the surface of the cells, sometimes giving the microvillous brush border a spotted granular appearance (Fig. 1a and 2). They are spherical to elliptical in shape measuring from 2-6 μm in diameter and protrude from the cell surface (Fig. 1c-f). Scanning electron microscopy may also be used to confirm infections because the parasites bulge outwards from the epithelial cell surface (Fig. 2a and b). Because little morphological details can usually be determined from histological sections, recourse is often made to transmission electron microscopy to confirm the identity of the organisms. Transmission electron microscopy has confirmed the intracellular but (extracytoplasmic) location within parasitophorus vacuoles formed by a continuous covering of microvillous membranes (O'Donoghue, 1995). Other sporozoan parasites which develop within parasitophorus vacuoles are located deeper within the cytoplasm next to the host cell nucleus. Nearly, all endogenous developmental stages of *Cryptosporidium* spp. have been confined to the apical surface of epithelial cells. The parasites contain a unique "Attachment" or "Feeder" organelle which is prominent at the base of each parasitophorus vacuole (Fig. 2c and d). The parasite pellicle is repeatedly folded to form a comb-like lamella closely associated with a dense adhesion zone formed by fusion of the outer microvillous membrane and the epithelial plasma membrane. This organelle is thought to facilitate the uptake of nutrients by the parasite from the host cell (Ramirez *et al*., 2004). Electron microscopic studies have revealed many features for different developmental stages of parasite. Sporulated oocysts each contain 4 sporozoites and a residium composed of numerous small granules and a spherical or ovoid membrane-bound globule. Most authors reported no sporocyst wall within the oocyst (Olson *et al*., 2004). Other morphological features often observed in coccidian oocysts, such as a micropyle and polar granules have not been found in oocysts of *Cryptosporidium* spp. A suture that dissolves during excystation has been identified with transmission electron microscopy (Fig. 2c).

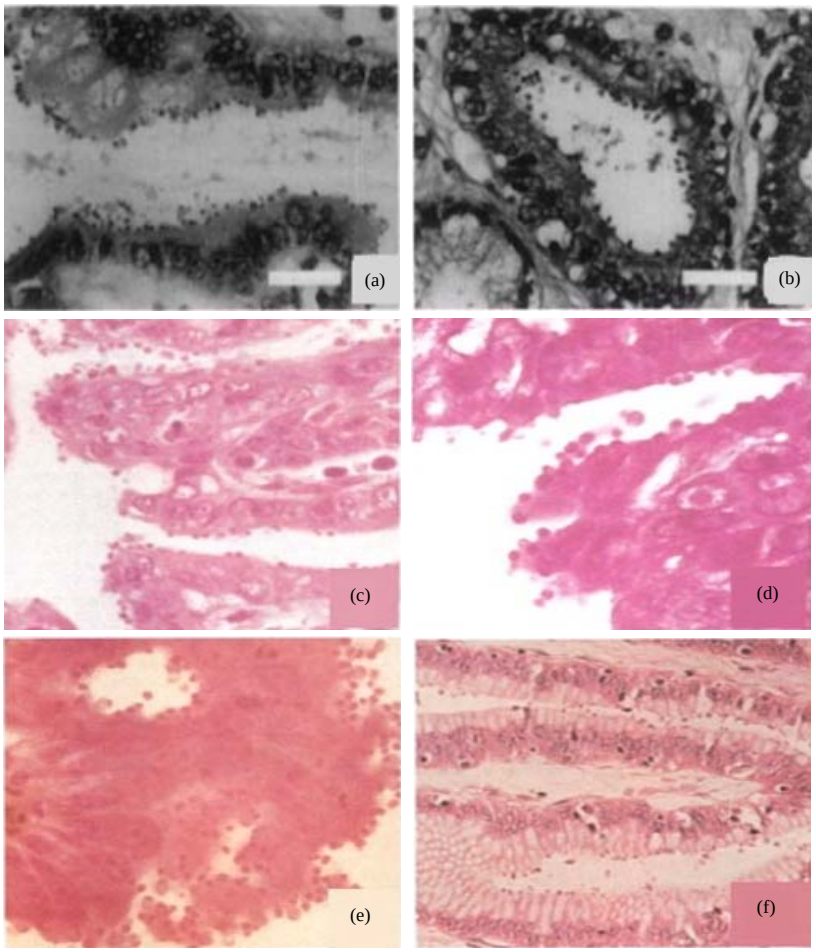

Fig. 1(a-f): (a) Endogenous developmental stages of *Cryptosporidium* apparent as small basophilic bodies lining the epithelium of the small intestine, (b) *Cryptosporidium* stages lining the epithelium of a gastric pit, (c) Mouse intestines infected with *Cryptosporidium* sp. round organisms are on the epithelial surface, (d) Gallbladder infected with *Cryptosporidium* sp. epithelial cells are squamous mucosa, (e) Trachea infected with *Cryptosporidium* sp. and (f) Stomach infected with *Cryptosporidium* sp.

Sporozoites are crescent-shaped with the anterior end slightly pointed and the posterior end rounded. Sporozoites of *C. parvum* average 4.9 by 1.2 μm. Each sporozoite contains a prominent nucleus in the posterior third of the body. Unlike other coccidia, the sporozoites are free within the oocysts and not surrounded by sporocysts (Spano and Crisanti, 2000). Trophozoites are rounded to oval intracellular forms, 2-2.5 μm in diameter (Fig. 2c and f). They are further characterized by a large nucleus which contains a large nucleolus. Type I meronts produce eight merozoites that bud from an "Attachment zone" or "Feeder organelle", where the meront interfaces with the host cell (Fig. 3a-c). Type II meronts produce 4 merozoites (Fig. 3d). Merozoites are crescent shaped with rounded anterior and posterior ends and measure about 1-5 μm. They contain a single vesicular nucleus, endoplasmic reticulum and a variety of unidentified granules (Fig. 3e). Like most other coccidian merozoites, at the anterior end are organelles such as the conoid, a polar ring, rhoptries

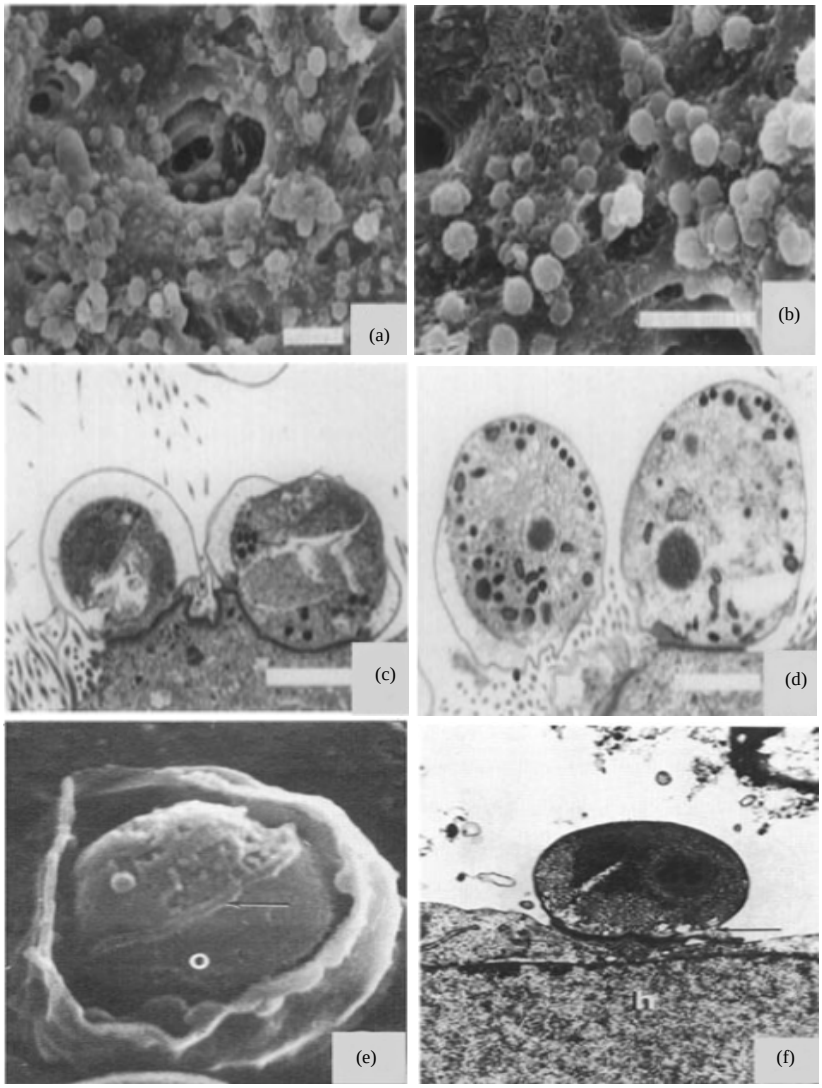

Fig. 2(a-f): (a and b) Scan e/m of *Cryptosporidium* stages attached to the epithelium of the trachea, (c) Transmission e/m of trophozoites of *Cryptosporidium* located within parasitophorous vacuoles on the surface of the small intestinal epithelium from a goat, (d) Transmission e/m of macrogametocytes attached to the bursal epithelium, (e) Scanning e/m of an oocyst (o) and (f) Transmission e/m of a trophozoite, (t). A feeder organelle (arrow) is developing above the dense attachment zone with the host cell

and micronemes but not refractile bodies, mitochondria, microspores or polysaccharide granules (Dubey *et al*., 1990). The microgamont (male stage) contains many condensed parts of nuclei, ribosomes, endoplasmic reticulum and membrane-bound vacuoles. Macrogamonts (female stage) are nearly spherical, contain a large single nucleus and endoplasmic reticulum and are surrounded by a double membrane called a pellicle. Beneath the macrogamont cytoplasm is a feeder organelle (Fig. 3f and g) (O'Donoghue, 1995).

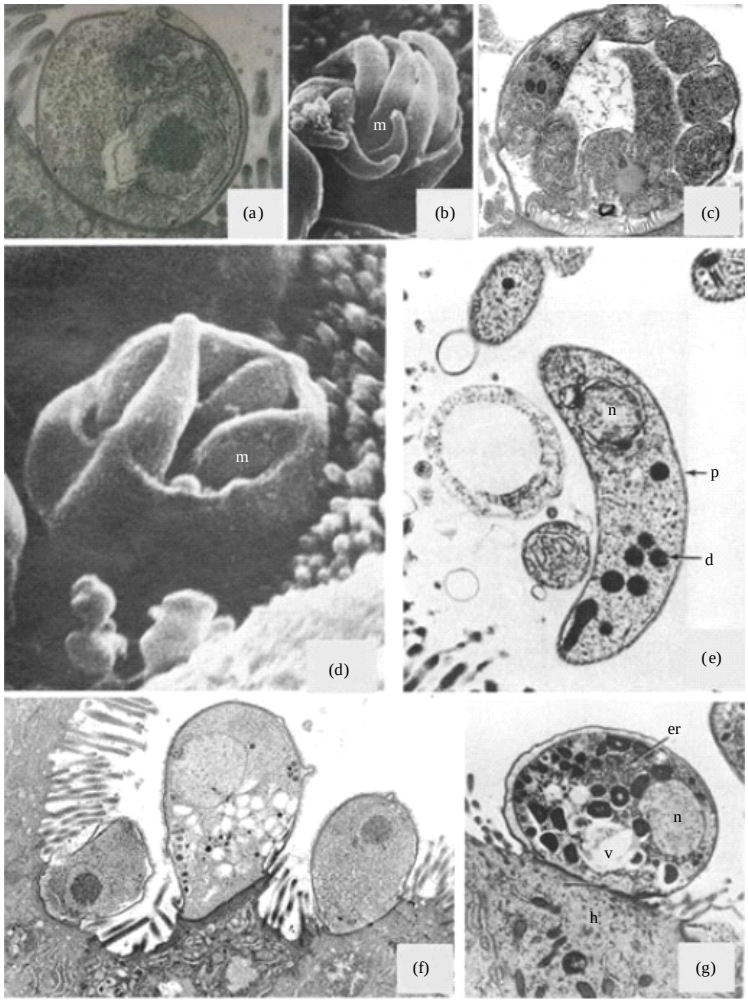

Fig. 3(a-g): (a) Transmission e/m of a trophozoite attached to epithelial cells, the nucleus has a large nucleolus and a feeder organelle is found in the attachment zone, (b) Scanning e/m of a type 1 meront with 8 merozoites (m), (c) Transmission e/m of a type1 meront with 8 merozoites, meronts have a dense band of attachment with a feeder organelle, (d) Scanning e/m of a type 2 meront with 4 merozoites (m) (e) Transmission e/m of a merozoite on the luminal surface adjacent to microvilli, merozoite is covered by a typical double-unit membrane pellicle, (p) dense granules, (d) are prominent, (f) Transmission e/m of two trophozoites and a macrogamont attached to epithelial cells and (g) Transmission e/m of a macrogamont, large nucleus (n), endoplasmic reticulum (er), a vacuole (v) and other granules are prominent

Detection of oocysts

Preservation and storage of specimens containing oocysts: For diagnosis of cryptosporidiosis, stool specimens should be submitted as fresh material or in 10% formalin or sodium acetate-acetic acid formalin (SAF) preservatives. Fresh or preserved stool specimens can be examined as wet mounts (Fig. 4a-c) or they can be concentrated or stained. Potassium dichromate solution (2.5%) is used routinely as a storage medium to preserve oocyst viability.

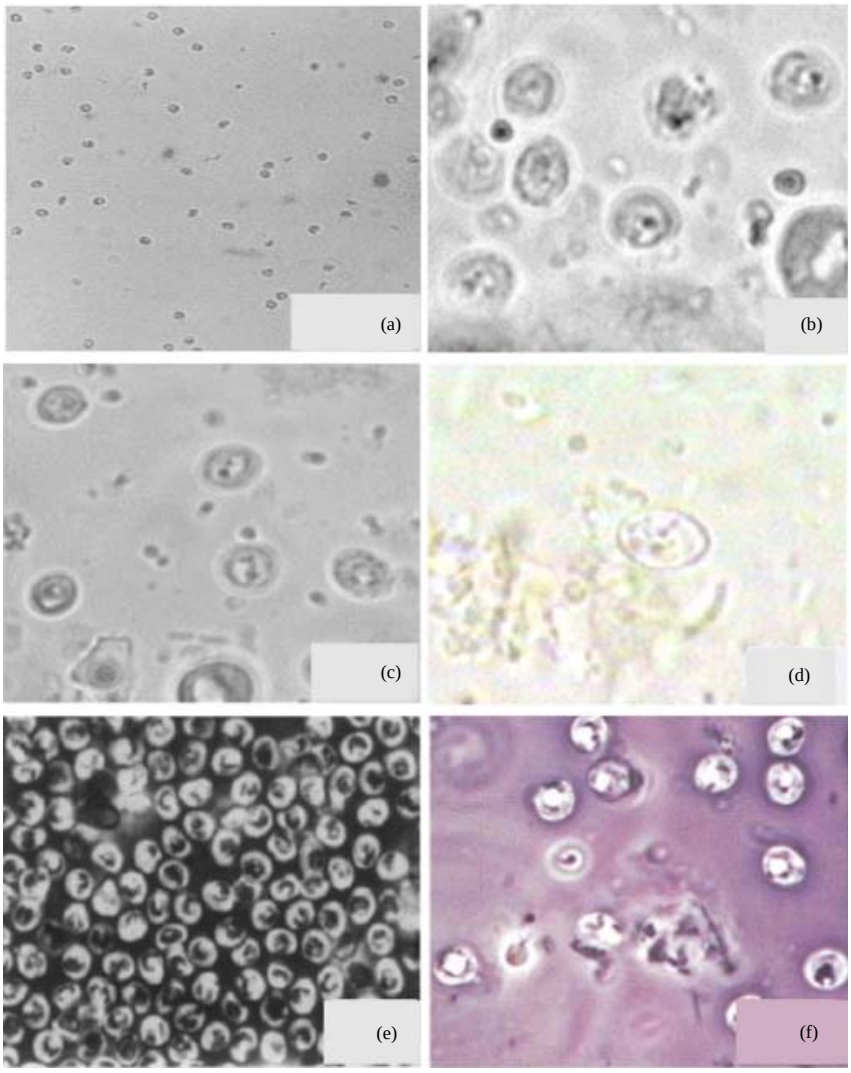

Fig. 4(a-f): (a) *C. parvum* oocysts (wet mount) oocysts are spherical organisms contain four sporozoites and some dark granules (b, c, and d) Oocysts appear as spherical organisms, faint pink in color with Sheather's sucrose concentration technique (e) *Cryptosporidium* oocysts harvested from the feces, light micrograph, phase-contrast and (f) phase-contrast photomicrograph of oocysts (white) suspended in Sheather's sucrose solution and yeast cells appear light brown

Cryptosporidium spp. oocysts remain viable for 3-12 months when stored at 4°C in potassium dichromate solution (Dubey *et al*., 1990).

Concentration and purification of Cryptosporidium oocysts: Concentration of stool specimens is important in non-acute illness with small numbers of oocysts. Oocysts have been successfully concentrated by centrifugal sedimentation in formalin-ether and formalin-ethyl acetate solutions and by centrifugal flotation in saturated sodium chloride (sp. gr. 1.20), magnesium sulphate (1.30), Sheather's sucrose (1.34) (Fig. 4d and f), zinc sulphate (1.49) and potassium iodide

(1.72) solutions (Current and Garcia, 1991; O'Donoghue, 1992; Kvac *et al*., 2003) or by sequential sedimentation and flotation techniques (Weber *et al*., 1992). Comparative studies on the sensitivities of different concentration techniques have yielded conflicting results. Some studies found few differences between concentration techniques (Kvac *et al*., 2003), whereas others found the most sensitive to be sucrose flotation (Fig. 4d and f) (MacPherson and McQueen, 1993) or formalin-ether sedimentation (Mtambo *et al*., 1992). A modified formalin-ethyl acetate sedimentation technique was also reported to be quite sensitive, although recent studies have shown the technique to result in poor oocyst concentration (Weber *et al*., 1991). *Cryptosporidium* oocysts are smaller than those of other coccidian parasites and they are best pelleted by longer and/or faster centrifugation (>500 g for at least 10 min) than commonly used for most coprological sedimentation techniques. Samples must be examined soon after preparation (within 10 min.) because longer exposure to the solutions results in oocyst distortion and collapse (O'Donoghue, 1995).

Faecal concentrates are best examined for oocysts by phase-contrast microscopy at 200-400 times magnification. The oocysts appear as phase-bright, briefringent bodies against a dark background and they usually contain one to several eccentric dark granules (Fig. 4e). In comparison, yeasts and other fecal debris are not phase-bright but remain dull and dark in appearance. When examined by normal bright-field microscopy, the oocysts (wet mounts) appear as small, non refractile bodies which are difficult to detect even though they sometimes appear light pink color (Fig. 4a-c). Nomarski interference contrast microscopy has also been found to be suitable for discerting internal structures within oocysts (i.e. sporozoites and crystalline residual bodies) (Fig. 5a). The identity of oocysts in faecal concentrates can also be checked by washing them soon after flotation or sedimentation and preparing smears for histochemical staining or immunolabelling (Dubey *et al*., 1990). Stool concentration is a diagnostic procedure that is essential since it allows detection of seven times more oocysts than unconcentrated stool smears (Quilez *et al*., 1996b).

Several specialized centrifugation techniques have also been developed to purify oocysts from faecal material; including isopycnic or discontinuous density gradient centrifugation in sucrose, caesium chloride and Percoll or Ficoll- sodium diatrizoate solutions (Kvac *et al*., 2003). Pure oocyst suspensions can be obtained free of fecal contaminants but such techniques are not suitable for routine diagnostic use due to time and cost constrains.

Various filtration techniques have been used to recover oocysts from water samples. Basically, they have involved filtering large volumes of water through spun polypropylene or polycarbonate filters (down to 1 μm pore size), eluting the sediment from the filter and then examining it for oocysts by various staining or immunolabelling techniques (Sterling, 1990). The efficiency of oocyst recovery by this method has generally been low, ranging from 9-59% (Smith and Rose, 1990). More recently, an alternative means of harvesting oocysts by calcium carbonate flocculation has been described with improved recovery ranging from 68-79% (Vesey *et al.,* 1993a). Specialized flow cytometry and cell sorting techniques have also been developed to detect oocysts in water samples with greater sensitivity than conventional epifluorescence microscopy (Vesey *et al*., 1993b).

Staining procedures for Cryptosporidium oocysts: Many specialized staining procedures have been described to stain the walls and/or contents of mature oocysts (Table 1). The oocysts are similar in size and shape to other faecal components especially some yeast (Fig. 4a-f), therefore, differential staining techniques are more desirable to avoid confusion. Some expertise in the

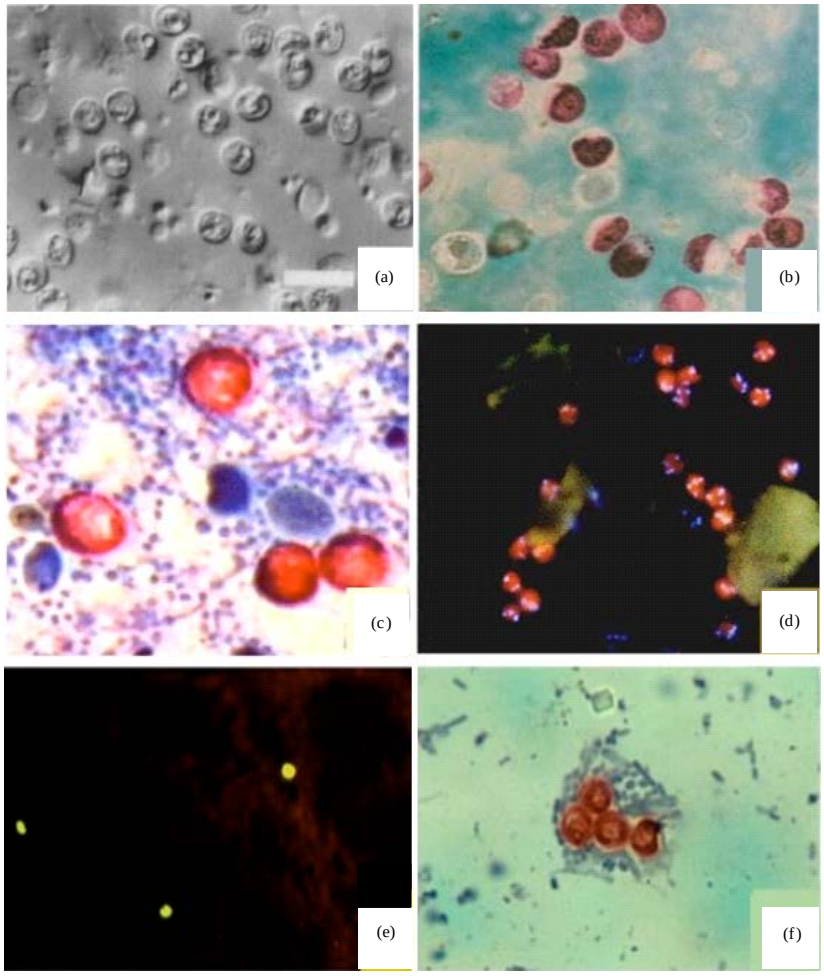

Fig. 5(a-f): (a) *Cryptosporidium* oocysts harvested from the feces, light micrograph, Nomarski interference-contrast, (b) Oocysts using modified acid fast stain appear as red containing some dark granules and usually have a central clear area while yeast cells, bacteria and fecal debris appear green or blue in color, (c and d) *Cryptosporidium parvum* oocysts stained with a combination of two fluorochromes (DAPI and PI), (e) *Cryptosporidium parvum* oocysts stained with auramine-carbol fuchsin fluorescence method, oocysts stained orange and (f) The 4 oocysts stained by Safranine-methylene blue

identification of oocysts is required because they can exhibit some variability in their staining characteristics depending on their age, viability and stage of development. Both fresh and fixed faecal material can be processed for staining although many laboratories recommend samples be fixed because of biohazard consideration. Acceptable fixatives include 10% formalin and Sodium-acetic Acid-Formalin (SAF) (Ramirez *et al*., 2004). The stain of choice for many diagnostic laboratories has been acid-fast staining (e.g. Kinyoun, Ziehl-Neelson and dimethyl sulfoxide (DMSO)-carbol fuchsin). Oocysts stain bright red whereas yeasts, bacteria and other faecal debris only take up the counterstain (green or blue) (Fig. 5a-c). Nonetheless, auramine-rhodamine staining techniques which stain only oocysts have been found to be very sensitive but rather

Table 1: Staining procedures for *Cryptosporidium* oocysts (O'Donoghue, 1995)

Staining technique	Appearance of oocysts	Background
Direct staining		
Giemsa	Blue	Blue
Gram	Red	Purple
Kohn's stain	Dark green	Grey
Methylene blue	Light blue	Dark blue
Aniline-carbolmethyl violet	Blue	Unstained
Safranine-methylene blue	Orange	Counter stained
Acid-fast stains		
Kinyoun	Red	Counter stained
Ziehl-Neelsen	Red	Counter stained
DMSO-carbol fuchsin	Red	Counter stained
Fluorochrome stains		
Auramine-rhodamine	Orange	Unstained
Auramine-carbol fuchsin	Orange	Unstained
Acridine orange	Orange/green	Orange
Diamidino-phenylindole (DAPI)	Blue	Unstained
Mepacrine	Orange	Unstained
Propidium iodide (PI)	Red	Unstained
Negative staining		
Carbol fuchsin	Unstained	Blue
Iodine	Unstained	Brown
Light green	Unstained	Green
Merbromine	Unstained	Orange
Methanamine silver	Unstained	Black
Nigrosin	Unstained	Unstained
Periodic acid-Schiff	Unstained	Red
Phosphotungstic acid	Light brown	Black
Uranyl acetate	Light brown	Black

expensive (MacPherson and McQueen, 1993). A recent study has found that the inclusion of diamidino-phenylindole (DAPI) and the exclusion of propidium iodide appear to be good indicators of oocyst viability (Fig. 5d) (Leav *et al*., 2003). Flourescent stains also include acridine orange which causes fluorescence in both oocysts, yeast forms and auramine-carbol fuchsin which distinguishes between yeasts and oocysts (Fig. 5e). Fluorescent staining does not allow visualization of detail of the oocyst and confirmatory staining may be necessary. Negative staining techniques, stain background yeasts and bacteria but not oocysts (Fayer *et al*., 2000). Other direct staining techniques include Giemsa stain which does not differentiate oocysts from yeasts, Gram stain which stains yeasts purple and oocysts red, aniline-carbol-methyl violet which stains oocysts blue but not yeasts, methylene blue which stains yeasts darker than oocysts and safranine-methylene blue which compares well with the modified acid fast techniques (Fig. 5f) (Leav *et al*., 2003).

Immunological assays: Most humans and animals with cryptosporidiosis pass enough oocysts in their stool, so that most of the concentration and/or staining techniques are adequate for detection and diagnosis. However, more sensitive techniques are sometimes needed to detect oocysts in specimens that contain few parasites and large amount of debris. These specimens include faecal samples from asymptomatic carriers or filtrates from surface or drinking water samples. More recently, several immunolabelling techniques using polyclonal or monoclonal antibodies have been developed to detect oocysts. Polyclonal rabbit antisera have been used to detect oocysts in fecal and water samples by fluorescent antibody tests and latex agglutination reactions (Ramirez *et al*., 2004). Mouse monoclonal antibodies have also been used to detect oocysts in faecal and water samples by immunofluorescence (Garcia *et al*., 1992; O'Donoghue, 1995) and

enzyme immunoassays (Anusz *et al*., 1990; Chapman *et al*., 1990; Robert *et al*., 1990; Siddons *et al*., 1992; Rosenblatt and Sloan, 1993) and several diagnostic kits are now commercially available. Monoclonal antibodies have also been used to detect endogenous developmental stages in tissue sections (Bonnin *et al*., 1990). While recent evaluation studies have shown that many immunolabelling tests have high sensitivities for the detection of *C. parvum* oocysts, particularly in samples containing large amounts of debris and few oocysts, further studies on the specificities of the antibodies are required to determine their cross-reactivity with other *Cryptosporidium* spp. which is important for the identification of veterinary and environmental isolates.

New rapid immunoassays designed for simple diagnostic testing with minimal training are commercially available (e.g. Immuno Card STAT and MERIFLUOR Direct Fluorescent-Antibody (DFA) test, (both from Meridian Bioscience, Inc.), the Pro Spec T *Cryptosporidium* microplate assay (Alexon-Trend, Inc.), Beckton Dickinson Color PAC and BIOSITE Diagnostic Triage Parasite Panel). MERIFLUOR DFA had the highest sensitivity but specificity was equal to or greater than 99% for all the tests (Johnson *et al*., 2003). Their suitability for use in individual laboratories depends on the balance between the assay costs, the reduced time and the number of specimens processed daily (Chan *et al*., 2000).

The most widely used antigen detection immunoassays for *Cryptosporidium* are the Direct Fluorescent Antibody (DFA) tests, which detect intact organisms and enzyme immunoassays (EIAs) which detect soluble stool antigen. DFA tests utilize fluorescein labelled antibodies directed against cell wall antigens of *Cryptosporidium* oocysts and allow visualization of the intact parasites, providing a definitive diagnosis. The sensitivity and specificity of the most commonly used commercial DFA test and the MERIFLUOR DFA test have been reported to be 96-100% and 99.8-100%, respectively. This test has a greater sensitivity than that of traditional examination of permanent smears prepared from concentrated stool specimens for *Cryptosporidium* (Johnson *et al*., 2003). Commercially available EIAs use antibodies for the qualitative detection of *Cryptosporidium*-specific antigens in preserved stool specimens. The reported sensitivities of EIAs (e.g. Alexon ProSpec T and Beckton Dickinson Color PAC-*Cryptosporidium* enzyme immunoassays) ranged from 99-100% and specificities ranged from 98.6-100%. The advantages of the EIAs are that numerous samples can be screened at one time and tests can be read objectively on a spectrophotometer instead of subjectively on a fluorescence microscope (Katanik *et al*., 2001). Immuno Card STAT; *Cryptosporidium*/*Giardia* rapid assay (Meridian Bioscience, Inc.) is a solid-phase qualitative immuno chromatographic assay that detects and distinguishes between *Giardia* and *C. parvum* in aqueous extracts of fecal specimens (fresh, frozen, unfixed or fixed in 5 or 10% formalin or SAF). By using specific antibodies, antigens specific for these organisms are isolated and immobilized on a substrate. After addition of appropriate reagents, a positive test is detected visually by the presence of a gray black color bar next to the organism name printed on the test device (Garcia *et al*., 2003). The assay can be performed in approximately 12 min.

Concerning immuno serology, specific antibodies against *Cryptosporidium* have been detected in host serum samples using several immuno serological test procedures. Indirect fluorescent antibody tests were first described using endogenous developmental stages in tissue sections as antigens (O'Donoghue, 1995), whereas later studies employed intact oocysts as antigens (De Graaf *et al*., 1999). More recently, enzyme immunoassays have been developed using crude soluble oocyst preparations as antigens (Ramirez *et al*., 2004). However, the detection of specific antibodies should not be regarded as being indicative of active infection but rather as providing presumptive evidence of prior exposure. In most cases, specific antibodies were only detected after

infections had become patent (with or without clinical signs) and significant antibody titres have been found to persist in many cases for up to 12 months after infection. Immuno serological tests therefore appear to be unsuitable for routine diagnostic use but they do provide tools for immunological and sero-epidemiological studies. However, indirect Immuno Fluorescent Antibody (IFA) procedures have been used to detect *Cryptosporidium* specific antibodies in the sera of humans and animals. IFA has been developed using histologic sections of infected mouse intestine or purified oocysts. Infected mouse intestine can be used to detect antibodies to all life cycle stage of the parasite, whereas intact oocysts are used for detection of antibodies to the outer oocyst wall (Dubey *et al*., 1990).

Comparative diagnostic studies were performed to evaluate different immunological assays. Direct immunofluorescence (IF) assay with monoclonal antibodies was compared with the Modified Ziehl Neelsen (MZN) acid-fast technique for the detection of *Cryptosporidium* oocysts in fecal samples from cattle and pigs (Quilez *et al*., 1996a). It is concluded that the monoclonal antibody-based immunofluorescence assay is more sensitive and efficient than MZN technique, especially for detecting a low number of *Cryptosporidium* oocysts in fecal samples (Table 2 and 3). However, MZN staining is specific and sufficiently sensitive to detect moderate or high number of *Cryptosporidium* oocysts in stool specimens from cattle and pigs. We therefore recommend that for routine diagnosis of *Cryptosporidium* infection, fecal specimens should be first screened with the MZN staining method. A recent study was performed to evaluate 4 immunological assays for diagnosis of clinical cryptosporidiosis in diarrheic calves; an Immunofluorescence Assay (IFA), two ELISA tests and an immuno chromatographic (dipstick) assay (Geurden *et al*., 2008). All diagnostic assays were found to be relatively specific and sensitive (Table 4).

Molecular diagnosis: Although current diagnostic procedures appear to be suitable for the detection of oocysts which are relatively abundant in clinical samples, more sensitive and specific

Table 2: Comparison between the percentage of cattle positive fecal samples recorded by Modified Ziehl-Neelsen and monoclonal antibody-based immunofluorescence techniques

Age range	MZN	IF	Discrepant cases
Suckling calves	19/29 (65.5%)	24/29 (82.7%)	5/29 (17.2%)
Weaned calves	4/35 (11.4%)	5/35 (14.3%)	1/35 (2.8%)
Heifers	0/44	0/44	0/44
Total	23/108 (21.3%)[a]	29/108 (26.8%)	6/108 (5.5%)

[a]None faecal sample was positive by MZN and negative by IF, MZN: Modified Ziehl-Neelsen, IF: Immunofluorescence

Table 3: Comparison between the percentage of pig positive fecal samples recorded by Modified Ziehl-Neelsen and monoclonal antibody-based immunofluorescence techniques

Age range	MZN	IF	Discrepant cases
Suckling piglets	0/14	0/14	0/14
Weaned piglets	7/16 (43.7%)	14/16 (87.5%)	7/16 (43.7%)
Fattening pigs	14/30 (46.7%)	17/30 (56.7%)	3/30 (10%)
Adults	0/30	0/30	0/30
Total	21/90 (23.3%)[a]	31/90 (34.4%)	10/90 (11.1%)

[a]None faecal sample was positive by MZN and negative by IF, MZN: Modified Ziehl-Neelsen, IF: Immunofluorescence

Table 4: Sensitivity and specificity of diagnostic assay for detection of *Cryptosporidium* spp

Parameters	Se	Sp
IFA	97.4 (91.7-99.9)	94.8 (90.3-99.7)
Tetra-ELISA	93.6 (87.1-98.3)	95.9 (90.4-99.3)
Techlab-ELISA	95.4 (90.0-98.6)	92.7 (86.6-97.8)
Dipstick	87.8 (81.9-92.7)	91.5 (85.5-96.5)

(Se): Sensitivity, Sp: Specificity

techniques are required for the detection of small numbers of oocysts passed by asymptomatic carriers or present in water samples. The application of Polymerase Chain Reaction (PCR) procedures for the amplification of defined DNA sequences has shown considerable promise for the development of highly sensitive and specific diagnostic tests. PCR amplification targets have been selected from sequence information derived from genomic libraries (Abrahamsen *et al*., 2004; Xu *et al*., 2004).

Gene sequences have also been used for polygenetic analyses to determine evolutionary relationships between *Cryptosporidium* spp. and other protozoan and metazoan organisms. Reverse transcription of small subunit ribosomal RNA and sequence analyses have revealed that *Cryptosporidium* does not exhibit any specially close phylogenetic relationships with other protozoan parasites (Bialek *et al*., 2002).

Originally, species assignment within the genus *Cryptosporidium* was based on phenotypic characteristics such as host specificity and oocyst morphology. Recently, genetic characterization using PCR techniques and sequence analysis have been employed to discriminate between *Cryptosporidium* species, which in turns helps determine the most likely source of origin and the real risks to human and animal health. Recently developed PCR protocols have proven to be very specific and sensitive; PCR-restriction Fragment Length Polymorphism (PCR-RFLP), PCR-Single Strand Conformation Polymorphism (PCR-SSCP), Reverse-Transcriptase-PCR (RT-PCR), Real-Time PCR, PCR-heteroduplex analysis incorporating DNA sequencing and single or multilocus mini and microsatellite analysis (Ramirez *et al*., 2004). Analysis of the small subunit of the ribosomal DNA gene (18S rDNA) has revealed existence of different genotypes of *C. parvum* assigned human, bovine, cattle, pig, cat, mouse, dog, monkey and ferret types (Xiao *et al*., 1999). Sequence analysis of additional genes such as ribosomal Internal Transcribed Spacer (ITS rDNA) regions, oocyst wall protein (COWP), dihydrofolate reductase (DHFR), B-tubulin, thrombospondin-related adhesive protein 1 and 2 (TRAP-C1, TRAP-C2), 60 kDa glycoprotein (GP 60) and 70-kDa heat shock protein (HSP70) have been successfully used for detection and differentiation between *Cryptosporidium* species (e.g. *C. parvum*, *C. hominis*, *C. meleagridis*, *C. baileyi*, *C. muris*, *C. felis*, etc) and *C. parvum* genotypes (e.g. pig, ferret, mouse and monkey) (Lowery *et al*., 2000; McLauchlin *et al*., 2000; Enemark *et al*., 2002; Chalmers *et al*., 2005). At least 2 genomic loci from an isolate should be analyzed at the same time to provide reliable identification. One of these should target a universal coding region and the other should be suitable for species identification and subtyping analyses (Caccio *et al*., 2002; Kato *et al*., 2003). Single gene and multilocus genotyping studies of isolates from different geographical localities and hosts have demonstrated considerable inter-species diversity between human and animal isolates and high levels of intra-species identity (specially among *C. parvum* and *C. hominis*) across countries (Mallon *et al*., 2003). The advantage of molecular identification techniques is that they are able to detect genus, species or genotypes-specific nucleic acid sequence of *Cryptosporidium*. Achieving collective agreement on criteria for species assignment within this genus will help physicians, veterinarians and epidemiologists to determine the impact of each species on human and animal health, the zoonotic potential, transmission mechanisms and preventive measures.

Mini and microsatellites are polymorphic sequence repeats in eukaryote genomes. Finger printing techniques targeting mini and microsatellite loci are available for *Cryptosporidium* and can be used to identify subgenotypes and clonal lineages e.g. in defining the epidemiology of an infection. For instance, by sequencing the amplified mini or microsatellite product, *C. parvum* and *C. hominis* subtypes can be identified (Caccio *et al*., 1999; Caccio *et al*., 2001; Chalmers *et al*., 2005).

Nucleic Acid Sequence Based Amplification (NASBA) is an isothermal transcription-based amplification system specifically designed for detection of RNA targets (Cook, 2003). Baeumner *et al*. (2001) described the use of NASBA for the amplification of the *Cryptosporidium* hsp 70 mRNA with a detection limit of five oocysts. This technique may offer the advantage of identifying viable oocysts because it is based on RNA detection, however, it is more complex than PCR because it requires three enzymes (reverse transcriptase, RNase H and T7 RNA polymerase). Additionally, the incorporation of enrichment culture or oocysts directly to the NASBA reaction, as possible with PCR can not be done because the temperature (42°C) is too low to lyse the cells and release the nucleic acids. NASBA research is new compared to PCR and its potential for the routine use for *Cryptosporidium* oocysts detection needs further developmental effort. More detection and typing tools are expected to be developed with the reported *C. parvum* and *C. hominis* genomes (Abrahamsen *et al*., 2004; Xu *et al*., 2004).

More advanced molecular studies have constructed *C. parvum* and *C. hominis* DNA libraries in different vectors and succeeded in cloning and expression of genes encoding for different parasite proteins. Several studies have used polyclonal and monoclonal antibodies to identify recombinant or partial fusin antigens; including a sporozoite protein of 140000 mol.wt (Dykstra *et al*., 1991), a large glycoprotein (>900000 mol.wt) from sporozoites which appears to be encoded by a single copy gene located on the largest chromosome (Petersen *et al*., 1992), an oocyst wall protein (190 000 mol.wt) with distinctly repetitive amino acid motifs (Ranucci *et al*., 1993) and an antigenic epitope shared by 2 recombinant proteins (15000 and 60000 mol.wt.) from sporozoites (Jenkins *et al*., 1993). Despite the reactivities of most of these antigens with immune sera, antisera, clostral antibodies or monoclonal antibodies, their immunogenic potential remains to be determined. Those which are recognized by hyper immune bovine colostrum may be suitable for the future production of high titer colostrum preparations.

PCR Protocols have proven to be very specific and highly sensitive. Using PCR, 1-10^5 oocysts have been detected. Compared with IF microscopy, the PCR has shown to increase detection sensitivity 10-10^4 fold in environmental and fecal samples in some studies (Hallier-Soulier and Guillot, 2000; Lowery *et al*., 2000). A twostep nested-PCR protocol was used to amplify a fragment of the SSU-rRNA gene of *Cryptosporidium* (~830 bp) and compared with IFA in detection of *Cryptosporidium* in ewes and lambs (Santin *et al*., 2007). The prevalence was significantly higher by PCR (50.8%) compared with IFA (20.6%), (Table 5). Another nested-PCR approach, using COWP gene primers, was adopted on 1 mL negative fecal material inoculated 1, 10 and 100 *C. parvum* oocysts (Kato *et al*., 2003). The limit of detection was recorded to be 100 oocysts. The 75% of DNA extracted samples spiked with 1 and 10 oocysts were positive by PCR (Fig. 6a). Based on this, small sample size using the COWP gene primers with a nested-PCR analysis could reliably identify infected animals. Zhou *et al*. (2007) developed a PCR diagnostic kit for molecular detection of *C. andersoni*. A portion of ITS-1 sequence of *C. andersoni* was chosen as the target DNA for designing the species-specific primers. The results showed that only *C. andersoni* were amplified specific band of about 500 bp, while other *Cryptosporidium* and protozoal species could

Table 5: Prevalence of *Cryptosporidium* spp. in ewes and lambs by nested-PCR and IFA

	No. of animals examined	No. of positives (prevalence)	
		IFA	PCR
Ewes	32	3 (9.4)	8 (25)
Lambs	31	10 (32.25)	24 (77.4)
Total	63	13 (20.6)	32 (50.8)

Values in parenthesis denote percentage, IFA: Immunofluorescence assay, PCR: Polymerase chain reaction

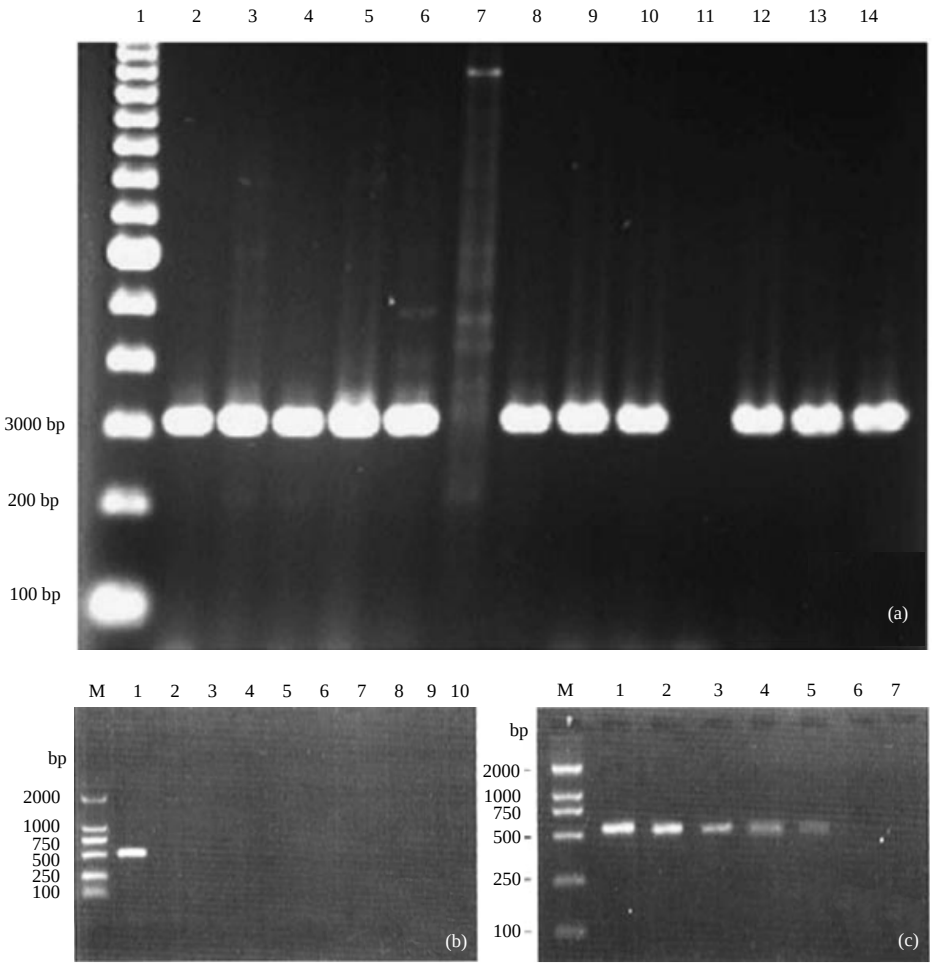

Fig. 6(a-c): (a) Agarose gel electrophoresis of the nested PCR using the COWP gene primers (b) Specificity of the PCR kit, M, DL-2000 DNA marker, lanes 1-10: Control samples, including *C. andersoni*, *C. parvum*, *C. baileyi*, *Eimeria* sp of dairy cattle, *Toxoplasma gondii*, *Eimeria* sp of pig, *Ascaris suum*, *Cyclospora* sp, *E. coli* and (c) Sensitivity of the PCR kit. M, DL-2000 DNA marker, lanes 1-7 represent $2.54' 10^6$, $2.54' 10^5$, $2.54' 10^4$, $2.54' 10^3$, $2.54' 10^2$, $2.54' 10^1$ and 0 oocysts, respectively

Table 6: Comparison of the detection rates of the PCR with that of routine methods

	Modified acid-fast stains			Saturation sucrose float			PCR assay		
Localities in China	Fecal samples	Positive number	Detection rates (%)	Fecal samples	Positive number	Detection rates (%)	Fecal samples	Positive number	Detection rates (%)
Xiangmanlu	48	2	4	48	2	4	48	3	6
Wenshi	86	3	3	86	3	3	86	5	6
Huamei	48	3	3	48	2	4	48	4	8
Huanong	36	2	5	36	2	5	36	3	8
Henan	16	4	25	16	3	18	16	5	31

PCR: Polymerase chain reaction

not be amplified (Fig. 6b). The lowest limit of detection was 254 oocysts (Fig. 6c). The positive rate of the PCR was 2-13% higher than other routine methods as acid-fast staining and sucrose flotation techniques (Table 6).

CONCLUSION

Different diagnostic methods were discussed in the current review article. Clinical examination revealed that diarrhea begins in infected neonates at 3-5 days post-infection and last for 4-17 days. However, histological and ultrastructural examination techniques are costly, time consuming and lack sensitivity because only relatively small pieces of tissues can be examined. The stain of choice for many diagnostic laboratories has been acid-fast staining and safranine-methylene blue. More recently, several immunolabelling techniques using polyclonal or monoclonal antibodies have been developed to detect oocysts. The monoclonal antibody-based Immunofluorescence Assay (IFA) is more sensitive and efficient than the Modified Ziehl-Neelsen (MZN) acid fast staining technique, especially in fecal and water samples containing few oocysts and large amount of debris. We therefore recommended that for routine diagnosis of *Cryptosporidium* infection, fecal specimens should be first screened with the MZN staining method. Negative samples, or incase where the number of oocysts is suspected to be low, IFA method should be used. Recently, genetic characterization using PCR techniques (e.g. PCR-RFLP, PCR-SSCP, RT-PCR, Real Time-PCR) and sequence analysis of genes (e.g. 18SrDNA, COWP, DHFR, TRAP-C1, TRAP-C2, GP60 and HSP70) have been successfully employed to discriminate between *Cryptosporidium* species, genotypes and other protozoan parasites, which in turns helps determine the most likely source of origin and the real risks to human and animal health.

REFERENCES

Abrahamsen, M.S., T.J. Templeton, S. Enomoto, J.E. Abrahante and G. Zhu *et al*., 2004. Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. Science, 304: 441-445.

Anusz, K.Z., P.H. Manson, M.W. Riggs and L.E. Perryman, 1990. Detection of *Cryptosporidium parvum* oocysts in bovine feces by monoclonal antibody capture enzyme-linked immunosorbent assay. J. Clin. Microbiol., 28: 2770-2774.

Baeumner, A.J., M.C. Humiston, R.A. Montagna and R.A. Durst, 2001. Detection of viable oocysts of *Cryptosporidium parvum* following nucleic acid sequence based amplification. Anal. Chem., 73: 1176-1180.

Bialek, R., N. Binder, K. Dietz, A. Joachim, J. Knobloch and U.E. Zelck, 2002. Comparison of fluorescence, antigen and PCR assays to detect *Cryptosporidium parvum* in fecal specimens. Diagn. Microbiol. Infect. Dis., 43: 283-288.

Bonnin, A., T. Petrella, J.F. Dubremetz, J.F. Michiels, D. Puygauthier-Toubas and P. Camerlynck, 1990. Histopathological method for diagnosis of cryptosporidiosis using monoclonal antibodies. Eur. J. Clin. Microbiol. Infect. Dis., 9: 664-666.

Bouzid, M., P.R. Hunter, R.M. Chalmers and K.M. Tyler, 2013. *Cryptosporidium* pathogenicity and virulence. Clin. Microbiol. Rev., 26: 115-134.

Caccio, S., E. Pinter, R. Fantini, I. Mezzaroma and E. Pozio, 2002. Human infection with *Cryptosporidium felis*: Case report and literature review. Emerg. Infect. Dis., 8: 85-86.

Caccio, S., F. Spano and E. Pozio, 2001. Large sequence variation at two microsatellite loci among zoonotic (genotype C) isolates of *Cryptosporidium parvum*. Int. J. Parasitol., 31: 1082-1086.

Caccio, S., W. Homan, K. van Dijk and E. Pozio, 1999. Genetic polymorphism at the β-tubulin locus among human and animal isolates of *Cryptosporidium parvum*. FEMS Microbiol. Lett., 170: 173-179.

Chalmers, R.M., C. Ferguson, S. Caccio, R.B. Gasser and Y.G.A. El-Osta *et al*., 2005. Direct comparison of selected methods for genetic categorisation of *Cryptosporidium parvum* and *Cryptosporidium hominis* species. Int. J. Parasitol., 35: 397-410.

Chan, R., J. Chen, M.K. York, N. Setijono, R.L. Kaplan, F. Graham and H.B. Tanowitz, 2000. Evaluation of a combination rapid immunoassay for detection of *Giardia* and *Cryptosporidium* antigens. J. Clin. Microbiol., 38: 393-394.

Chapman, P.A., B.A. Rush and J. McLauchlin, 1990. An enzyme immunoassay for detecting *Cryptosporidium* in faecal and environmental samples. J. Med. Microbiol., 32: 233-237.

Cole, D.J., N.D. Cohen, K. Snowden and R. Smith, 1998. Prevalence of and risk factors for fecal shedding of *Cryptosporidium parvum* oocysts in horses. J. Am. Vet. Med. Assoc., 213: 1296-1302.

Cook, N., 2003. The use of NASBA for the detection of microbial pathogens in food and environmental samples. J. Microbiol. Methods, 53: 165-174.

Current, W.L. and L.S. Garcia, 1991. Cryptosporidiosis. Clin. Microbiol. Rev., 4: 325-358.

De Graaf, D.C., E. Vanopdenbosch, L.M. Ortega-Mora, H. Abbassi and J.E. Peeters, 1999. A review of the importance of cryptosporidiosis in farm animals. Int. J. Parasitol., 29: 1269-1287.

Dubey, J.P., C.A. Speer and R. Fayer, 1990. Cryptosporidiosis of man and animals. CRC press, USA, ISBN: 9780849364013, Pages: 199.

Dykstra, C.C., B.L. Blagburn and R.R. Tidwell, 1990. Construction of genomic libraries of *Cryptosporidium parvum* and identification of antigen-encoding genes. J. Protozool., 38: 76S-78S.

Enemark, H.L., P. Ahrens, C.D. Juel, E. Petersen and R.F. Petersen *et al*., 2002. Molecular characterization of Danish *Cryptosporidium parvum* isolates. Parasitology, 125: 331-341.

Fayer, R., L. Gasbarre, P. Pasquali, A. Canals, S. Almeria and D. Zarlenga, 1998. *Cryptosporidium parvum* infection in bovine neonates: Dynamic clinical, parasitic and immunologic patterns. Int. J. Parasitol., 28: 49-56.

Fayer, R., U. Morgan and S.J. Upton, 2000. Epidemiology of *Cryptosporidium*: Transmission, detection and identification. Int. J. Parasitol., 30: 1305-1322.

Garcia, L.S., A.C. Shum and D.A. Bruckner, 1992. Evaluation of a new monoclonal antibody combination reagent for direct fluorescence detection of *Giardia* cysts and *Cryptosporidium* oocysts in human fecal specimens. J. Clin. Microbiol., 30: 3255-3257.

Garcia, L.S., R.Y. Shimizu, S. Novak, M. Carroll and F. Chan, 2003. Commercial assay for detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens by rapid solid-phase qualitative immunochromatography. J. Clin. Microbiol., 41: 209-212.

Geurden, T., E. Claerebout, J. Vercruysse and D. Berkvens, 2008. A Bayesian evaluation of four immunological assays for the diagnosis of clinical cryptosporidiosis in calves. Vet. J., 176: 400-402.

Ghazy, A.A., F.A.M. Khalil and H. Soufy, 2004. Studies on equine cryptosporidiosis among farm horses in Egypt. J. Egypt. Vet. Med. Assoc., 64: 201-214.

Hallier-Soulier, S. and E. Guillot, 2000. Detection of cryptosporidia and *Cryptosporidium parvum* oocysts in environmental water samples by immunomagnetic separation-polymerase chain reaction. J. Applied Microbiol., 89: 5-10.

Harp, J.A., D.B. Woodmansee and H.M. Moon, 1990. Resistance of calves to *Cryptosporidium parvum*: Effects of age and previous exposure. Infect. Immun., 58: 2237-2240.

Jenkins, M.C., R. Fayer, M. Tilley and S.J. Upton, 1993. Cloning and expression of a cDNA encoding epitopes shared by 15-and 60-kilodalton proteins of *Cryptosporidium parvum* sporozoites. Infect. Immunity, 61: 2377-2382.

Joachim, A., T. Krull, J. Schwarzkopf and A. Daugschies, 2003. Prevalence and control of bovine cryptosporidiosis in German dairy herds. Vet. Parasitol., 112: 277-288.

Johnson, S.P., M.M. Ballard, M.J. Beach, L. Causer and P.P. Wilkins, 2003. Evaluation of three commercial assays for detection of *Giardia* and *Cryptosporidium* organisms in fecal specimens. J. Clin. Microbiol., 41: 623-626.

Kaminjolo, J.S., A.A. Adesiyun, R. Loregnard and W. Kitson-Piggott, 1993. Prevalence of *Cryptosporidium* oocysts in livestock in Trinidad and Tobago. Vet. Parasitol., 45: 209-213.

Katanik, M.T., S.K. Schneider, J.E. Rosenblatt, G.S. Hall and G.W. Procop, 2001. Evaluation of ColorPAC *Giardia/Cryptosporidium* rapid assay and ProSpecT *Giardia/Cryptosporidium* microplate assay for detection of *Giardia* and *Cryptosporidium* in fecal specimens. J. Clin. Microbiol., 39: 4523-4525.

Kato, S., G. Lindergard and H.O. Mohemmed, 2003. Utility of the *Cryptosporidium* oocyst wall protein (COWP) gene in a nested PCR approach for detection infection in cattle. Vet. Parasitol., 111: 153-159.

Kvac, M., D. Kvetonova, G. Puzova and O. Ditrich, 2003. Comparison of selected diagnostic methods for identification of *Cryptosporidium parvum* and *Cryptosporidium andersoni* in routine examination of faeces. J. Vet. Med. Ser. B, 50: 405-411.

Leav, B.A., M. Mackay, H.D. Ward and D. Acheson, 2003. *Cryptosporidium* species: New insights and old challenges. Clin. Infect. Dis., 36: 903-908.

Lowery, C.J., J.E. Moore, B.C. Millar, D.P. Burke, K.A.J. McCorry, E. Crothers and J.S.G. Dooley, 2000. Detection and speciation of *Cryptosporidium* spp. in environmental water samples by immunomagnetic separation, PCR and endonuclease restriction. J. Med. Microbiol., 49: 779-785.

MacPherson, D.W. and R. McQueen, 1993. Cryptosporidiosis: Multiattribute evaluation of six diagnostic methods. J. Clin. Microbiol., 31: 198-202.

Majewska, A.C., A. Wemer, P. Sulima and T. Luty, 1999. Survey on equine cryptosporidiosis in Poland and the possibility of zoonotic transmission. Ann. Agric. Environ. Med., 6: 161-165.

Mallon, M.E., A. MacLeod, J.M. Wasting, H. Smith and A. Tait, 2003. Multilocus genotyping of *Cryptosporidium parvum* type 2: Population genetics and sub-structuring. Infect. Genet. Evol., 3: 207-218.

McLauchlin, J., C. Amar, S. Pedraza-Diaz and G.L. Nichols, 2000. Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: Results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal samples from livestock animals. J. Clin. Microbiol., 38: 3984-3990.

Mtambo, M.M.A., A.S. Nash, D.A. Blewett and S. Wright, 1992. Comparison of staining and concentration techniques for detection of *Cryptosporidium oocysts* in cat faecal specimens. Vet. Parasitol., 45: 49-57.

O'Donoghue, P.J., 1992. The Cross-Transmission Potential of *Cryptosporidium* spp. In: Zoonoses: Australian Veterinarians in Public Health, Post Graduate Committee in Veterinary Science (Ed.). Post Graduate Committee in Veterinary Science, University of Sydney, Australia, ISBN: 9781875582167, pp: 283-300.

O'Donoghue, P.J., 1995. Cryptosporidium and cryptosporidiosis in man and animals. Int. J. Parasitol., 25: 139-195.

O'Handley, R.M., C. Cockwill, T.A. McAllister, M. Jelinski, D.W. Morck and M.E. Olson, 1999. Duration of naturally acquired giardiosis and cryptosporidiosis in dairy calves and their association with diarrhea. J. Am. Vet. Med. Assoc., 214: 391-396.

Olson, M.E., C.L. Thorlakson, L. Deselliers, D.W. Morck and T.A. McAllister, 1997. *Giardia* and *Cryptosporidium* in Canadian farm animals. Vet. Parasitol., 68: 375-381.

Olson, M.E., R.M. O'Handley, B.J. Ralston, T.A. McAllister and R.C.A. Thompson, 2004. Update on *Cryptosporidium* and *Giardia* infections in cattle. Trends Parasitol., 20: 185-191.

Petersen, C., J. Gut, P.S. Doyle, J.H. Crabb, R.G. Nelson and J.H. Leech, 1992. Characterization of a> 900,000-M(r) *Cryptosporidium parvum* sporozoite glycoprotein recognized by protective hyperimmune bovine colostral immunoglobulin. Infect. Immunity, 60: 5132-5138.

Quilez, J., C. Sanchez-Acebo, A. Clavel, E. del Cacho and F. Lopez-Bernad, 1996a. Comparison of an acid-fast stain and a monoclonal antibody-based immunofluorescence reagent for the detection of *Cryptosporidium* oocysts in faecal specimens from cattle and pigs. Vet. Parasitol., 67: 75-81.

Quilez, J., C. Sanchez-Acedo, E. del Cacho, A. Clavel and A.C. Causape, 1996b. Prevalence of *Cryptosporidium* and *Giardia* infections in cattle in Aragon (northeastern Spain). Vet. Parasitol., 66: 139-146.

Ralston, B.J., C.L. Cockwill, N.J. Guselle, F.H. van Herk, T.A. McAllister and M.E. Olson, 2003. Prevalence of *Giardia* and *Cryptosporidium andersoni* and their effects on performance in feedlot beef cattle. Can. J. Anim. Sci., 83: 153-159.

Ramirez, N.E., L.A. Ward and S. Sreevatsan, 2004. A review of the biology and epidemiology of cryptosporidiosis in humans and animals. Microbes Infect., 6: 773-785.

Ranucci, L., H.M. Muller, G. La Rosa, I. Reckmann and M.A. Morales *et al*., 1993. Characterization and immunolocalization of a *Cryptosporidium* protein containing repeated amino acid motifs. Infect. Immunity, 61: 2347-2356.

Robert, B., A. Ginter, H. Antoine, A. Collard and P. Coppe, 1990. Diagnosis of bovine cryptosporidiosis by an enzyme-linked immunosorbent assay. Vet. Parasitol., 37: 1-8.

Rosenblatt, J.E. and L.M. Sloan, 1993. Evaluation of an enzyme-linked immunosorbent assay for detection of *Cryptosporidium* spp. in stool specimens. J. Clin. Microbiol., 31: 1468-1471.

Rotkiewicz, T., Z. Rotkiewicz, A. Depta and M. Kander, 2001. Effect of *Lactbacillus* and *Bifidobacterium* sp. on the source of *Cryptsporidium parvum* invasion in new born piglets. Bull. Vet. Inst. Pulawy, 45: 187-195.

Santin, M., 2013. Clinical and subclinical infections with *Cryptosporidium* in animals. N. Z. Vet. J., 61: 1-10.

Santin, M., J.M. Trout and R. Fayer, 2007. Prevalence and molecular characterization of *Cryptosporidium* and *Giardia species* and genotypes in sheep in Maryland. Vet. Parasitol., 146: 17-24.

Shaapan, R. and F. Khalil, 2014. Zoonotic Significance of Cryptosporidiosis. LAMBERT Academic Publishing, Saarbrucken, Germany, ISBN-13: 978-3-659-53506-2, Pages: 68.

Shaapan, R., F.A.M. Khalil and M.T.A.E.E. Nadia, 2011. *Cryptosporidiosis* and *Toxoplasmosis* in native quails of Egypt. Res. J. Vet. Sci., 4: 30-36.

Siddons, C.A., P.A. Chapman and B.A. Rush, 1992. Evaluation of an enzyme immunoassay kit for detecting *Cryptosporidium* in faeces and environmental samples. J. Clin. Pathol., 45: 479-482.

Smith, H.V. and J.B. Rose, 1990. Waterborne cryptosporidiosis. Parasitol. Today, 6: 8-12.

Spano, F. and A. Crisanti, 2000. *Cryptosporidium parvum*: The many secrets of a small genome. Int. J. Parasitol., 30: 553-565.

Sterling, C.R., 1990. Waterborne Cryptosporidiosis. In: Cryptosporidiosis of Man and Animals, Dubey, J.P., C.A. Speer and R. Fayer (Eds.). CRC Press, USA., pp: 51-58.

Sturdee, A.P., A.T. Bodley-Tickell, A. Archer and R.M. Chalmers, 2003. Long-term study of *Cryptosporidium* prevalence on a lowland farm in the United Kingdom. Vet. Parasitol., 116: 97-113.

Vesey, G., J.S. Slade, M. Byme, K. Shepherd and C.R. Fricker, 1993a. A new method for the concentration of *Cryptosporidium* oocysts from water. J. Applied Bacteriol., 5: 82-86.

Vesey, G., J.S. Slade, M. Byme, K. Shepherd, P.J. Dennis and C.R. Fricker, 1993b. Routine monitoring of *Cryptosporidium* oocysts in water using flow cytometry. J. Applied Bacteriol., 75: 87-90.

Weber, R., R.T. Bryan and D.D. Juranek, 1992. Improved stool concentration procedure for detection of *Cryptosporidium* oocysts in fecal specimens. J. Clin. Microbiol., 30: 2869-2873.

Weber, R., R.T. Bryan, H.S. Bishop, S.P. Wahlquist, J.J. Sullivan and D.D. Juranek, 1991. Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: Evidence for low sensitivity of current diagnostic methods. J. Clin. Microbiol., 29: 1323-1327.

Xiao, L. and R.P. Herd, 1994. Review of equine *Cryptosporidium* infection. Equine Vet. J., 26: 9-13.

Xiao, L., U.M. Morgan, J. Limor, A. Escalante and M. Arrowood *et al*., 1999. Genetic diversity within *Cryptosporidium* parvum and related *Cryptosporidium* species. Applied Environ. Microbiol., 65: 3386-3391.

Xu, P., G. Widmer, Y. Wang, L.S. Ozaki and J.M. Alves *et al*., 2004. The genome of *Cryptosporidium hominis*. Nature, 431: 1107-1112.

Zhou, R.Q., G.Q. Li, S.M. Xiao and W.H. Li, 2007. Development of a PCR diagnostic kit for *Cryptosporidium andersoni* in dairy cow. Agric. Sci. China, 6: 493-498.