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Cryptosporidium parvum (Apicomplexa: Cryptosporidiidae) Oocyst and Sporozoite Antigens Recognized by Bovine Colostral Antibodies†

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Colostral whey from seven hyperimmunized and two control cows (hyperimmune bovine colostrum) was examined by Western immunoblotting for the presence of antibody against oocysts and sporozoites of *Cryptosporidium parvum*, using rabbit anti-bovine immunoglobulin A (IgA), IgG1, IgG2, and IgM antibodies, followed by a horseradish peroxidase goat anti-rabbit polyvalent antibody. Although considerable variation was found in binding activity between cows on different immunization protocols, IgA and IgG1 in whey recognized a greater variety of *C. parvum* antigens than did IgG2 and IgM. A band at 9 to 10 kilodaltons appeared unique in that it was recognized only by IgA.

Cryptosporidium parvum (Apicomplexa: Cryptosporidiidae) is a parasitic protozoan that infects a wide range of mammals, including humans. Infections in immunocompetent individuals are usually of short duration, with intestinal involvement resulting in moderate to severe diarrhea and possible weight loss. In immunocompromised individuals, however, infections can become persistent and life threatening.

Although all known available chemotherapeutic agents have been unsuccessful for treatment of the disease (3), recent evidence has suggested parasite susceptibility to immune modulators. In suckling mice, monoclonal antibodies against several sporozoite surface proteins have limited the extent of parasite development (1, 22, 23). Prophylactic administration of hyperimmune bovine colostrum (HBC) resulted in significant reduction in parasite numbers in *C. parvum*-infected mice and calves (1, 4, 6, 22). Direct duodenal perfusion of HBC resulted in abrogation of life-threatening infections in three immunocompromised patients (27, 28, 30).

Despite the successful use of HBC under experimental conditions for immunoprophylaxis and immunotherapy, the biologically active substances in HBC have not been determined. This study was designed to identify the ability of specific HBC antibodies to bind antigens on Western immunoblots of *C. parvum*.

MATERIALS AND METHODS

Production of colostrum. HBC was produced in seven multiparous Holstein-Friesian cows. At 10 weeks before expected parturition (WBP), cows received an intramuscular injection of an emulsion of 5 ml of Freund complete adjuvant and 5 ml of *C. parvum* oocysts in Hanks balanced salt solution (HBSS) that had been frozen and thawed three times immediately before mixing. Two animals served as nonimmunized controls. Oocysts used for immunization were obtained from 1- to 2-week-old calves in Beltsville, Md., and Auburn, Ala., and were cleaned from fecal debris by sucrose flotation. Oocysts were then sterilized externally by incubation in a 1.31% (vol/vol) solution of sodium hypochlorite in HBSS for 5 min at 5°C. At 8, 6, and 4 WBP, a 2-ml mixture of Freund incomplete adjuvant (1 ml) and frozen-thawed (three times) oocysts of *C. parvum* in HBSS (1 ml) was infused into the teat canal of each quarter of the mammary gland and massaged to distribute antigen. The exact number of oocysts administered at each time period for the cows varied (Table 1). Colostrum from the first milking after parturition was collected, and 50-ml samples were centrifuged at 31,000 × *g* for 30 min. After removal of fat, whey was aspirated and frozen at −70°C. Whey collected from nonimmunized animals was processed similarly.

Production and purification of oocysts and sporozoites for Western blotting. Oocysts and sporozoites of *C. parvum* (KSU-1 isolate) were obtained from experimentally infected, 1- to 2-week-old goats, *Capra hircus*, as described previously (26. Purification of oocysts by using sucrose and cesium chloride gradients, excystation procedures, and purification of sporozoites by DE-52 anion-exchange column chromatography have been described previously (M. Tilley and S. J. Upton, Can. J. Zool., in press).

Experimental design. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins and glycoproteins of *C. parvum*. Both 7.5 and 12% gels were used in a Mini Gel apparatus (Bio-Rad Laboratories, Richmond, Calif.) with a discontinuous buffer (10). Both SDS-PAGE and transfer of proteins onto nitrocellulose were done as described previously (Tilley and Upton, in press) except for the use of a two-dimensional rather than multiwell comb. Either 1.9×10^7 frozen-thawed (three times) oocysts or 7.5×10^7 sporozoites were layered on the stacking gel. Prestained molecular weight markers were run placed in separate wells.

After transfer of parasite proteins to nitrocellulose, blots were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS). Nitrocellulose sheets were then placed in a Miniblotter 16 manifold (Immunetics Inc., Cambridge,

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TABLE 1. Protocol for immunizing cows with *C. parvum* oocysts

Cow no.	No. of oocysts (10^6)			
	Intramuscular, 10 WBP	Intramammary gland/quarter		
		8 WBP	6 WBP	4 WBP
8442	20	24	5	5
8445	20	24	5	5
8455	20	24	5	5
8685	0	0	0	0
8801	20	3	3	0.6
8804	20	3	3	0.6
8809	0	0	0	0
8825	20	3	3	0.6
8826	20	3	3	0.6

Mass.), and residual liquid was aspirated from each lane. Colostrum samples were diluted 1:10 in PBS, 120 μl was added to each lane, and the apparatus was incubated for 3 h at room temperature (ca. 23°C) with gentle agitation. Blots were then washed with 250 ml of PBS and probed with specific rabbit anti-bovine immunoglobulin A (IgA), IgG1, IgG2, or IgM diluted 1:1,000 in PBS containing 1% bovine serum albumin (Table 2). Production of these anti-immunoglobulins has been described previously (4). After 1.5 h, blots were rewashed with 250 ml of PBS, removed from the manifold, and placed in a tray with blocking solution containing a 1:1,000 dilution of goat anti-rabbit polyvalent immunoglobulin conjugated to horseradish peroxidase (Organon Teknika, Malvern, Pa.). After 1.5 h, blots were washed with blocking solution for 30 min, with fresh changes of blocking solution at 5-min intervals. Blots were then rinsed twice with PBS and developed by using 4-chloro-1-naphthol (Bio-Rad) as a substrate. After development, blots were rinsed in distilled water and photographed, and bands were analyzed by exponential regression analysis.

RESULTS AND DISCUSSION

Figure 1 illustrates the binding of HBC immunoglobulins from nine cows to oocyst and sporozoite proteins and glycoproteins of *C. parvum*. Table 3 summarizes the molecular sizes of the antigens recognized by each of the four classes and subclasses of antibody. Although considerable individual variation was found, blots revealed that IgA and IgG1 bound a greater number of antigens than did IgG2 and IgM. Whey from nonimmunized animals demonstrated little antibody that bound parasite antigens.

Several studies have suggested that infants fed breast milk may be at less risk of acquiring cryptosporidiosis than are bottle-fed infants (2, 7, 9, 16–18, 21, 32). Whereas three reports indicate a lack of efficacy of colostrum for treating cryptosporidiosis (8, 20, 25), other studies have shown pronounced efficacy of HBC on the course of the disease (1, 4, 6, 22, 27, 28).

As reported previously in one serologic study (28), experimental *C. parvum* infections result in varying levels of immunoglobulins in an animal. Variation in the intensity of the reaction on Western blots using whey increased with the number of oocysts administered as the immunizing dose. In addition, IgA and IgG1 in HBC reacted more intensely with *C. parvum* than did IgG2 or IgM. These data are explained in the accompanying report by Fayer et al. (5), in which whey from cow 8801 (Table 1) was fractioned into the same immunoglobulin classes and subclasses examined in this study and used therapeutically to treat suckling mice infected experimentally with *C. parvum*. Histologic examination (24) of all treatment groups revealed that all mice had significantly fewer developmental stages than did positive control animals and that mice treated with whole whey or the IgA or IgG1 fraction had even fewer developmental stages than did those treated with IgG2 or IgM. Although whey immunoglobulin fractions only from cow 8801 have been examined thus far (5), it is likely that fractions from more highly immunized cows (i.e., 8442, 8445, and 8455) will be found to be as efficacious or more so.

A highly reactive 9- to 10-kilodalton (kDa) band was observed only when anti-IgA was used to detect bound antigens. Immunoreactivity of this band has not been clearly defined, although it is possible that this band appears in a previously published Western blot with sera from two recovered patients (19); it is also possible, however, that the band (19) simply represents the dye front. Although of interest because it reacted only with IgA, the importance of this molecule during the immunologic response to *C. parvum* infections is unknown. However, a more immunologically important band that was bound strongly by all four antibodies was a 14.5- to 16.5-kDa protein. This molecule appears to represent a sporozoite surface glycoprotein (23; Tilley and Upton, in press) and has appeared in several published immunoblots (13, 14, 19; Tilley and Upton, in press).

The KSU-1 isolate has about 20 sporozoite surface proteins susceptible to ^{125}I labeling (Tilley and Upton, in press). The most notable are at 102 to 105, 83, 73 to 76, 48, 44, 42, 37 to 39, 29 to 33, 23, and 15 kDa with SDS-PAGE (Tilley and Upton, in press). These appear to correspond to the 100-, 88- or/and 82-, 68- to 72-, 49- or/and 47-, 45- to 46-, 41- to 43-, 38- to 40-, 30- to 31- or/and 31.5- to 32-, 23-, and 14.5- to 16.5-kDa bands, respectively, in this study. Although several studies have regarded P20 and P23 as immunodominant protein(s) (1, 14, 15, 19, 29; Tilley and Upton, in press), careful examination of blots from various publications indicates that 44- to 50- and 68- to 76-kDa molecules are also consistently recognized (11, 13, 14, 19; Tilley and Upton, in press).

Several high-molecular-weight bands were found to be unique in blots using whole oocysts as antigen, indicating

TABLE 2. Protocol for Western blotting of *C. parvum* antigens using HBC[a]

Lane[b]	Initial probe	Secondary antibody
A	1:10 dilution of 5% nonfat dry milk	None; 1% BSA[c] in PBS
B	1:10 dilution of 5% nonfat dry milk	Rabbit[d]
C	8455[e]	None; 1% BSA in PBS
D	8442	Rabbit[d]
E	8445	Rabbit[d]
F	8455	Rabbit[d]
G	8685	Rabbit[d]
H	8801	Rabbit[d]
I	8804	Rabbit[d]
J	8809	Rabbit[d]
K	8825	Rabbit[d]
L	8826	Rabbit[d]

[a] Each protocol was visualized by using a horseradish peroxidase-conjugated goat anti-rabbit polyvalent antibody.
[b] Corresponds to the respective lane in Fig. 1.
[c] BSA, Bovine serum albumin.
[d] Anti-bovine class- or subclass-specific polyvalent antibody.
[e] Cow number from which HBC was derived. Corresponds to numbers presented in Table 1.

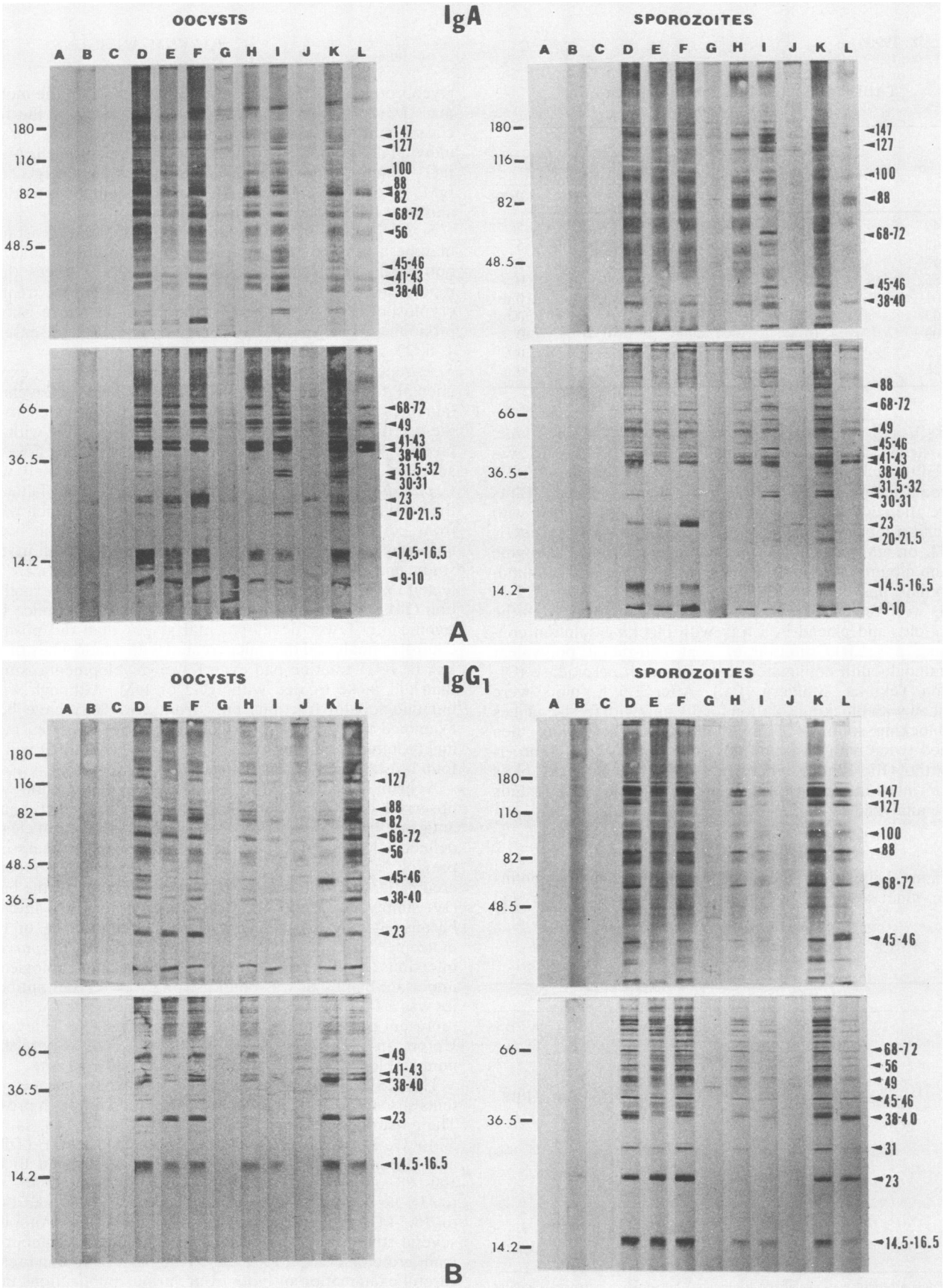
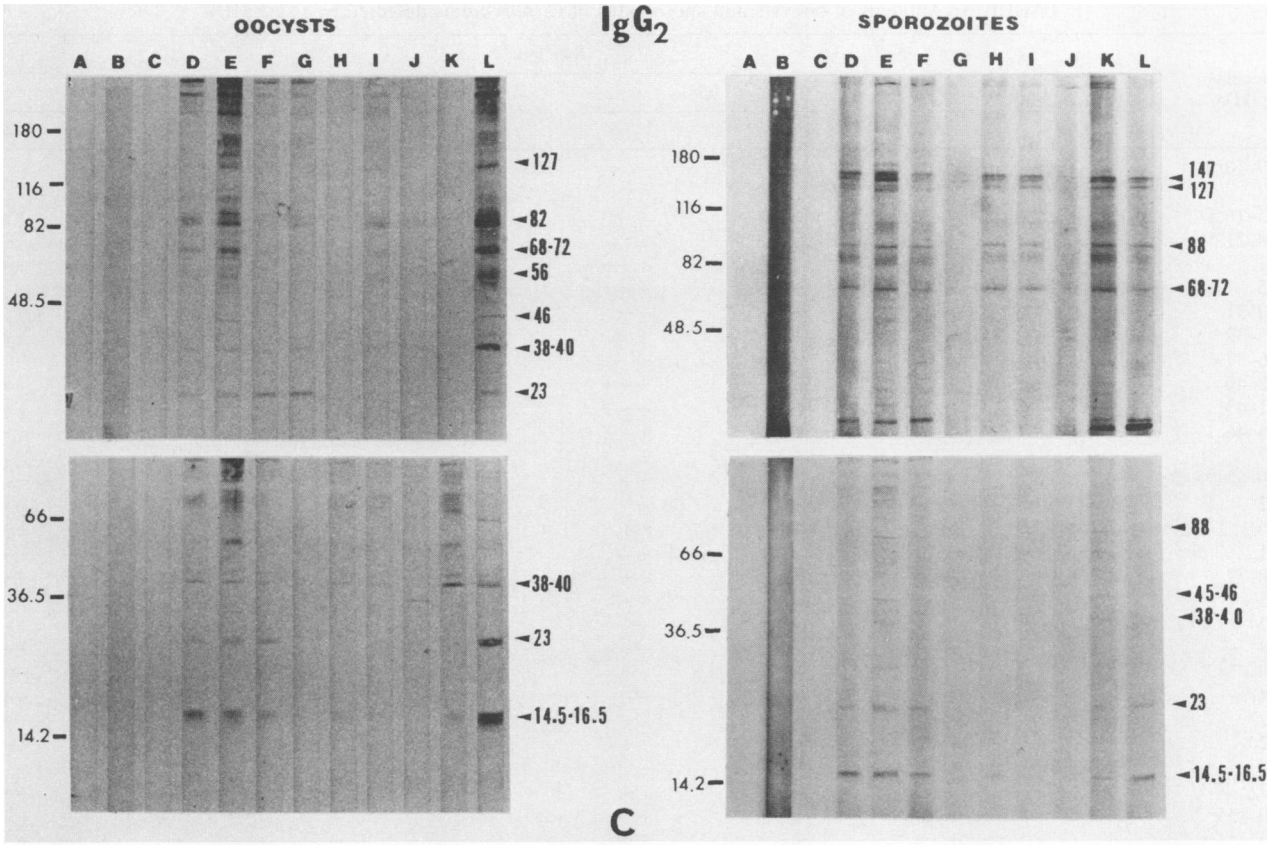


FIG. 1. Western blots of oocysts and sporozoites of *C. parvum* probed with hyperimmune bovine colostrum. Each set of four blots (A to D) represents 7.5% (top) and 12% (bottom) gels run on frozen-thawed oocysts (left side) and sporozoites (right side). The immunization protocol for animals represented in individual lanes can be found in Table 1; the antibody detection procedures for each lane are presented in Table 2. Molecular size (in kilodaltons) markers are represented to the left of each blot; select antigens are indicated by arrowheads to the right.



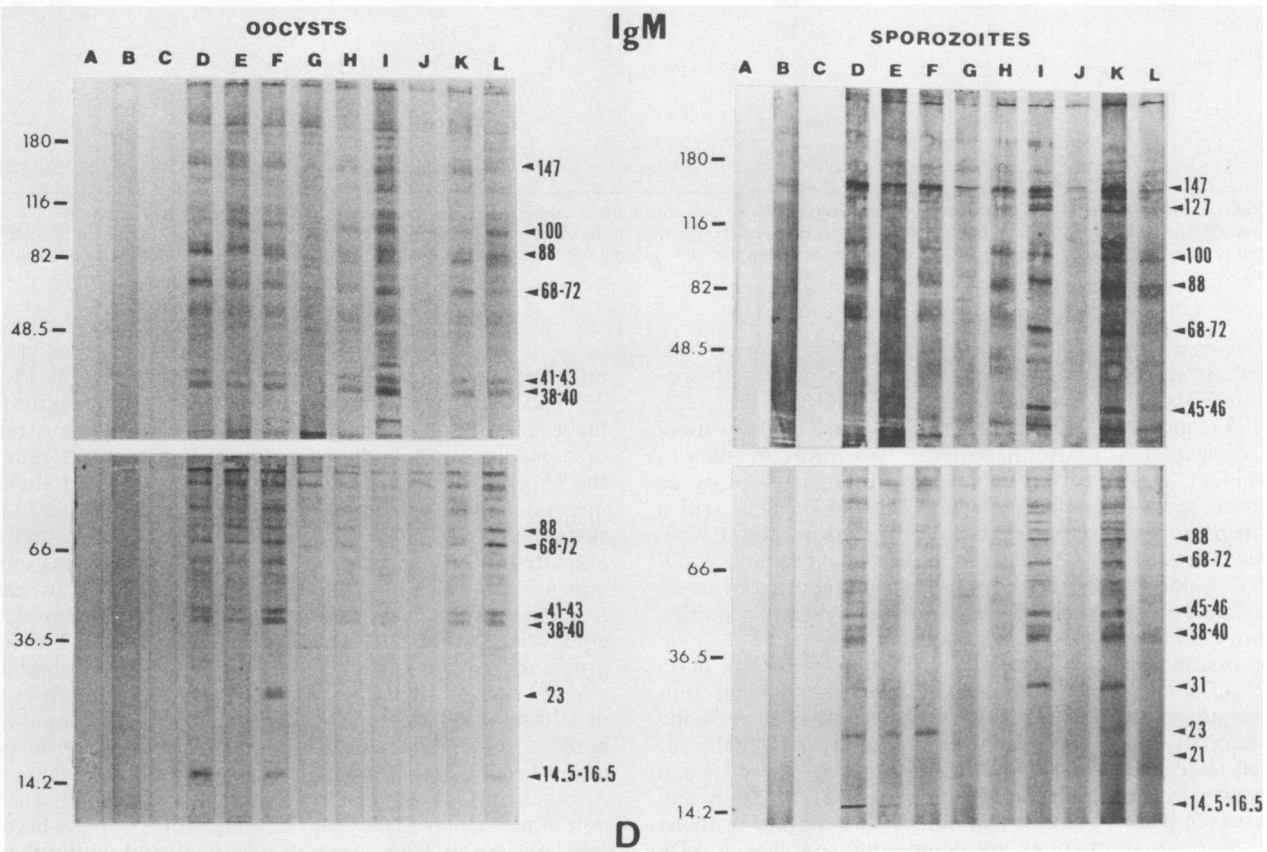


FIG. 1—*Continued.*

TABLE 3. Antigens of oocysts and sporozoites of *C. parvum* as detected by using HBC

Molecular size (kDa)	Reaction[a]							
	IgA		IgG1		IgG2		IgM	
	OO	SZ	OO	SZ	OO	SZ	OO	SZ
9–10	+++	++++	−	−	−	−	−	−
13	++	−	+	−	−	−	−	−
14.5–16.5	++++	++++	++	+	++++	++++	++	+++
20–21.5	++	++	+	+	−	−	−	−
23	++++	++++	++++	++++	+++	++	+++	++
25	++	−	++	−	+	−	++	−
30–31	+++	++++	++	++	+	+	−	−
31.5–32	+++	+++	+	+	+	+	−	+++
36–37	+	+	+	+	−	−	−	−
38–40	++++	+++	++++	++++	+++	++	++++	+++
41–43	++++	++	+++	+++	++	++	++++	+++
45–46	+++	+++	+++	++++	+	++	++	+++
47	+++	+	++	+	−	−	−	−
49	++	+++	+++	++	−	−	−	−
52	++	++	+	+	−	−	+	−
56	+++	++	+++	++	++	+	+	++
61	−	−	+	+	+	+	+	++
68–72	++++	+++	++++	+++	++++	++++	+++	++++
76	++	++	++	++	+	−	−	−
82	+++	++	+++	++	+++	+++	++	+
88	+++	+++	+++	+++	+++	+++	+++	+++
100	++	++	++	++	++	+++	+++	+++
114	++	+	+	−	−	−	++	++
127	++	++	+++	++	++	++	++	++
132	++	++	++	++	+	+	++	++
137	+	+	−	−	−	−	−	−
147	+++	+++	++	++	++	++	++	++
150–155	++	++	+++	++	++	+	++	++
160	+	+	+	+	−	−	+	+
167–177	++	−	++	++	++	++	−	−
190–205	++	−	++	+	−	−	++	−
211	++	−	−	−	−	−	−	−
223	++	−	−	−	+	−	−	−
227	++	−	−	−	−	−	+	−
235	++	−	−	−	−	−	−	−
240	++	−	−	−	−	−	−	−
≥250	++	+	+	+	++++	+	−	−

[a] Relative intensities of bands that were optimally recognized by cows for a given antibody. −, No reactivity; +, band recognized by antibody; ++, band well recognized by antibody; +++, band highly recognized by antibody; ++++, band intensely recognized. Although samples were analyzed identically, we present relative intensities as a means of examining bands within the same gel and not as a means of quantitating antibody concentrations. OO, Oocysts; SZ, sporozoites.

that these molecules are probably not associated with sporozoites. Most bands were of high molecular size (211, 223, 227, 235, and 240 kDa), whereas two (13 and 25 kDa) were of low molecular size. Neither of the two lower bands was prominent, and the 25-kDa band was often masked by an intensely reactive 23-kDa molecule. The 23-kDa band, which has been reported by various investigators to migrate between 20 and 23 kDa, appears to be a surface protein (15, 19, 23; Tilley and Upton, in press). It has been reported by many investigators to be recognized by most individuals acquiring cryptosporidiosis (14, 15, 19, 29).

Sporozoite proteins of *C. parvum* within the range of 15 to 25 kDa are highly immunogenic, and monoclonal antibodies to some of these have been effective in reducing the severity of *C. parvum* infections in experimentally infected mice (1, 22, 23). However, caution is advised when comparing reported Western blots. Results of this study and others (see below) suggest that at least two surface proteins of *C. parvum* sporozoites are being confused. The 23-kDa molecule, which appears to be weakly iodinated (Tilley and Upton, in press) but highly immunogenic (14, 15, 19, 29) may have epitopes that cross-react with a variety of higher-molecular-size species (1, 15). Oral administration of monoclonal antibodies against this protein has reduced the severity of disease in experimentally infected suckling mice compared with controls (1). However, an 18- to 20-kDa surface protein, often referred to as P20, though intensely iodinated (Tilley and Upton, in press), appears less immunogenic than P23. We have detected galactose or galactosamine residues on this molecule but have failed to detect glycosylation on P23 (Tilley and Upton, in press). Effects of preparation techniques on the degree of glycosylation or perhaps strain differences may explain the variation in size of P20 reported by various investigators. Monoclonal antibodies against P20 are also effective at lessening the severity of disease in experimentally infected suckling mice (1). Another surface molecule of similar size, which was reported previously (Tilley and Upton, in press) and which we refer to here as P26, could also be confused with either or both of the aforementioned proteins.

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LITERATURE CITED

1. **Arrowood, M. J., J. R. Mead, J. L. Mahrt, and C. R. Sterling.** 1989. Effects of immune colostrum and orally administered antisporozoite monoclonal antibodies on the outcome of *Cryptosporidium parvum* infections in neonatal mice. Infect. Immun. **57**:2283–2288.
2. **Cruz, J. R., F. Cano, P. Caceres, F. Chew, and G. Pareja.** 1988. Infection and diarrhea caused by *Cryptosporidium* sp. among Guatemalan infants. J. Clin. Microbiol. **26**:88–91.
3. **Dubey, J. P., R. Fayer, and C. A. Speer.** 1990. Cryptosporidiosis in man and animals. CRC Press, Inc., Boca Raton, Fla.
4. **Fayer, R., C. Andrews, B. L. P. Ungar, and B. Blagburn.** 1989. Efficacy of hyperimmune bovine colostrum for prophylaxis of cryptosporidiosis in neonatal calves. J. Parasitol. **75**:393–397.
5. **Fayer, R., A. Guidry, and B. L. Blagburn.** 1990. Immunotherapeutic efficacy of bovine colostral immunoglobulins from a hyperimmunized cow against cryptosporidiosis in neonatal mice. Infect. Immun. **58**:2962–2965.
6. **Fayer, R., L. E. Perryman, and M. W. Riggs.** 1989. Hyperimmune bovine colostrum neutralizes *Cryptosporidium* sporozoites and protects mice against oocyst challenge. J. Parasitol. **75**:151–153.
7. **Guderian, R., C. A. Sandoval, and C. D. Mackenzie.** 1986. Cryptosporidiosis in Ecuadorian children with acute diarrhea. J. Trop. Pediatr. **32**:290–292.
8. **Harp, J. A., D. B. Woodmansee, and H. W. Moon.** 1989. Effects of colostral antibody on susceptibility of calves to *Cryptosporidium parvum* infection. Am. J. Vet. Res. **50**:2117–2119.
9. **Hojlyng, N., K. Molbak, and S. Jepsen.** 1986. *Cryptosporidium* spp., a frequent cause of diarrhea in Liberian children. J. Clin. Microbiol. **23**:1109–1113.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Lazo, A., O. O. Barriga, D. R. Redman, and S. Bech-Nielsen.** 1986. Identification by transfer blot of antigens reactive in the enzyme-linked immunosorbent assay (ELISA) in rabbits immunized and a calf infected with *Cryptosporidium* sp. Vet. Parasitol. **21**:151–163.
12. **Lopez, J. W., S. D. Allen, J. Mitchell, and M. Quinn.** 1988. Rotavirus and *Cryptosporidium* shedding in dairy calf feces and its relationship to colostrum immune transfer. J. Dairy Sci. **71**:1288–1294.
13. **Luft, B. J., D. Payne, D. Woodmansee, and C. W. Kim.** 1987. Characterization of the *Cryptosporidium* antigens from sporulated oocysts of *Cryptosporidium parvum*. Infect. Immun. **55**: 2436–2441.
14. **Lumb, R., J. A. Lanser, and P. J. O'Donoghue.** 1988. Electrophoretic and immunoblot analysis of *Cryptosporidium* oocysts. Immunol. Cell Biol. **66**:369–376.
15. **Lumb, R., P. S. Smith, R. Davis, P. J. O. O'Donoghue, H. M. Atkinson, and J. A. Lanser.** 1989. Localization of a 23 000 MW antigen of *Cryptosporidium* by immunoelectron microscopy. Immunol. Cell Biol. **67**:267–270.
16. **Mata, L., H. Bolanos, D. Pizarro, and M. Vives.** 1984. Cryptosporidiosis in children from some highland Costa Rican rural and urban areas. Am. J. Trop. Med. Hyg. **33**:24–29.
17. **Mata, L., H. Bolanos, D. Pizarro, and M. Vives.** 1984. Cryptosporidiosis en niños de Costa Rica: estudio transveral y longitudinal. Rev. Biol. Trop. **32**:129–135.
18. **Mathan, M. M., S. Venkatesan, R. George, M. Mathew, and V. I. Mathan.** 1985. *Cryptosporidium* and diarrhoea in southern Indian children. Lancet **ii**:1172–1175.
19. **Mead, J. R., M. J. Arrowood, and C. R. Sterling.** 1988. Antigens of *Cryptosporidium* sporozoites recognized by immune sera of infected animals and humans. J. Parasitol. **74**:135–143.
20. **Moon, H. W., D. B. Woodmansee, J. A. Harp, S. Abel, and B. L. P. Ungar.** 1988. Lacteal immunity to enteric cryptosporidiosis in mice: immune dams do not protect their suckling pups. Infect. Immun. **56**:649–653.
21. **Pape, J. W., E. Levine, M. E. Beaulieu, F. Marshall, R. Verdier, and W. D. Johnson.** 1987. Cryptosporidiosis in Haitian children. Am. J. Trop. Med. Hyg. **36**:333–337.
22. **Perryman, L. E., M. W. Riggs, P. H. Mason, and R. Fayer.** 1990. Kinetics of *Cryptosporidium parvum* sporozoite neutralization by monoclonal antibodies, immune bovine serum, and immune bovine colostrum. Infect. Immun. **58**:257–259.
23. **Riggs, M. W., T. C. McGuire, P. H. Mason, and L. E. Perryman.** 1989. Neutralization-sensitive epitopes are exposed on the surface of infectious *Cryptosporidium parvum* sporozoites. J. Immunol. **143**:1340–1345.
24. **Riggs, M. W., and L. E. Perryman.** 1987. Infectivity and neutralization of *Cryptosporidium parvum* sporozoites. Infect. Immun. **55**:2081–2087.
25. **Saxon, A., and W. Weinstein.** 1987. Oral administration of bovine colostrum anti-cryptosporidia antibody fails to alter the course of human cryptosporidiosis. J. Parasitol. **73**:413–415.
26. **Tilley, M., S. J. Upton, B. L. Blagburn, and B. C. Anderson.** 1990. Identification of outer oocyst wall proteins of three *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) species by ^{125}I surface labeling. Infect. Immun. **58**:252–253.
27. **Tzipori, S., D. Robertson, and C. Chapman.** 1986. Remission of diarrhoea due to cryptosporidiosis in an immunodeficient child treated with hyperimmune bovine colostrum. Br. Med. J. **293**: 1276–1277.
28. **Tzipori, S., D. Robertson, D. A. Cooper, and L. White.** 1987. Chronic cryptosporidial diarrhea and hyperimmune cow colostrum. Lancet **ii**:344–345.
29. **Ungar, B. L. P., and T. E. Nash.** 1986. Quantification of specific antibody response to *Cryptosporidium* antigens by laser densitometry. Infect. Immun. **53**:124–128.
30. **Ungar, B. L. P., D. J. Ward, R. Fayer, and C. A. Quinn.** 1990. Cessation of *Cryptosporidium*-associated diarrhea in an acquired immunodeficiency syndrome patient after treatment with hyperimmune bovine colostrum. Gastroenterology **98**:486–489.
31. **Williams, R. O., and D. J. Burden.** 1987. Measurement of class specific antibody against *Cryptosporidium* in serum and faeces from experimentally infected calves. Res. Vet. Sci. **43**:264–265.
32. **Wolfson, J. S., J. M. Richter, M. A. Waldron, D. J. Weber, D. M. McCarthy, and C. C. Hopkins.** 1985. Cryptosporidiosis in immunocompetent patients. N. Engl. J. Med. **312**:1278–1282.