CRYPTOSPORIDIOSIS IN HUMANS AND CATTLE IN MANITOBA: EPIDEMIOLOGICAL, SEROLOGICAL, AND TRANSMISSION STUDIES

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BY

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ABSTRACT

Cryptosporidiosis was recently recognized as a prominent cause of diarrhea in humans and cattle, but its extent in Manitoba was unknown. This study was undertaken to determine its prevalence in humans and cattle in Manitoba, based on the examination of fecal specimens available at the Cadham Provincial Laboratory, and the Provincial Veterinary Laboratory. Questionaires were submitted to physicians and to owners of positive herds, to collect clinical and epidemiological data from humans and cattle respectively. A serosurvey was conducted on cattle from infected farms, and on randomly selected adult cattle slaughtered in Manitoba abattoirs, using an indirect immunofluorescence test with feces derived oocysts as antigen.

Cryptosporidium was detected in approximately 1% of human diarrheal specimens, and in approximately 25% of specimens from diarrheal calves. No link was established between cases in humans and calves, but was suspected on several occasions. Asymptomatic infections, not previously described, were detected in 18 adult cattle on several farms. Sixteen of 24 sera (66.7%) from adult cattle on infected farms, and 76/186 (40.9%) of randomly collected sera had antibody to cryptosporidial oocysts ($p<0.025$).

Bovine cryptosporidiosis was successfully transmitted to mice, and the ultrastructure of oocysts in feces was studied by electron microscopy.

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LIST OF ABBREVIATIONS

Ab	=	Antibody
Ag	=	Antigen
AIDS	=	Acquired immune deficiency syndrome
C	=	Degrees Celcius
CRP	=	Cryptosporidiosis
EM	=	Electron microscopy
FES	=	Formalin ether sedimentation
g	=	gravity
gm	=	gram(s)
hr	=	hour
IIF	=	Indirect immunofluorescence
KAF	=	Kinyoun's acid-fast
KOH	=	Potassium hydroxide
l	=	liter(s)
M	=	Molar
MW	=	molecular weight
min	=	minute(s)
ml	=	milliliter(s)
pid	=	post infection day
PSS	=	Physiological saline solution
rt	=	room temperture
SAF	=	Sodium acetate-Acetic acid-Formalin
sec	=	seconds
sp gr	=	specific gravity
um	=	micrometer(s)

CHAPTER 1 INTRODUCTION.

CHAPTER 1 INTRODUCTION

On a world-wide basis diarrheal disease is the greatest cause of illness and mortality, and in the United States it is considered to be the second most common class of illness. In the underdeveloped world, death rates as high as 13% in the first 5 years of life, are associated with diarrheal disease in the majority of cases (Guerrant, 1985).

Many infectious agents have been identified as causes of diarrheal disease. Bacterial agents include the classical causes such as Vibrio cholerae, Salmonella sp., Shigella sp., and Escherichia coli, as well as more recently recognized bacteria such as Campylobacter jejuni & coli, Aeromonas hydrophila, and Yersinia enterocolitica. Viruses such as Rotavirus also cause diarrhea, especially in young children, and protozoa such as Giardia sp. have been associated with traveller's diarrhea (Guerrant, 1985). Many cases of diarrheal disease are still undiagnosed, due either to low sensitivity of the diagnotic methods, or to the presence of unrecognized agents.

Neonatal diarrhea is a major cause of economic loss in farm livestock, and is of special significance in beef and dairy cattle (Morin et al, 1980; Morin et al, 1976). Some researchers have indicated mortality rates as high as 11% of calves, in the first 15 days of life (Larivière et al, 1980; House, 1978; Speicher and Hepp, 1973; Oxender et al, 1973). Many agents, both infectious and non-infectious, have been

implicated. Infectious agents include bacteria, viruses, fungi and protozoa (Morin et al, 1976; Snodgrass et al, 1982; Morin et al 1980). Many secondary factors such as nutrition, immunity and stress have also been implicated in the disease syndrome, and several infections are often found simultaneously (Morin et al, 1980; Snodgrass and Sherwood, 1983). Many outbreaks of neonatal diarrhea are still undiagnosed. Morin et al (1976) and Snodgrass and Sherwood (1983) failed to identify an agent, in 20/55 and in 8/34 outbreaks respectively.

Cryptosporidium sp. was recognized in a calf (Panciera et al, 1971), and has been associated with approximately 1/3 of the herd outbreaks of neonatal diarrhea, in a number of bovine surveys (Morin et al, 1976; Snodgrass and Sherwood, 1983; Sanford and Josephson, 1982; Heine and Boch, 1981). Since 1976 Cryptosporidium sp. has been identified as a cause of diarrhea, in both immunodeficient and immunocompetent persons (Tzipori, 1983; Navin and Juranek, 1984; Current et al, 1983). Its overall significance relative to other enteropathogens has not been determined, but it has major significance to immunodeficient persons, such as those having Acquired Immune Deficiency Syndrome (AIDS). Cryptosporidiosis (CRP) has been considered to be the cause of death in many of these patients, due to the absence of any effective therapy (Centers for Disease Control, 1982).

CRP has been diagnosed in persons diarrheic after contact with calves excreting the organism, indicating its zoo-

notic potential (Anderson et al, 1982; Current et al, 1983). The infection has been identified in many other species, including mammals, reptiles, and birds, in association with a number of enteric and respiratory syndromes (Angus, 1983). There is a need to learn more about this organism, and to determine the extent of its role in these syndromes.

The economic importance of the beef and dairy industry to Manitoba, and the large number of persons who are in regular contact with cattle, give CRP considerable significance here. Farmers, veterinarians, and meat handlers have continuous contact with cattle, and virtually all members of the population consume meat and/or milk.

The infection had been recognized in calves in Manitoba before 1983, but its extent was not known. It had not been diagnosed in humans. Therefore the first goal of this study was to confirm the presence of CRP, in humans and cattle in Manitoba, and to estimate its prevalence in both hosts. The possibility of cattle as a source for the infection in humans received special attention. The survey was designed to also provide information on the seasonal incidence, association with other pathogens, and reservoirs of infection in either humans or animals.

Cases of CRP in humans and animals were identified by the examination of available diarrheic fecal specimens, followed up by sending questionaires to the attending physicians and to the owner of infected cattle herds. Some infected farms were visited to assess management factors, and to

collect serum and fecal specimens from "in contact" animals.

An indirect immunofluorescence (IIF) test using feces derived oocysts as antigen (Ag), was used to detect antibody (Ab) in sera of persons and animals. A sero-survey was conducted comparing prevalence of Ab to Cryptosporidium, in sera collected on infected farms, with that in sera randomly collected from Manitoba market cattle.

In an attempt to fulfill Koch's postulates, and to demonstrate the cross-species transmissibility of CRP, infant white mice were infected, using a crude oocysts suspension, prepared from the feces of an infected calf. Ultrastructural studies were performed by electron microscopy (EM), on the infected intestinal tracts.

CHAPTER 2 LITERATURE REVIEW.

CHAPTER 2. LITERATURE REVIEW

1. The Organism:

a. Classification:

Tyzzer (1907) first described Cryptosporidium as a protozoan of the class Sporozoa and later called it a coccidian parasite. Table 2.1 relates Cryptosporidium to other protozoa according to the classification of Levine (1973), with some modifications.

b. Life-Cycle and Morphology:

In his description of Cryptosporidium parvum of the mouse, Tyzzer (1912) described all the major stages of the life-cycle using nomenclature identical to that used for other sporozoa. The life-cycle is completed within one host, and presumably begins with the ingestion of oocysts in fecally contaminated feed. When sporozoites are released from the oocysts, (mechanism not fully known), they attach to enterocytes, and develop into trophozoites and then into first stage schizonts, containing 8 merozoites, which are released into the lumen of the intestine. These merozoites again attach to enterocytes (photographed by Sanford and Josephson, 1982) to develop into second stage schizonts each containing 4 merozoites. The second stage merozoites also attach to enterocytes and differentiate into either macro- or microgametocytes. Each mature microgametocyte contains 14-16 microgametes which are released into the lumen and fertilize macrogametocytes forming a zygote which then deve-

TABLE 2.1 THE CLASSIFICATION OF CRYPTOSPORIDIUM IN RELATION TO SOME OTHER SPOROZOANS OF VETERINARY AND MEDICAL INTEREST. ADAPTED FROM LEVINE, (1973)

Subphylum: Apicomplexa

Class: Sporozoasida

Order: Eucoccidiorida

Suborder: Eimeriorina

Family: Cryptosporidiidae

Genus: *Cryptosporidium* sp.

Family: Eimeriidae

Genus: *Eimeria* sp.

Isospora sp.

Order: Endodyococcidorida

Family: Sarcocystidae

Genus: *Sarcocystis* sp.

Family: Toxoplasmatidae

Genus: *Toxoplasma gondii*

Suborder: Hemospororina

Family: Plasmodiidae

Genus: *Plasmodium* sp.

Order: "unknown"

Genus: *Pneumocystis carinii*

lops into oocysts (Goebel and Braendler, 1982). Oocysts are released into the lumen and pass out with the feces. Vetterling et al (1971) disagreed with Tyzzer, and suggested that the "oocysts" described were really second stage schizonts. They failed to identify any structure they could consider an oocyst. Slavin (1955) described oocysts in the turkey. These authors may have believed they were observing differences in the organism, related to the host species being studied, but more recent studies indicate no difference in life stages in various host species.

Bird and Smith (1980) first diagrammed the life-cycle, but did not include the extra-intestinal portions, as they were unable to demonstrate oocysts. They did however publish a photograph of what they believed to be an early zygote. Current and Long (1983) studied the life-cycle in chicken embryos infected with Cryptosporidium, and proposed a more complete life-cycle (Fig 2.1). They described two types of oocysts, thick walled and thin walled, suggesting that the thick walled oocysts are passed in the feces for transmission of the infection to new hosts, while the thin walled oocysts release their sporozoites within the intestinal tract, to auto-infect the present host. Auto-infection can explain the chronic CRP described by many authors in immunocompromised humans (Pitlik et al, 1983a), in contrast to the self-limiting nature of other coccidial infections. Navin and Juranek (1984) suggested a form of continuous schizogony to explain this unusual auto-infection.

FIGURE 2.1 The life-cycle of Cryptosporidium (Current, 1984).

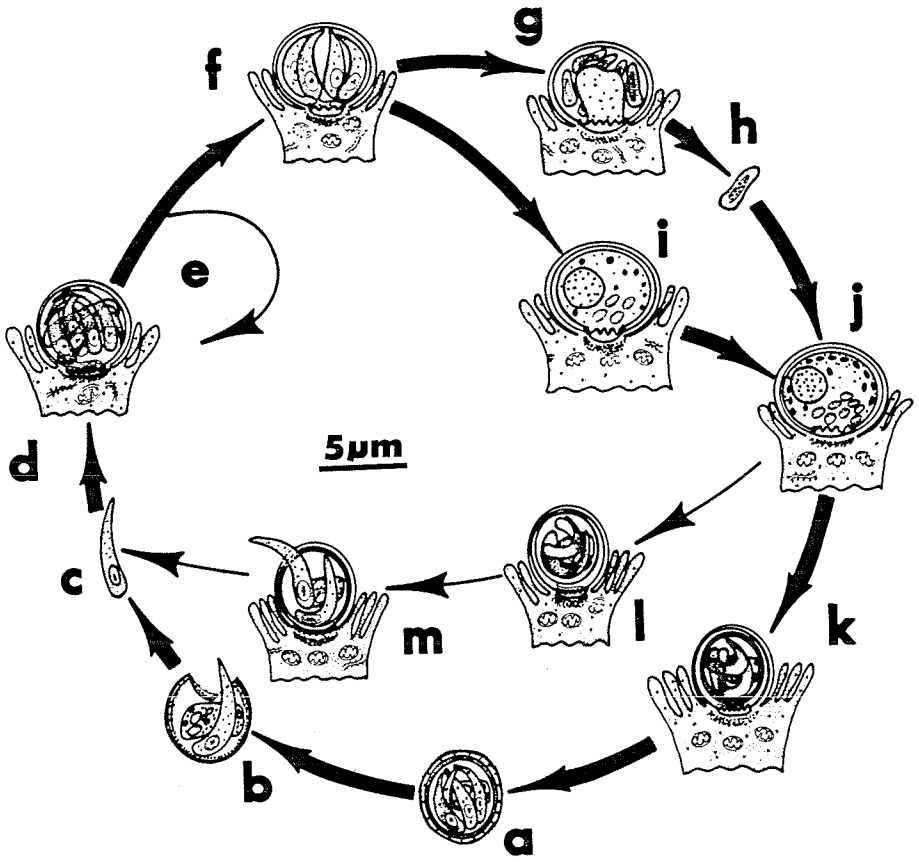

Figure 1. Proposed life cycle of Cryptosporidium. (a) Sporulated oocyst in feces. (b) Excystation in intestine. (c) Free sporozoite in intestine. (d) Type I meront (6 or 8 merozoites). (e) Recycling of Type I merozoite. (f) Type II meront (4 merozoites). (g) Microgametocyte, with ∿ 16 microgametes. (h) Microgamete fertilizes macrogamete (i) to form zygote (j). Approximately 80% of the zygotes form thick-walled oocysts (k) which sporulate within the host cell. About 20% of the zygotes do not form an oocyst wall, their sporozoites are surrounded only by a unit membrane (ℓ). Sporozoites within "autoinfective", thin walled oocysts (ℓ) are released into the intestinal lumen (m) and reinitate the endogenous cycle (at c).

Several publications have described the morphology of various stages of the life-cycle of Cryptosporidium, both by light and electron microscopy. The description by Tyzzer (1912), is very similar to those in more recent studies except for the greater detail described in EM studies. Most life stages have been seen in sections of infected intestinal tracts in the majority of hosts studied (Slavin, 1955; Bird and Smith, 1980; Pearson and Logan, 1983; Vetterling et al, 1971; Iseki, 1979; Goebel and Braendler, 1982; Pohlenz et al, 1978b).

All tissue stages are found within a parasitophorous vacuole formed by the microvilli fusing around the sporozoites or merozoites (Goebel and Braendler, 1982), and all differentiations occur within this vacuole. All fixed stages are bounded by a double layered pellicle which fuses with the cytoplasmic membrane in the area of the attachment zone to form a feeder organelle. A description of the morphology of the stages follows:

Sporozoite: A crescent shaped structure approximately 1 micrometer (um) by 5 um, with a prominent nucleus in the anterior or posterior portion. It has a double membrane pellicle (Iseki, 1979). Current and Long (1983) described a prominent nucleus near the posterior end.

Trophozoite: An oval or round structure approximately 2-2.5 um in diameter with the nucleus, containing a prominent nucleolus, occupying most of the internal space. It develops an attachment organ and has other cellular organel-

les such as endoplasmic reticulum and golgi complex (Iseki, 1979; Pohlenz et al, 1978b).

Schizonts: They are of two types, although not all authors agree. First generation schizonts develop 8 merozoites, while second generation develop only 4. The merozoites develop in the parasitophorous vacuole, by budding through the parasitic pellicle. The space remaining inside the pellicle is taken up by a small residual body near the feeder organelle. The schizonts are approximately 4-5 um in diameter (Iseki, 1979).

Merozoites: They are very similar to the sporozoites, and are 1 um x 5 um and crescent shaped, having a double membrane pellicle (Iseki, 1979).

Macrogametocyte: These are spherical or ovoid in shape with a relatively large nucleus and have a prominent lipid vacuole at one side of the nucleus. They also have a large number of polysaccharide granules and wall forming organs within their cytoplasm (Goebel and Braendler, 1982).

Microgametocyte: These are spherical about 4.5 um in diameter. They may be seen in early stages with multiple nuclear divisions (ie. chromatin around the edges of the pellicle). Later these nuclear division form 14-16 microgametes, which acquire a pellicle by budding into the parasitophorous vacuole (Goebel and Braendler, 1982).

Microgametes: The motile gametes are released into the lumen of the gut and become attached to the macrogametocytes, penetrating the pellicle to fuse with the nucleus in

the fertilization process. They are 0.25 um x 1.0 um in size, wedge shaped, with a large nucleus taking up most of the internal space. Various other organelles have been described (Goebel and Braendler, 1982).

Oocysts: These are ovoid structures, 4.5-5 um in diameter having an oocyst wall and containing 4 C-shaped sporozoites, each surrounded by a pellicle, and some residual material. Sporogony takes place within the oocyst before it is released into the lumen of the gut, and sometimes sporozoites are released from the oocyst at this time (Iseki, 1979). Current and Long (1983) identified thin and thick walled oocysts (fig 2.1).

Barker and Carbonell (1974) published photographs of oocysts "in situ" in mucosal cells, surrounded by a parasitophorous vacuole enclosed in a microvillar derived membrane. The oocyst is surrounded by an oocyst wall containing sporozoites, and a residual body including polysaccharide granules. They also described an outer membrane of a parasitic pellicle lying outside the oocyst wall. The residual body is separated from the attachment zone by the oocyst wall, thus differentiating oocysts from second stage schizonts.

Pohlenz et al (1978b) and Baxby et al (1984) have published EM photographs of oocysts in feces, showing the oocyst wall and the parasitic pellicle surrounding the sporozoites and residual material, and a prominent vacuole. Several authors described lipid vacuoles within the residual body (Angus, 1983; Bronsdon, 1984). Sporocysts have not

been described within oocysts, this characteristic forming part of the definition of Cryptosporidium (Tyzzer, 1912; Levine, 1980).

c. Transmission

The normal mode of transmission of this infection, like that in other coccidia, is believed to be by the fecal-oral route. Epidemiological studies which show clusters of cases in day care centers (Centers for Disease Control, 1984), and cases in humans or animals in association with other infected hosts, indicate transmission by direct contact (Reese et al, 1982, Anderson et al, 1982). Baxby et al (1983) described CRP in a nurse, who had been caring for a 13 month old boy with prolonged cryptosporidial diarrhea. Collier et al (1984) described a case in an immunosuppressed mother, infected while caring for her two young children who had CRP. Oral infection has been presumed in the infection of a researcher, when a rabbit being orally inoculated with Cryptosporidium sneezed in his face (Blagburn and Current, 1983). Weber and Philip (1983) suggested transmission by sexual contact to a homosexual man, after identifying the infection in his sexual partner. Experimental infection of numerous species has been accomplished by oral inoculation with feces derived oocysts, or scrapings from infected intestinal tracts (Anderson, 1982; Angus et al, 1982b; Current et al, 1983; Glisson et al, 1984; Moon and Bemrick, 1981; Moon et al, 1982a; Pavlàsek, 1983a; Tzipori et al, 1980a; Schloemer, 1982).

d. Environmental Resistance and Disinfection:

The oocysts released in the feces appear to be quite resistant to environmental factors. Pohlenz et al (1978a) described new cases of CRP on one farm, 10 months after previous cases were diagnosed. Two outbreaks occurred in animals kept in pens, occupied 6-7 months previously by infected animals (Snyder et al, 1978; Tzipori et al, 1981d). Both incidents suggest the organism may have survived in the environment during the interval.

Oocysts in fecal samples stored in potassium dichromate (Willson and Acres, 1982), or in phosphate buffered saline (Sherwood et al, 1982), at 4 degrees Celsius (C) for several months remained infective. Storage of undiluted fecal samples at 4 C, and later at room temperature for a total of 45 days did not result in autolysis of the oocysts (Pohlenz et al, 1978b). Willson and Acres (1982) stored oocysts in potassium dichromate for up to 120 days, without loss of morphology. Freezing has been shown to destroy infectivity of CRP oocysts for mice (Sherwood et al, 1982), and Current (1983) indicated that oocysts were killed by freezing, or heating at 65 C for 30 minutes (min).

Many common laboratory disinfectants have been found ineffective in inactivating cryptosporidial oocysts. Campbell et al (1982) found oocysts resistant to iodophore, cresylic acid, sodium hypochlorite, benzylkonium chloride, and sodium hydroxide, but 10% formalin and household ammonia inactivated the organism. Angus et al (1982c) found oocysts

resistant to 2 aldehyde disinfectants: Tegodor (Th. Goldschmidt) and Formula H (Hoechst). Stein et al (1983) found 2 hr exposure to phenol, chloroform, alcohol and perchlorethylene inadequate to render oocysts non-infective for mice.

e. In Vitro Cultivation:

Current and Long (1983) succeeded in cultivating cryptosporidia, in embryonated hens eggs, with the production of oocysts infective to mice. Woodmansee and Pohlenz (1983) cultivated Cryptosporidium in a human rectal tumor cell line, but oocysts were not produced. Current and Haynes (1984) reported completion of the life-cycle in human fetal lung cells. These methods facilitate the production of pure cultures of Cryptosporidium for biochemical studies, transmission studies, and drug evaluation.

f. Geographic Distribution:

Table 2.2 shows the geographic ubiquity of this infection, in several host species as described by numerous authors in recent years.

2. Species Affected and Clinical Syndromes:

Tyzzer (1907) recognized no clinical disease associated with CRP in mice. In 1912, he described another species of Cryptosporidium asymptomatically infecting the small intestine of mice, and suggested a lack of immune protection after infection as he saw it in "old" as well as "young" mice in the colony. Pivont and Antoine (1982) reported that Tyzzer described asymptomatic CRP in rabbits and in chickens. This parasite has now been described in many species

TABLE 2.2 IDENTIFICATION OF CLINICAL CRYPTOSPORIDIOSIS AT VARIOUS GEOGRAPHIC LOCATIONS

COUNTRY	AUTHOR*	DATE	SPECIES
USA	Panciera	1971	cattle
Australia	Barker	1974	lambs & calves
Canada	Morin	1976	calves
Ireland	Pearson	1978	calves
Japan	Iseki	1979	cats
South Africa	Howerth	1981	calves
France	Antoine	1981	calves
Czechoslavakia	Pavlàsek	1982	calves
Argentina	Magnasco	1982	calves
Isreal	Nobel	1982	calves
Holland	Peeters	1982	calves
Romania	Dan	1983	calves
USSR	Pavlàsek	1983b	calves
China	Tsai	1983	chickens
Denmark	Holten-Anderson	1983	humans
Wales	Casemore	1983	humans
Finland	Jokipii	1983	humans
New Zealand	Carter	1984	humans
Liberia	Hojlyng	1984	humans
Rwanda	Bogaerts	1984	humans
Costa Rica	Mata	1984	humans
Bangladesh	Rahaman	1984	humans & calves
Brazil	Weikel	1985	humans

* Name of first author only.

including mammals, birds and reptiles, although many of these infections were subclinical(table 2.3).

CRP was first described in cattle by Panciera et al (1971) who identified it in the intestinal tract of an 8 month old calf with chronic diarrhea. Barker and Carbonell (1974) associated CRP with diarrhea in lambs and calves.

Nime et al (1976) reported the first human Cryptosporidium infection in an immunologically normal child having diarrhea, vomiting and other symptoms of enterocolitis. The second was diagnosed the same year in a man who developed massive watery diarrhea after immunosuppression therapy (Meisel et al, 1976).

Until recently Cryptosporidium was considered species and site-specific. Each isolate from a new host was given a new specific name (Tyzzer, 1907, 1912; Slavin, 1955; Vetterling et al, 1971; Barker and Carbonell, 1974; Proctor and Kemp, 1974). Vetterling et al (1971) failed to transmit an isolate from guinea pigs to mice, rabbits, chickens or turkeys. In contrast Tzipori et al (1980a) managed to transmit a bovine isolate to seven different species and suggested Cryptosporidium may be a single species genus. Schloemer (1982) and Pavlàsek (1983a) also transmitted CRP to several mammalian species simultaneously, but both failed to transmit a mammalian isolate to chickens. Transmission between mammalian species has been repeated many times; from calves to pigs (Moon and Bemrick, 1981; Tzipori et al, 1981c), from humans to pigs (Moon et al, 1982a), from calves

TABLE 2.3 FIRST IDENTIFICATION OF CRP IN VARIOUS SPECIES.

SPECIES	CLINICAL SIGNS AND/OR PATHOLOGY	REFERENCE
mouse	none / none	Tyzzer, 1907
snakes[1]	none / none	Triffit, 1925
dingo[1]	none / none	Bearup, 1954
turkey	respiratory, diarrhea / ileitis	Slavin, 1955
jungle cat[1]	none / none	Dubey and Panda, 1963
guinea pig	none / chronic enteritis	Jervis et al, 1966
cattle	chronic diarrhea / villous atrophy	Panciera et al, 1971
rhesus monkey	chronic diarrhea / biliary hyperplasia	Kovatch and White, 1972
goose	none / villous atrophy	Proctor and Kemp, 1974
lambs	diarrhea, death / villous atrophy	Barker and Carbonell, 1974
humans	diarrhea, vomiting / ileocolitis	Miesel et al, 1976, Nime et al, 1976
pigs	none / villous atrophy	Kennedy et al, 1977
horses	diarrhea and death /enteritis	Snyder et al, 1978
goats	diarrhea and death / villous atrophy	Mason et al, 1981
cats	none / villous atrophy	Iseki, 1979
dogs	diarrhea, respiratory / villous atrophy	Wilson, 1983
deer	diarrhea, death / villous atrophy	Tzipori et al, 1981d
raccoon	none / villous atrophy	Carlson and Nielsen, 1982
quail	respiratory / tracheobronchitis	Tham et al, 1982
gazelle	death / ileocolitis	Fenwick, 1983

1. These identifications were made on the basis of oocysts alone and were considered invalid by Vetterling (1971) and Levine (1984).

to lambs (Tzipori et al, 1981a), from humans to rodents (Current et al, 1983).

Levine (1980) considered cryptosporidia host-specific. In 1984, he reviewed their classification, proposing 4 species: C. muris (Tyzzer, 1907) from mammalian hosts, C. meleagridis (Slavin, 1955) from birds, C. crotali (Triffit, 1925), from reptiles, and C. nasorum (Hoover, 1981) from fish.

Clinical Syndromes:

a. Immunosuppressed humans:

The man described by Meisel et al (1976) had abdominal pain, massive watery diarrhea and dehydration. Symptoms continued until the immunosuppression therapy was discontinued. Several cases of CRP associated with AIDS were reviewed by the Centers for Disease Control (1982), in which similar symptoms were described. Some patients had severe protracted watery diarrhea which continued till death. Bowel movements varied from 6-25/ day in number, and 1-17 liters/ day in quantity. Additional cases have been described with symptoms of nausea, vomiting, severe watery diarrhea, abdominal pain, and dehydration (Guarda et al, 1983; Forgacs et al, 1983; Koch et al, 1983; Miller et al, 1983; Lefkowitch et al, 1984). Symptoms were sometimes intermittent over very long periods of time (Guarda et al; Lefkowitch et al; Current et al 1983).

b. Immunocompetent Humans:

The child described by Nime (1976) had been admitted to hospital vomiting everything ingested, and having severe

watery diarrhea which continued for 9 days, resolving spontaneously with only supportative therapy. Additional cases in immunocompetent persons were described between 1980 and 1983 (Tzipori et al, 1980b; Babb et al, 1982; Reese et al, 1982; Fletcher et al, 1982). All these patients had a severe watery self-limiting diarrhea, varying in duration from several days to 2 weeks. Sometimes the diarrhea was followed by several days of constipation. Other symptoms included vomiting, nausea, severe headaches, abdominal pain, weakness and lethargy. Relapses were sometimes reported (Anderson et al, 1982).

c. Calves:

In calves the clinical picture in CRP is generally described as part of the neonatal diarrhea syndrome. The frequent involvement of other agents makes it difficult to separate signs due solely to CRP. Characteristic signs include watery diarrhea which is often malodorous, frothy and unresponsive to therapy, beginning between 7 and 21 days of age. The morbidity and mortality are often high (Barker and Carbonell, 1974; Pohlenz et al, 1978a; Morin et al, 1978; Heine and Boch, 1981; Sanford and Josephson, 1982). In experimental CRP, Tzipori et al (1983a) described depression and anorexia, as the earliest and most consistent signs. These were followed by profuse diarrhea within 2-3 days of the first signs, and progressed to intermittent diarrhea or loose feces. The calves often exibited tenesmus, and frequently became dehydrated and emaciated. Heine et al (1984a)

showed a direct correlation between the shedding numerous oocysts, and a similar clinical syndrome in experimental CRP in gnotobiotic calves, thus establishing its pathogenicity.

d. Lambs and Goats:

Barker and Carbonell (1974) described diarrhea with a high mortality rate in 1 week old lambs with CRP. Tzipori et al (1981a) described CRP in lambs with diarrhea, depression and inappetence, starting at 5-12 days of age and lasting 7-16 days. Mortality was high in chronic cases. Angus et al (1982a) reported diarrhea, stiffness, hyperpnea and shivering from 10 days of age, resulting in death in 2-3 days, or spontaneous recovery after 7 days. Some had relapses, and response to therapy was poor.

Tzipori et al (1982a) also described CRP and acute diarrhea, with high morbidity and mortality in goat kids. Nagy et al (1984) also detected CRP in many diarrheic kids.

e. Chickens:

Dhillon et al (1981) recognized respiratory distress and high mortality associated with CRP in chickens. Some authors did not recognize any clinical disease (Fletcher et al, 1975; Ikakura et al, 1984). Some infected birds may have been immunodeficient due to pathology of the Bursa of Fabricius (Randall, 1982; Tsai et al, 1983; Dhillon et al, 1981).

f. Turkeys:

Slavin (1955) diagnosed CRP in 10-14 day old turkey poults, having diarrhea and moderate mortality rates. Hoerr et al (1978) described CRP associated with respiratory dis-

tress, conjunctival exudate and serous nasal discharge, chronic in at least one bird. Glisson et al (1984) described severe bilateral facial sinusitis with external swelling, and identified only Cryptosporidium in these lesions.

g. Pigs:

Kennedy et al (1977) described CRP as an incidental pathological finding in 3 clinically normal pigs. Links (1982) and Schimdt and Nienhoff (1982) recognized CRP with diarrhea in young pigs, but other pathogens were also identified. Experimentally, Moon and Bemrick (1981) and Moon et al(1982a) reproduced CRP in neonatal pigs; diarrhea starting 3-5 days post infection and continuing for 5-14 days, coinciding with shedding of oocysts. Tzipori et al (1981c) also infected young pigs with CRP, and produced inappetence, vomiting and diarrhea, in association with oocyst excretion.

h. Horses:

Snyder et al (1978) and Gibson et al (1983) reported watery, fatal diarrhea in Arabian foals, born with severe combined immunodeficiency. Gajadhar et al (1985) described diarrhea, associated with the excretion of cryptosporidial oocysts, in two immunologically normal foals. This condition was self-limiting in one. The other died, but also had a septicemia of undetermined origin.

i. Cats:

Iseki (1979) and Koch et al (1983) described incidental subclinical CRP in cats, and Augustin-Bichle et al (1984) detected no clinical signs in experimentally infected

cats. However, Poonachia and Pippin (1982) and Bennet et al (1985) described anorexia, weight loss and chronic diarrhea in cats having CRP.

j. Dogs:

Four publications have described CRP in dogs. Wilson (1983) diagnosed the infection in a pup that died with acute diarrhea and repiratory signs. Fukusima and Helman (1984) recognized CRP as an incidental finding in one pup from a litter, dying with typical signs of canine distemper. Sisk et al (1984) found the infection incidentally on post mortem examination of 2 dogs. Experimental CRP in dogs remained subclinical (Augustin-Bichle et al, 1984).

k. Monkeys:

Kovatch and White (1972) described persistent diarrhea, dehydration and coma in rhesus monkeys, and Wilson et al (1984) described similar symptoms which were unresponsive to therapy in juvenile macaques.

3. Lesions and Host Parasite Relationship.

a. Light microscopy;

Grossly visible lesions are rarely found in CRP, but similar microscopic intestinal lesions have been described in many species, even though clinical syndromes differ widely. Pearson et al (1982) found intestinal lesions in calves most common in the ileum, with lesser lesions in the jejunum and colon. The location of lesions in various species has been reviewed by Anderson (1984). The histopathology of CRP in calves has been described by Meuten et al (1974), Morin

et al (1976), Pohlenz et al (1978a), Sanford and Josephson (1982), and most recently in gnotobiotic calves by Heine et al (1984a).

Microvilli in infected areas of mucosa are displaced by numerous cryptosporidia. The major lesions are severe villous atrophy, cross-bridging between villi, and cuboidal or low columnar metaplasia of mucosal cells. The crypts of Lieberkuen are often dilated with epithelial debris, and the lamina propria is infiltrated with neutrophils and/or mononuclear cells. Similar lesions have been described in humans (Meisel et al, 1976; Nime et al, 1976), in lambs (Tzipori et al, 1981a; Angus et al, 1982b) and in pigs (Moon and Bemrick, 1981; Tzipori et al, 1981c). Meuten et al (1974) indicated that mucosal lesions were proportional to the severity of infection, but Angus et al (1982b) found no lesions in some heavily infected areas of the proximal jejunum of lambs. Infected crypts in the caecum and colon were devoid of mucous secreting cells. Heine et al (1984a) indicated that some parasitized epithelial cells had disrupted apical plasma membranes.

In respiratory CRP of poultry Dhillon et al (1981), and Hoerr et al (1978) described thickening of the tracheal mucosa, cuboidal or squamous metaplasia of the epithelium, and infiltration of the lamina propria with a variety of inflammatory cells. Glisson et al (1984) described similar lesions in the facial sinuses of infected turkeys, and noted the absence of cilia from the infected mucosa.

Forgacs et al (1983) observed metaplastic changes on the respiratory epithelium, without inflammation, in a man dying with respiratory CRP. Brady et al (1984) described chronic inflammation in respiratory CRP, in a man having AIDS, but other pathogens were also present in the lesion. Kovatch and White (1972) described folded mucosa with thickened lamina propria, and inflammatory infiltration, in the bile ducts of a rhesus monkey with biliary CRP. The crypts were filled with debris, and mucosal cells were flattened. Pitlik et al (1983b) described similar lesions of the gall bladder of a homosexual man with intestinal CRP.

b.Electron microscopy:

The host-parasite relationship and the fine structural pathology of CRP have been studied, by scanning and transmission electron microscopy, in many species. Tyzzer (1912) considered cryptosporidia to be extracellular organisms, but Vetterling et al (1971) showed them to be enclosed by modified microvillous membranes of host origin, identifying them as intracellular parasites. Most authors agreed with these findings (Barker and Carbonell, 1974; Kovatch and White, 1972; Kennedy et al, 1977; Bird and Smith, 1980; Pearson and Logan, 1978). Pohlenz et al (1978b) published a diagram in which they suggested an extracellular position for the organism in calves, however Pearson and Logan (1983) demonstrated the presence of host derived membranes, enclosing the parasitophorous vacuole, in calves also.

c. Pathogenesis and immunological factors:

Jervis et al (1966), studying CRP in guinea pigs, suggested the parasite derived some of its nutrition from the lumen of the gut. Withholding feed led to a marked decrease in the number of organisms seen on the mucosa, but when feeding was resumed, the numbers were quickly restored. They indicated that this is a well recognized phenomenon among free-living protozoa of the guinea pig gut. He also observed that the tissue reaction in the gut was proportional to the number of organisms present, except in severe cases, in which he noted villous atrophy with few cryptosporidia in the upper small intestine. Jervis et al also found intestinal mucosal enzyme levels decreased, proportional to the severity of mucosal lesions. The loss of absorptive area and the lowering of enzyme levels suggested malabsorption as the pathogenesis of diarrhea. They also postulated a toxic metabolite of Cryptosporidium, passing into the epithelial cells through the attachment zone, causing enteritis.

Kennedy et al (1977) showed cryptosporidia were phagocytosed by leukocytes in intestinal crypts. They recognized organisms inside the phagocytes, including the host derived parasitophorus membrane, indicating that the action of these phagocytes on parasitized epithelial cells may contribute to the intestinal lesion.

Pohlenz et al (1978b) observed destruction of the terminal web, and an increase in the number of lysosomes in infected cells, and indicated that the organisms were not

highly toxic as these cells were not destroyed. They suggested that the diarrhea produced in calves was likely due to loss of absorptive area brought about by the absence of microvilli, the villous atrophy and epithelial cross-bridging. Angus et al (1982b) also suggested malabsorption as the pathogenesis of diarrhea in CRP, but their observation of bacteria adhering to the damaged epithelium, added the possibility that these may have contributed to the clinical signs by the production of toxins.

Pohlenz et al (1983) studied the pathogenesis in gnotobiotic calves infected only with Cryptosporidium. By measuring the ratio of the absorptive surface of the mucosa to the lamina muscularis mucosae, they determined a marked reduction in absorptive area due to the fusion of villi. They also found an increase in the number of mitotic figures in the crypts of Lieberkuen. This occurred earlier than the fusion of villi, and they proposed a possible hypersensitivity reaction in addition to the loss of cells, as the stimulus. This suggestion is further supported by the finding of large numbers of eosinophils in the affected mucosa. They also found mucosal enzymes greatly reduced, and combining this finding with the decrease of absorptive area, concluded that malabsorption was the most likely cause of the diarrhea.

Tzipori (1983) also reported studies of intestinal mucosal enzymes, indicating a marked decrease in lactase activity in infected as compared to control calves, espec-

ially in the mid small intestine. Wilson et al (1984), studying the infection in monkeys, suggested that the loss of microvilli and disruption of the terminal web, caused malfunction of the microvilli leading to malabsorption. They also suggested that there may be increased permeability of epithelial cells, resulting in excessive secretion of fluids.

Heine et al (1984a) also concluded that the diarrhea occurring in some species in association with CRP was because of epithelial damage and loss of absorptive area. They also suggested a cell mediated immune response as a possible cause of the villus atrophy, as was shown to occur in helminth infections (Ferguson and Jarrett, 1975), and in graft-versus-host disease (MacDonald and Ferguson, 1977). In a separate study, Heine et al (1984b) were able to reproduce CRP in nude mice (congenitally deficient in cell mediated immunity) and concluded that a hypersensitivity reaction could not be the cause of the lesion.

Lefkovitz et al (1984), found little or no mucosal lesion in tissues from an infected man, and suggested an enterotoxin as the cause of the severe diarrhea.

4. Diagnostic Techniques:

Until recently the only method of diagnosis of CRP in humans, was the examination of biopsy specimens from the ileal mucosa. The difficulty in locating the lesion, and the major intervention involved, limited this procedure to chronic cases of diarrheal disease, negative for all other

examinations. Similarly, the diagnosis was only rarely made in calves, when intestinal specimens were obtained soon after death for histological examination (Sanford and Josephson, 1982). With the recognition of oocysts in feces, and the development of methods for demonstrating them, the diagnosis of CRP was greatly simplified.

The demonstration of oocysts in feces has been accomplished by either fecal floatation or differential staining of fecal oocysts. Iseki (1979) used concentration by zinc sulfate floatation, and (Anderson, 1981) used sucrose floatation, prior to microscopic examination of wet mounts. Pohlenz et al (1978a) had success in staining the oocysts using Giemsa procedure on direct fecal smears, and Henriksen and Pohlenz (1981), described a modified Zeil-Neelsen acid-fast procedure. This demonstration, that cryptosporidial oocysts retained the carbolfuchsin stain, led to the development of several adaptations. Ma and Soave (1983) described a three step procedure including the Kinyoun's acid-fast (KAF) stain in one of the steps. Garcia et al (1983) compared 15 different techniques, and concluded that a modified (hot) carbolfuchsin procedure was most sensitive. Miller et al (1983) observed that only a small proportion of oocysts in feces retained carbolfuchsin. This finding led to the development of other staining procedures now in common use by some workers. Baxby and Blundell (1983), described two methods using either safranine counterstained with methylene blue, or methylene blue counterstained with basic fuchsin. These

methods are also considered to lack sensitivity when oocyst numbers are low (Hart et al, 1984). Cross et al (1983) described a method using methylene blue counterstained with eosin. Others attempted to overcome the failure of carbolfuchsin to stain all oocysts by using penetrating agents such as dimethyl sulfoxide (Bronsdon, 1984; Pohjola et al, 1985).

Several negative staining procedures have also been described for screening fecal specimens. Heine (1982) used carbolfuchsin as a negative stain on drops of unfixed fecal specimens, smearing the feces and stain together and air drying, before examination under oil immersion at 40x magnification. The oocysts appeared as refractile unstained spheres on a solid red background. Horen (1983) described a procedure using periodic acid-Schiff to stain the background, leaving the oocysts unstained. Staining with fluorescing dyes is being successfully used in some laboratories (Nichols and Thom, 1984), and the indirect immunofluorescence (IIF) procedure is being used for serological identification of oocysts in fecal smears(Current, personal communications).

The large number of new procedures indicate the difficulty in staining cryptosporidial oocysts. The KAF procedure appears to be the most convenient, and may be more specific than negative staining, as it shows some of the internal structure of the oocysts. However it may lack sensitivity, if only 10-25% of oocysts retain the carbolfuchsin as suggested by Miller et al (1983).

5. Epidemiological Surveys:

a. calves (Table 2.4):

The prevalence of CRP was first studied in neonatal calves. Surveys were designed to relate the prevalence of CRP, to that of other organisms associated with neonatal diarrhea. Morin et al (1978) surveyed 47 dairy herds in Quebec and found Cryptosporidium in 33% of diarrheic calves, compared to only 14% of non-diarrheic calves. Co-infection with Rota- and Corona-viruses were common. Sanford and Josephson (1982) found 42/161 (26%) calves with fatal diarrhea, positive for CRP in an Ontario survey. Sixty-three percent of diarrheic calves submitted for necropsy while still alive had CRP. They suggested that this difference was due to a lower sensitivity of histological diagnosis, if postmortem examination was delayed. It may also have reflected the greater willingness of owners with very severe losses, to allow the sacrifice of calves for examination. Most cases occurred between December and February, and they associated the seasonality with the close confinement necessary during that season.

Heine and Boch (1981) described a study conducted on dairy farms in Germany, in which they found 13.7%, 39.6% and 60.9% of calves CRP positive among non-diarrheic, diarrheic and fatally diarrheic calves respectively. During repeated examination of veal calves at one farm in Holland, Peeters et al (1982) found CRP in 85% of 20 calves by 16 days of age. Only 4/17 calves in this study had diarrhea, but fecal

specimens containing oocysts were usually less firm, and contained mucous. Anderson and Hall (1982) found CRP positive calves on 67% of dairy farms in Idaho.

Snodgrass and Sherwood (1983) found high rates of excretion of oocysts in both diarrheic and non-diarrheic calves, but CRP was found in only one non-diarrheic calf, on a farm where diarrheic calves did not have CRP. De Leeuw et al (1984) surveyed 11 dairy herds, having a total of 375 calves, in the Netherlands. CRP occurred, sometimes intermittently, in all the herds, with infection rates varying from 19 to 85%. No other pathogens were present in 25% of the cases of diarrhea, and CRP was subclinical in 15% of infected calves.

These surveys had several deficiencies. Specimens were collected on only one occasion, non-diarrheic calves studied were on farms already found positive for CRP, and only herds having neonatal diarrhea were studied. Myers et al (1984), who studied 22 herds not having neonatal diarrhea, found them all negative for CRP. He concluded that the high prevalence of CRP, found by other authors in non-diarrheic calves, was likely due to the greater probability of finding subclinical infections in herds where the organism has already been identified, compared to that for the general population. These surveys indicate a high prevalence of CRP infection in cattle in many geographic locations.

TABLE 2.4 SURVEYS OF BOVINE CRYPTOSPORIDIOSIS ASSOCIATED WITH NEONATAL DIARRHEA.

AUTHOR/DATE	# HERDS	# POSITIVE/ # EXAMINED WITH DIARRHEA	NO DIARRHEA	UNSPEC.	COMMENTS
Morin et al, 1978	47	18/51(33)	3/21(14)	-	
Heine and Boch, 1981	-	88/266(40)	44/331(14)	-	14/23(61%) of calves dying with diarrhea were CRP positive
DeLeeuw et al, 1984	11	85/347(25)	ND	-	Cryptosporidium was the sole pathogen in these cases.
Sanford and Josephson, 1982	26	42/161(26)	ND	-	
Peeters et al, 1982	1	-	-	17/20(85)	only 4/17 had diarrhea.
Dan et al, 1983	8	(33-100)	-	-	from seven districts of Romania
Jungman and Hippe, 1983	6	-	-	87/172(51)	45% of infected calves had acute diarrhea.
Redman et al, 1983	20	-	-	135/839(16)	all 20 herds were CRP positive
					there was a strong association
					between infection and fluid stool.
Snodgrass and Sherwood, 1983	34	64/280(23)	8[1]/57(14)	-	neonatal diarrhea was a problem on all these farms.
Myers et al, 1984	22	ND	0/136	-	only farms with no neonatal diarrhea were studied.
Stein et al, 1984	4	-	-	69/72(96)	60(87%) had severe diarrhea.

1. only one of these was from a farm where calves were CRP negative.

b. Human surveys (Table 2.5):

Four human surveys of cryptosporidial infection were published in 1983. Tzipori et al (1983b) examined hospital patients over a period of 16 months in Austalia, finding 36/884 (4.1%) patients with gastroenteritis, and 0/320 without gastroenteritis, excreting cryptosporidial oocysts. Only 5/36 CRP patients had other enteric pathogens. The infection was most common in the late summer and fall, and the rate for children under 15 years of age was 4.8% compared to 1.6% for older persons.

In Finland, Jokipii et al (1983) found cryptosporidial oocysts in 14/1422 (0.98%) fecal specimens sent for routine parasitological examination. All their patients were adults and 13/14 had been travelling abroad; 12 to Leningrad. Giardia and CRP occurred simultaneously in 3 (21%) patients.

In Denmark, Holten-Anderson et al (1983) detected CRP in the absence of other pathogens in 10/800 consecutive stool specimens from patients with gastroenteritis. Six were children, of whom 3 were associated with calves having the infection; 2 were tourists returning from the USSR.

The Contagious Diseases Report (1984) reviews 61 cases of CRP occurring in Britain in 1983 indicating, that they occurred in various regions, were associated with diarrhea and vomiting in 53/61, and that two-thirds of the patients were under 15 years of age. Only 4 were immunodeficient, 2 were travellers returning from Spain, 2 were nurses caring for infected children and 2 had direct contact with animals.

Mata et al (1984) examined specimens from rural and urban children in Costa Rica, and found CRP in 4.3% of all diarrheic specimens examined, and in none of the specimens from non-diarrheic children. Infections occurred only during a warm wet season, between May and August. They found no cases in children <1 year of age in rural areas, whereas there was a rate of 3% in this age group in urban areas. This was attributed to the difference in breast feeding patterns between these two populations.

Wolfson et al (1984) found 2.6% of 1290 stool specimens positive for CRP, and 20-23% of these were also positive for Giardia. They suggested an association between these two protozoans.

In North Wales, Casemore and Jackson (1983, 1984) diagnosed CRP in 1.4-1.6% of diarrheal stool specimens. The rate in children <16 years of age was 2.8% compared with 0.34% in older persons. They found the severity of symptomes directly related to the oocyst count, when they examined consecutive specimens from patients and their families.

In Rwanda, Bogaerts et al (1984) found 10.4% of liquid stools from children, and 3% of those from adults, positive for CRP. The overall prevalence of CRP (7.8% of 293 specimens) was lower than that of Salmonella, but among children CRP was more prevalent. This survey lasted only 3 months. If a variable seasonal incidence occurs in that location, than the annual rate could have been higher or lower, depending whether the survey was taken at high or low season.

Four marasmic children had very severe diarrhea and one of these died. No oocysts were found in 94 formed stools.

De Mol et al (1984) compared children having measles and diarrhea, with 3 other groups in Rwanda. They found 25% of patients having both conditions excreting cryptosporidial oocysts, compared with 5.2% or less in the other 3 groups, and concluded that measles was a predisposing factor for CRP.

The Centers for Disease Control (1984) reported 5 outbreaks of CRP, in day care centers in the United States. In a center in Georgia, 5 cases were identified in children with recent diarrhea, none of whom were hospitalized. In another center in Georgia seven cases of CRP were identified in association with an outbreak of diarrhea. Only 2 of these children were asymptomatic. None were hospitalized. In Pennsylvania 20/59 (34%) of children in a day care center had diarrhea. Eleven of 17 (65%) diarrheic children and 3/28 (11%) non-diarrheic children had CRP. In a Michigan day care center 12/38 (55%) children examined during an outbreak of diarrheal disease had CRP. In California 6/11 classmates of the 2 year old index case, and 3 parents of these children, had CRP. All infected persons had mild to moderate diarrhea, but none were hospitalized. In a New Mexico day care center 18/38 children had diarrhea. Five had CRP, and 6 had giardiasis; 1 having both infections. Four of the Giardia positive specimens were not examined for CRP. In another room 7/13 children with recent diarrhea had CRP, and 6 had giardiasis.

Hunt et al (1984) examined 867 stools during the fall and winter of 1983 in Bristol, England. They found CRP in 7-8% of children <5 years of age, a rate equal to the sum of the rates of Salmonella, Shigella, Campylobacter, and Giardia during the same time period. Persons >25 years of age had only one half this incidence. They recognized two clusters in day care centers. Seventy-two percent of patients were in contact with household pets, but this was considered similar to the rate of pet contact in the general population. Some family contacts also had CRP. Most cases occurred in the age group consuming milk, but none were consuming unpasteurized milk. No cases occurred in infants <10 months of age, and the possibility that this was related to the low rate of cow's milk consumption in this group, was considered.

In Bangladesh, Rahaman et al (1984), studied cases of diarrhea among calves and their attendants on a dairy farm, during a 1 year period. They found 14% of 208 diarrheic calves <28 days of age positive for CRP, in contrast to only 1% of age matched non-diarrheic calves. Most cases occurred between 7 and 21 days of age during August and September. All 88 animal attendants had diarrhea in the same months, and at least 7 of these had CRP. Seven of 77 family contacts, associated with 2 positive animal attendants, also had CRP and diarrhea, while all 155 non-diarrheic family members examined were negative for CRP. This study suggested zoonotic as well as person-to-person transmission of CRP.

TABLE 2.5 ASSOCIATION OF CRYPTOSPORIDIOSIS WITH GASTROENTERITIS IN HUMANS

COUNTRY	WITH GASTROENTERITIS # POS/# EXAM(%)	WITHOUT GASTROENTERITIS # POS/# EXAM	AGE PROFILE	COMMENTS	REFERENCE
Australia	36/884 (4.1)	0/320	3x rate in children	summer and fall	Tzipori et al, 1983b
Finland	14/1422 (0.98)	ND	all adults	13/14 travellers	Jokipii et al, 1983
Denmark	10/800 (1.25)	ND	6 children	associated with calves	Holten-Anderson et al, 1983
Britain	61/?	ND	2/3 children	most immunocompetent	Cont. Dis. Report, 1983
Costa Rica	12/278 (4.3)	0/90	only children	more urban	Mata et al, 1984
USA	33/1290 (2.6)	ND	-	20-23% Giardia positive	Wolfson et al, 1984
N. Wales	25/1500 (1.6)	ND	19/25 children	see note 1.	Casemore and Jackson, 1984
England	7/800 (0.88)	3/?	-	see note 2.	Nichols and Thom, 1984
Rwanda	23/293 (7.8)	0/94	3 x rate in children		Bogaerts et al, 1984
England	43/867 (5.0)	ND	2 x rate < 5 yrs	milk associated?	Hunt et al, 1984
Bangladesh	14/165 (8.5)	0/155		see note 3.	Rahaman et al, 1984
New Zealand	10/407 (2.5)	ND	8/10 <10 yrs,	most frequent pathogen	Carter, 1984
Liberia	22/278 (7.9)	ND	only children	see note 4.	Hojlyng et al, 1984

Notes:
1. The clinical severity varied directly with the oocyst count.
2. Indicated that asymptomatic infections occur.
3. Indicated both zoonotic and person-to-person spread of the infection.
4. Some excreted oocysts for at least 1 month.

c. Evidence of Zoonotic Infection:

The first indication of zoonotic transmission of CRP was the infection of a veterinary student, who was caring for infected calves (Anderson et al, 1982). Later, a researcher in Alabama was infected, while surveying calves for CRP (Reese et al, 1982), and further investigation of other animal handlers identified several additional cases (Current et al 1983). Other evidence for transmission from calves comes from Rahaman et al (1984), as mentioned above. Two authors have implicated cats as a source of human CRP. Koch et al (1983) identified the infection in a companion cat, after CRP was diagnosed in an AIDS patient. Bennet et al (1985) found a child with leukemia, the owner of a CRP positive cat, shedding cryptosporidial oocysts.

6. Serology:

The indirect immunofluorescence (IIF) test has been used to detect Ab to Cryptosporidium, in sera from animals and humans. Tzipori and Campbell (1981) used cryostat sections of infected lamb intestine, as Ag, to test sera from 10 different species, including humans. They screened a varying number of sera, from a variety of sources, at a 1:10 serum dilution, and found Ab in 80-100% of nine of the species. Mice from a specific-pathogen-free colony were all negative, as were colostrum-deprived neonatal calves, lambs and pigs. Sero-conversion, between post infection day (pid) 7 and 14, was demonstrated in experimentally infected calves and lambs. Control sections from uninfected lambs,

were consistently negative, as were sections from specific-pathogen-free mice. None of the fluorescein conjugated, anti-species antisera reacted directly with either the infected or non-infected sections.

Campbell and Current (1983) tested sera from 5 groups of persons, using a similar procedure, with parrafin-embedded intestinal sections from infected mice as Ag. Sections from uninfected mice were used as controls, and the sera were titrated. All reactions at <1:40 were considered negative. The five groups were: 1. Infected with CRP, and recovered, 2. Exposed to a source of CRP, but no infection detected, 3. No known exposure to CRP. 4. Having AIDS with prolonged CRP. 5. Hypogammaglobulinemic with prolonged CRP. Sero-conversion was demonstrated in 7/12 persons in group 1. All persons in groups 3 and 5 were negative.

In order to demonstrate the specificity of their test, they included several control procedures. Only 2/6 sera with high titers for Toxoplasma gondii were weakly positive for CRP. One serum, having a high titer against CRP, was adsorbed using a variety of protozoal antigens. Adsorption with cryptosporidial oocysts massively decreased the titer of this serum, whereas adsorption with sporozoites of Isospora sp. did not decrease it, and adsorption with tachyzoites of T. gondii or bradyzoites of Sarcocystis sp. only slightly decreased it. The Ag used in each of these adsorption procedures was reacted with fluorescein conjugated anti-human serum. Only the CRP antigen fluoresced.

Koch et al (1985) used oocysts derived from feces as Ag, and detected Ab in hospital personnel, in contact with a patient having chronic CRP. Gastroenteritis occurred in some contacts, but no fecal specimens were examined for CRP.

7. Treatment and Control:

Treatment of CRP in immunodeficient persons has met with little or no success (Centers for Disease Control, 1982), however several authors have indicated improvement in patients after treatment with Spiramycin. Remission of symptoms occurred in one patient on Spiramycin (Collier et al, 1984), but the simultaneous reduction of immunosupression therapy, may have had a significant effect on the infection. Portnoy et al (1984) reviewed the effect of Spiramycin therapy in 9 immunodeficient patients. They reported remission of symptoms after one week of therapy in 5, and improvement in the condition of the other 4. Relapse occurred in some of these, and only 3 became negative for fecal oocysts in the post therapeutic period. Miller et al (1984) also reported improvement in one patient after spiramycin, but relapse occurred when therapy was discontinued.

The accepted methods of treatment in the neonatal diarrhea syndrome of calves, have been unsuccessful in the treatment of CRP. A wide variety of coccidiostats have been field tested against Cryptosporidium by veterinary practitioners, because of its similarities to coccidia. Without conducting controlled experiments, some practitioners suggested they could control outbreaks, by prophylactic use of

sulfamethazine, amprolium or decoquinate (Sanford and Josephson, 1982). Tzipori et al (1982b) tested 16 antimicrobials experimentally in mice, and found them completely ineffective in arresting or modifying the course of the infection. Moon et al (1982a) found an ornithine decarboxylase inhibitor ineffective, in experimentally infected pigs.

Of nine drugs tested by Moon et al (1982b), only Lasalocid (Roche) showed some reduction in oocyst excretion when used prophylactically, but it was toxic to the calves at effective doses. Angus et al (1984) also tested a number of drugs prophylactically in experimental CRP in mice. Arprinocin (MSD Agvet) produced a greater than 99% reduction of oocysts excretion, and only a few organisms remained in histological sections. Salmonomycin (Hoecht), Sulfaquinoxaline (May + Baker), and Dinitomide (Cheminex) also caused lower reductions in oocyst excretion.

Guenther (1983, 1984) found sulfamethazine, sulfadimadine, sulfaquinoxaline, oxytetracycline and metronidazole ineffective, and suggested that hygienic measures offered the only hope of control in an outbreak. Contrepois and Vallet (1984) indicated that therapy with sulfonamides, colistin, B vitamins and oral electrolytes led to recovery in calves with CRP associated diarrhea. Therapy with antibiotics, prior to the diagnosis of CRP, had apparently aggravated the condition. Tzipori (1983) reviewed the literature on CRP including a table which identified all drugs previously found ineffective against this protozoan.

CHAPTER 3. MATERIALS AND METHODS.

CHAPTER 3 MATERIALS AND METHODS

I. Prevalence Studies on CRP in Manitoba:

1. Collection of Fecal Specimens:

A. human.

A total of 3656 fecal specimens, obtained from 3593 persons, were examined by Mr. C. Koschick and the author. Initially, examinations were done on all fecal samples from patients with diarrhea, submitted to Cadham Provincial Laboratory in SAF preservative (Yang and Scholten, 1976) for parasitic examination. After the first month some specimens from patients with diarrhea, submitted unpreserved for bacteriological and virological examinations were also included. All suitable specimens (approximately 50/week) were collected on Tuesday of each week. Specimens were selected on the basis of their appearance as being diarrheal (ie. they took the shape of their container), and being in sufficient quantity to carry out the examination. The study included specimens submitted from October 1, 1983 to October 31, 1984.

B. bovine.

All fecal specimens submitted to the Provincial Veterinary Laboratory for the diagnosis of neonatal diarrhea of calves were examined, as well as some fecal specimens or mucosal scrapings obtained from calves submitted for post mortem examination. The study included specimens submitted from October 1, 1983 to October 31, 1984. A total of 182

bovine fecal specimens, representing 148 herds in which neonatal diarrhea was occurring were examined. These included 152 submissions from beef calves and 30 from dairy calves, representing 124 and 24 herds respectively. Each specimen from a calf represented a problem in a herd where a variable number of animals were affected by neonatal diarrhea. Ten farms were visited to collect additional specimens from neonates and from older animals. This was done to assess the management on these farms, and to determine whether asymptomatic infections, which could serve as a source for the neonates, were present. Seven had previously reported diarrheal illness, while 3 had not.

2. Examination Procedures.

A. Acid-fast staining:

Preliminary studies on the acid-fast staining techniques described in the literature (Henriksen and Pohlenz, 1981; Ma and Soave, 1983; Garcia et al, 1983), found them satisfactory for the diagnosis of acute cases in both humans and calves, although the internal structure of cryptosporidia was only partially demonstrated by these methods. The Kinyoun's acid-fast (KAF) procedure used by Ma and Soave (1983) (fig 3.1 A. and C.) was adopted for the examination of all fecal specimens, because of the shorter staining time and the inconvenience of using heat in the method of Garcia et al (1983). The method used was as follows: (Instructions for the preparation of the solutions used are included in Appendix 1).

a. Wash out preservative with PSS, and smear fecal specimen on glass slide and air dry.
b. fix slide in 100% methanol for 10 min.
c. Immerse slide in Kinyoun's carbolfuchsin for 2 min.
d. Wash in tap water.
e. Decolorize by dropping acid alcohol over smear until alcohol flows colorless.
f. Wash in tap water.
g. Counterstain for 20-30 sec. in Kinyoun's Methylene blue.
h. Wash in tap water, and air dry.
i. Scan slides for typical acid-fast spherical organisms, 2-6 um in diameter using 400X or 1000X magnification.

When large numbers of oocysts were present in a specimen, they were easily identified on KAF smears, however in some specimens a large proportion of unstained cryptosporidia were seen (fig 3.1 C.), confirming similar reports in the literature, that many cryptosporidial oocysts do not stain acid-fast (Miller et al, 1983). Organisms of varied morphology were also seen in some specimens, particularly those from asymptomatic cattle. I therefore attempted to develop a more sensitive staining procedure, to confirm the identity of the organisms being observed on KAF smears.

B. In-solution Acid-fast Staining:

A number of authors reported the ability to stain sporozoites within oocysts. I attempted to repeat a Giemsa "in solution" staining technique described by Pavlàsek (1982), in which sporozoites cytoplasm stained blue, while

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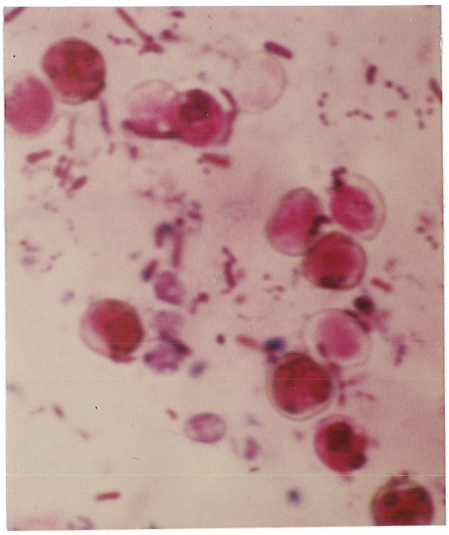
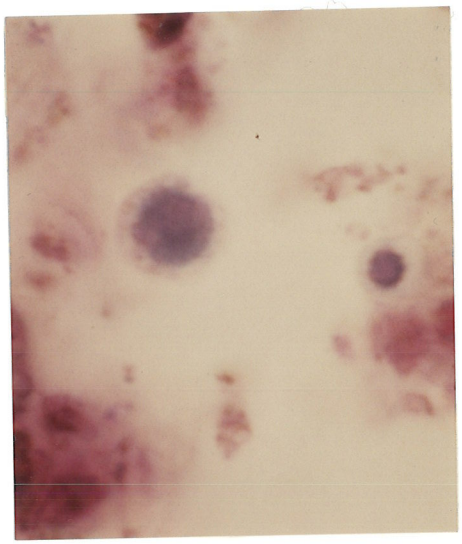
FIG. 3.1 VARIATIONS IN STAINING CHARACTERISTICS AND SIZE OF CRYPTOSPORIDIA FROM CLINICAL CASES.

A. Oocysts in calf feces, typically stained with KAF. B. Variation in size noted in cryptosporidia from a chronically ill bull (KAF "in solution" stain). C. A KAF preparation from calf feces. Note numerous unstained oocysts. Magnification: x 3,500.

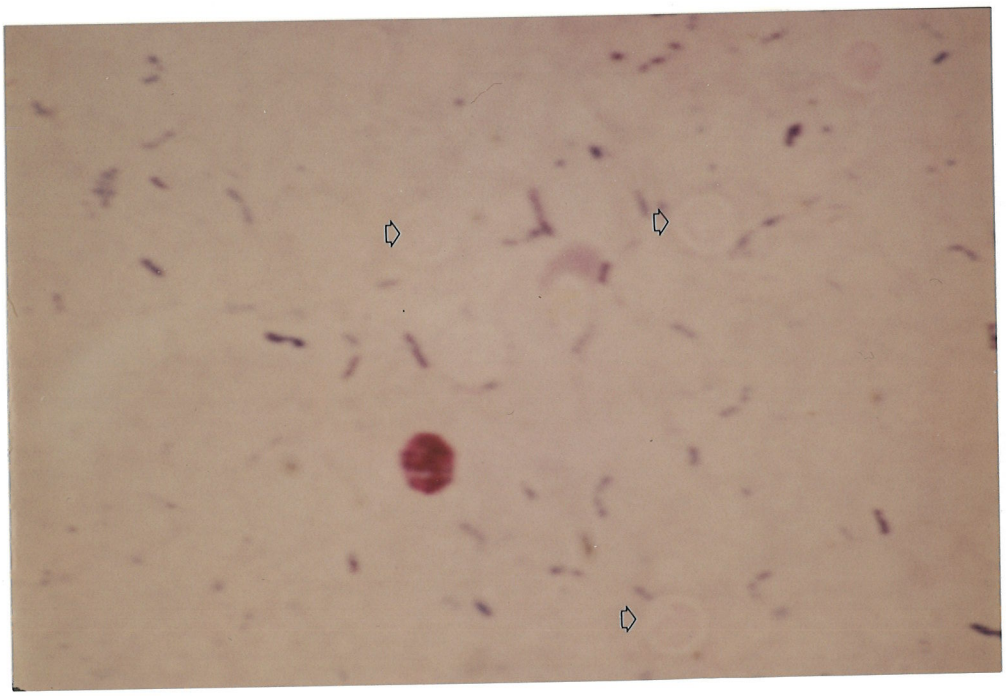
A.



B.



C.



nuclei stained red, but met with only minor success. I then attempted to adapt the KAF procedure for use in an "in solution" technique. This procedure proved successful only on one specimen, containing oocysts of a larger than average size, collected from a chronically ill bull. In this specimen large as well as small oocysts stained similarly; the carbolfuchsin was retained weakly by vacuoles and the residual body, while the sporozoites stained with methylene blue (fig 3.1 B). The staining procedure was as follows:

a. strain 3-5 ml of the preserved fecal specimen through 2 layers of gauze into a 15 ml centrifuge tube.
b. add PSS to fill the tube, mix and centrifuge 3-5 min at 800 x g.
c. discard supernatant, and wash several times with PSS to remove preservative.
d. add 5 ml methanol to sediment; fix for 10 min, centrifuge and discard supernatant.
e. wash out methanol with 2 additional PSS washes.
f. add 5 ml Kinyoun's carbolfuchsin; stain for 2-3 min, dilute with PSS, centrifuge and discard supernatant.
g. wash 3 times with PSS.
h. decolorize with 5 ml acid alcohol for 1 min; dilute with PSS, centrifuge and discard supernatant.
i. wash twice with PSS.
j. add 5 ml of Kinyoun's Methylene blue; stain for 1-2 min, and dilute with PSS.

k. centrifuge 5 min at 800 x g; remove supernatant.

At this point smears may be made on glass slides, which can be examined microscopically after air drying. Alternately, sugar floatation (Willson and Acres, 1982) can be carried out to separate the oocysts from fecal debris, and allow better visualization of the oocysts.

C. Fluorescent Staining with Specific Antiserum:

A crude suspension of the fecal specimen was prepared by removal of the gross debris, by simple sedimentation of a 1:10 dilution of feces in PSS for a 1 hr period, and discarding the sediment. The supernatant was centrifuged, and the pellet was smeared on glass slides. The slides were air dried, fixed in acetone for 10 min, and again air dried before storage at -56 C until examination.

The smears were exposed for 30 min to a rabbit antiserum to Cryptosporidium oocysts (obtained from Dr. W. Current), in a moist chamber, washed with phosphate buffered saline, and then exposed for 30 min to goat anti-rabbit IgG -fluorescein conjugate (Boehringer-Mannheim Bioch.) in a moist chamber. The slides were washed in phosphate buffered saline containing 7-10 drops of Evan's blue counterstain per liter, and air dried. Coverslips were mounted with glycerol solution and slides were examined under, epifluorescence using a 50x water immersion lens, for the presence of fluorescing spheres 2-6 um in diameter.

D. Electron Microscopy on Fecal Specimens:

In an attempt to resolve the problem associated with

the presence of a variety of oocyst morphologies on KAF smears, EM was performed with the help of Mr P. Hazelton, on three fecal specimens, previously diagnosed as CRP by stained smears. The first was from an acute case in a child, and contained mostly typical oocysts. The second was from an acute case in a calf, containing many typical oocysts, but also a large number that failed to stain by KAF. The third was from a chronically ill 2 year old bull. The organisms in this specimen had a much larger average size, than that of typical cryptosporidia. Fig. 3.1 shows photographs of some of these variations. The specimens were prepared for examination by EM according to procedures described by Hayat (1981), as follows:

a. Pellet fecal sample by centifugation an 1500 x g for 5 min. in a conical centrifuge tube.
b. Suspend pellet in 3 ml 2.5% Glutaraldehyde in 0.05 M Sodium Cacodylate, 0.01 M Calcium Chloride, pH 7.4 (Appendix 2). Fix for 10 min.
c. Pellet as in step 1.
d. Suspend pellet in 0.05 M Sodium Cacodylate, 0.01 M Calcium Chloride, pH 7.4 (Appendix 2), and wash for 5 min.
e. Pellet as in step 1.
f. Prepare Agarose cores (Yuan and Gulyas, 1981; Appendix 2) and treat as tissue blocks in subsequent steps.
g. Wash for 5 min in 0.05 M Sodium Cacodylate, 0.01 M Calcium Chloride, pH 7.4.

h. Fix for 1 hr by immersion in Osmium Tetroxide in 0.05 M Sodium Cacodylate, 0.01 M Calcium Chloride, pH 7.4 (Appendix 2).

i. Wash twice for 5 min each in 0.05 M Sodium Cacodylate, 0.01 M Calcium Chloride, pH 7.4.

j. Dehydrate through graded acetone as per the following schedule:

Concentration Acetone(%)	Time (min)
10	rinse
30	3
50	5
70	5
80	5
90	10
100	10 x2

k. Replace acetone with Propylene Oxide transition medium as per the following graded series:

Concentration Acetone(%)	Concentration Propylene Oxide(%)	Time(min)
75	25	10
50	50	10
25	75	10
0	100	15 x2

l. Infiltrate with plastic (Polysciences DER 332-732) as per the following schedule:

Concentration Propylene Oxide(%)	DER 332-732(%)	Time (min)
75	25	30
50	50	30
25	75	30
0	100	Overnight at 4 C
0	fresh 100	1 hr @ 37 C uncovered

m. Embed in freshly prepared plastic as per the following polymerizing shedule:

Temperature	Time (hrs)
37 C	24
48 C	24
60 C	24

n. Sections were cut at 1 um on the ultramicrotome, using a freshly fractured glass knife, and mounted on 200 mesh, formvar-carbon coated, copper grids.

o. They were then stained with Ethanolic Uranyl Acetate (50%)(Stempek and Ward, 1964) for 10 min, and counter-stained with Lead Citrate (Venable and Coggeshall, 1965) for 5 min in a carbon dioxide free atmosphere, and were immediately examined in a Phillips Model 201 Electron Microscope.

3. Collection of Clinical Data

Questionaires (Appendix 3) were sent to the attending physicians of CRP patients, and to the owners of infected cattle herds. For human cases we requested information regarding age, sex, onset and duration of diarrhea, frequency of bowel movements, association with fever, vomiting, colic and other illnesses, and history of travel or contact with animals. For bovine cases we requested information regarding morbidity and mortality in the herd, association with recent purchase of calves, onset and duration of diarrhea, response to treatment, history of neonatal diarrhea problems in previous years, and indication of any associated diarrheal illness in humans. Data concerning associated virus infections were obtained from the Provincial Veterinary Laboratory, and were based on examination by EM.

When responses suggested a possible animal source of human infections, and when such information was obtained quickly enough to be useful, fecal specimens were requested from the suspect animals. When questionaires submitted to herd owners suggested, that there may have been human diarrheal disease in association with the occurrence of CRP in calves, we requested fecal specimens and serum samples from such patients.

4. Serological Studies:

A. Sources of Antigen:

Four sources of fecal oocysts were evaluated. Ag 137 prepared from the feces of an acutely infected calf, excreting large numbers of oocysts, was used only in preliminary studies. Ag D was prepared from a chronically ill 2 year old bull, found to be excreting many oocysts shortly before death. Some oocysts were twice as large as the average oocyst in acute human or calf infections (Dr. W. Current had confirmed our identification of these organisms as cryptosporidia). Because some sera, negative with other cryptosporidial antigens, were found to have antibody to this antigen, it was used only as a comparison with the others, particularly against bovine sera. Ag 1838, prepared from the feces of an acutely infected calf, shedding large numbers of oocysts, contained large numbers of yeasts, which proved difficult to separate from the oocysts, and led to some difficulties in the serological tests. Ag 8/85 was prepared by Mrs. Ruhangiz Taghi Kilani Damavandi, from the feces of

an experimentally infected calf, contained a very high concentration of oocysts, and was used in most of the tests described in this thesis.

B. Preparation of the Antigen.

Oocyst suspensions were prepared using a combination of floatation and sedimentation procedures, adapted from a procedure obtained from Dr. D. Snodgrass (personal communications). The procedure used is as follows:

a. A calf fecal sample, containing large numbers of oocysts, was distributed (10 ml. per tube) to several 50 ml. centrifuge tubes.
b. Add 10 ml of PSS; stand for 60 min at room temperature.
c. Pour off and save supernatant.
d. Add 10 ml PSS and repeat the previous 2 steps.
e. Combine the 2 supernatants and discard the sediment.
f. Centrifuge combined supernatants at 800x g for 10 min; remove and discard the supernatant.
g. If sediment contains mucous, add several drops 10% KOH and mix until homogeneous.
h. Add PSS to dilute, centrifuge as before and discard supernatant.
i. Wash once or twice with PSS.
j. Resuspend sediment from each tube in ~ 3 ml of PSS, and transfer to a 15 ml centrifuge tube.
k. Add 6-7 ml of saturated sucrose solution (Willson and Acres, 1982), fill tube to 13 ml mark and centrifuge at 800x g for 20 min.

l. Remove surface 3-4 ml to a new tube, add PSS, mix and recentrifuge, discarding supernatant.

m. Wash 2 additional times to remove remaining sucrose.

n. Resuspend sediment in 2-3 ml of PSS to use as antigen.

o. Count the oocysts in the resulting final sediment in a hemocytometer, and dilute to a concentration of 450,000 oocysts/ ml.

p. Spot the oocyst suspension on to multiwell slides at the rate of 10 ul. per well, air dry, and fix in acetone for 15 min.

q. Air dry the slides and store at -56 C. until use.

C.Collection of Sera for Test.

(i) Sera from convalescent patients were collected by the attending physician of those patients who agreed to participate in our study. (ii) Approximately 50 sera from infected calves and their herd mates, were collected during visits to farms identified as having a problem with neonatal diarrhea, associated with CRP. Sera were obtained from diarrheic calves, their dams, non-diarrheic calves, and sometimes yearling animals in the herd. (iii) In addition, random samples of sera from mature Manitoba market cattle were obtained, by selecting every 100th serum submitted to the Animal Pathology Laboratory in the "market cattle surveillance" program for brucellosis. Those sera traceable to cattle originating on Manitoba farms, were included in the sero-survey for antibody to CRP. Fetal calf serum (Flow

Laboratories) was used as a negative control in the testing of bovine sera. Positive and negative human control sera, and an immune serum from a rabbit inoculated with cryptosporidial oocysts, were obtained from Dr. W. C. Current. All sera were stored at -20 C until testing.

D. Indirect Immunofluorescence Procedure:

The IIF procedure for Toxoplasma gondii (Gull Toxo; Gull Laboratories) in use at Cadham Provincial Laboratory was adapted to CRP by Mrs. G. Eibisch.

Reagents:

Buffer: The buffer included in "Gull Toxo" kits was used for serum dilution and slide washing.

Fluorescein Conjugates:

i. Anti-human combined IgG and IgM; The conjugate supplied with the "Gull Toxo" kit, and premixed with Evan's blue was used at the working dilution as supplied.

ii. Anti-bovine IgG (Miles-Yeda Ltd.) prepared in rabbits was used at 1:80 dilution (The optimum dilution was determined by performing a checker board titration using several dilutions of positive serum and conjugate).

iii. Anti-rabbit IgG (Boehringer Mannheim Bioch.) prepared in goats was used at 1:20 dilution (the optimum dilution was determined as for the bovine conjugate).

Evan's Blue counterstain was added to the wash water at a concentration of 7-10 drops per liter, after the incubation with conjugate.

Procedure:

a. Defrost antigen and sera, and air dry slides before use.
b. Make serum dilutions in preparation for desired test (ie. screening or titration). Screening was attempted first at 1:10 and 1:40 dilutions.
c. Place controls and test sera on antigen wells.
d. Incubate slides in moist chamber for 30 min and rinse off serum using a wash bottle containing buffer solution, taking care not to cross contaminate between wells.
e. Wash with buffer for 10 min using magnetic stirrer.
f. Air dry and apply appropriate dilution of conjugated anti-species serum to all wells.
g. Incubate slides in moist chamber for 30 min, and rinse off conjugate as in step d.
h. Wash with buffer (containing Evan's blue for bovine or rabbit tests) for 10 min using magnetic stirrer.
i. Air dry and mount cover slips with glycerol solution.
j. Read under epifluorescence using 50x water immersion objective (Read fluorescence only on morphologically characteristic oocysts; <1:40 reactions were considered negative).

Note: Titration was performed only on a few selected sera.

5. Data Analysis

In humans, an estimate of the incidence of CRP by age and sex was made by comparing the number positive, with the total number of specimens examined. The rate on the basis of the population in each region of Manitoba, and in each age

group was also calculated, using population data obtained from the Manitoba Health Services Commission. In cattle, age and sex were not considered in the fecal examination surveys, but association with certain management factors was evaluated. Since all bovine specimens were also examined for viruses, association of CRP and viruses was assessed. Serological results from sera collected on infected farms, were compared to those from the randomly collected sera. The Chi square test for significance was used to establish differences between groups. Relative Risk was calculated with the formula R.R.= Number positive/Number examined (group A) : Number positive/Number examined (group B).

II. Transmission Studies:

1. Experimental Animals:

Four litters of newborn Swiss (Webster) white mice were purchased, 2 to be infected and 2 to act as controls. Preinfection fecal specimens from the adult mice were examined for the presence of CRP.

2. Inoculum Preparation:

A fecal specimen collected from a diarrheic calf, was shown to contain large numbers of cryptosporidial oocysts by examination of stained smears. This specimen had been stored in potassium dichromate solution for a period of 5 months. Oocysts were separated from the fecal material by series of floatation and sedimentation procedures, adapted by a system of trial and error, from methods of purifying

oocysts obtained from Dr. D. R. Snodgrass (personal communications), and the fecal floatation procedures of Willson and Acres, (1982). The method used was as follows:

a. Place 10 ml of the preserved fecal specimen in a 15 ml centrifuge tube.
b. Centrifuge for 10 min at 800x g and discard the supernatant.
c. Wash out preservative with four consecutive washes with physiological saline solution (PSS), each being followed by centrifugation at 800x g. for 5 min and removal of the supernatant.
d. To approximately 3 ml of sediment add 6 ml of saturated sucrose solution (825 gr in 500 ml of distilled water), and fill tube with PSS solution to yield a final specific gravity of approximately 1.13.
e. Centrifuge at 800x g for 20 min.
f. Remove top 3-4 ml to another tube, fill with saline and sediment at 800x g for 10 min.
g. Discard supernatant, saving approximately 4 ml of the sediment.
h. Divide resuspended sediment into 2 equal portions to be labelled "A" and "B".

The infective inoculum "A" was stored at 4 C, and the control inoculum "B" was frozen overnight at -56 C to inactivate the oocysts. An oocyst count was done on inoculum "A" prior to use, by making smears using a known quantity of the preparation, staining with KAF and microscopically coun-

ting all oocysts in the smear under 40x magnification. This preparation contained approximately 300 oocysts per ul. No recognizable oocysts were seen on smears of inoculum B.

3. Infection Procedure:

Each infant mouse in litters 1 and 2 was inoculated per os with 10-20 ul of inoculum "A", using a 10 ul Eppendorf pipette. Each infant mouse in litters 3 and 4 was inoculated similarly with inoculum "B". The adult mice in litters 1 and 2 were inoculated with 200 ul of inoculum "A".

4. Specimen Collection:

All feces were collected from each of the four cages on alternate days from post infection day (pid) 2 to pid 16. Several additional collections were made, up to 4 weeks pid. Most of these specimens were presumed to have come from the adult mice, as no baby size fecal pellets were found.

On pid 5 and 8, three infant mice from each litter were euthanized, using a carbon dioxide chamber for 7-10 min. Each mouse was pinned out in dorsal recumbency, and the body cavities were opened by a ventral midline incision. The intestinal tract was removed and extended to full length. Five millimeter portions of the lower ileum and the mid colon were smeared on opposite ends of a labelled glass slide (the intestinal tracts were too small to allow opening and scraping of the mucosa), fixed in methanol, stained by KAF and examined for the presence of CRP. Similar portions of ileum and colon were also placed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate, 0.01 M calcium chloride (pH

7.4) for E.M. studies, and in 95% ethanol for possible use as Ag in immunofluorescence studies. The remaining intestinal tract, as well as portions of liver and lung, were preserved in 10% formalin for histopathology. On pid 19, the adult mice were euthanized and similar specimens were collected. On pid 40, the remaining mice were euthanized and again similar specimens were collected.

5. Processing of Smears:

The smears prepared from ileum and colon were fixed in methanol for 10 min. and stained by KAF in an identical manner to fecal smears. These were then examined by light microscopy using 400x and 1000x magnification.

6. Histopathological Procedures:

After fixing in 10% formalin, tissues were trimmed and processed through an automatic tissue processor, mounted in paraffin blocks, and sectioned at 6 um. The sections were stained with hematoxylin and eosin and examined by 400x light microscopy. Epoxy fixed tissue were sectioned at 1 um, stained with toluidine blue (Mercer, 1963) and examined at 400x and 1000x magnification by light microscopy.

7. Electron Microscopy:

Tissues collected from infected and control mice were processed as follows:

a. Place tissue samples in 2.5% Glutaraldehyde in 0.05 M Sodium Cacodylate, 0.01 M Calcium Chloride, pH 7.4. Allow to fix for at least 1 hr.

b. Follow steps g. to o. as found on pages 49 and 50.

CHAPTER 4. RESULTS.

CHAPTER 4 RESULTS

I. Prevalence Studies:

1. Confirmation of Diagnostic Methods:

a. Fluorescent Staining with Immune Sera:

On two occasions, 4/5 and 2/3 specimens, from asymptomatic animals in contact with diagnosed cases, previously found to contain a few oocysts by KAF examination, also reacted with the rabbit antiserum, serologically confirming the diagnosis of CRP. Six of 9 similar specimens containing few, and atypical oocysts, sent to Dr. Current for confirmation, were positive by a specific fluorescence test.

b. Electron Microscopy of Fecal Oocysts:

We could not demonstrate any differences between the morphologies of the oocysts in the three specimens examined. Fig 4.1 shows a cross section of a typical oocyst as seen in fecal specimens by EM. Figs 4.2, and 4.3 are oocysts as seen in fecal specimens from a person, and from an acutely diarrheic neonatal calf respectively. All have similar internal structures, but differences in the sectioning plane result in some structures not appearing in some specimens. Sporozoite nuclei were not identified in oocysts seen in feces, but presumably this was also due to the plane of sectioning in the particular organisms seen. Fig 4.1 and 4.3 demonstrate the lipid vacuole and the residual body well, whereas fig 4.2 has only cross sections of sporozoites. Fig 4.4 is also an oocyst seen in a human fecal specimen and is similar to the others, but this organism contains

FIG. 4.1 A cryptosporidial oocyst from a human fecal specimen. sp = sporozoite, lv = lipid vacuole, rb = residual body, ow = oocyst wall. Magnification: x 40,000.

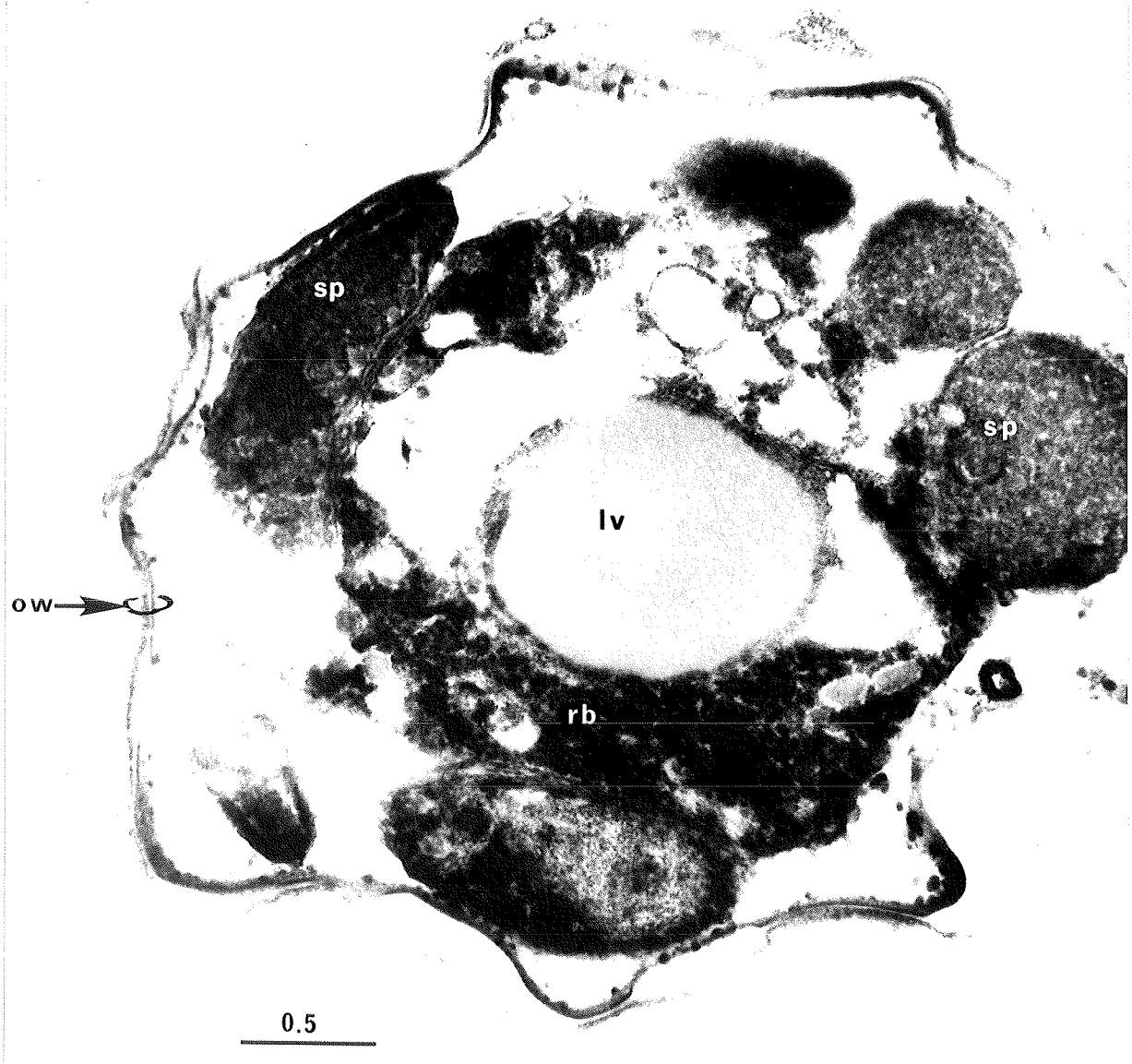


FIGURE 4.2 Cryptosporidial oocyst seen in human feces. It has cross sections of 4 sporozoites containing numerous unidentified granules. Note the similarities to oocyst "in situ" in the mouse intestine (fig 4.14).
g = granules, sp = sporozoite, ow = oocyst wall.
Magnification: x 50,000.

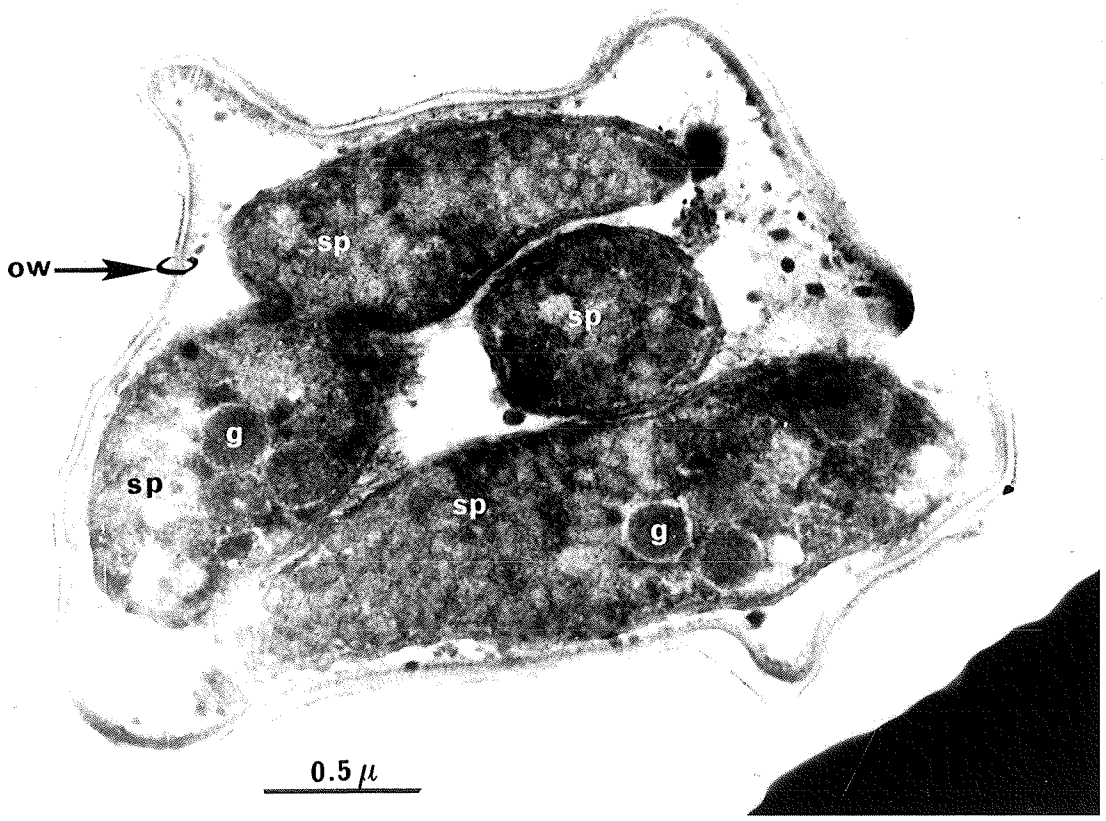


FIGURE 4.3 An oocyst as seen in feces from a diarrheic calf. sp = sporozoite, lv = lipid vacuole, ow = oocyst wall, rb = residual body. Magnification: x 40,000.

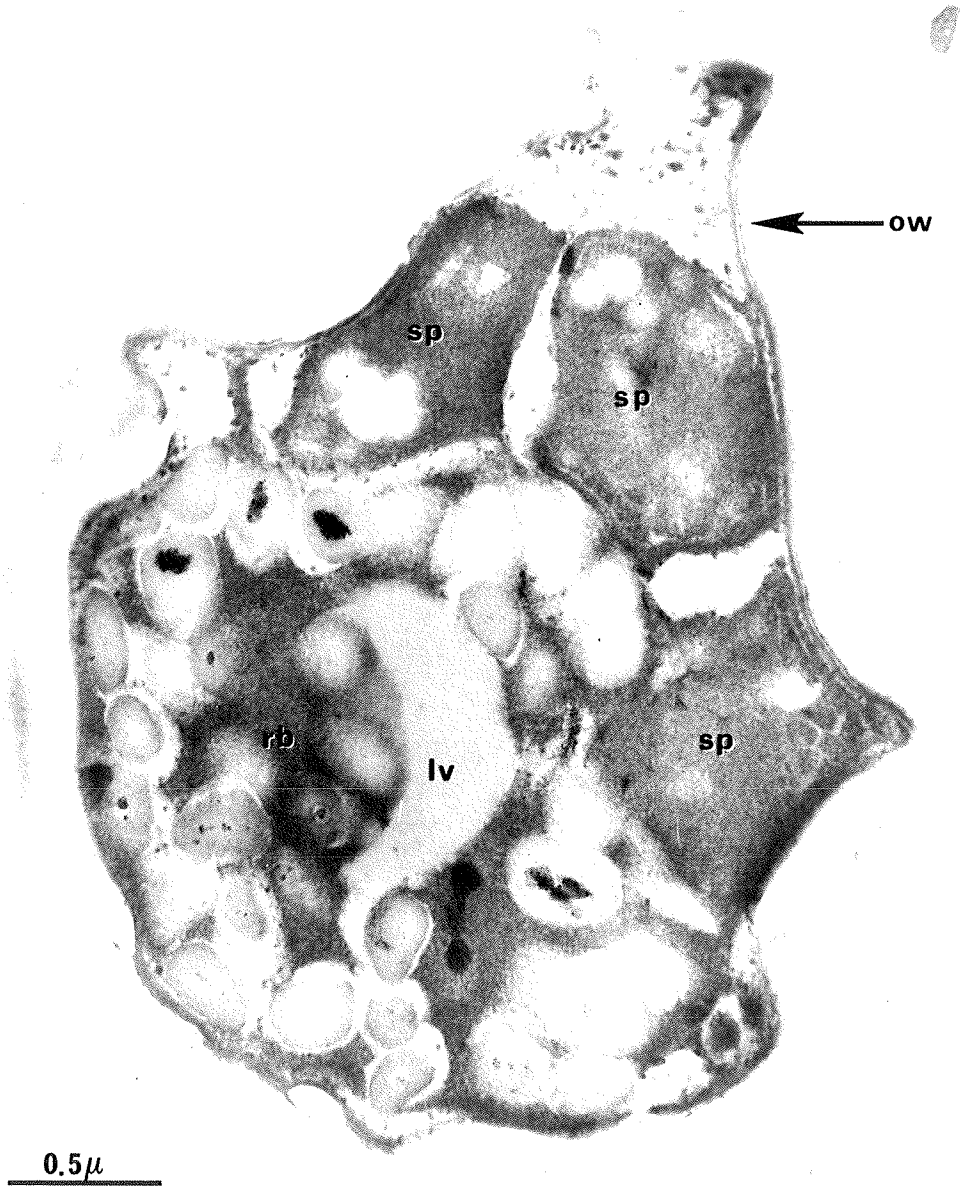
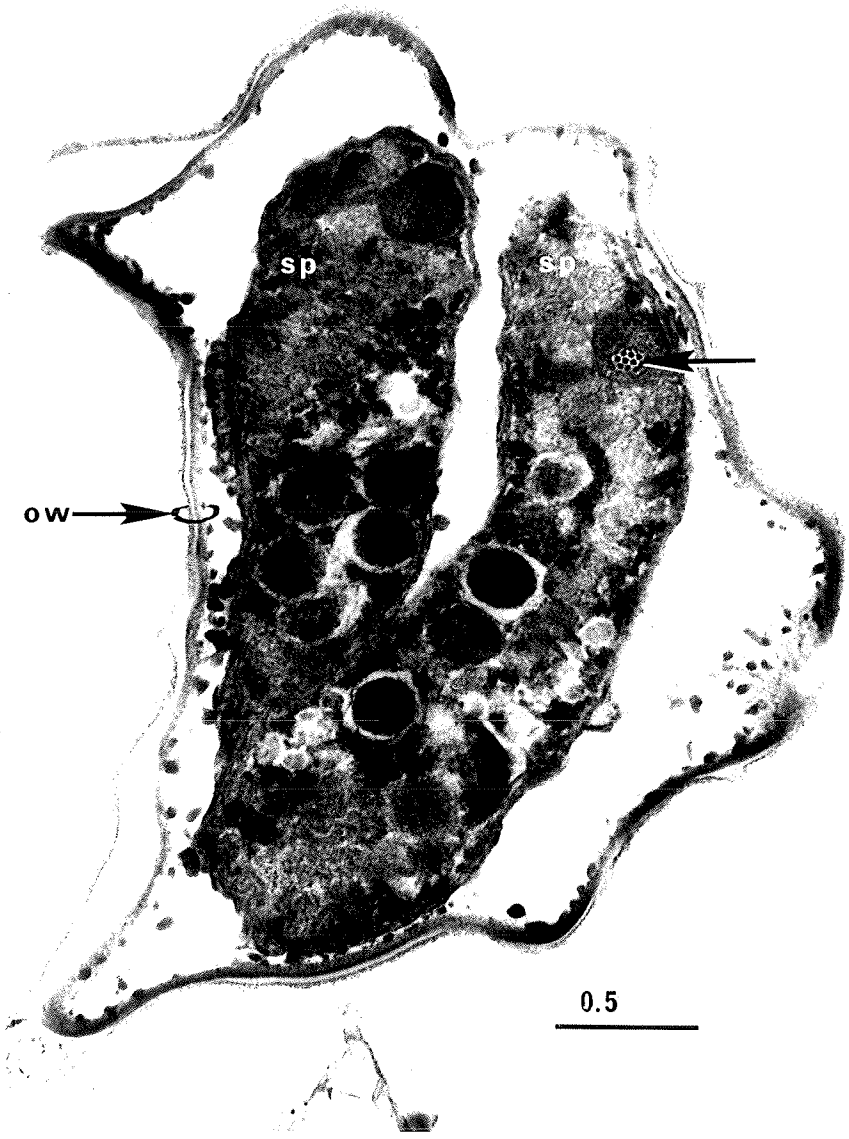


FIGURE 4.4 An oocyst from a human fecal specimen. This section shows only 2 of the 4 sporoziotes. Note the crystalline array of particles (25 nanometers) indicated by arrow. ow = oocyst wall, sp = sporozoite. Magnification: x 40,000.



an unidentified crystalline array of particles approximately 25 nanometers in diameter, seen on this one occasion only.

2. Prevalence of Human Cryptosporidiosis

During the period from October 1, 1983 to October 31, 1984, 39 persons, approximately 1% of those examined, were found to be shedding cryptosporidial oocysts. Table 4.1 gives the age and sex distribution of the human infections in Manitoba. The apparent preferences for males in the <5 age group and for females in the >5 age group were not significant. The overall preference for the <5 age group as compared to the >5 age group was statistically significant ($p < 0.025$). The relative risk for <5's as compared with >5's was 2.29. The age range was from about 2 months to 71 years but there were only 4 cases in persons over 30 years of age.

The age specific rates of CRP for the year ending October 31, 1984 (table 4.2) indicated a significantly higher risk for children <5 years old as compared to persons >5 years old ($p < 0.0005$). A significantly higher risk was also shown for a very defined group of children who are >1 and <2 years of age, as compared to any other group within the <5's ($p < 0.005$), or the average of the <5's ($p < 0.05$).

The geographic locations where human cases occurred, and the locations of positive cattle herds is shown on fig 4.5. Human CRP occurred province-wide, however 24 of the 39 patients (61.5%) were living in rural areas. Sixteen persons (13 < 2 years of age) were from northern native reserves and 8 were from farms. Fourteen were identified as urban, of

TABLE 4.1 AGE AND SEX DISTRIBUTION OF HUMAN CRYPTOSPORIDIOSIS IN MANITOBA.

AGE[1]	MALES[2]	FEMALES[2]	TOTAL[2]
<5	17/918(1.85)	8/787(1.16)	25/1605(1.5)[a]
>5	5/900(0.56)	9/1088(0.82)	14/1988(0.7)[a]
Total	21/1818(1.15)	17/1775(0.96)	39/3593(1.06)

1. in years.
2. number positive /number examined (% positive).
a. $p < 0.025$, and Relative Risk = 2.29

TABLE 4.2 CASES OF HUMAN CRYPTOSPORIDIOSIS PER HUNDRED THOUSAND POPULATION IN MANITOBA FOR THE YEAR ENDING OCTOBER 31, 1984.

	AGE (YEARS)	# OF CASES	AGE SPECIFIC POPULATION*	RATE/100,000 POPULATION
I	<5	22	83,494	26.3[a,b]
	1) <1	4	16,847	23.7[c]
	2) 1	12	16,885	71.0[b,c]
	3) 2	2	16,676	12.0
	4) 3	2	16,623	12.0
	5) 4	2	16,463	12.0
II	>5	11	1,015,268	1.1[a]
Total		33	1,098,763	3.0

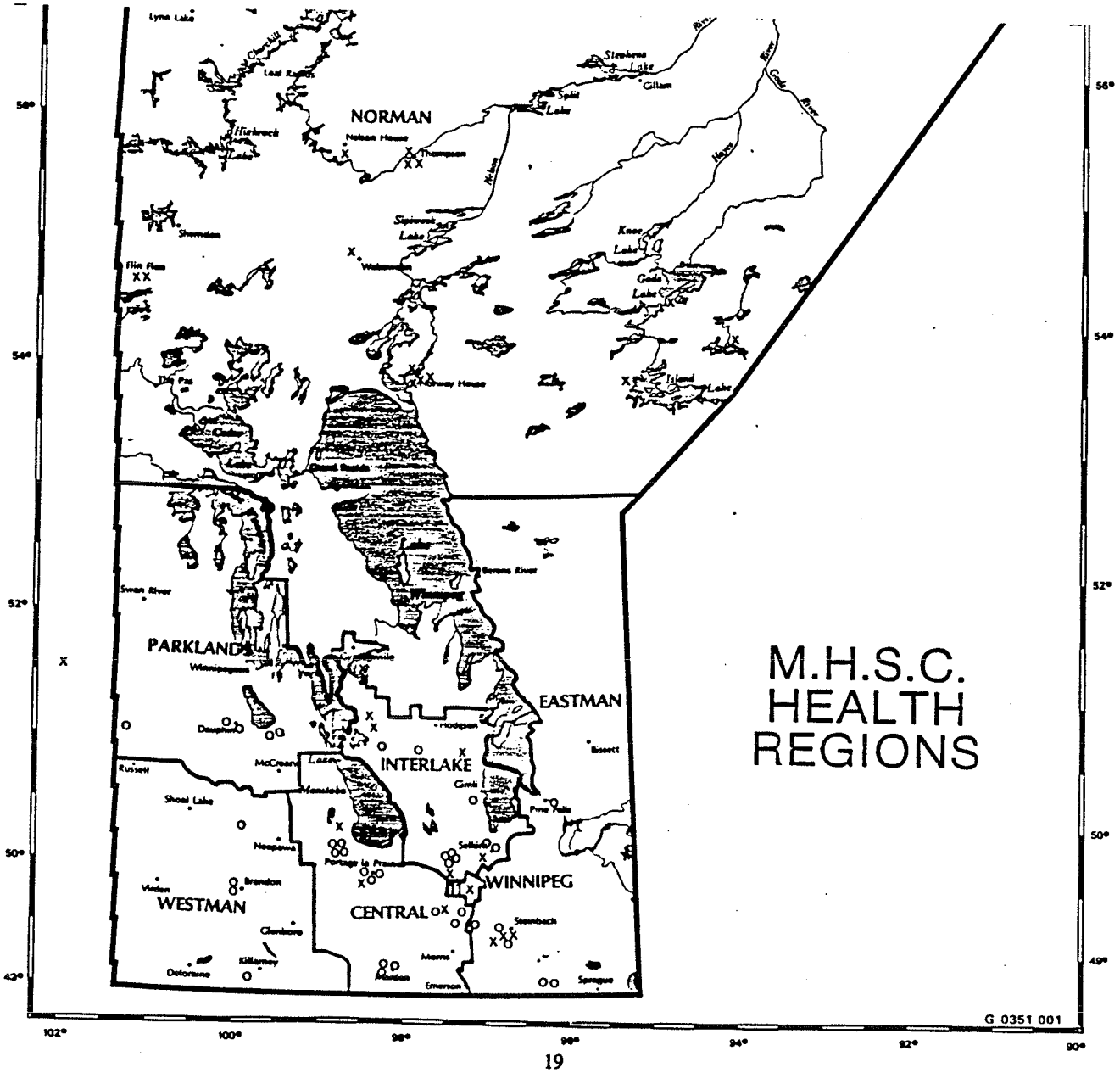
* Manitoba Health Services Commission, Statistics 1984.

a. significantly different ($p < 0.0005$).

b. significantly different ($p < 0.005$).

c. significantly different ($p < 0.05$).

FIGURE 4.1 Geographic location of human and bovine cases of cryptosporidiosis within Manitoba between October 1, 1983 and October 31,1984. x = human case. o = a positive cattle herd. Note: Eleven human cases occurred in the city of Winnipeg.



which 11 occurred in Winnipeg; 4 of these were children aged 1-3 years, all of whom attended the same day care center. One patient was a pediatrician and 1 was a nurse attending sick children. The rates per 100,000 population for the year ending October 31, 1984 on the basis of the region of Manitoba where patients lived (table 4.3), indicated a major difference between northern and southern communities. The former had a rate of 13.6/100,000 whereas the latter had only 3.09/100,000 in rural areas and 1.15/100,000 in Winnipeg city. The rate in the northern as compared to both rural and urban southern populations was significantly higher ($p < 0.0005$), but rates in rural and urban southern populations were not significantly different. The regional distribution of cases in children <5 years of age (table 4.4) was similar to that for the entire population (table 4.3), but these data indicated a very high risk of infection (92.4/100,000 population) in northern children. The rate in 1 year old northern children was 293.0/100,000, compared to 23.8/100,000 in Winnipeg (table 4.5; $p < 0.0005$).

Most human cases occurred in the late summer and fall (fig 4.6).

Twenty of 38 questionaires sent to physicians, were returned for a response rate of 52%. Only 2 persons gave a history of travel outside of Manitoba. Two persons believed their illness was water related, and 3 children from farms had a history of having consumed unpasteurized milk. Fourteen of the 20 patients had animal exposure; three were in

TABLE 4.3 HUMAN CRYPTOSPORIDIOSIS BY MANITOBA REGION FOR THE YEAR ENDING OCTOBER 31, 1984.

MANITOBA REGION	# OF CASES	POPULATION[1]	RATE[2]
A. Winnipeg	7	610,391	1.15[b]
B. Norman	17	124,800[a]	13.62[b,c]
C. Non urban southern regions	9	291,147	3.09[c]
1) Eastman	3	78,569	3.81
2) Central	2	92,395	2.16
3) Interlake	3	69,716	4.30
4) Parkland	1	50,467	1.80

1. Manitoba Health Services Commission, Statistics 1984.
2. per hundred thousand population.

a. Population of Norman Region plus "Status Indian" population of Manitoba.

b. significantly different ($p < 0.0005$).

c. significantly different ($p < 0.0005$).

NOTE: No data are included from Westman Region since stool specimens are not received in Cadham Provincial Laboratory.

TABLE 4.4 REGIONAL DISTRIBUTION OF CASES OF CRYPTOSPORIDIOSIS IN CHILDREN 5 YEARS OF AGE AND UNDER IN MANITOBA, FOR THE YEAR ENDING OCTOBER 31, 1984.

MANITOBA REGION	# OF CASES[1]	POPULATION[2]	RATE[3]
A. Winnipeg	3	41,262	7.3[b]
B. Norman	15	16,235[a]	92.4[b,c]
C. Non-urban southern regions[4]	4	24,337	16.4[c]

1. in children less than 5 years of age.
2. Manitoba Health Services Commission, Statistics 1984.
3. per hundred thousand population.
4. Eastman, Central, Interlake and Parkland. No data are included from Westman Region since stool specimens are not received in Cadham Provincial Laboratory.

a. Population of Norman Region plus "Status Indian" population of Manitoba.

b. significantly different ($p < 0.0005$).

c. significantly different ($p < 0.001$).

TABLE 4.5 RISK OF CRYPTOSPORIDIOSIS FOR 1 YEAR OLD CHILDREN IN TWO REGIONS OF MANITOBA FOR THE YEAR ENDING OCTOBER 31, 1984.

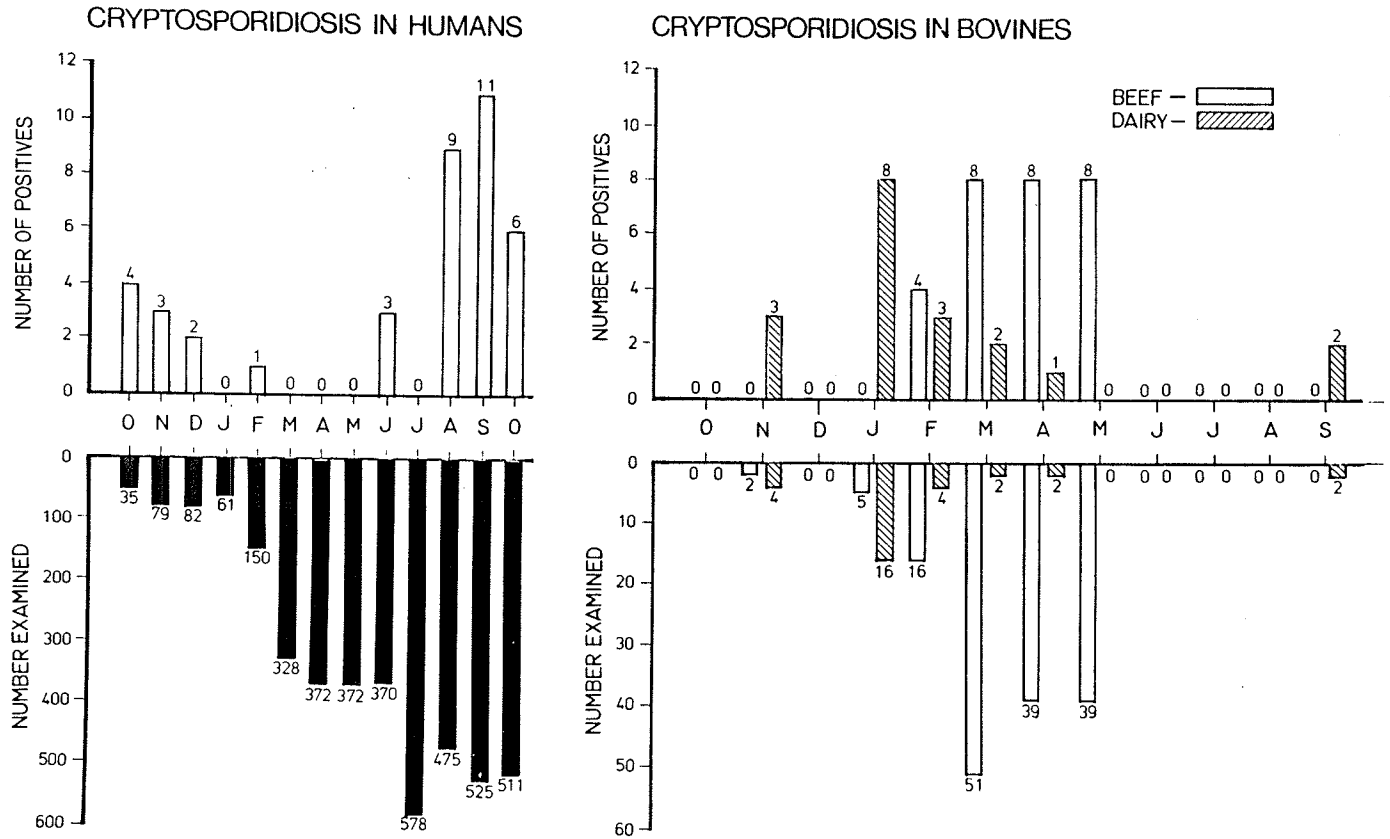
	# OF CASES[1]	POPULATION[2]	RATE[3]
Norman	10	3,413[a]	293.0[b]
Winnipeg	2	8,381	23.8[b]

1. in children >1 and <2 years of age.
2. Manitoba Health Services Commission, Statistics 1984.
3. per hundred thousand population.

a. Population of Norman region plus the "Status Indian" population of Manitoba.

b. significantly different ($p < 0.0005$).

FIGURE 4.6 The monthly distribution of cases of cryptosporidiosis in humans and calves, in relation to the number of specimens examined from October 1, 1983 to October, 31, 1984.



contact with cattle, 5 with cats, 8 with dogs, and 1 with a budgie. Two infected children had contact with a puppy found to be shedding CRP oocysts. Since this puppy had been obtained from the Winnipeg Humane Society, examinations were also made on fecal specimens from 8 young dogs from that institution, but no oocysts were demonstrated. Diarrhea was reported among family contacts of some human cases. On 5 occasions when CRP was diagnosed in cattle herds, owners admitted that there had been coincident human diarrheal disease in either animal attendants or family members. We were not able to determine the etiology of these illnesses, as no fecal specimens were obtained.

Some patients had other associated illnesses, including one 14 year old male with Ewing sarcoma and a 71 year old female with chronic lymphocytic leukaemia, two conditions regularly associated with immunodeficiency, but both patients were considered immunocompetent. One child had been hospitalized for an ocular condition and another had an upper respiratory infection. Only 4 of the 20 patients had other enteric pathogens detected; one had Aeromonas hydrophila, one had Shigella sonnei, one had recently been successfully treated for Campylobacter jejuni and one had Taenia saginata.

All 20 patients had an acute watery diarrhea which was self-limiting. The duration varied from 4 days to 12 weeks and was less than 2 weeks in 12 of the 20 cases. The number of bowel movements per day also varied from 4 to more than 20 but was less than 13 in three quarters of the cases. Two

patients had blood in their stool, two persons had recurrent diarrhea, and 4 children were hospitalized due to dehydration. Diarrhea was associated with fever in 12/20, vomiting in 12/20 and colic in 9/20.

2. Prevalence of Bovine Cryptosporidiosis

Forty-seven (25.8%) of the bovine specimens, representing 38 (25.7%) of the herds examined, were found positive for CRP. We found 28(18.4%) of 152 submissions from beef calves, and 19(63.3%) of 30 from dairy calves positive (table 4.6). Fig 4.6 compares the seasonal occurrence of CRP in beef and dairy calves to that of humans. The CRP season for humans overlaped somewhat with that for dairy calves, but not with that for beef calves.

CRP in calves was commonly associated with enteric viruses. Ten of the 47 cases (21.0%) were positive for Rotavirus, 11(23.4%) for Coronavirus, and 4(8.5%) for Toga-like viruses. Approximately 27% of the 47 CRP positive calves were also infected by one or more pathogenic viruses.

Twenty-five (65.8%) of 38 questionaires sent to owners of positive herds were returned. Three owners indicated that there may have been a link between diarrhea among their calves, and the purchase of calves from other farms or cattle markets. This practice occurred most commonly in herds which also had other indicators of poor management, such as co-maintainance of beef and dairy animals, and animals of various ages and species (table 4.7). The same class of herds also had the highest rate of mortality associated with

TABLE 4.6 DISTRIBUTION OF CRYPTOSPORIDIOSIS IN DIARRHEIC BEEF AND DAIRY CALVES IN MANITOBA HERDS, FROM OCTOBER 1, 1983 TO OCTOBER 31, 1984.

	SPECIMENS[1]	HERDS[1]
BEEF	28/152 (18.4)[a]	24/124 (19.4)[b]
DAIRY	19/30 (63.3)[a]	14/24 (58.3)[b]
TOTAL	47/182 (25.8)	38/148 (25.7)

1. number positive / number examined (% positive).

a. significantly different ($p < 0.0005$).

b. significantly different ($p < 0.0005$).

TABLE 4.7 THE ASSOCIATION OF THE PURCHASE OF CALVES WITH THE OCCURRENCE OF CRYPTOSPORIDIOSIS IN VARIOUS CLASSES OF FARMS.

Class[1]	Purchase Calves (%)[2]
a	6(54.5)
b	2(40.0)
c	1(50.0)
d	6(86.0)

1. a. producing only beef(11 farms), b. producing primarily dairy products(5 farms), c. producing beef and dairy products in segregated herds(2 farms),and d. producing beef and dairy products in unsegregated herds(7 farms).
2. The number of herds in the class that purchase calves from undefined sources.

neonatal diarrhea (table 4.8). Table 4.8 shows the mortality rate in 4 defined classes of cattle herds. Cryptosporidium associated diarrhea led to an ~15% case fatality rate in beef herds. In dairy or mixed herds, case fatality rates ranged from about 35-60%.

Eight owners reported that neonatal diarrhea had been worse in 1984 than in previous years. An additional 4 indicated no neonatal diarrhea problem in the previous 2 years. Onset of diarrhea occurred during the first week of life, and had a duration of less than 2 weeks in the majority of herds. However, there was considerable variability in both time of onset and duration, within as well as between herds, this possibly being due to multiple etiologies involved in the syndrome on some of these farms, as indicated by the high rate of virus co-infection. Specimens were not specifically examined for "enteropathogenic" Escherichia coli, a well known cause of neonatal diarrhea in calves that may have occurred on some of these farms.

Clinical signs in calves included yellow to green watery diarrhea leading to rapid dehydration and death within 1 to 2 days in many cases. If recovery occurred the diarrhea tended to become brown and there was a long convalescence with some relapses. A wide variety of antimicrobials, fluids, and electrolytes were used orally, parenterally or often both simultaneously, in the treatment of neonatal diarrhea in calves. Results of treatment were disappointing on many

TABLE 4.8 MORTALITY RATE IN NEONATAL DIARRHEA ASSOCIATED WITH CRYPTOSPORIDIOSIS IN CATTLE.

Class[1]	Mortality(%)
a	62/414 (15.0)[2,3]
b	23/41 (56.0)[2]
c	4/11 (36.4)
d	22/37 (59.5)[3]

1. a. producing only beef(11 farms), b. producing primarily dairy products(5 farms), c. producing beef and dairy products in segregated herds(2 farms),and d. producing beef and dairy products in unsegregated herds(7 farms).
2. significantly different ($p < 0.0005$).
3. significantly different ($p < 0.0005$).

farms, but one owner reported a dramatic improvement after injecting affected calves with Ivermectin.

On 9 of 10 farms visited, additional cases of cryptosporidial infection were identified, including 10 neonatal calves with diarrhea, 15 without diarrhea and 3 older calves that had diarrhea previously. In 18 instances, oocysts were also found in the feces of asymptomatic adult cattle. A chronically ill 2 year old bull in a beef herd, and 2 neonatal calves in a dairy were found excreting large numbers of oocysts. No neonatal diarrhea problem was reported from either farm, but the calves had loose stools at the time the specimens were collected. Oocysts were also detected in asymptomatic yearling and adult animals on two farms where CRP had not already been associated with neonatal diarrhea. On two farms with the infection in cattle, fecal specimens from barnyard hens contained cryptosporidial oocysts.

4. Serological Surveys:

a. Results of Preliminary Serological Studies:

The oocysts of all antigen preparations fluoresced specifically with the rabbit antiserum provided by Dr. W. Current for antigen control. This serum did not react with most fecal debris, and therefore it was possible to recognize the specific size, shape and staining characteristics of oocysts. Fig 4.7 shows an example of a positive and a negative reaction using Ag 137, and human sera.

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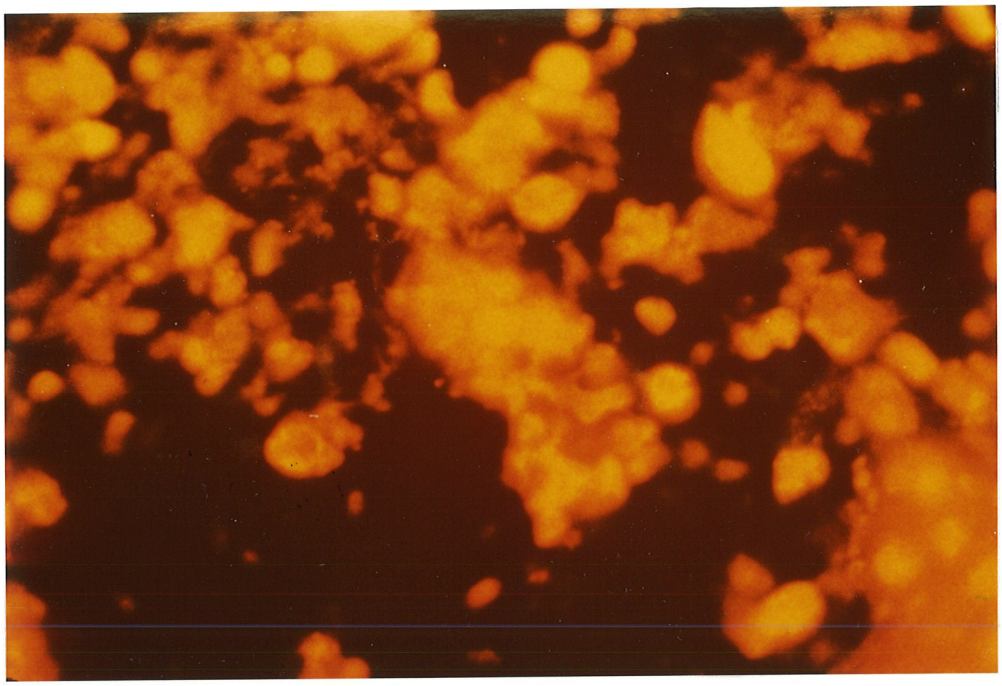
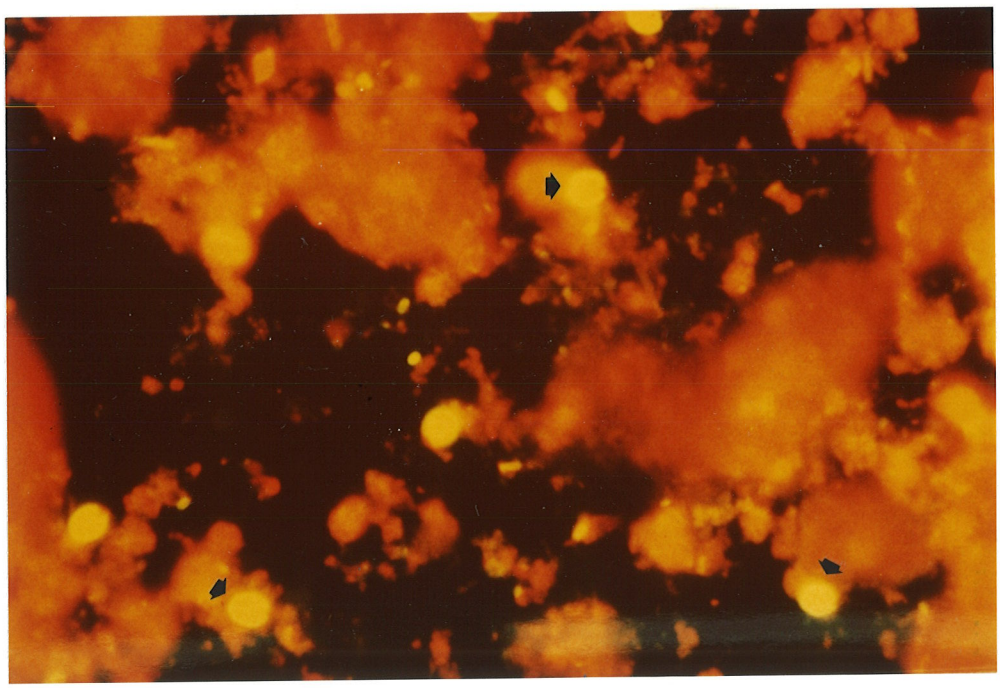
FIGURE 4.7 A. negative, and B. positive reactions in the indirect immunofluorescence test for cryptosporidiosis. Magnification: x 1800. (Ag 137; Human sera).

A.



B.



The buffer wells were consistently negative, indicating that none of the 3 conjugates used reacted directly with any of the 4 antigen preparations. Cerebrospinal fluid used as a control in the human system, and fetal calf serum in the bovine system were consistently negative for all antigens. Consistently negative sera were identified for each system. Seven bovine sera and 5 human sera titrated on several occasions had consistent titers against each antigen, demonstrating the repeatability of the test. Maximum titers ranged between 1:160, and 1:320.

Three of 4 Toxoplasma positive sera from sheep were negative, and the 4th had only a low titer for CRP. Four human Toxoplasma positive sera were negative for CRP. Four CRP positive sera from humans and 3 from cattle were negative against Toxoplasma antigen. These findings indicate low cross-reactivity between these two sporozoa.

b. Results of Human Serum Testing:

Five patients positive for CRP by KAF also had Ab against Ag 137, and Ag 1838. Two additional sera negative for Ag 137, and Ag 1838, reacted consistently with Ag D. The 5 reacting with all antigens, were positive at higher dilutions, against Ag D.

c. Results of Bovine Serum Testing:

A significantly larger proportion of sera collected on infected farms reacted with the calf derived antigens (Ag 1838 and Ag 8/85) than with Ag D (table 4.9), supporting

TABLE 4.9 COMPARISON OF NUMBER OF BOVINE SERA, FROM FARMS WITH CRYPTOSPORIDIOSIS, HAVING ANTIBODY TO THREE DIFFERENT CRYPTOSPORIDIUM ANTIGEN PREPARATIONS.

	# TESTED	# POSITIVE (%)		
		AG D[a]	AG 1838[b]	AG 8/85[c]
CALVES	26	20(77)	14(54)	13(50)
ADULTS	24	24(100)	16(67)	16(67)
TOTAL	50	44(88)[d]	30(60)	29(58)[d]

a. recovered from feces of bull.

b. recovered from a naturally infected calf.

c. recovered from an experimentally infected calf.

d. significantly different ($p < 0.001$).

evidence of antigenic difference between Ag D, and the other 2 antigens, as was suggested by the human sera tests. Fifty-eight to 88% sera collected on farms having CRP in calves, had antibody to Cryptosporidium. It is difficult to interpret differences found between the various subgroups of animals on farms where CRP occurred (table 4.10), since the number of sera tested was small. The high prevalence of Ab in very young calves, may indicate passive transfer in colostrum, as reported by Current (1983). Approximately 41% of random sera from Manitoba market cattle had Ab to CRP (table 4.11), indicating a high prevalence of the infection in Manitoba herds. Table 4.12 shows a higher percentage of adult cattle, in known infected herds, were sero-positive, demonstrating a correlation between exposure and Ab prevalence.

Table 4.11 also compares percentages of antibody positive sera in cattle from the veterinary districts in Manitoba. A statistically significant difference was found between Boissevain and Emerson districts, but the number of sera tested was small, making interpretation difficult.

TABLE 4.10 PREVALENCE OF ANTIBODY TO *CRYPTOSPORIDIUM* IN BOVINE SERA, ACCORDING TO CLASS OF ANIMAL, ON INFECTED FARMS (Using Ag 8/85).

CLASS	NUMBER TESTED	NUMBER POSITIVE(%)
Ai	5	4 (80.0)
Aii	6	2 (33.3)
Bii	12	5 (41.7)
Biii	2	1 (50.0)
Cii	1	1 (100.0)
Beef Cows	7	5 (71.4)
Dairy Cows	14	8 (57.1)
Dairy Heifers	3	3 (100.0)
TOTAL	50	29 (58.0)

A = < 3 weeks of age. i = diarrheic.

B = 3-8 weeks of age. ii = history of diarrhea.

C = 9 weeks to 1 year. iii = no history of diarrhea.

TABLE 4.11 THE PREVALENCE OF ANTIBODY TO CRYPTOSPORIDIUM IN MARKET CATTLE ORIGINATING IN VARIOUS MANITOBA VETERINARY DISTRICTS.

DISTRICT	NUMBER EXAMINED	NUMBER POSITIVE (%)
Swan River	7	3 (42.9)
Dauphin	24	8 (33.3)
Interlake	7	3 (42.8)
Carman	36	14 (38.9)
Portage la Prairie	11	5 (45.5)
Emerson	5	0 (0.0)[a]
Winnipeg	27	12 (44.4)
Brandon	29	13 (44.8)
Boissevain	15	8 (53.3)[a]
Minnedosa	16	8 (50.0)
Shoal Lake	9	2 (22.2)
TOTAL	186	76 (40.9)

a. significantly different ($p < 0.05$).

TABLE 4.12 THE PREVALENCE OF ANTIBODY TO CRYPTOSPORIDIUM IN SERA FROM ADULT CATTLE ON KNOWN INFECTED FARMS, AS COMPARED TO SERA RANDOMLY COLLECTED FROM MANITOBA MARKET CATTLE.

	NUMBER EXAMINED	NUMBER POSITIVE (%)
Random Sera	186	76 (40.9)[a]
Infected Herds	24	16 (66.7)[a]

a. significantly different ($p < 0.025$).

II. Transmission Studies:

Table 4.13 summarizes the observations made on intestinal smears from mice sacrificed on pid 5 and pid 8. Cryptosporidia were identified in the ileum and the colon of all infected mice (fig 4.8), and were absent in the control groups on both occasions. These results were confirmed by histopathology (table 4.14), which revealed the presence of tissue stages of the organism (fig 4.10), thus excluding the possibility that the organisms seen on smears were those in the infective inoculum. Some ileal sections contained large numbers of various life stages of Cryptosporidium (fig 4.10). There was extensive villous atrophy, and loss of microvilli from the ileal mucosa, compared to the normal mucosa (fig 4.9). This difference is also evident on electron micrographs, as shown by comparing fig 4.12 with fig 4.11.

No cryptosporidia were seen in fecal specimens, taken from the adult mice prior to infection, or in 13 specimens taken from other adult mice in the colony. The adult mice were not observed to be shedding cryptosporidial oocyst, at any time post infection, and no cryptosporidia were detected in the adult mice when they were sacrificed on pid 17. The remaining young mice, examined on pid 38, were also found negative for CRP. No clinical signs of disease were noted in any of the mice at any time.

TABLE 4.13 THE OCCURRENCE OF CRYPTOSPORIDIA ON INTESTINAL SMEARS OF EXPERIMENTALLY INFECTED AND CONTROL MICE.

LITTER	INOCULUM[1]	MOUSE	PID* #5		PID #8	
			ILEUM	COLON	ILEUM	COLON
1	A	1	3+	4+	5+	4+
		2	1+	1+	2+	2+
		3	4+	3+	2+	3+
2	A	1	1+	4+	2+	5+
		2	3+	4+	3+	5+
		3	2+	2+	3+	5+
3	B	1	-	-	-	-
		2	-	-	-	-
		3	-	-	-	-
4	B	1	-	-	-	-
		2	-	-	-	-
		3	-	-	-	-

1. Inoculum A, given to litters 1 and 2 contained viable oocysts recovered from the feces of calf infected with CRP. Inoculum B, given to litters 3 and 4 was a portion of inoculum A, which had been frozen over night at -56 C to inactivate the oocysts.

* post infection day.

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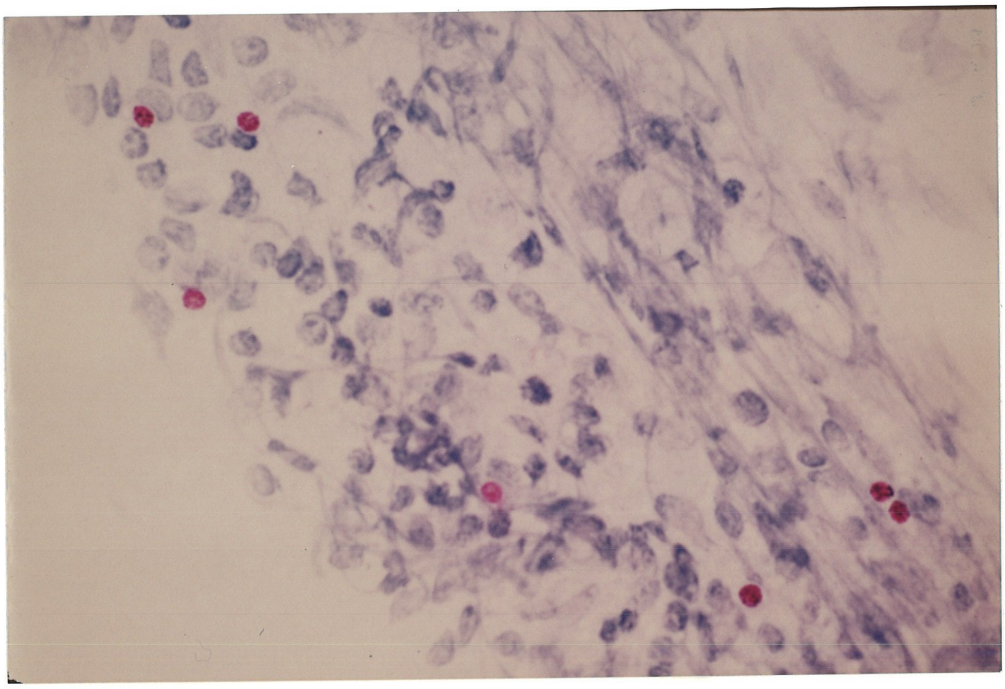
FIGURE 4.8 Light micrograph of cryptosporidia in the intestinal smears of infant mice infected with cryptosporidiosis (pid 5). Stained by KAF. Magnification: x 1,400. A. ileum. B. colon.

A.



B.

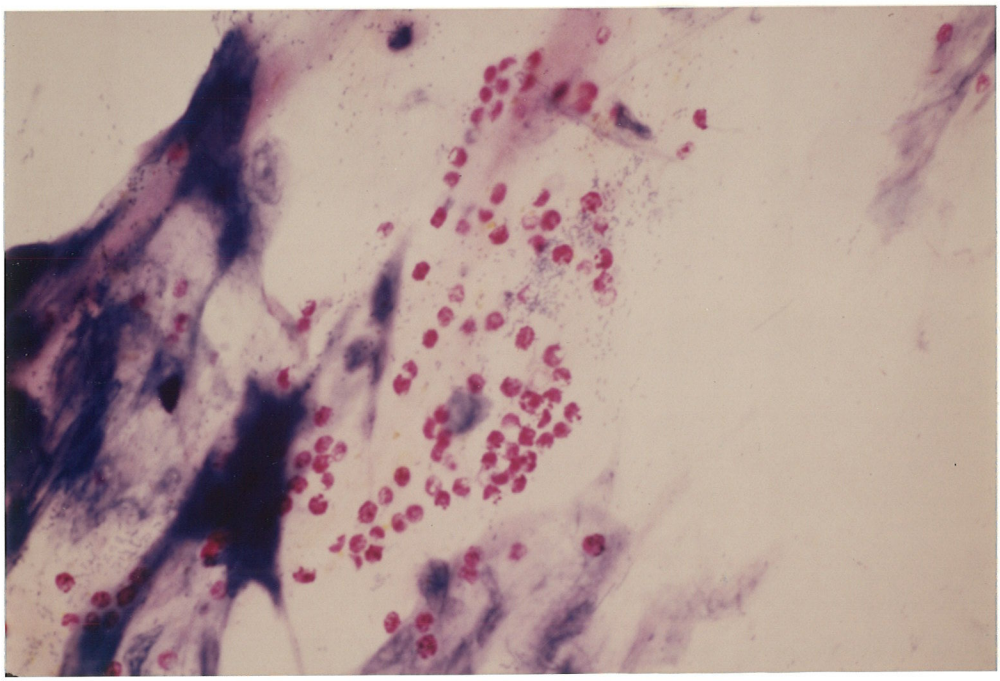


TABLE 4.14 DETECTION OF TISSUE STAGES OF CRYPTOSPORIDIUM ON HISTOPATHOLOGICAL SECTIONS FROM EXPERIMENTALLY INFECTED AND CONTROL MICE.

LITTER #	INOCULUM[1] #	PID[2] # 5		PID #8	
		ILEUM	COLON	ILEUM	COLON
1	A	+	+	-	-
2	A	+	+	+	+
3	B	-	-	-	-
4	B	-	-	-	-

1. Inoculum A, given to litters 1 and 2 contained viable oocysts recovered from the feces of calf infected with CRP. Inoculum B, given to litters 3 and 4 was a portion of inoculum A, which had been frozen over night at -56 C to inactivate the oocysts.
2. post infection day.

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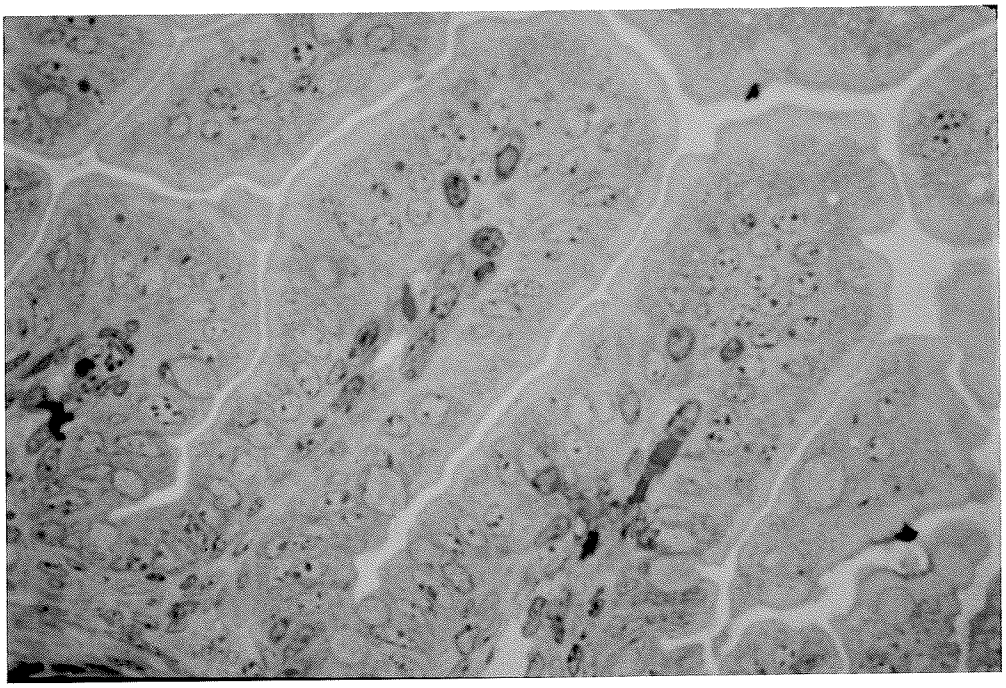
FIGURE 4.9 Light micrograph of normal ileal mucosa. Note the uniform microvillar border. Stained with Toluidine blue. Magnification: A. x 1,400. B. x 3,500.

A.



B.

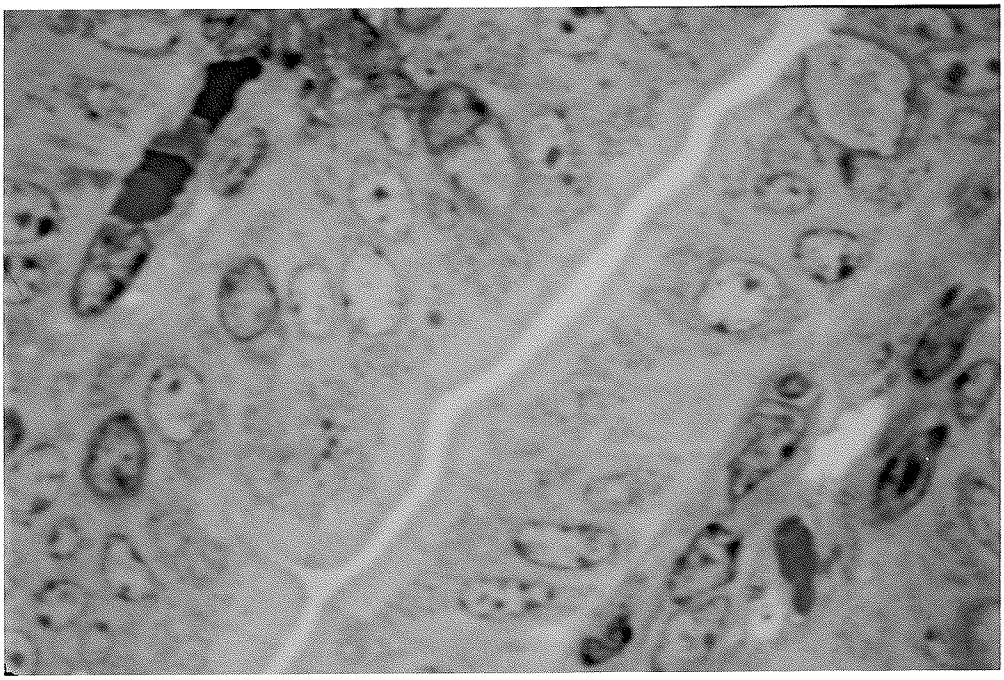
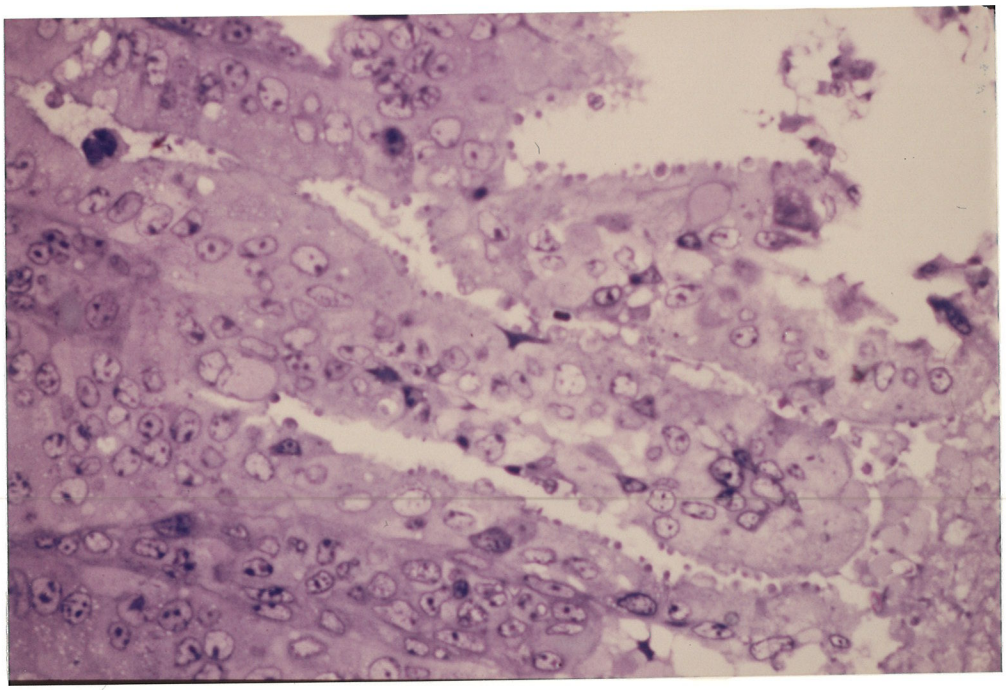


FIGURE 4.10 Light micrograph of ileal mucosa of a *Cryptosporidium* infected infant mouse. Magnification: A. x 1,400. B. x 3,500. Stained with Toluidine blue. Note the numerous cryptosporidia attached to the mucosa, and extensive disruption of the microvilli.

A.



B.

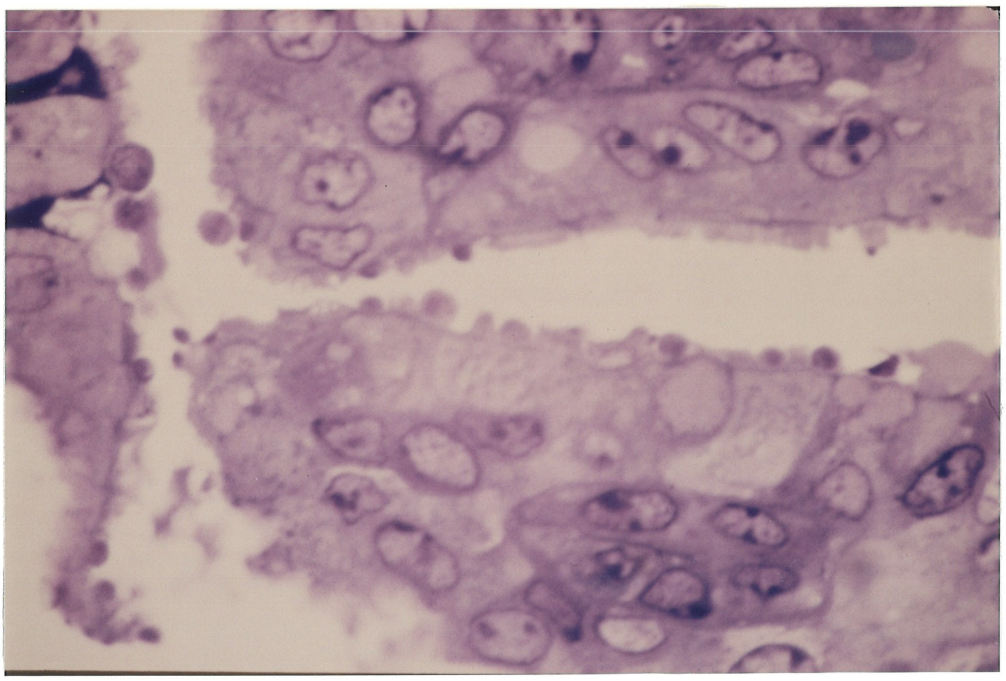


FIGURE 4.11 Two adjacent villi in the ileal mucosa of a control mouse. Note the uniform brush border and tall columnar epithelial cells. Magnification: x 12,000.

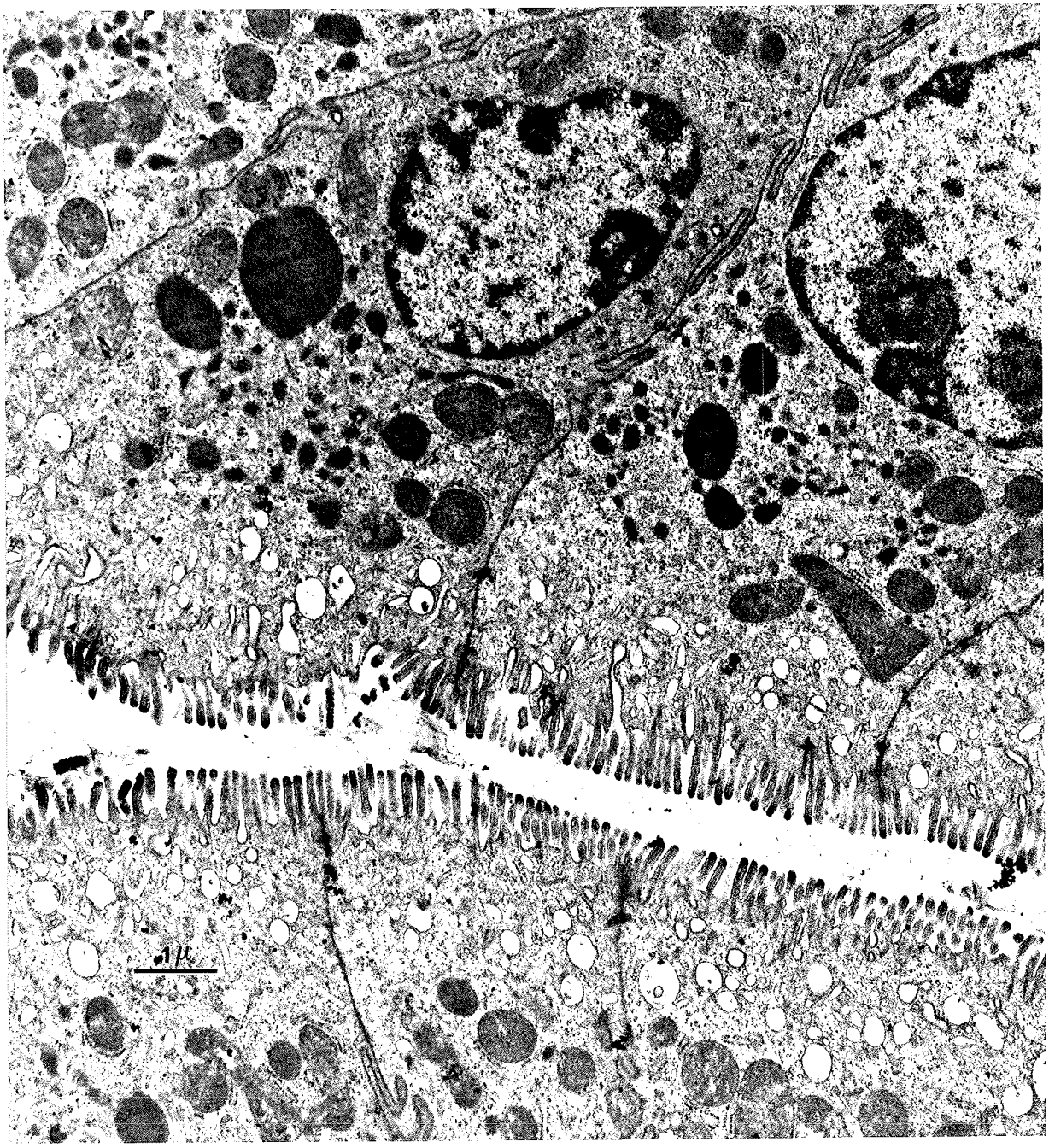


FIGURE 4.12 Cryptosporidia on ileal mucosa cells of two adjacent villi in an experimentally infected infant mouse (pid 5). Several stages of the life-cycle are present. T = trophozoite, M = macrogametocyte, S = 1st stage schizont, me = merozoite, mi = microvilli, n = nucleus, jz = junction zone, rb = residual body, pv = parasitophorous vacuole. Note the significant loss of microvilli as compared to a similar intervillar space shown in fig 4.11. Also note the two cryptosporidia-like structues marked "P" within the epithelial cells. Are these ingested parasites? Magnification: x 12,000.

FIGURE 4.12 (See legend on facing page).

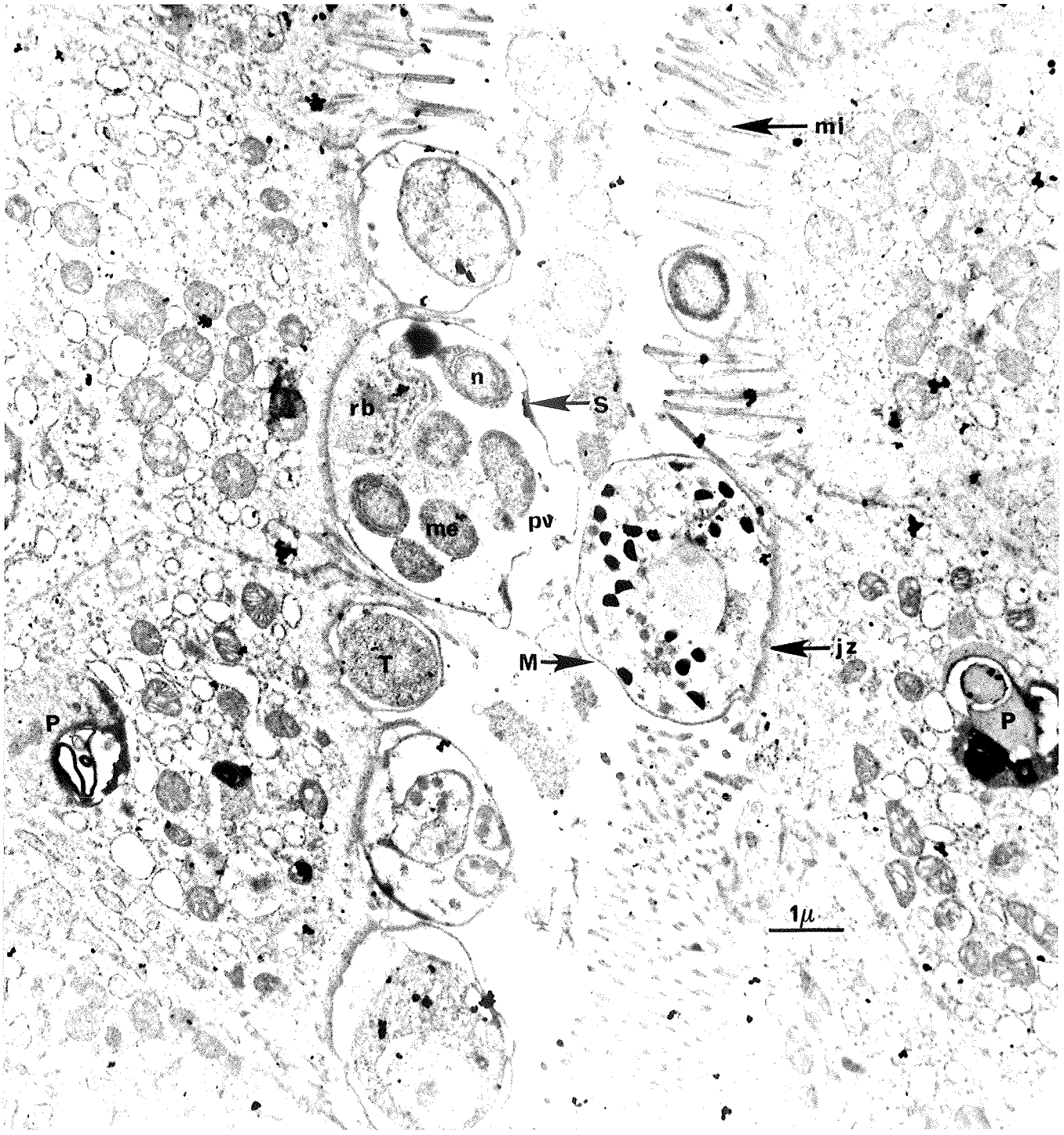


Fig 4.12 -4.15 show a variety of life stages of Cryptosporidium in the ileal mucosa of infected infant mice. These stages have been identified according to the descriptions and photographs published by other authors. Fig 4.12 shows a first stage schizont, with cross sections of at least 6 of the enclosed merozoites. It appears that the parasitophorous vacuole may have ruptured, to release merozoites into the lumen of the ileum. There is also a macrogametocyte, containing a lipid vacuole, as well as numerous granules, which have been reported to consist of polysaccharides (Bird and Smith, 1980). Several trophozoites are also present, as well as some intermediate stages. There are 2 cryptosporidia-like structures within the epithelial cells. These structures were not identified, and cryptosporidia have not previously been reported within enterocytes.

Fig 4.13 also has a first stage schizont, 2 macrogametocytes and some trophozoites. The oocyst appears to have collapsed, and is staining very densely, making it difficult to identify sporozoites or other internal structures. Many published photographs of oocysts "in situ", also appear collapsed, and the reason why they differ from other life stages in this regard, is of interest. In contrast the oocyst "in situ" in fig 4.14 has not collapsed, and sporozoites are easily identifiable within it. Oocysts seen in feces (fig 4.1- 4.4) also did not collapse, and the reason for the difference is not obvious.

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FIGURE 4.13 Numerous cryptosporidia in various stages of the life-cycle in the crypts of Lieberkuen of an experimentally infected infant mouse ileum (pid 5). O = oocyst, S = 1st stage schizont, T = trophozoite, M = macrogametocyte, n = nucleus, mi = microvilli, me = merozoite, pg = polysaccharide granules, lv = lipid vacuole. Magnification: x 12,000.

FIGURE 4.13 (See legend on facing page).

FIGURE 4.14 Cryptosporidial oocyst "in-situ" in the ileal mucosa of an experimentally infected infant mouse (pid 5). Note the 4 sporozoites, two of which are sectioned through the nuclei. n = nucleus, sp = sporozoite, jz = junction zone, pv = parasitophorous vacuole, fo = feeder organelle, ow = oocyst wall. Magnification: x 28,000.

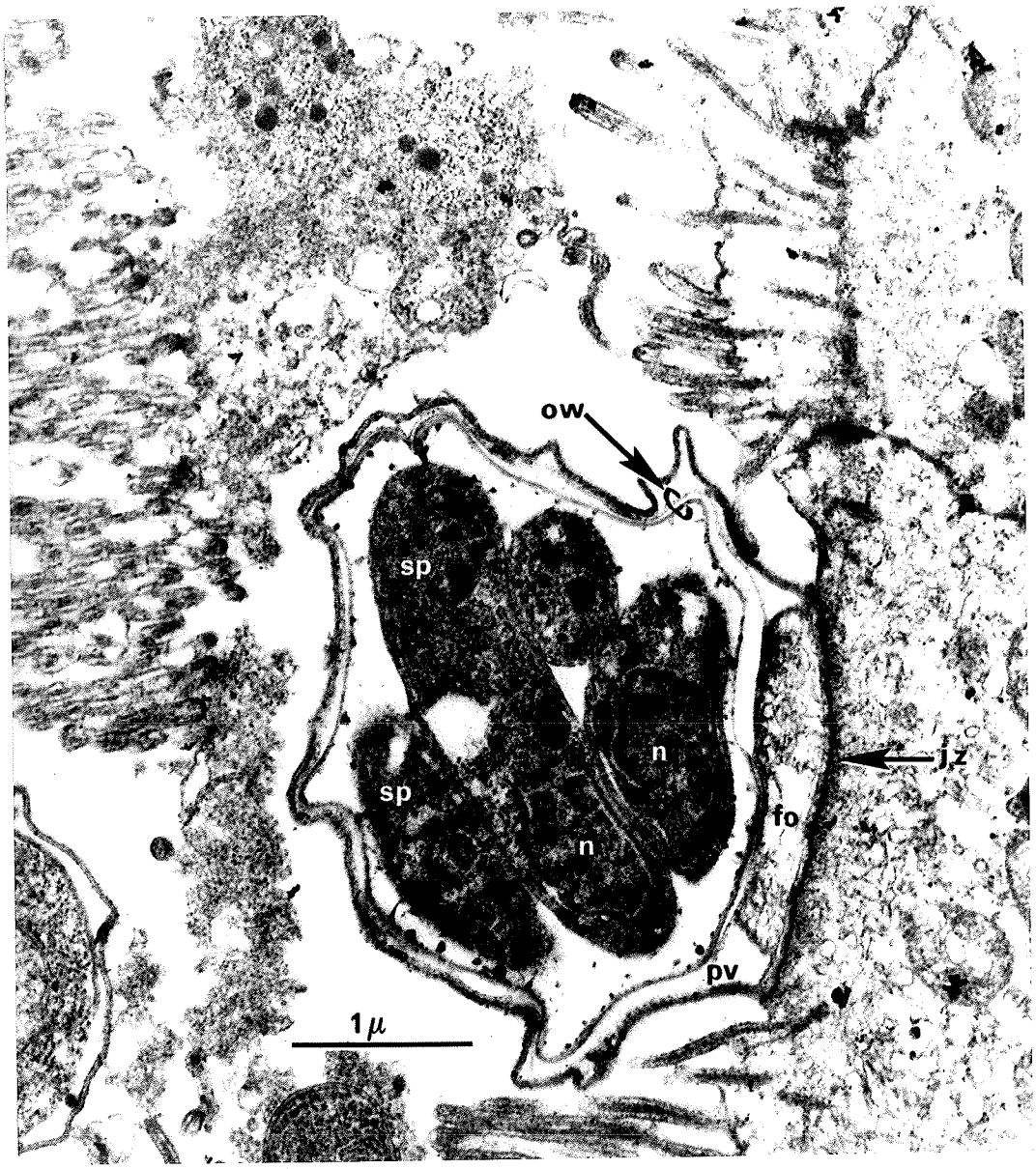

Fig 4.15 shows an intermediate life stage. It demonstrates the parasitophorous vacuole and the junction zone very clearly, but the dense staining granules inside the margin of the parasitic pellicle were not identified. If this is a developing macrogametocyte, these could be wall forming bodies, that would be instrumental in the formation of the oocyst wall. However, if this organism is a developing microgametocyte these granules could be aggregations of chromatin that would become nuclei in microgametes, as described by Goebel and Braendler (1982).

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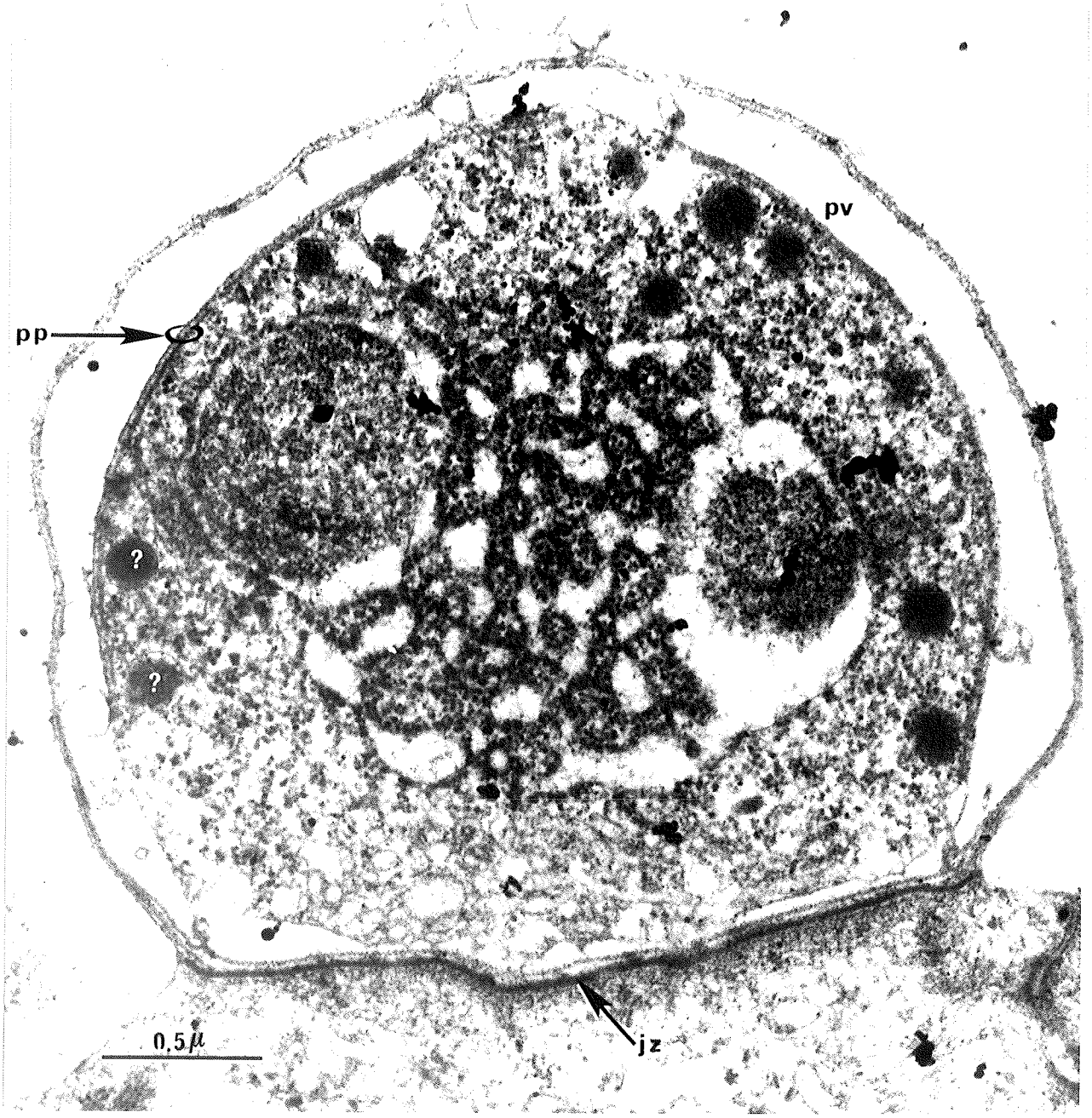
FIGURE 4.15 An intermediate stage in the life-cycle of Cryptosporidium, "in situ" in the ileum of an infected infant mouse. pv = parasitophorous vacuole, pp = parasitic pellicle, jz = junction zone, ? = unidentified granules. Magnification: x 50,000.

FIGURE 4.15 (See legend on facing page).



CHAPTER 5. DISCUSSION OF RESULTS.

CHAPTER 5 DISCUSSION

During the period of time in which 1% of human specimens examined for CRP were positive, approximately 1.5% and 4% respectively, of diarrheal specimens examined at the same laboratory, for Campylobacter jejuni & coli, and Salmonella sp. were positive. Therefore CRP appears to have a frequency comparable with these well established enteric pathogens. As indicated in Table AP.B (Appendix 4), a similar number of cases of CRP were diagnosed in Cadham Provincial Laboratory in the year following our survey, reinforcing the data presented in this study.

Since the completion of this study, and its preliminary reporting (Mann et al, 1984), similar results have been reported in two other surveys conducted in Canada. In British Columbia, Montessori and Bischoff (1984) examined stool specimens from 7300 patients, finding 46(0.63%) positive, most cases occurring in late summer and fall. In Newfoundland, Ratnam et al, (1984) examined specimens from 1317 patients during a period from May to September, and identified 15 cases (1.14%). Cryptosporidial oocysts have been reported in similar proportions of diarrheal specimens in other geographic locations (Jokipii et al, 1983; Tzipori et al, 1983b; Casemore et al, 1984; Hojlyng et al, 1984; Mata et al, 1984).

Many authors have associated CRP with immunodeficiency in humans (Meisel et al, 1976; Guarda et al, 1983; Pitlik et al, 1983a; Sloper et al, 1982). Our data, and the other

Canadian surveys, support workers who have described this infection in patients with no recognized immunodeficiency (Reese et al, 1982; Anderson et al, 1982; Jokipii et al, 1983; Tzipori et al, 1983b). Only 2 of the 39 patients identified here, had conditions which are regularly associated with immunodeficiency, but at the time they were examined both were considered immunocompetent by their attending physicians. The high prevalence found in young children as compared to adults, presumably was partially due to the more lax hygienic habits characteristic of this age group. An age related difference in resistance can also be postulated, since a similar age distribution occurred in cattle, not explainable by hygiene differences. These data are also reinforced by a similar age distribution of cases, in the year ending October 31, 1985 (table AP.B; Appendix 4). The large number of surveys indicating CRP in immunologically normal persons, eliminates immunodeficiency as a prerequisite for CRP, however it is presumably a significant predisposing factor, and certainly does affect the severity and prognosis (Navin and Juranek, 1984).

The source of infection in humans is still unknown in most instances. Our data suggested both person-to-person and zoonotic transmission, with the possibility of water or unpasteurized milk acting as a vehicle. Contamination of milk with feces of asymptomatically infected animals, could occur during the milking procedure, due either to inadequate cleaning of the udder prior to machine milking, or more

likely during hand milking into open pails. Although cows are no longer hand milked on most commercial dairies, this practice is not uncommon on mixed farms, where one or more cows are milked for home use. It was not determined whether this occurred on the Manitoba farm. Cryptosporidium has been shown to have considerable environmental resistance (Sherwood et al, 1982), and should survive well in unpasteurized milk. Mammary infection with CRP could be another source of milk contamination. Although no such suggestion has been made, Pavlàsek (1984) recognized cryptosporidial infection in liver, lung and heart smears of infected mice and calves. If extra-enteric infections occur in these organs, then infection of the mammary gland can also be postulated. Hunt et al (1984) suggested Cryptosporidium may be able to survive the usual pasteurization procedure, which would have much broader epidemiological implications.

Two patients believed their infections to have been related to water. The environmental resistance of Cryptosporidium, and the association between CRP and traveller's diarrhea (Jokipii et al, 1983; Saove and Ma, 1985;Ma et al, 1985) suggest water as a possible source of infection.

Considering that transmission of CRP from infected calves to humans has been demonstrated (Anderson et al, 1982; Current et al, 1983), it was remarkable that only one human case was confirmed during the spring, when 25% of the submissions from diarrheic calves were positive. Replies to the questionaire suggested some links between human and

animal CRP, but transmission was not established conclusively. The lack of significant difference in prevalence, between rural and urban communities in southern regions, also decreases the likelihood of bovine-to-human transmission. Mata et al (1984), found higher prevalence in urban as compared to rural areas, but this was from a geographic location where socio-economic differences between the two populations, were presumably smaller than here. He attributed the difference to the higher rate of breast feeding, with its concomitant decrease in exposure to the natural environment, observed in the rural population.

Further studies are required to explain why this organism, which has been shown to be transmissible from cattle to humans, and is very common in cattle in Manitoba, does not appear to be frequently transmitted, to persons who are in regular contact with these animals.

Two children may have been infected by contact with a dog found to be excreting oocysts. The dog was acquired only 4-5 days prior to the occurrence of diarrhea in the first child, and the basement of the home was widely contaminated with dog feces at the time the specimens were collected.

Schultz (1983) described this infection as "an emerging zoonosis", and there is sufficient evidence to justify his conclusion, but this study also supports the opinion expressed by Casemore and Jackson (1984) that CRP should not be considered primarily a zoonotic infection.

The occurrence of a cluster of cases in a day care center in this study, was similar to reports from 5 states of the United States (Centers for disease Control, 1984), and indicated the likelihood of person-to-person transmission. Since the incubation period of CRP is reported to be 3-7 days (Anderson et al, 1982), and the child having an eye condition had been hospitalized for more than a month when the specimen was taken, CRP may have been acquired as a nosocomial infection. Cross-infection between persons in institutions has been shown by Baxby et al (1983), and cases identified here in health care workers support his findings.

The rate of CRP infections in northern settlements was of particular interest, as it was more than 4 times as high as in the non-urban southern areas, and almost 7 times as high as that in Winnipeg city. These data are again reinforced by a similar regional distribution of cases, in the year following our survey (table AP.A; Appendix 4). This is the first report of higher prevalence of CRP in northern as compared to southern communities, although reports from Denmark (Holten-Anderson et al, 1983) and from Finland (Jokipii et al, 1983) report CRP patients living in similar latitudes. Whether the regional difference was due to climatic, socio-economic or other factors was not determined. The northern populations are of lower socio-economic groups, and their sanitary facilities are of a lower standard than those of the southern and urban populations. The possibility that there may be a higher rate of specimen submission

to Cadham Laboratory from northern regions, where no other laboratory facilities are available, should also be considered.

Contact with cattle and other farm livestock is presumably minimal in the northern communities, but contact with dogs, wild animals and wild animal products is more common than in southern and urban communities. Person-to-person transmission of other enteric pathogens is well documented in these communities, and may also occur in CRP, however if this mode of transmission was predominent major seasonal differences in prevalence would be less likely, as people are in contact with each other during all seasons.

The age specific data indicated a higher risk of CRP in young children. The extremely narrow age range, of the group having the highest risk, was unexpected (ie infants <1 year have a lower risk than 1 year olds), however similar findings have been reported in other surveys (Casemore and Jackson, 1983; Mata et al, 1984). This finding also incriminates the environment as the source of infection, because children <1 year of age usually have less contact with the natural environment than older children. Data from Cadham Provincial Laboratory for the year ending October 31, 1985, are somewhat different, in that age specific incidence does not decrease until after 4 years of age (table AP.B; Appendix 4). Data in Table AP.C (Appendix 4), similar to that in our survey, again shows the very significant risk of CRP to young children who live in northern Manitoba.

We could not determine the incubation period in any of the human cases, as we did not confirm any specific source of infection. The cases in the day care center were all diagnosed within a month of each other. One adult CRP patient, the mother of another child who attended the same day care center, was also diagnosed during the same month. Her child also had diarrhea, but was not examined for CRP. The above findings suggest only, that the incubation period is less than 1 month. The occurence of diarrhea in a child 3 days after drinking unpasteurized milk, while visiting a farm, suggested a short incubation period, as did the incident in which a CRP positive puppy arrived on a farm only a few days before two "in contact" children became ill with cryptosporidial diarrhea. Other workers reported incubation periods of 5-7 days (Blagburn and Current, 1983; Anderson et al, 1982)

The source of infection in herd outbreaks in cattle is also unknown. The presence of oocysts in adults and yearlings on positive farms, and the high prevalence of antibody to Cryptosporidium in Manitoba cattle, suggested this organism may have a high prevalence in the bovine population. Clinical cases are certainly not all diagnosed, and many subclinical infections may also occur, and could act as an important source of infection.

The finding of cryptosporidial oocysts in specimens obtained from hens wandering in the barnyard, on two farms, led to speculation that these birds may have been a source

for the calves. Tzipori et al (1980a) were able to transmit the infection to 6 mammalian species and chickens, using oocysts recovered from calf feces, and suggested that Cryptosporidium may be a single species genus. However Pavlàsek (1983a), and Schloemer (1982) failed to transmit bovine CRP to chickens. Levine (1984) considered mammalian and avian isolates to be separate species.

Co-infection with other enteric pathogens was not common in human CRP. This finding supported other published evidence that Cryptosporidium is a primary pathogen in humans (Jokipii et al, 1983; Tzipori et al, 1983b; Hojlyng et al, 1984; Mata et al, 1984), although a 20% association between CRP and giardiasis has been suggested (Wolfson et al, 1984). Casemore et al (1984) related the severity of the syndrome directly to the number of oocysts being excreted. In this study we found absence or decreased numbers of oocysts in recovering patients. However two publications (Hart et al, 1984; Ratnam et al, 1984) indicated continued excretion of oocysts, for up to 11 days after symptoms have subsided. This finding requires further investigation and if confirmed, certainly is a major factor in the epidemiology of this infection.

In calves 27% of cases occurred coincident with other enteric pathogens. Other authors reported similar findings, however, the pathogenicity of Cryptosporidium for calves has been established by reproduction of the syndrome in gnotobiotic calves (Heine et al, 1984a). Some workers reported

clinical diarrhea in calves, coinciding with the shedding of oocysts, and its absence in the postpatent period (Moon and Bemrick, 1981), but Stein et al (1983) observed diarrhea often continuing, after oocyst shedding had stopped, and was unable to draw any direct parallels between shedding and clinical signs. It is possible that this interpretation was due to the low sensitivity of the examination procedures or to other coincident infections.

The finding of oocysts in one herd, not reporting a diarrhea problem, indicated that CRP does not always lead to clinical disease in cattle. The finding of oocysts in adult and yearling cattle on some farms, suggested these animals may have been carriers, and a source of infection for calves. Alternately they may have been asymptomatically infected simultaneously with, or after contact with infected calves. CRP in cattle is apparently similar to many other endemic infections, which survive within a population, causing clinical disease only in particular subsets of the population, under specific conditions.

Numerous experimental transmissions, within as well as between species, with the characteristic development of lesions and clinical syndromes has, established the pathogenicity of Cryptosporidium, however severity of the infection appears to vary considerably between individual animals in some trials. Current (1983), showed no protection from infection in the presence of colostral antibody. Fayer et al (1985) also found equal rates of CRP in colostrum-depriv-

ed and colostrum-fed calves, and further indicated no direct relationship between infective dose and the severity of the infection. They found a toxigenic Clostridium perfringens and Rotavirus contaminating the inocula, and concluded that Cryptosporidium interacted with other agents, in causing a clinical effect. Tzipori et al (1981b) demonstrated a predisposition for clinical CRP in artificially reared lambs, also indicating a multifactorial syndrome. The basic interaction of host, environment and infectious agent is the only adequate explanation, covering all these observations, and variations in any one of the interacting factors, can result in differences in the severity of an infection.

The seasonality observed in human CRP is also difficult to explain. Previous reports indicated a higher incidence of the infection in warm wet months (Tzipori et al, 1983b; Mata et al, 1984), but findings in Canadian conditions are not easy to relate to those in tropical climates. August and September are however, the best combination of warmth and wetness in Manitoba. In the first fall of this survey, cases continued into the winter and this is very difficult to relate to the findings of others. Explanation is further complicated by the higher rates found in northern regions, where normal tempertures range between 4 C and -4 C in October, and well below 0 C in November. Cryptosporidium is reported not to survive well in freezing temperatures (Sherwood et al, 1982).

The overlap of the CRP season in dairy calves, and

that of humans, could suggest either the involvment of climatic factors, or an association between the infection in these two populations. In beef calves the seasonality was certainly related to the presence of a large susceptible population of neonates, as most beef calves in Manitoba are born in the winter and spring. Sanford and Josephson (1982) found the seasonal incidence in dairy calves in Ontario, to be similar to ours. Most cases occurred in the winter months, and they attributed this to the close confinement practiced during that period of the year. They particularly implicated the use of hutches in which calves congregated for shelter. Many of the dairy calves in this study, were also confined in crowded stalls during the winter months, and many beef calves were managed in crowded conditions because of the stressful weather conditions, during the calving season. Close contact between calves facilitates direct transmission of many infections, including CRP.

The mortality data indicated the economic effects of neonatal diarrhea in calves. The purchase of young calves, from undefined sources, was most common in the class of farms having the highest losses. In some instances, the purchased calves, may have introduced the organism to the herd. This suggestion becomes very plausible, when considering the findings of Stein et al (1983), who recorded oocyst counts in calves as high as 5 $x10^6$/gm of feces.

The results of treatment were similar to those reported by other workers. Whereas most neonatal diarrhea out-

breaks in calves, respond reasonably well to the use of antibiotics, fluids, and electrolytes, Cryptosporidium associated diarrhea seemed to be refractory to therapy. Stein et al (1983) suggests, that antibiotics may actually exacerbate CRP. This could be explained by the theory of "competitive exclusion", whereby the removal of normal bacterial flora by antibiotics, allows the resistant cryptosporidia to proliferate freely. Barker and Carbonell (1974) described a fatal case in a calf, which relapsed suddenly after apparent response to antibiotic therapy. Their inability to isolate bacteria from any organ, suggests death may have been due to the uninhibited proliferation of cryptosporidia. The use of antibiotics may be contraindicated in CRP, and other drug resistent enteric infections. The case seen in a patient, after treatment for Campylobacter jejuni, could also be an example of antibiotic therapy predisposing to CRP, although there is no specific evidence for such a hypothesis.

Angus et al (1984) obtained a prophylactic effect, using Arprinocin on experimental CRP in mice, suggesting a possible means of controlling this infection. The value of their findings were limited, because the attempt to apply the mouse model to lambs failed. Apparently species differences are significant, and further investigations are indicated. The report of dramatic improvement following injection of "Ivermectin" also needs further study. The reported mechanism of action of this drug on metazoan parasites, involving interference with neuro-transmission, should have

no effect on protozoa, but an unknown mechanism may be functioning.

Transmission of calf CRP to infant mice, confirmed the interspecies transmissibility of Cryptosporidium, and partially fulfilled Koch's postulates, although no clinical disease was produced in the mice. This transmission also supported the suggestion of Tzipori (1980a) that Cryptosporidium may be a single species genus. The infective dose used in this study was high, but lower than the dose used by some other workers (Sherwood et al, 1982; Current et al, 1983). The exact number of oocysts or sporozoites, required to infect mice or other species, has not been determined. Fayer et al (1985) found that the oocyst sediment, and the supernatant over the pellet, were equally able to transmit the infection to calves. They did not recognize any oocyst in the supernatant. Therefore an undetectably small number of oocysts, or sporozoites, present and unrecognized, were transmitting the infection. In contrast, Schloemer (1982) indicated that, the prepatent period in mice was inversely proportional to the infective dose. The determination of the infective dose for various species, would provide a basis for comparison of isolates and strains, for an estimation of their relative pathogenicity.

The extensive disruption of microvilli, seen in the ilea of the infant mice, was similar to that described by other authors (Pohlenz et al, 1978b; Heine et al, 1984a), and gives credence to malabsorption, as a major factor in

the pathogenesis of the diarrhea, resulting from this infection in many species. The reason CRP does not produce diarrhea in some species, could be due to a greater ability to compensate for failure of absorption in the ileum, (for example by reabsorption in the lower gut), or by affecting a smaller portion of the gut.

The two cryptosporidia-like structures seen within the mucosal cells in fig 4.12, were not identified. Kennedy et al (1977) described a cryptosporidial organism, in the process of degradation, within a leucocyte, but the structures seen here appear to be within enterocytes.

There is still a question regarding the sensitivity of the staining procedures. Procedures having a low sensitivity, may only be able to detect oocysts, in specimens containing large numbers of them. When the excretion rate is low, a very sensitive diagnostic method is required, to detect the few organisms present. Stein et al (1983) detected no difference between the sensitivity of Giemsa staining, and the negative carbolfuchsin technique of Heine (1982). Hart et al (1984) indicated, that the sensitivity of the Safranine-Methylene blue method of Baxby and Blundell (1983), decreased rapidly in the late stages of human infections. If the sensitivity of the diagnostic methods in use, is still low, then the prevalence of CRP is presumably higher than that indicated by many of the reported surveys. Cases in the very early stages, late stages and asymptomatic carriers would often be considered negative.

The variations in size, shape and staining characteristics, within the set of organisms meeting the criteria, of identification as cryptosporidia, raise some questions. Are these all variations of oocysts within one species of Cryptosporidium? Do other unrelated organisms have similar characteristics? Are other life stages also present in the feces? Since cryptosporidia are so superficial in the intestinal tract, and are presumably causing accelerated loss of enterocytes from the mucosa, tissue stages may be shed with these cells. The presence of other life stages, could account for some of the differences in morphology and staining characteristics observed, but some could also be due to a difference in stain uptake by viable and non-viable organisms.

The results of EM studies of oocysts in feces do not explain the variations in size, shape, and staining characteristics seen by light microscopy of these specimens. Some of the structures observed by light microscopy may not have been seen, or not recognized on EM preparations. Further EM studies on individual organisms, which have already been identified by staining and light microscopy, would be useful to help interpret the observed variations. Although it was not identified, the crystalline array of particles seen in one fecal oocyst suggest, that other infectious agents (ie. endosymbionts) may be present within cryptosporidia, and may contribute to the pathogenicity of these organisms. Bird and Smith (1980) reported adenovirus, in association with

CRP, in some human infections, but the structures seen here are apparently too small to be adenoviruses.

The high prevalence of Ab to Cryptosporidium in adult market cattle was expected, and correlated well with the proportion of neonatal calves having diarrhea associated with the organism. Also, as expected, the prevalence of Ab was higher in known infected herds than in random sera, which presumably represented both infected and non-infected herds. Since differences between districts were virtually insignificant, there seemed to be no local preferences for the infection. This finding is also similar to the apparent random geographic distribution of infected herds, detected by fecal examination of diarrheic calves. A significant difference in antibody prevalence was detected between Boissevain and Emerson districts, but has no obvious explanation. There are differences in physical features, the former being an arid plain, whereas the latter is forested.

Isolates from different sources need to be studied, to detect antigenic markers, which may be correlated to apparent differences in virulence. Some such antigenic variation was suggested by serological tests performed in this study. If virulence varies in different isolates, there will be a need to determine the virulence factors responsible for such variation. The debate, regarding whether Cryptosporidium is single species genus, is continuing, and the epidemiology of this infection cannot be fully explained, until this question is resolved.

There is also still uncertainty regarding the normal mode of transmission of CRP, and although the existence, in feces, of sporulated oocysts capable of transmitting the infection by oral inoculation, has been demonstrated, the factors involved in excystation have only recently been examined (Reduker and Speer, 1985). These factors are very important when attempting to explain extra-enteric infections, described in a number of host species. If excystation depends on enteric enzymes and/or pH conditions, as is the case in many other enteric protozoa, what is the stimulus when Cryptosporidium infects the respiratory tracts and biliary tracts? One child in our study, also had a respiratory infection simultaneously, but we have no indication that respiratory CRP , a condition usually only seen in immunodeficient patients(Forgacs et al, 1983) was present in this case. The respiratory and biliary tracts have direct connections with the alimentary canal, and entry of excysted sporozoites through these junctions can be postulated, but Pavlàsek (1984) claimed to have demonstrated stages of Cryptosporidium in the liver, lung, and heart of infected mice and calves, and in the blood of calves. If this claim can be verified, all the proposed epidemiological and transmission models for CRP, will require revision. Fenwick (1983) described a case of CRP in a one day old gazelle. Considering an oral route of infection, and a prepatent period of 4-5 days, as have been reported in CRP, postpartum infection of this neonate appears improbable. There-

fore this case also suggests a systemic phase of the infection and may be the first indication of "in-utero" transmission of CRP.

A number of topics have been addressed in this study with varying success, but many questions about CRP are still unanswered. The cost of neonatal diarrhea to the livestock industry, still requires solution in a number of its facets. This thesis, as well as the work of numerous other authors, demonstrates that CRP contributes significantly to that syndrome. Of much greater interest, is the effect of CRP on human health. The discomfort and loss in productivity, resulting from bouts of diarrhea in otherwise healthy persons, is adequate justification for studying CRP, but the mortality resulting from this infection in immunodeficient persons, and perhaps in malnurished third world children, demands an urgent solution.

CHAPTER 6. SUMMARY OF CONCLUSIONS..

CHAPTER 6 SUMMARY OF CONCLUSIONS

1) CRP occurs in both calves and humans in Manitoba.
2) During the study period ~1% of diarrheic stools submitted to Cadham Provincial Laboratory were positive for CRP.
3) The infection in humans occurred mostly in the late summer and fall and was significantly more common in children five years and under as compared to older persons. Children between 1 and 2 years of age were at significantly higher risk than any other age group.
4) The rate per 100,000 population was significantly higher ($p < 0.0005$) in the northern region as compared to southern regions of the province. There was no significant difference between rural and urban southern regions.
5) During the study period ~ 25% of fecal specimens from diarrheic neonatal calves were positive for CRP. Twenty-seven percent of specimens also contained pathogenic viruses.
6) The case fatality rate in CRP associated with diarrhea was ~15% in beef herds and 35-60% in dairy herds.
7) Asymptomatic CRP was recognized in yearling and adult animals on infected farms. One severe infection was seen in a two year old bull. CRP had not been previously described in mature cattle.
8) Transmission from cattle to humans could not be established although it was suspected on several occasions.
9) Transmission of bovine CRP to mice was demonstrated; the

infection was established and pathology was demonstrated, but no clinical disease was evident.

10) No ultra-structural differences between oocysts from humans, calves or the 2 year old bull, could be demonstrated by electron microscopy.

11) An indirect immunofluorescence test, using feces derived oocysts as antigen, was used to conduct a sero-survey on bovine sera. Twenty-nine of 50 (58%) sera collected from animals on farms where calves had CRP, had antibody titers of > 1:40 against the oocysts. Seventy-six of 186 (40.9%) sera collected at random from Manitoba slaughter cattle contained antibody against Cryptosporidium sp.

12) Five human sera collected from CRP patients had antibody against Cryptosporidium sp.

The results of this study give CRP a prominent place among the causes of enteric infections, in both humans and cattle, and indicate its significant economic importance in the latter. Several areas have been highlighted as needing further research. Among these, the identification of sources of infection, allowing a greater understanding of the epidemiology may be foremost. Effective means of control cannot be instituted in the absence of such knowledge. Of similar urgency is the development of effective therapeutic agents, to prevent the severe economic effects of CRP, but more particularly, to halt the mortality caused by this infection in immunologically compromised patients.

APPENDIX

APPENDIX 1

STAINING SOLUTIONS:

KINYOUN'S ACID FAST STAIN (Paik, 1980).

Kinyoun's Carbolfuchsin:

Basic Fuchsin	4.0 gm
95% Ethanol	20 ml
Phenol	8.0 gm
Distilled water	100 ml

Acid Alcohol:

Concentrated HCl	3 ml
95% Ethanol	97 ml

Methylene blue counterstain:

Methylene blue	0.3 gm
distilled water	100 ml

APPENDIX 2

Solutions Used in Electron Microscopy:

A. 0.05 M Sodium Cacodylate, 0.01 M Calcium Chloride (Hayat, 1981).

1. Combine:
   a. 1.07 gm Sodium Cacodylate-trihydrate. MW 214.03
   b. 0.147 gm Calcium Chloride-dihydrate. MW 147.02
   c. glass distilled water to 75 ml.
3. Adjust pH to 7.4 with HCl.
4. Bring to a final solution of 100 ml with glass distilled water.
5. Filter through a 0.45 um pore filter.
6. Store at 4 C for up to 4 months.

B. 2.5% Glutaraldehyde in 0.05 M Sodium Cacodylate, 0.01 M Calcium Chloride pH 7.4 (Hayat, 1981).

1. Combine:
   a. 1.07 gm Sodium Cacodylate-trihydrate. MW 214.03
   b. 0.147 gm Calcium Chloride-dihydrate. MW 147.02
   c. glass distilled water to 75 ml.
2. Stir until dissolved.
3. Add 5.0 ml 50% Glutaraldehyde.
4. Add 2.5 gm Barium Carbonate.
5. Stir 1 hr at room temperature.
6. Pellet the Barium Carbonate by centrifuging at 1000x g for 5 min in a bench top centrifuge.
7. Adjust pH of supernatant to 7.4 with HCl.

Appendix 2 (continued).

8. Bring to a final volume of 100 ml with glass distilled water.
9. Filter through a 0.45 um pore filter.
10. Store at 4 C for up to 4 months.

C. Osmium Tetroxide in 0.05 M Sodium Cacodylate, 0.01 M Calcium Chloride pH 7.4 (Hayat, 1981). (ALWAYS WORK IN A FUME HOOD, WEARING RUBBER GLOVES. DO NOT INHALE THE FUMES OR OTHERWISE EXPOSE YOURSELF TO THEM. EXTREMELY TOXIC).

1. Combine:
   a. 0.428 gm Sodium Cacodylate-trihydrate MW 214.03
   b. 0.0588 gm Calcium Chloride-dihydrate MW 147.02
   c. Glass distilled water to 20 ml.
2. Stir until dissolved.
3. Add 1.44 gm Glucose. MW 180.00
4. Stir until dissolved.
5. IN THE FUME HOOD, score a 10 ml vial of 4% Osmium Tetroxide.
6. Carefully break the vial and add the contents to the solution.
7. Adjust pH to 7.4 with HCl.
8. Bring to a final volume of 40 ml with glass distilled water.
9. Store at 4 C for up to 4 months.

Appendix 2 (continued).

D. 3% Agarose. (Yuan and Gulyas, 1981)(PREPARE FRESH FOR EACH USAGE).

1. In a small test tube, combine:
   a. 30 mg indubose agarose.
   b. 1 ml 0.45 um filtered buffer.
2. Heat in boiling bath until dissolved (3-5 min).
3. Cool to 50 C before use.

PREPARATION OF AGAROSE CORES (Yuan and Gulyas, 1981).

1. Combine equal volumes of pelleted material and 3% molten agarose (T= 45-50 C).
2. Resuspend pellet in the Agarose with a fine tipped glass rod.
3. Draw suspension-Agarose mixture 1-2 cm into a pastuer pipette.
4. Allow the mixture to cool and solidify (1 min).
5. Expell the Agarose core onto a piece of parafilm.
6. Cut cores into 1 mm blocks.
7. Treat as tissue blocks in E.M. procedures.

APPENDIX 3

A samples copy of the questionaire sent to the physicians of cryptosporidiosis patients is included on page 126, and that sent to the owners of cryptosporidiosis positive cattle herds, appears on pages 127 and 128.

Cryptosporidiosis Study

Date Form Completed _____ Dr. L. Sekla

Questionnaire - Parasitology
Cadham Provincial Laboratory

I Name: _____ Telephone Number: _____

Address: _____

Age: _____ Sex: _____

Have you recently moved? _____

If so, from where? _____

Have you travelled recently? _____

If so, where? _____

Occupation: _____

Do you or family members have any regular contacts with animals? _____

If so, please list what animals and in what connection (e.g. pets, farm animals, zoo, etc.) Also note whether any of these animals had diarrhea recently.

_____

_____

_____

II This section may be completed with the help of your doctor.

Doctor's name: _____

Address: _____

_____ Telephone Number: _____

When did the diarrhea first start (give date)? _____

How long has it been going on? _____

How many bowel movements occur in a 24 hour period at its worse? _____

Other diagnosis made in the lab. or in examination _____

_____

	Yes	No
Blood in stool	___	___
Fever	___	___
Colic	___	___
Vomiting.	___	___

Please return the completed questionnaire in the attached self-addressed envelop.

CRYPTOSPORIDIOSIS IN CALVES

Date: _____

QUESTIONNAIRE FOR OWNERS

Owner's Name: _____

Address : _____

_____ Telephone _____

Is your cattle operation primarily: A. Beef ☐; B. Dairy ☐; C. Both ☐ .

Please answer the questions that are appropriate for your operation.

If you have checked C above, please answer the questions in both A and B boxes.

A. Beef

- How many breeding cows in your herd (approx.) _____
- How many calves are born between Jan. 1 and May 1 (approx.) _____

	Yes	No
- Are the cows kept outside during winter?	____	____
- Are cows taken into calving pens for delivery?	____	____
- Are newborn calves and dams placed back into the herd after calving?	____	____
- Do you have calf shelters in the lot?	____	____
- Did you have a Scours problem last year?	____	____
- Did you have a Scours problem the previous year?	____	____
- Do you buy any: - Breeding Cows	____	____
- Calves	____	____
- Feeders	____	____

B. Dairy

- How many cows are you milking (average)? _____
- How many calves under 3 weeks of age do you normally have? _____

	Yes	No
- Do you raise your own replacements?	____	____
- Do you raise your own steer calves?	____	____
- Do you buy any young calves?	____	____
- Do you buy any cows?	____	____
- Are calves kept in the cow barn?	____	____
- Do cows calve in special calving stalls?	____	____

- How are calves fed (pail, automatic feeders, etc.)? _____
- Do they get milk or artificial milk replacers? _____
- Is Scours more common in any particular season? _____
  If so, which season? _____

History of Present Problem

How many calves have had Scours in the past 3 months (approx.)? _____

How old are the calves when they get Scours (days)? _____

Do they get sick enough to stop drinking? _____

Is the diarrhea bloody? _____

How long does the diarrhea last (days)? _____

Are you treating the scouring calves? _____

- With what? _____

Does treatment seem to help? Yes ☐ No ☐

How many calves have died from Scours in the last three months? _____

Would you be willing to allow me to visit your farm to collect more samples as mentioned in the letter? _____

Have any family members or farm workers had diarrhea during the time the problem was occurring among the calves? _____

Comments: _____

APPENDIX 4

TABLE AP.A HUMAN CRYPTOSPORIDIOSIS BY MANITOBA REGION FOR THE YEAR ENDING OCTOBER 31, 1985.*

	# CASES	POPULATION[1]	RATE[2]
A. Winnipeg	8	610,391	1.3[b,d]
B. Norman	22	124,800[a]	17.6[b,c]
C. non-urban southern regions	10	291,197	3.4[c]
1) Eastman	6	78,569	7.6[d]
2) Central	0	92,395	-
3) Interlake	3	69,716	4.3
4) Parkland	1	50,467	1.8

1. Manitoba Health Services Commission, Statistics 1984.
2. per hundred thousand population.

a. Population of Norman Region plus "Status Indian" population of Manitoba.

b. significantly different ($p < 0.0005$).

c. significantly different ($p < 0.0005$).

d. significantly different ($p < 0.0005$).

NOTE: No data are included from Westman Region since stool specimens are not received in Cadham Provincial Laboratory.

* data courtesy of Mr. C. Koschick (Cadham Provincial Laboratory).

APPENDIX 4 (continued).

TABLE AP.B AGE DISTRIBUTION OF HUMAN CRYPTOSPORIDIOSIS IN MANITOBA IN THE YEAR ENDING OCTOBER 31, 1985.*

AGE(YEARS)	# CASES	POPULATION[1]	RATE[2]
<1	8	~17,000	47.0
>1-2	8	16,848	47.5
>2-3	8	16,885	47.4[b]
>3-4	4	16,676	24.0
>4-5	1	16,623	6.0[b]
<5	29	84,031	33.3[a]
>5	11	1,015,449	1.2[a]
TOTAL	40	1,099,480	3.6

1. Manitoba Health Services Commission, Statistics 1984.
2. per hundred thousand population.

a. significantly different ($p < 0.0005$).

b. significantly different ($p < 0.025$).

* data courtesy of Mr. C. Koschick (Cadham Provincial Laboratory).

APPENDIX 4 (continued).

TABLE AP.C RATES OF CRYPTOSPORIDIAL INFECTION IN CHILDREN UNDER 3 YEARS OF AGE IN THREE MANITOBA REGIONS FOR THE YEAR ENDING OCTOBER 31, 1985.*

	# CASES	POPULATION[1]	RATE[2]	
Norman	16	8,158	196.1	
Eastman	2	4,332	46.1	
Winnipeg	3	24,956	12.0	
Total	21	37,446		56.1

1. Manitoba Health Services Commission, Statistics 1984.
2. per hundred thousand population.

a. Population of Norman Region plus "Status Indian" population of Manitoba.

b. significantly different ($p < 0.05$).

* data courtesy of Mr. C. Koschick (Cadham Provincial Laboratory).

NOTE:

Of 22 cases in Norman region 16 were < 3 years of age.

Of 24 cases in children < 3 years, 16 were in Norman region.

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