Enteric protozoa in ruminants: diagnosis and control of Cryptosporidium, the role of the immune response

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Summary: Cryptosporidiosis is diagnosed by histological examination of tissues or microscopic examination of faecal material to reveal large numbers of endogenous or exogenous parasitic stages. The contribution made by Cryptosporidium to a diarrhoeal disease outbreak should be decided after testing for the presence of other known enteropathogens. Comparison between immunocompetent and immunodeficient animals with cryptosporidiosis indicates that immunity does influence the outcome of the disease; the latter group suffers persistent infection whereas the disease is transient in normal individuals. Experimental evidence from ruminant infections has shown that titres of secretory IgM and IgA rise in association with declining oocyst output; this evidence suggests that these antibodies act by preventing penetration of motile stages. Furthermore, experimental prevention of infection in infant mice and rats indicates that the role of passive lacteal immunity in ruminants deserves investigation.

KEYWORDS: Cryptosporidium - Diagnosis - Immunity.

INTRODUCTION

Cryptosporidium is a small apicomplexan protozoan inhabiting the respiratory and gastro-intestinal tracts of a wide range of vertebrates. Infection of the respiratory tract is more common in birds while gastro-intestinal infection is more important in mammals.

Acute infectious diarrhoea remains an important medical and veterinary health problem especially in preweaned age groups. In addition to being less able to tolerate fluid loss caused by diarrhoeal diseases, these age groups are more susceptible to infection, especially when overwhelming environmental contamination combines with inadequate maternal and acquired immunity (8, 56). While several agents including Escherichia coli, Salmonella spp. and rotavirus have been extensively studied, Cryptosporidium has only in recent years received attention as an enteropathogen causing acute diarrhoea in man and animals.

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There are three main reasons for the increased attention given to cryptosporidiosis. First, the disease rapidly became known as a complication of host immunodeficiencies. For humans the most important of these is caused by infection with the human immunodeficiency virus (HIV) (73). Secondly, a number of studies have shown apparent lack of host specificity among the mammalian species and this was a feature which set Cryptosporidium apart from other coccidia (77). Its importance lies in the ability of the organism to take advantage of a large number of host species. Thirdly, there is no known effective therapy for cryptosporidial enteritis.

Most of the work done on cryptosporidiosis has dealt with epidemiology, studies of parasite morphology and life cycle, pathogenic mechanisms and diagnosis of the disease. Investigation of treatment and control has been hindered by the inability to culture the organism in vitro and the absence of a symptomatic small animal model.

**LIFE CYCLE**

Many details of the now accepted life cycle were first described by Tyzzer in 1910 and 1912 (74, 75). Since these reports, most advances have come from ultrastructural studies. Numerous studies of the life cycle in man, calves, lambs, mice, chickens and cell culture have consolidated an accepted outline similar to other coccidia: asexual followed by sexual endogenous stages, resulting in production of oocysts that can survive outside the host and constitute the infective stage. A schematic representation of the life cycle, as is understood to occur in intestinal epithelium, is shown in Fig. 1.

**ASSOCIATED PATHOGENS**

Despite numerous reports of Cryptosporidium being associated with diarrhoea in neonatal animals, its specific role is often made obscure by the presence of other known enteropathogens (2, 43, 46, 65, 71). It has been shown that Cryptosporidium, in the absence of normal intestinal flora or known enteropathogens, can cause diarrhoea in monoinfected, gnotobiotic calves and lambs (24, 70).

Associated primary enteropathogens include enteric viruses such as rotavirus and coronavirus and bacteria such as enterotoxigenic Escherichia coli (ETEC) and Salmonella spp. Infection with ETEC is the most likely cause of diarrhoea in calves and lambs less than 4-5 days of age. All the remaining agents including Cryptosporidium must be considered in cases up to 2-3 weeks of age (33). Coronavirus and ETEC can result in high mortalities in untreated calves due to dehydration, acidosis and hyperkalaemia. In comparison, rotavirus tends to produce a less severe transient diarrhoea (33). Salmonellosis does not always cause diarrhoea and is often associated with calves brought into rearing units (59). In uncomplicated cryptosporidiosis calves tend not to suffer severe dehydration and acidosis, and mortality is low (84). All of these enteropathogens can often be isolated from the one outbreak.
FIG. 1
Schematic representation of the life cycle of Cryptosporidium

1 = first generation
2 = second generation
Ex = excystation
M = merozoite
Mac = macrogamont
Mic = microgamont
Mig = microgamete

Oo = oocyst
S = schizont
Sp = sporulated
Spz = sporozoite
T = trophozoite
Th = thin-walled
Tk = thick-walled
Studies of mixed enteric infections in lambs indicate that Cryptosporidium induces a more severe disease in lambs aged at least six days than ETEC or rotavirus acting alone or in combination (83). While coronavirus-like particles have been detected in the faeces of sheep with transient diarrhoea (85), the infection is not recognised as an important enteropathogen in this species (39).

**RESISTANCE TO CRYPTOSSPORIDIUM INFECTION**

There are several reports in the literature which indicate that immunity does influence the outcome of Cryptosporidium infection. Cryptosporidium is rarely associated with neonatal equine scour (14, 58, 76). In contrast, Arabian foals with combined immunodeficiency have been reported to suffer severe Cryptosporidium infection (21, 72). Similarly, other genetic (congenitally athymic nude mice), infectious (AIDS resulting from HIV infection) or drug induced (cyclophosphamide) immunodeficiencies are associated with persistent cryptosporidiosis (23, 57, 73). Cryptosporidium infection is transient in normal immunocompetent humans and animals (67, 73, 84).

**Passively acquired immunity**

Observations from field outbreaks of Cryptosporidium-associated diarrhoea neither support nor deny a protective role for colostral antibody. Higher morbidity and mortality has been described for Cryptosporidium-associated diarrhoea in artificially reared neonates and those born to inexperienced dams (4, 71, 78). However, there has been no field study of susceptibility to cryptosporidiosis in relationship to specific passively acquired antibody.

Neonatal piglets and ruminants lose the capacity to absorb large quantities of colostral antibody into the circulation after approximately 30 hours of life (35). Hence, an investigation of colostral antibody protection must consider the role of antibody remaining unabsorbed and that which is secreted back into the lumen of the intestine. In bovine mammary secretions there is a rapid fall in immunoglobulin content over the first few days. Although functional significance may not be related to abundance, it is interesting to relate this decline to the far greater occurrence of cryptosporidiosis in neonatal ruminants compared to piglets. During the first three days of bovine lactation, the levels of all immunoglobulin classes (IgG₁, IgG₂, IgM and IgA) fall rapidly. By four days after parturition the concentration of each immunoglobulin class has been found to be less than approximately 1 mg/ml of milk (53). While IgM and IgG have a similar rate of decline in porcine lactation, that of IgA is not as marked (54). After the first four days IgA becomes the predominant immunoglobulin in sow milk, remaining at a level of approximately 10 mg/ml during the first four weeks of lactation (54). Hence, enteric protection by unabsorbed lacteal antibody may be short-lived in ruminants compared to pigs.

Colostral antibody to surface epitopes may function to block attachment receptor sites on motile stages of Cryptosporidium. This role for antibody was suggested in a report of heat-inactivated hyperimmune bovine serum against Cryptosporidium parvum sporozoites, neutralising their capacity to infect sucking mice (60). Using infant rats in a similar experimental design it was shown that sporozoite neutralisation
by hyperimmune lamb serum was associated with parasite-specific IgG which had been separated by affinity chromatography (29). However, removing total IgG from hyperimmune lamb serum did not eliminate its capacity to reduce sporozoite infectivity; the remaining activity was associated with an unidentified heat-stable, non-dialysable component which was also found to be present in uninfected gnotobiotic lamb serum (29). Hence, neutralisation by serum has been shown to have both specific (associated with parasite-specific IgG) and non-specific sporozoite neutralising components; the latter may reach the bowel lumen via inflammatory exudation and serve to limit early parasite proliferation until specific components of the immune response are generated. The protection afforded infant rats (29) by sporozoite-specific IgG has important implications for passive protection of young ruminants.

The major immunoglobulin of ruminant colostrum and post-colostral serum is IgG1, accounting for approximately 80% of the total (53). The half-life of bovine post-colostral IgG1 is approximately 18 days while that of IgM and IgA is 4 and 2 days respectively (38, 53). In young calves the clearance of serum IgG1 can mostly be accounted for by transfer into the intestine (7). In lambs, similar figures have been found for IgM and IgA but IgG was found to have a half-life of approximately 14 days (68). Although IgM, IgA and IgG are all secreted into the intestines of cattle and sheep (15, 32, 55), the greater quantity and longer half-life of post-colostral IgG make it a better candidate for protecting against neonatal cryptosporidiosis.

Actively acquired immunity

For a wholly enteric infection, presentation of antigen to gut-associated lymphoid tissue would be expected as the first step in mounting a specific immune response. Cryptosporidium antigen, both in a degraded form and as morphologically recognisable parasites, has been described deep within M cells overlying Peyer's patches and in subjacent macrophages (34, 40).

The kinetics and specificity of antibody isotypes produced in response to enteric cryptosporidiosis

Both immunofluorescent and enzyme immuno-assays have been used to detect antibody against Cryptosporidium in sera from animals and from immunocompetent and immunocompromised humans (12, 13, 80, 87). These studies indicate that there is a high incidence of exposure to, or infection with, Cryptosporidium in human and animal populations.

The kinetics of the endogenous antibody response in relation to oocyst shedding has only recently been investigated (29, 30). In these studies 5-day-old colostrum-deprived conventional and gnotobiotic lambs were each infected orally with 10^6 Cryptosporidium parvum oocysts. The oocyst prepatent period, as detected by phenol auramine staining of faecal smears (48), ranged from 3 to 5 days. Immunofluorescent assay (IFA) of sera from conventional lambs, using sporozoites as antigen, revealed an early rise of IgM and a slower but steady rise of IgG accompanied by a peak titre of IgA (30). The experiment was subsequently repeated using gnotobiotic lambs (29) and the same results were obtained (Fig. 2). The group mean titres of specific immunoglobulins detected in faecal extracts of these gnotobiotic lambs are shown in Fig. 3 (29). It is interesting to note that while IgG was not detected in faecal extracts, the titres of IgA and IgM rose in association with declining oocyst output. Similarly, analysis of intestinal mucus from these lambs revealed the presence of parasite-specific IgA and IgM at 8 days after infection, and of IgA alone at 16 days, but specific IgG was not detected on either occasion (Fig. 4).
Group mean serum titres, expressed as $\log_n$ IFA titre (±SEM), from gnotobiotic lambs infected with $10^6$ Cryptosporidium oocysts at five days of age and uninfected control lambs.

Serum assays were conducted with FITC-labelled pig anti-sheep IgM, IgA and IgG. Values represent the mean of ten gnotobiotic (six principal and four control) lambs. Cryptosporidium sporozoites were used as IFA antigen.

Immunoblot analysis has shown that IgA and IgM from convalescent gnotobiotic lambs recognised the same sporozoite antigens. Six antigens, with estimated molecular weights of 180 (I), 93 (II), 67 (III), 47 (IV), 23 (V) and between 12.3 and 17.2 (VI) kD were detected on immunoblots developed with these antibodies (29). Titres of IgG rose slowly during the oocyst-shedding period but, in comparison to IgM and IgA, it was found only in the serum and, on immunoblot analysis, recognised relatively few sporozoite antigens (29). Immunoblot studies probing merozoite proteins with hyperimmune sera prepared against sporozoites have revealed numerous shared antigens between these two motile stages (28). The antigens include a 23 kD band which is also evident when blots are probed with sera from infected people (86). Most
Group mean oocyst excretion and faecal immunoglobulin titres expressed as $\log_{10}$ total oocysts/day/lamb and $\log_{10}$ IFA titre (±SEM), respectively from gnotobiotic lambs infected with $10^6$ Cryptosporidium oocysts at five days of age and uninfected control lambs

Values represent the mean of measurements from six principal and two control male lambs. Cryptosporidium sporozoites were used as IFA antigen.

merozoite antigens recognised by IgA, from intestinal secretions of convalescent lambs, appeared in the molecular weight range of 66-180 kD, as did most of the sporozoite antigens recognised by this antibody. However, several sporozoite antigens between 45 and 66 kD, detected by IgA, were not detected by this antibody on merozoite blots. These differences may be useful in determining which stage of the parasite's life cycle is the target of protective immunity (29).
Examination of soluble extracts, prepared from mucus collected from the intestinal tracts of gnotobiotic lambs, for specific antibody to *Cryptosporidium* sporozoites

Principal lambs were infected at five days of age, killed at either eight (Fig. 4a) or sixteen (Fig. 4b) days after infection and compared with age-matched controls. Antibody was assayed with FITC-labelled pig antisheep IgM, IgA and IgG conjugates on mucus from control and infected lambs. Intestinal segments 1-7 denote proximal to distal small intestine respectively; 8 is caecum; 9 is colon. Values represent the means (± SEM) of four principal and two control lambs (eight days after infection) and six principal and four control lambs (sixteen days after infection).
Possible roles for secretory antibodies in immunity to cryptosporidiosis

In lambs and probably other young ruminants, IgM and IgA are the major immunoglobulins available for activity against endogenous stages of Cryptosporidium attached to or present in mucus coating host enterocytes (29, 30). Both may act by agglutinating and hindering attachment of motile stages of the parasite. If complement proteins were present in serum exudates resulting from enteritis, then IgM may be able to participate in complement-mediated parasite destruction.

Studies with experimental cryptosporidiosis in mice and gnotobiotic lambs (29) have shown that the quantity of intestinal surface mucus increased during the infection in association with increasing numbers of free merozoites. Another role for IgA may be in increasing mucus viscosity and thereby hindering the progress of motile stages. Both IgA and albumin have been shown to increase the viscosity of pig and dog gastric mucin glycoproteins (20, 47). This effect on viscosity might be expected with increasing amounts of albumin and IgA present on inflamed intestinal surfaces.

Inhibition of sporozoite penetration of host cells by IgA has been noted in studies of Eimeria tenella infection in chickens (17, 18). Similarly, expulsion of Giardia muris by mice was associated with increasing concentrations of parasite-specific IgA in intestinal secretions (69). Whereas crude caecal and colonic mucus from normal mice was without effect on the sporozoites of the coccidial parasite Eimeria falciformis, immune mucus caused them to agglutinate; immune mucus, unlike that from normal mice, contained sporozoite-specific IgA but not IgG or IgM as detected by an immunofluorescent antibody test (19).

The relative importance of antibody and cell-mediated immunity in cryptosporidiosis has been given little attention; this deserves some discussion since studies of immunity to other enteric coccidia have shown a dependence on the presence of functional T-cells. Comparisons made between normal and congenitally athymic (nude) mice or rats have indicated: a primary role for “effector” T-lymphocytes in immunity to E. falciformis var. pragensis in mice (41) and a thymic dependence for the architectural changes and cellular infiltrations seen in the intestines of E. nieschulzi infected rats (31, 63). In contrast, T-cells do not appear to be essential in the development of lesions in athymic mice with cryptosporidiosis (23).

Adoptive transfer of immunity to E. vermiformis infection in mice was accomplished with dividing mesenteric lymph node cells (64). Recipients developed earlier and sometimes higher serum titres of specific antibodies but there appeared to be no correlation between these titres and protection. Antibody titres in intestinal secretions were not examined. The chronicity of Giardia muris infection in athymic mice results from lack of intestinal trophozoite-specific IgA (26, 27). Similarly, during cryptosporidiosis in conventional and gnotobiotic lambs (30, 29) rising faecal IgA titres coincided with falling oocyst output. These experiments have highlighted the need to interpret, in investigations of enteric protozoan infections, the possible involvement of cell-mediated immune mechanisms alongside the kinetics of secretory antibody response as well as that in serum. The serum response may not reflect the concentration or isotype of antibody available at the site of intestinal infection, thus influencing any correlation between antibody titre and protection.
Age-related resistance

Natural and experimental infections indicate that diarrhoea due solely to Cryptosporidium would not be expected beyond 30 days of age in normal calves (1, 25, 42). Other studies have shown a similar age susceptibility in lambs (2, 79). Between three and thirteen days after infection of gnotobiotic lambs with Cryptosporidium parvum, depressed group mean daily milk intakes were associated with elevated group mean faecal weights (Fig. 5) (29). Endogenous stages of Cryptosporidium observed histologically in the intestines of normal young adult sheep (4) and oocysts recovered from faeces of adult sheep and cattle (50) indicate that a low level carrier status exists in the adult population. Hence, resistance in adult animals may relate only to clinical disease.

Age-related resistance does not appear to be influenced by T-cell deficiency. Infection of either normal or athymic mice at 42 days of age produces only mild subclinical infection with no histological changes observed in the intestinal mucosa (23). The mechanism for age-related resistance operating in the gastro-intestinal tract does not apply to other mucosal systems since patent infection is able to establish in uterine epithelium of adult immunocompetent mice (36). Similarly, it does not depend solely upon the presence of a normal intestinal microflora. Normal adult conventional mice remained resistant to Cryptosporidium infection after treatment with antibiotics designed to deplete intestinal microflora (22).

It would be interesting to know if infection with merozoites protected against subsequent challenge with sporozoites. This type of experiment has yielded valuable information in studies of Eimeria spp. (37, 61, 62). While most domestic and laboratory animal species are susceptible to Cryptosporidium infection as infants, by the time a primary infection has subsided, interpreting the results of a challenge infection may be difficult due to the onset of age-related resistance.

Innate age resistance to clinical disease may come from an ability to avoid severe infection of the small intestine. In this way malabsorption, resulting from villous atrophy, would not cause overloading and increased lumenal osmolality in the large intestine that resulted in neonatal diarrhoea. How the population dynamics of various endogenous stages might influence the outcome of infection has not been studied. Physiological conditions in the adult small intestine might not be optimal for synchronous excystation and localised infection. Spread of infection along a much expanded adult small intestine may interfere with mechanisms governing recycling of asexual generations and sexual reproduction.

DIAGNOSIS OF CRYPTOSPORIDIOSIS

Direct demonstration of the parasite

Histological examination of infected tissues, obtained as a biopsy or at necropsy, and microscopic examination of faecal material provide reliable means of diagnosis. The mere detection of endogenous or exogenous stages by these methods is not diagnostically significant. Large numbers of endogenous or exogenous stages, in association with typical clinical signs and enteric lesions, would constitute a diagnosis
FIG. 5

Group mean daily milk intake and faecal weight of gnotobiotic lambs infected with $10^6$ Cryptosporidium oocysts at five days of age and age-matched controls

Mean values were calculated from measurements on control (two females, two males) and principal (six males) gnotobiotic lambs.
in the absence of other complicating enteropathogens. Because *Cryptosporidium* infection can be subclinical or associated with other enteropathogens, its distribution in healthy animals should be considered in interpreting results (10, 43).

Tissue autolysis, resulting in dislodgement of parasites from cells (52), and choice of stain (21), can influence histological diagnosis. Similarly, choice of stain and use of various oocyst concentration techniques can influence microscopic diagnosis. Oocysts can be detected in smears of faecal material by direct staining with: Giemsa (1), carbol-fuchsin (3), methylene blue-eosin (16), phenol auramine (48) or by negative staining with nigrosin (51). Concentration by flotation can be done using saturated sugar (1) or salt solutions (49, 88).

**Indirect demonstration of the parasite**

Methods which include demonstration of specific antibodies and inoculation of infected host material into laboratory animals are generally unsatisfactory. Specific antibodies have been widely detected in healthy and unhealthy animals from several species (12, 80). The likelihood of maternal antibody confounding the kinetics of endogenous antibody response in neonatal animals makes serological diagnosis unsuitable. The use of laboratory animals suffers from variations in parasite infectivity (9) and host susceptibility (6). The variability and lack of interlaboratory standardisation associated with indirect diagnostic tests could make them unsuitable for routine use.

**TREATMENT AND CONTROL**

Neonatal animals suffering from debilitating diarrhoea, caused by *Cryptosporidium* infection, are treated palliatively with rehydration therapy and intestinal absorbents and may be given antimicrobial agents (81). Surface disinfectants are available for farm buildings and laboratories (11) but specific chemotherapy is not available for parenteral or oral administration.

Remission of cryptosporidiosis has been reported in a child with congenital agammaglobulinaemia after treatment with hyperimmune bovine colostrum administered both orally and by nasogastric tube (82). However, in other human cases oral administration of bovine colostral anti-*Cryptosporidium* antibody has failed to alter the course of the infection (66).

A variety of antimicrobial and antiprotozoal agents have been tested in calves (45), pigs (44) and mice (5) without success. As greater understanding of the parasite's biology and composition become available, particularly from studies of molecular biology, drugs may emerge which are able to target vulnerable points in the life cycle. The need for such drugs is nowhere more evident than in cases of persistent *Cryptosporidium* infection in AIDS patients where attempts at therapy have been similarly unsuccessful (73).
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PROTOZOAIRE INTESTINAUX CHEZ LES RUMINANTS: DIAGNOSTIC ET PROPHYLAXIE DE LA CRYPTOSPORIDIOSE, IMPORTANCE DE LA RÉPONSE IMMUNITAIRE. - B.D. Hill.

Résumé: Le diagnostic de la cryptosporidiose s’effectue grâce à des examens histologiques ou coproscopiques qui mettent en évidence les différents stades de développement, endogènes ou exogènes, du parasite. Le rôle de Cryptosporidium dans un syndrome diarrhéique ne peut être éluclidié que lorsque les autres agents entéropathogènes connus ont été recherchés. Les comparaisons effectuées entre animaux immunocompétents et immunodéficients montrent que le statut immunitaire influe sur l’issue de la maladie. Chez les sujets immunodéficients, la maladie persiste, alors qu’elle rétrocède chez les sujets normaux. Des études expérimentales, menées sur des ruminants, montrent que l’augmentation des titres d’IgM et d’IgA sécrétoires s’accompagne d’une baisse du nombre d’ookystes. Ces résultats laissent à penser que les anticorps empêchent les stades mobiles du parasite de pénétrer dans les cellules hôtes. En outre, la prévention expérimentale de l’infection chez des souriceaux et des jeunes rats indique que le rôle de l’immunité colostrale chez les ruminants mérite d’être approfondi.

MOTS-CLÉS : Cryptosporidium - Diagnostic - Immunité.

PROTOZOARIOIS INTESTINALES EN LOS RUMIANTES: DIAGNÓSTICO Y PROFILAXIS DE LA CRIPTOSPORIDIOSIS, IMPORTANCIA DE LA RESPUESTA INMUNITARIA. - B.D. Hill.

Resumen: El diagnóstico de la criptosporidiosis se realiza mediante exámenes histológicos o coproscópicos que revelan las diferentes fases del desarrollo, endógenas o exógenas, del parásito. El papel de Cryptosporidium en un síndrome diarréico sólo puede elucidarse cuando se han buscado los demás agentes enteropatógenos. Las comparaciones entre animales inmunocompetentes e inmunodeficientes muestran la influencia de la inmunización en la evolución de la enfermedad, que retrocede en los inmunocompetentes y persiste en los inmunodeficientes. Estudios experimentales en ruminantes muestran que el aumento de títulos de IgM y de IgA secretorios es acompañada de una disminución del número de ooquistes, lo que permite pensar que los anticuerpos impiden entrar a las fases móviles del parásito en las células huéspedes. Por otra parte, la prevención experimental de la enfermedad en ratoncillos y ratas jóvenes indica que el papel de la inmunidad colostral en los ruminantes debe ser mejor estudiado.

PALABRAS CLAVE: Cryptosporidium - Diagnóstico - Inmunidad.
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