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REVIEW

Recent advances in the diagnosis in livestock of *Cryptosporidium*, *Toxoplasma*, *Giardia* and other protozoa of veterinary importance

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PROTOZOA are ubiquitous organisms and are frequently encountered in domesticated animals. Many animals are host to intestinal protozoa which are commensals of the digestive tract and aid in the breakdown of plant material. Ruminants, for example, have large numbers of ciliate protozoa in the rumen and reticulum; in horses similar organisms are found in the caecum and colon.

Of the parasitic protozoa (Table 1), coccidian parasites belonging to the phylum Apicomplexa can be the most significant in terms of morbidity and mortality, especially in intensively reared animals. Included in this group are members of the genera *Eimeria* and *Isospora* which cause 'coccidiosis' in livestock and companion animals respectively. Animals such as the pig, may be infected with species of both genera. These parasites are characteristically highly host-specific undergoing development within a single host species (Levine 1985). Other protozoa may be less host specific and may be of particular concern where lack of host-specificity may lead to zoonotic transmission and infections in man. Under this category, the most important parasites are *Cryptosporidium*, *Giardia* and *Toxoplasma*. *Cryptosporidium* and *Giardia* are increasingly recognised as significant causes of intestinal disease in animals and man (Taylor 1995). *Toxoplasma* may cause a range of clinical signs but is perhaps of greatest significance as a cause of congenital infection and abortion in pregnant women (Dubey 1986a). Coccidiosis in domestic animals has been extensively reviewed and the reader is therefore referred to several texts (Long 1990, Shirley 1992, Barta and Fernando 1993). For the purposes of this review, the incidence and clinical effects of *Cryptosporidium*, *Giardia* and *Toxoplasma* will be discussed in detail, as will advances in methods of diagnosis. Reference will also be made to other protozoa, including *Balantidium*, *Blastocystis*, *Entamoeba*, *Neospora*, *Pneumocystis* and the microsporidia.

TABLE 1: Parasitic protozoa – classification based on Levine (1985)

Phylum	Genera*	General characteristics
Apicomplexa	*Eimeria* *Isospora* *Cryptosporidium* *Neospora* *Sarcocystis* *Toxoplasma*	Apical complex (visible with electron microscope) generally consisting of polar ring, rhoptries, microneme, conoid; all species parasitic
Sarcomastigophora		
Subphylum **Mastigophora**	*Giardia*	One or more flagella usually present in trophozoites; asexual reproduction longitudinal
Subphylum **Sarcodina**	*Entamoeba*	Pseudopoda or locomotive protoplasmic flow without discrete pseudopodia; asexual reproduction by fission
(subphylum **Blastocysta**)†	*Blastocystis*	
Microspora	*Encephalitozoon* *Enterocytozoon* *Nosema*	Unicellular spores with imperforate wall containing a uninucleate or binucleate sporoplasm and extrusion apparatus with polar tube or cap; obligatory intracellular parasite
Unclassified	*Pneumocystis*	

* Genera of parasitic protozoa referred to in text only
† Jiang and He (1993) suggest that *Blastocystis* should be classified in its own subphylum within the phylum sarcomastigophora

CRYPTOSPORIDIUM

Parasites of the coccidial genus *Cryptosporidium* are small intracellular parasites which occur throughout the animal kingdom and have been reported in many species of mammals, birds, reptiles and fish (Fayer and Ungar 1986). Since the discovery of the organism (Tyzzer 1907), the importance was for a long time overlooked, until the recognition of infection in various species of domesticated animals, especially cattle and sheep, in the 1970s. The taxonomic status of *Cryptosporidium* is still not fully resolved but it is generally accepted that the species that occur in mammals are *C parvum* and *C muris* (Current and Blagburn 1990). *Cryptosporidium wrairi* has also been reported from the guinea pig (Chrisp et al 1992).

Cryptosporidiosis in mammals

Infection in man and domesticated livestock, particularly calves, is usually associated with *C parvum*. Cryptosporidiosis is prevalent in young calves and appears to be age related. Seasonal peaks of disease have been reported to coincide with birth peaks in spring and autumn (Angus 1988). Infection is predominantly seen in young calves less than three weeks old (Fayer and Ungar 1986). The first calves to be born often become infected without showing clinical signs. In these calves the parasite multiplies producing large numbers of oocysts in the faeces which contaminate the environment for calves that follow. Infection spreads rapidly, and later-born calves can become so heavily infected that clinical disease results. Symptoms of profuse watery diarrhoea, abdominal pain and dehydration may be followed by recovery and immunity to further clinical episodes. Disease is often associated with the pres-

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ence of other organisms, notably enterotoxogenic *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens*, rotavirus and coronavirus (Current and Blagburn 1990), all of which may contribute to the neonatal diarrhoea complex (calf scours) although evidence suggests that *C parvum* is a primary pathogen in its own right (Angus 1988). Disease associated with *C parvum* has also been reported in neonates of sheep, goats and deer and in non-domesticated ruminants including antelope and orynx (Gregory 1990). Symptoms in all species included diarrhoea, dehydration and death in a number of cases. In young calves infected with *C parvum*, pathological signs include inflammation and distension of the intestines with enlargement of the mediastinal lymph nodes. Histopathological changes occur mainly in the jejunum and ileum with parasitic stages seen on the surface of epithelial cells lining the jejunum, ileum and caecum and occasionally in severe infections, the colon and rectum (Fayer and Ungar 1986).

Pigs, goats and horses can also be infected. Most porcine cryptosporidial infections are asymptomatic with the majority of infections occurring in six- to 12-week-old pigs (Lindsay and Blagburn 1991). Clinical signs, where they do occur, include diarrhoea and unthriftiness although these are usually associated with the presence of other pathogens such as *Salmonella* spp., *E coli*, adenovirus or *Isospora suis*. Cryptosporidiosis has been reported in immunodeficient foals (Gibson et al 1983) as a cause of diarrhoea. Other findings indicate that apparently immunocompetent horses can develop patent infections and that *Cryptosporidium* can contribute to mortality in the young foal (Coleman et al 1989). There are few reports of *Cryptosporidium* infections in companion animals. Dogs, cats and other pets are occasionally infected but they do not seem to be an important source of infection to other hosts. Given their ubiquitous occurrence, there is a large potential reservoir of infection to man and domesticated animals. A survey in cats in Scotland suggested that infection is common among young and newborn kittens but that the disease was usually asymptomatic (Mtambo et al 1991). As such, cats may be potential carriers of infection for human or other animal hosts. Clinical cryptosporidiosis has been reported in immunosuppressed cats infected with feline leukaemia virus (FeLV) or feline immunodeficiency virus (FIV) (Angus 1988).

Cryptosporidium muris, which was first described in the stomach of mice (Tyzzer 1907), has also been reported from cattle although its significance is unknown (Anderson 1991). As in mice, the predilection site is the glandular stomach (abomasum or ruminant 4th stomach). Parasite stages are found attached predominantly to cells of the gastric gland mucosa (Anderson 1987).

Avian cryptosporidiosis

In birds, two species *C baileyi* and *C meleagridis* have been reported although other species may exist. *Cryptosporidium baileyi* is usually associated with infections of the cloacal bursa, cloaca and respiratory tract in a number of species of birds (Lindsay et al 1986). In infected birds, parasites are on epithelial cells of the cloaca and Bursa of Fabricius (the site of B cell maturation in the bird), and occasionally the caecae and trachea. Heavy infections produce epithelial hyperplasia and hypertrophy, particularly of the bursa, and the ensuing inflammatory exudate occasionally produces bursal casts. *Cryptosporidium meleagridis* is thought to be the species associated with gut-related infections in turkeys (Goodwin et al 1988). The avian cryptosporidia appear to have a high host specificity for birds. However, a report of infection with *C baileyi* in an immunosuppressed human patient (Ditrich et al 1991) suggests that under certain circumstances cross-transmission to mammalian hosts may occur.

Epidemiology of cryptosporidiosis

In many instances where *Cryptosporidium* is diagnosed, it appears that infections usually originate from the same host species. In animals, the primary route of infection is likely to be the direct animal-to-animal faecal-oral route. Thus, in calves for example, overcrowding, stress of early weaning, transport and marketing, together with low levels of hygiene will increase the risk of clinical infections. In lambs, chilling due to adverse weather conditions in the neonatal period, intercurrent infections or nutritional or mineral deficiencies could exacerbate or increase the likelihood of disease. Infection in these cases is likely to occur through grooming, nuzzling, coprophagy, faecal soiling by direct contact with infected animals, or indirectly through consumption of contaminated foods or environmental sources including pasture and water (Taylor 1995). Heavy infections can lead to high levels of environmental contamination which influences the rate of infection in susceptible hosts. *Cryptosporidium muris* has been isolated from wild mice in the UK (Chalmers et al 1994) but the role of rodents in transmission of cryptosporidiosis is still unclear.

In man, person-to-person transmission is now recognised to be common, thus indicating that cryptosporidiosis is not necessarily a zoonosis (Casemore 1990). Zoonotic transmission has been reported from calves and lambs particularly following educational visits to farms. Companion animals such as cats and dogs have also been implicated in human disease. In Australia, Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) has demonstrated geographical differences in strain isolates from different hosts which suggests local endemnicity and zoonotic transmission (Morgan et al 1995). Polymorphic areas have been identified in the 18S rRNA and the internal transcribed spacer 1 regions (Carraway et al 1996). Bonnin and colleagues (1996) have used a repetitive DNA element to genotype human and bovine isolates of *C parvum*. Isoenzyme analysis in the UK, has shown differences in human isolates and animal isolates (MacDonald and Awad El-Kariem 1995). It is not clear at this stage whether this represents isolate variation in cross-species transmission between Australian or UK isolates, or just differences in the strain typing methods.

Diagnosis

The infective stage of *Cryptosporidium parvum* is an oocyst, 4-6 μm in diameter, which is excreted in the faeces of the host animal. Current laboratory methods of diagnosis of cryptosporidiosis rely on the direct examination of faecal smears, or faecal concentration and subsequent microscopic examination. Several strains have been used to differentiate oocysts from surrounding debris, including acid-fast stains, Giemsa, methenamine silver, period acid shift (PAS) and others. These methods, which are summarised in Table 2, are generally considered to be adequate for detection of *Cryptosporidium* oocysts in samples from acute infections where many oocysts are present. Research has shown that the detection limits using such techniques may be as high

TABLE 2: Summary of laboratory methods available for detection of *Cryptosporidium*

Technique	Method(s)	Sensitivity (oocysts/g)	Speciation	Use
Smear/stains	Ziehl Neelson (ZN)	>100,000	Based on morphology	Clinical diagnosis
	Auramine Phenol (AP)	>50,000		
Concentration	Flotation – NaCl – Sucrose Formalin-ethyl acetate	>500,000	Based on morphology	Clinical diagnosis
	Flotation + stain (eg sucrose + AP)	>5000	Based on morphology	Clinical diagnosis
	Cartridge/membrane filtration	N/A	Based on morphology	Monitoring of water and environmental samples
Immunological	IFA	>50,000	Based on morphology	Clinical diagnosis
	ELISA	>50,000	Not possible	Clinical diagnosis
	Sample concentration and immunofluorescence	>5000	Based on morphology	Clinical diagnosis
	Flow cytometry	N/A	Based on morphology	Monitoring of water and environmental samples; tracing asymptomatic carriers
Biophysical biochemical techniques	Isoenzyme analysis	N/A	Based on banding patterns	Isolate characterisation
	Two-dimensional electrophoresis	N/A	Based on banding patterns	Isolate characterisation
DNA based techniques	PCR	<100	Specific probes	Monitoring of water and environmental samples; tracing asymptomatic carriers. Isolate characterisation
	RFLP analysis	N/A	Based on banding patterns	Isolate characterisation
	RAPD-PCR	N/A	Based on banding patterns	Isolate characterisation
	DNA sequencing	N/A	Based on analysis of sequence data	Isolate characterisation

Abbreviations: IFA – Immunofluorescence assay; ELISA – Enzyme-linked immunosorbent assay; PCR – polymerase chain reaction; RFLP – Restriction fragment length polymorphism; RAPD-PCR – Random Amplified Polymorphic DNA-Polymerase Chain Reaction; N/A – not applicable

as 500,000 oocysts per gram of faeces (Weber et al 1991).

In human diarrhoeal cases, a further problem faced is the microscopic misidentification of *Cryptosporidium* as *Cyclospora* (Wurtz 1994). Both organisms stain similarly using acid fast stains. *Cyclospora* cysts (8-10 µm in diameter, two sporocysts each with two sporozoites) are slightly larger than *C parvum* oocysts (4-6 µm, four sporozoites without sporocysts), but very similar in size to *C muris* (7·4 µm x 5-6 µm). The internal structures are not clearly visible without the use of electron microscopy. It is important to obtain a differential diagnosis as there is currently no effective treatment for cryptosporidiosis but trimethoprim/sulfamethoxazole has proved useful in treating *Cyclospora* diarrhoea in man (Colley 1996). The incidence of *Cyclospora* in domestic animals is unknown although it has been reported in insectivorous mammals (Ford and Duszynski 1988).

Recently, research effort has concentrated on the elucidation of the molecular composition of *Cryptosporidium*. Electrophoretically distinct patterns of oocyst wall and sporozoite antigens have been constructed for *C parvum*, *C muris* and *C baileyi* (Nina et al 1992). Monoclonal antibodies (mAbs) have been produced which are specific to some of these polypeptides and such mAbs have the ability to distinguish sporozoites of *C parvum*, which is potentially the most pathogenic species to mammals, from other cryptosporidia. Monoclonal antibodies have become the basis for alternative detection methods which involve the use of immunofluorescence (IF) or enzyme-linked immunosorbent assays (ELISA) (Rusnak et al 1989). Commercially available ELISA and IF kits, based upon genus specific mAbs to oocyst surface proteins have allowed the transfer of these techniques to diagnostic laboratories. Although, mAbs to genus-specific oocyst surface proteins are available, at present there are no mAbs that can be used to discriminate between species of intact oocysts. The detection limits of ELISAs and IF tests are similar to those stated above for the examination of stained faecal smears and depend primarily upon the concentration methods employed to prepare the sample (Weber et al 1991, Webster et al 1998).

As oocysts can be identified and species discriminated on the basis of protein composition, it is not too surprising that DNA-based techniques allow similar conclusions to be drawn. The polymerase chain reaction (PCR) is an obvious choice for a sensitive and specific diagnostic test. When coupled with non-radioactive detection techniques which are now readily available, the transfer of sophisticated technology to the diagnostic laboratory becomes possible. The results of a number of PCRs have been published (for example Laxer et al 1991, Webster et al 1993). They are based upon the amplification and subsequent detection of *Cryptosporidium* specific DNA. The latter authors introduced a differential probe which also allows discrimination between species. Currently only the first steps in diagnostic protocol development have been investigated. Before such techniques are adopted they require rigorous testing and comparison to orthodox methodologies. They do however, offer the advantage of increased sensitivity, with detection

limits in the order of 80-90 oocysts per gram, when used in conjunction with immuno-magnetic separation (IMS) (Webster et al 1996). Improved sensitivity, as offered by PCR, has implications for both epidemiological and clinical studies where the diagnosis of early or asymptomatic infection is important. It would assist the tracing of outbreaks, being applicable to the analysis of environmental samples and the identification of asymptomatic excretors and their potential role in the transmission of infection and disease.

GIARDIOSIS

Giardia infections are frequently overlooked in domesticated animals despite being isolated from a variety of mammalian, avian, reptilian, amphibian and fish hosts. The host specificity of *Giardia* is still undecided and as such the zoonotic potential of animal infection is the subject of much research. For a long time is was considered that *Giardia* spp. were host specific and this resulted in the description of over 40 species based on the host species in which they were found. This assumption is not universally accepted, however, and current thought is that there are just three structural types or species (Thompson et al 1993). The species affecting man and the majority of domesticated animals, is referred to as *Giardia duodenalis* although even here there are generic and specific variations with *G intestinalis*, *G lamblia* and *Lamblia intestinalis* being synonymous. The other morphologically different species are *G muris* identified in rodents, birds and reptiles and *G agilis* from amphibians.

Biochemical and immunological differences in *Giardia* isolated from humans suggest that there is a great deal of heterogeneity within these strains, whilst at the same time relatively close relationships exist between certain human and animal strains (Thompson et al 1993). Recent studies by Ruest et al (1995) described high levels (15-32 per cent) of natural prevalence amongst cattle with the highest level during the winter months. Additionally, this group produced an experimental infection in a calf with trophozoites of the WB laboratory strain (human origin). The calf developed intermittent diarrhoea after a five-day prepatent period.

Giardia are flagellate protozoans normally found adherent to epithelial surfaces of the small intestine especially the middle to lower areas of the villi. The life cycle is simple and direct, the trophozoite stage dividing by binary fission to produce further trophozoites. Species variation exists in the size of the trophozoite, and the shape and size of the median bodies. Intermittently, trophozoites encyst forming resistant cyst stages that pass out in the faeces of the host (Adam 1991).

Clinical signs

Giardia infections in many species of animals are often asymptomatic. When disease does occur, the signs often include chronic, pasty diarrhoea, weight loss, lethargy and failure to thrive. The diarrhoea may be continuous or intermittent. The existence of *Giardia* in domestic animals has been known for many years but during that time little information has become available on prevalence, pathogenicity and the disease caused by these parasites in their hosts. *Giardia* has been reported in cattle, sheep, goats, horses, dogs, cats, rodents and psittacines. Based on limited investigations, the incidence of these parasites varies but can be assumed to be higher in some species than has been reported. Studies in Canada (Buret et al 1990) indicate infections in sheep and cattle of 18 per cent and 10 per cent, respectively. Taylor et al (1993) reported that nearly 70 per cent of a flock of lambs were infected with *Giardia* although the presence of the organism was not necessarily associated with clinical signs of disease. Xiao et al (1993) reported a 100 per cent infection rate in young diarrhoeic calves on two farms. A survey of dogs attending a charity animal hospital in London (Sykes and Fox 1989) showed an overall prevalence of infection of 15 per cent with the highest prevalence in dogs less than 12 months of age (30 per cent). In aviary birds, *Giardia* is frequently encountered in psittacines such as cockatiels, budgerigars and lovebirds causing enteropathy, weight loss and death (Fudge and McEntee 1986).

Epidemiology

Limited epidemiological studies suggest that direct animal-to-animal contact and faecal soiling is the most likely method of transmission although water contamination can also be considered as a possible route. Human infection in the USA has been reported from drinking water contaminated with *Giardia* thought to have originated from beavers (Dykes et al 1980). Other wild animals may act as reservoirs of infection. As reported earlier, an infection was established with a human isolate in a calf (Ruest et al 1995). The role of farm animals in the overall epidemiology of human giardiosis has yet to be investigated.

Diagnosis

Giardia cysts can be detected in faeces by a number of methods. Traditional methods of identification of giardiosis involve direct examination of faecal smears, or faecal concentration by formalin-ethyl acetate or zinc sulphate methods and subsequent microscopic examination (Adam 1991). It is generally recommended that three consecutive samples are examined as cysts are excreted intermittently (Kirkpatrick 1989), and a single sample identifies only 50-75 per cent of positive cases (Heymans et al 1987, Adam 1991). Recent developments include the use of fluorogenic dyes such as fluorescein diacetate, which is taken up by viable cysts, and propidium iodide which is taken up by non-viable cysts (Smith and Smith 1989).

A number of immunological methods have been evaluated with the result that a number of immunoassays have become commercially available. These either involve the identification of cysts by IF (Sterling et al 1988) or detection of *Giardia* specific antigen by ELISA (Green et al 1985, Nash et al 1987, Ungar et al 1984). Barr et al (1992) evaluated the procedures used for the diagnosis of *Giardia* in dogs. They found that a commercially available ELISA gave 10 per cent false-negative and 13 per cent false-positive results compared with conventional zinc sulphate faecal concentration. The use of a per-oral string test was abandoned following practical and safety problems with implementation of the test. The use of the polymerase chain reaction (PCR) to amplify target DNA has produced a sensitive and specific assay for *Giardia* based on the detection of the giardin gene (Butcher and Farthing 1989). In addition, the PCR technique is reported to be able to distinguish between pathogenic and non-pathogenic *Giardia* strains (Mahbubani et al 1992) and between live and dead cysts by measuring PCR product from giardin mRNA (Mahbubani et al 1991).

TOXOPLASMA

Toxoplasma gondii is an obligate intracellular parasite with a typical apicomplexan life cycle in which gametogony and oocyst production occur in domestic and wild cats (Fayer 1981). The parasite differs from other coccidia however, in that it shows a complete lack of specificity for hosts and tissues during its asexual phase and can infect a wide range of animals including man. The parasite has world-wide distribution and is of both medical and veterinary importance. In humans, toxoplasmosis is usually asymptomatic or associated with mild symptoms only, often characterised by lymphadenopathy involving the posterior cervical lymph nodes. In immunocompromised individuals (eg AIDS patients) *T gondii* infections can be severe with cerebral involvement (Johnson 1990). In pregnant women infection can result in miscarriage or congenital malformations of the child.

In animals, clinical signs are not usually seen in the cat but can be severe in intermediate hosts. In sheep, abortion, stillbirth or birth of weak lambs may occur depending on when infection of the pregnant ewes occurs. Infection in mid pregnancy (60 to 90 days) usually results in abortion (Buxton 1989). Ewes infected during the later stages of pregnancy (after 110 days), can lead to stillborn or weak lambs at full term.

Humans can acquire toxoplasmosis by ingestion of sporulated oocysts or viable tissue cysts in meat from infected animals. Encysted *T gondii* are killed at temperatures above 60°C (Dubey et al 1990a), but eating raw or undercooked meat constitutes a risk (Fayer 1981). Among meat-producing animals, pigs, sheep and goats commonly harbour tissue cysts, whereas cattle appear to be relatively resistant and clear of infection. As such beef is not generally regarded as an important source of human infection (Fayer 1981). Milk from acutely infected animals has also been implicated as a source of infection (Reimann et al 1975). Occupational risk of infection may include certain farming practices, and pregnant women and other people in special risk groups should avoid contact with sheep at lambing time (Buxton 1989).

Diagnosis

Clinical signs and pathological changes in placenta and aborted foetuses are not pathognomonic for toxoplasmosis and detection of *Toxoplasma* tachyzoites by histological methods is not always easy, especially in autolysed material. Impression smears stained with Giemsa or Romanowsky (Dubey 1977) or Periodic Acid Schiff (PAS) (Dubey 1986b) may allow identification of tachyzoites or tissue cysts. Diagnosis may be confirmed by isolation of viable parasites by mouse inoculation or by demonstration of parasite stages by immunohistochemical methods.

Serological tests include the Sabin and Feldman dye test (DT) (Sabin and Feldman 1948), complement fixation test (CFT) (Sabin 1949), indirect fluorescent antibody test (IFAT) (Goldman 1957), enzyme linked immunosorbent assay (ELISA) (Uggla et al 1990), and by commercially available indirect haemagglutination test (IHA) and latex agglutination test (LAT). Detection of high antibody titres to *T gondii* at the time of abortion indicates a recent infection as a likely cause. However, since antibody titres may persist for months or even years, a firm diagnosis cannot be made on this basis only, especially in individual animals (Blewett et al 1983). Recent studies by Dubey and colleagues (1995) have compared a number of serological methods for the detection of *T gondii* infection in naturally infected sows. The modified agglutination test (MAT) appeared to be the most useful test with a sensitivity and specificity of 82·9 per cent and 90·3 per cent respectively. This compared with 29·4 and 98·3 per cent for IHA; 45·9 and 96·9 per cent for LAT and 85·9 per cent for ELISA. Similarly an ELISA, IFAT and MAT were compared for the detection of antibodies to *T gondii* in ovine foetal fluids. Eighteen per cent of samples were positive by MAT compared with 15 per cent by ELISA and 16 per cent by IFAT. The MAT was preferred because it is simple to perform and was more effective than IFAT for the detection of antibodies in autolysed foetuses (Seefeldt et al 1989). Trees and colleagues (1988) also compared a LAT with the methods described above for the detection of antibodies to *T gondii* in ovine foetal fluids and found it be very specific (ie it never gave a positive reaction without at least one of the other methods also being positive). However, this group concluded that IFAT was the single most reliable method. Recently diagnostic methods based on detection of *T gondii* specific DNA by PCR have been developed. These have been based upon the p30 gene and can detect a single organism in the presence of a million host cells (Savva and Holliman 1990). This PCR has also been applied to tissues from sheep (brain, amnion/chorion, peritoneal fluid and cotyledons) (Wheeler et al 1990). Other PCRs have been developed based upon sequences of ribosomal DNA (Guay et al 1993) and the B1 gene (Joss et al 1993). Wastling et al (1993) compared the p30 and the B1 PCRs and found that the B1 PCR was more sensitive, gave fewer false-negatives and the results correlated closely with conventional mouse inoculation methods. The few false-positive results recorded are thought to be due to the presence of non-viable parasites.

Greig and colleagues (1993) assessed methods for *Toxoplasma* diagnosis in sheep. They compared detection of antibodies in foetal fluids and histopathology on foetal tissues with PCR. While antibodies and histopathology showed 98 per cent agreement, the PCR agreed with traditional methods in only 68 per cent (34/54) of cases. In 14 of the discordant cases the PCR alone was positive, but in five cases the PCR was negative where the other two tests were positive.

NEOSPORA

Neospora is a *Toxoplasma*-like protozoan which was first reported from Norway in 1984 as a cause of encephalomyelitis and myositis in dogs. The parasite was morphologically similar to *T gondii* but *T gondii*-specific antibodies were not detected in the sera of infected dogs. A similar parasite found in the central nervous system of dogs in the USA in 1988, was named *Neospora caninum* (Dubey et al 1988a). Prior to 1988, it is likely that *N caninum* was misdiagnosed as *T gondii* because of their structural similarities (Dubey 1992). However, antigenic differences detectable by immunohistochemistry and serology, the thickness of the cyst wall, and the number of rhoptries in the tachyzoites enable these parasites to be distinguished at the generic levels (Dubey et al 1988b, Barr et al 1991a).

Later studies have shown that a variety of mammals including cattle, sheep, dogs, cats, rodents and primates can be experimentally infected with *N caninum* and neosporosis is found naturally in cattle, goats, sheep and horses. Neosporosis is a major cause of abortion in dairy cattle,

particularly in California, where since 1985 12-24 per cent of abortions have been due to *N caninum* (Anderson et al 1991, Barr et al 1991b). *Neospora caninum* has been recorded in 11/120 Scottish cattle which had recently aborted, but only in 1/97 cattle from herds in which there had been no recent abortions (Trees et al 1994). In sheep, Otter and colleagues (1997b) examined foetal tissues using histopathology and foetal fluids using IFAT for antibodies to *Neospora*. Non-suppurative myocarditis and encephalitis were present in 0·3 per cent (9/281) aborted lambs. Immunocytochemistry using antisera against *Neospora* and *Sarcocystis* resulted in no labelling, but anti-*T gondii* sera showed labelled organisms in four lambs. This suggests that neospora infection is not associated with significant numbers of ovine abortions in England and Wales. The complete life cycle of the parasite has yet to be elucidated. Only asexual stages of the life cycle (bradyzoites, tissue cysts and tachyzoites) have been described to date (Speer and Dubey 1989). The only recorded method of transmission is transplacental (Dubey et al 1990b) although it is not inconceivable that transmission may occur via ingestion.

Diagnosis

Diagnosis of *N caninum* induced abortion or congenital neosporosis is currently based on the identification of characteristic non-suppurative inflammation by histology, the detection of endozoites or cysts in tissues of infected host animals and the use of serological methods for the detection of specific antibodies in foetal fluids or maternal sera. Characteristic features may be identified using histology, but levels of infection are usually so low that detection of parasites in tissues may only be possible using immunohistochemical methods (Lindsay and Dubey 1989a) or by culture of the parasite in mice or tissue cultures (Lindsay and Dubey 1990). An indirect fluorescent assay (IFA) has been used to detect antibodies against trachyzoites in dogs (Dubey et al 1988b). This has been modified with bovine serum and *Neospora* tachyzoites from cattle (Barr et al 1993). More recently, a number of ELISA tests have been developed for the serological diagnosis of *Neospora* infections in cattle. An ELISA described by Paré et al (1995) had a sensitivity and specificity of 88·6 per cent and 96·5 per cent respectively compared with immunohistochemical diagnosis. Bjorkman et al (1994) reported 97·6 per cent sensitivity and 95·6 per cent specificity, using IFAT as the standard. Williams and colleagues (1997) reported 96 per cent specificity and 95 per cent sensitivity respectively using an assay which has now been modified and is available commercially.

Otter and colleagues (1995) reported non-suppurative inflammation of the brain and/or myocardium and placental cotyledons, identified by light microscopy in 10·5 per cent of aborted or stillborn foetuses from England and Wales. The diagnosis was confirmed by specific *N caninum* immunocytochemistry in 4·2 per cent of cases. Similarly McNamee and colleagues (1996) in Ireland found 6·3 per cent positive by histology and confirmed 4·2 per cent by immunocytochemistry. When maternal serology (IFAT) was used to assess exposure to *Neospora* a prevalence of 12·6 per cent positives was found in aborting cows compared to 3 per cent in age matched normally calving controls. Otter et al (1997a) in a study of over 250 cows concluded that IFATs on serum from aborting cows and on pleural fluid from aborted foetuses are useful diagnostic methods for *Neospora* but cautioned that preliminary investigations of abortions in a herd must include histological examination of aborted foetuses.

Marsh et al (1995) analysed the nuclear small subunit ribosomal RNA (nss-rRNA) from five bovine *Neospora* spp. isolates, two *N caninum* isolates and three *Toxoplasma gondii* isolates to each other and to other sequences available on Genbank (*Sarcocystis*). There were no nucleotide differences detected between the *Neospora* spp. isolates from cattle and dogs. This has subsequently been confirmed by sequencing of the 16S-like region and the internal transcribed spacer 1 regions of the ribosomal RNA of canine and bovine isolates, which were identical (Stenlund et al 1997). However, based on immunohistochemical staining, there may be antigenic differences between *Neospora* parasites found in the tissues from naturally infected bovine foetuses and *N caninum* parasites in canine or murine tissues (Barr et al 1991a). Tissue cysts from an infected calf reacted positively to anti-*N caninum* sera, but unlike *N caninum* they were positive to two out of four sera to *T gondii* and to antisera from *Hammondia hammondi*. The authors suggested that the differences in antigenic reactivity may reflect variations of a single organism dependent on factors such as host species infected. However, this study remains to be confirmed. Four nucleotide differences were consistently detected when sequences of *Neospora* spp. isolates were compared with *T gondii* isolates (Marsh et al 1995). These results indicate that *Neospora* and *Toxoplasma* are closely related but distinct species and provide the basis for sensitive differential PCR methods. Holmdahl and Mattsson (1996) described such a method which can specifically detect five organisms. Other protocols include those developed by Muller and colleagues (1996), Yamage et al (1996), Lally et al (1996) and Payne and Ellis (1996). Such methods offer exquisite sensitivity, however, as yet PCR remains a tool for research.

BALANTIDIUM

Balantidium coli is the only ciliate protozoan of known veterinary importance and occurs in pigs, man and other primates; occasionally in the dog, rat and ruminants. The pig appears to be the primary host, and in it *B coli* is generally regarded as a commensal of the large intestine where it lives on starch, ingesta and bacteria (Levine 1985). Occasionally, it may invade the mucosa and cause ulceration and mild to severe diarrhoea (Georgi and Georgi 1990). In man, *B coli* produces superficial to deep ulcers associated with dysentery. Human infection is a zoonosis acquired from pigs through faecal contamination. The cyst form is the source of infection and these can remain viable for days or weeks in moist pig faeces. *Balantidium* infection in monkeys and primates is normally an endemic infection maintained by the animals themselves.

Diagnosis

Balantidium can be readily identified in wet mount preparations stained with iodine in which they appear orange with black starch granules. Fresh faecal smears can be stained with trichrome or iron haematoxylin. Cultural isolation is not usually necessary although the organism has been successfully cultured in gastrin mucin media (Klaas 1974).

BLASTOCYSTIS

Blastocystis was for many years described as a yeast but is now considered to be a protozoan in the subphylum Blastocysta (Zierdt 1988). The organism is found in the intestinal tract of man and in many animals including monkeys, pigs, birds, rodents, snakes and invertebrates. Infection is generally asymptomatic but it is becoming increasingly associated with gastrointestinal disease in man (Zierdt 1991).

Reports of the prevalence of *Blastocystis* are very variable. Studies in man show varying levels of infection with *B hominis* in both healthy patients, and patients with diarrhoea (Kukoschke and Muller 1991, Martin-Sanchez et al 1992, Nimri 1993).

Blastocystis has been reported in a wide range of animals. McClure et al (1980) reported the presence of *B hominis* in a pig tailed macaque, and Yamada et al (1987) described *Blastocystis* spp. in monkeys. Pakandl (1991) describes the presence of the parasite in pigs, and notes that it occurs at quite high prevalence throughout life. The pathogenicity of *Blastocystis* in pigs is uncertain. Cross transmission studies have indicated a low host specificity for *Blastocystis* (Pakandl 1991), and it is not unlikely that the parasite will be found in other farm animals.

The organism has also been reported in chickens (Belova and Kostenko 1990, Yamada et al 1987), ducks (Pakandl and Pecka 1992), partridge and pheasants (Taylor et al 1996) and reptiles (Teow et al 1992).

In faecal samples, *B hominis* is brightly refractile, of widely variable diameter with a thin band of peripheral cytoplasm surrounding a central body. In severe infections an amoeboid form may be seen (Zierdt 1991).

Diagnosis

Diagnosis is based on microscopic identification of characteristic refractile, pleomorphic forms using either bright field optics or by differential interface contrast optics. Faecal smears can be heat fixed or gluteraldehyde fixed and stained with Giemsa, trichrome or iron haematoxylin. The organism can be successfully cultured in Boeck Drbohlav's, Dobell and Laidlaw's Medium or Robinson's Medium (Taylor et al 1996). Limited immunological methods of diagnosis have been developed. Zierdt (1991) described an immunofluorescent staining method using rabbit antiserum raised to whole *B hominis* antigen.

ENTAMOEBA

Entamoeba hystolytica is usually associated with tropical dysentery with blood or mucoid diarrhoea in humans. The parasite has been recorded in cattle, pigs, dogs, cats, monkeys as well as man (Levine 1985). The life cycle is similar to *Giardia* with trophozoites dividing by binary fission. Transmission occurs when the trophozoites round up, become smaller and form a cyst. Two forms of the parasite exist. Non-pathogenic forms of the organism normally live in the lumen of the large intestine. Pathogenic forms invade the mucosa causing ulceration and dysentery. From there they may be carried via the portal system to the liver and other organs where large abscesses may form. Isoenzyme markers can be used to differentiate the two forms, but there is some debate as to whether the two types represent different species or if they can change from one type to another under certain circumstances (Bruckner 1992).

Diagnosis

Trophozoites and cysts can be stained by methods described for *Balantidium*. The organisms can also be cultured in a number of media including Boeck and Drbohlav's, Dobell and Laidlaw's, TYI-S-33 (Diamond et al 1982) and Robinson's medium (Diamond 1987). A number of serological tests have been evaluated for the diagnosis of *E histolytica* infections. These include ELISA (Nogami et al 1991); latex agglutination, complement fixation (Lotter et al 1993) and indirect haemagglutination (Boisseau et al 1992). A number of PCRs have also been used to detect *E histolytica* in clinical samples (Romera et al 1992, Cheng et al 1993, Tachibana et al 1992, Acuna-Soto et al 1993). The PCRs are based upon the amplification of specific DNA sequences which correlate to the pathogenic/non-pathogenic isoenzyme analysis categorisation and appear to be very sensitive and specific.

PNEUMOCYSTIS

Pneumocystis carinii is an obligate intracellular protozoan parasite found in the lungs of infected hosts. Two major forms of *P carinii* have been consistently identified from histological and ultrastructural analysis of organisms found in human and rat lung. These are a trophic form and a larger cyst stage containing sporozoites (Yoshida 1989). Asexual and sexual reproductive stages have been identified. The organism is presumed to be transmitted by aerosol.

The organism has been reported from a range of animals. In Denmark, examination of lungs from carcases selected randomly in an abattoir detected *P carinii* pneumocysts in 3·8 per cent of calves, 3·6 per cent of sheep and 6·7 per cent of pigs (Settnes and Henriksen 1989). In Slovakia, three of 25 three-month-old calves were found to be infected with *P carinii* (Pavlina et al 1986). Studies in Japan detected *P carinii* in a wide range of animals including cattle (Shimizu et al 1985). The organism has been reported to have caused pneumonia in weaning pigs (Kondo et al 1993).

Human infection is usually seen in immunosuppressed individuals, for example those suffering from neoplasias, hypogammaglobulinaemia, or malnutrition, and patients with AIDS are high susceptible to this infection. Yinnon and Betts (1994) detected *P carinii* pneumonia in 11·3 per cent of AIDS patients with concomitant infections admitted to the medical centre in Jerusalem between 1980 and 1990.

Diagnosis

Toluidine blue (TBO) is the most effective for cyst stages while Giemsa stains are used to show trophozoites. Tissue sections are stained with methenamine silver (MS) (Latorre et al 1977). There are reports that the organism can be grown in cell culture. Bartlett et al (1985) used a monolayer of WI 38 diploid cells; Latorre et al (1977) described growth in three cell lines, Vero, Chang liver and MRC5 with an overlay of Eagle MEM with 10 per cent calf serum. Mirovsky and Fishman (1993) maintained *P carinii* in a culture of human lung fibroblasts with Eagle MEM for up to 42 days. Axenic culture methods have been described using RPMI-1640 (Le Marchand-Affret et al 1991). However, in vitro cultivation, especially from clinical samples is not always successful. A number of PCRs have been reported which amplify specific regions of DNA from *P carinii* and are approximately 100 times more sensitive than conven-

tional TBO staining (Honda et al 1994, Ishimine et al 1994). Elvin (1994) compared indirect immunofluorescence with the most commonly used chemical stains TBO and SM.

The use of (mAbs) 36F detected both cyst and trophozoite stages of *P carinii* and was more sensitive than the chemical stains when applied to clinical samples. However, a number of other commercially available mAbs stained only a proportion of cysts and no trophozoites. ELISA has been applied to the characterisation of the immune response to *P carinii* (Lundgren et al 1993) and also applied to survey birds in Denmark for the presence of *P carinii* (Settnes et al 1994). Specific antibodies to a 116 kDa antigen of rat pneumocysts were demonstrated in 13 per cent of chicken sera examined. The presence of *P carinii* could not be demonstrated from lung tissue by TBO staining or PCR using the dihydrofolate reductase gene as a specific probe. This would suggest that *P carinii* does not normally reside in the pulmonary tissue of birds, although they may be exposed to external sources.

MICROSPORIDIA

Microsporidia are obligate intracellular parasites which have been found in every major group of animals, and have been reported in man, dogs, guinea pigs, rabbits, hamsters, and a range of wildlife (Shadduck and Parkes, 1971, Canning and Hollister 1987). The infective stages are resistant spores which can survive in moist, cool conditions for one or more years. The clinical conditions caused by *Microsporidia* vary widely, some cause gastrointestinal disturbances, others cause systemic disease, nephritis and ascites, and others affect the nervous system. The route by which spores are released varies similarly.

In humans, *Microsporidia* have been found particularly in patients with immunosuppression such as AIDS patients. Recent evaluation of intestinal biopsies from patients with AIDS enteropathy showed 30 per cent to carry microsporidiosis (Sun 1993). As medical practitioners become aware of microsporidia they are increasingly reported, and the incidence is now up to 27 per cent in AIDS patients (Sun 1993). As new species of *Microsporidia* are identified in AIDS patients, the origin of these organisms is still in doubt. It remains uncertain whether these are previously unrecognised natural parasites of man, or zoonotic infections from another mammal (Canning and Hollister 1987). Serological evidence suggests that latent infections may be quite common in man (Canning and Hollister 1987). Singh et al (1982) investigated the presence of antibodies to *Nosema cuniculi* in human and animal sera. Normal blood donors in Singapore did not have detectable antibody titres, but six out of 69 apparently normal Caucasians had a low titre of antibodies. They also detected high titres of antibodies in laboratory rabbits, and significant titres in 38 of 40 sheep. In a field trial in New Zealand, mob stocking of pastures by sheep was found to increase the number of microsporidial infections in the flock (Stewart and Archibald 1987).

Diagnosis

A number of staining techniques have been described for microsporidia. Ryan et al (1993) describe a modified trichrome staining method. Giang et al (1993) used a modification of the standard one-step trichrome staining using a 10-fold concentration of chromotrope 2R. Van Gool et al (1990) used a rapid fluorescence technique using Uvitex 2B. *Encephalitozoan cuniculi* has been cultured in monolayer culture of rabbit choroid plexus cells. More recent work has shown that numerous cell lines will support the growth of *E cuniculi*. Madin Derby Canine Kidney (MDCK) cells have been shown to be particularly successful (Canning and Hollister 1991). Aldras et al (1994) have described the detection of formalin fixed tissue culture derived *E cuniculi*, and *E hellem* and two other human microsporidia, *Enterocytozoon bienusi* and *Septata intestinalis* in formalin fixed faecal and urine samples respectively. Canning and Hollister (1987) described an ELISA for detection of antibodies to *E cuniculi* infection. Antigen was produced from spores derived from tissue culture.

CONCLUSIONS

Since the identification of immunodeficiency viruses in human and animal hosts, a number of protozoan parasites have assumed increasing importance, either as secondary pathogens or as pathogens in their own right. Research on many of these organisms has increased considerably due mainly to the zoonotic implications and potential role of animals as reservoirs of infection. Many aspects relating to the epidemiology, pathogenesis and interactions with other disease agents require further investigation.

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