Parasitol Res (2002) 88: 412–420
DOI 10.1007/s00436-001-0586-z

ORIGINAL PAPER

J-R. Yu · S.P. O'Hara · J.L-C. Lin · M.E. Dailey
G. Cain · J.L-C. Lin

A common oocyst surface antigen of *Cryptosporidium* recognized by monoclonal antibodies

Received: 14 September 2001 / Accepted: 7 December 2001 / Published online: 6 February 2002

Abstract Two hybridoma clones, CMYL3 and CMYL30, were generated by immunizing Balb/c mice with excysted oocysts of *Cryptosporidium muris*. Both clones secreted monoclonal antibodies against an oocyst-wall antigen with apparent molecular mass of 250 kDa (called CM250) from *C. muris* and *C. parvum*. The epitope appeared to be periodate-sensitive, suggesting the involvement of a carbohydrate moiety. Immunofluorescence and confocal microscopy on purified oocysts and infected mouse tissues revealed staining confined to the oocyst wall of both *Cryptosporidium* species. Immunogold labeling further revealed the presence of the CM250 antigen in electron-dense vesicles and cytoplasm of developing macrogametocytes, and ultimately localized to the oocyst wall of mature oocysts. Both antibodies cross-reacted with *C. serpentis* oocysts but did not recognize the other enteropathogenic protozoans *Giardia muris*, *Eimeria falciformis* and *E. nischulz*. These antibodies may be valuable tools for the analysis of oocyst-wall formation in *Cryptosporidium* and characterization of the common antigen.

Introduction

Cryptosporidiosis is an opportunistic disease that can be fatal to immunocompromised patients through the induction of chronic diarrhea. The infection is transmitted by oocysts in a fecal-to-oral route, with parasite development occurring in the epithelial cells of the gastrointestinal tract. In the genus *Cryptosporidium*, six to eight species have been tentatively approved as valid, based on differences in host specificity, oocyst morphology and the site of infection (Fayer et al. 1997; O'Donoghue 1995). Recent phylogenetic analysis of small subunit rRNA sequences of several *Cryptosporidium* isolates revealed that at least four distinct species, *C. parvum*, *C. baileyi*, *C. muris*, and *C. serpentis*, are valid (Xiao et al. 1999). Although human infections are chiefly due to *C. parvum*, some reports have revealed that *C. baileyi* and *C. muris* are also capable of infecting humans (Ditrich et al. 1991; Katsumata et al. 2000), but these observations still have to be confirmed. Moreover, clinically, *C. muris* infection is less pathogenic than *C. parvum* (Taylor et al. 1999).

In addition to oocyst morphology and phylogenetic differences at the gene level, the different sites of infection support a valid distinction between *C. parvum* and *C. muris*. *C. parvum* infects the small intestine, preferentially in the ileum, while *C. muris* infects the stomach. The oocyst wall undoubtedly provides protection for sporozoites via a highly resistant barrier to external influences, such as the environment external to the host, as well as assaults from the host organism. The differential localizations of infections between *C. parvum* and *C. muris* raise questions about possible differences in oocyst-wall composition.

Monoclonal antibodies (mAbs) have proven useful, not only in the characterization of unique antigens, but also in the detection of oocysts from feces for diagnostic purposes (Anusz et al. 1990; McLauchlin et al. 1987; Sterling and Arrowood 1986), in neutralizing infectivity, and in providing relief from cryptosporidiosis symptoms in murine models (Langer and Riggs 1996; Perryman et al. 1990). Various parasitic protists are known to induce diarrhea. One of these, *Giardia lamblia*, is a human parasite with clinical symptoms similar to cryptosporidiosis (Roberts and Janovy 1996). The cyst of *G. lamblia* in human stool is about 8–12 μm long, only

J-R. Yu
Department of Parasitology, College of Medicine,
Konkuk University, Chungju 380-701, Korea

S.P. O'Hara · J.L-C. Lin · M.E. Dailey · G. Cain
J.L-C. Lin (✉)
Department of Biological Sciences,
University of Iowa, 138 Biology Building,
Iowa City, IA 52242-1324, USA
E-mail: jim-lin@uiowa.edu
Tel.: +1-319-3351075
Fax: +1-319-3351069

slightly larger than *Cryptosporidium* oocysts. In addition, many domestic animals are affected by *Eimeria* species (Roberts and Janovy 1996). This Apicomplexan parasite also induces diarrhea. Whether similar oocyst components exist between *Eimeria* and *Cryptosporidium* remains to be determined. Therefore, the identification and characterization of new parasite oocyst antigens could be useful for diagnosis and for understanding the molecular biology of the parasites.

Most antibodies developed to *Cryptosporidium* are against *C. parvum*. To date, no mAb against *C. muris* has been developed. In this study, we generated mAbs against a *C. muris* antigen that localizes to the oocyst wall of both immature and mature oocysts, as well as in cytoplasmic vesicles of developing macrogametocytes.

Materials and methods

Oocyst purification and antigen preparation

The oocysts of *C. muris* were obtained from the feces of C57BL6 female mice, which were orally infected with 2×10^6 oocysts per mouse, as described previously (Yu 1998). The oocysts of *C. parvum* were obtained by the same method as *C. muris*, but the mice were immunosuppressed by intramuscular injection of 0.1 mg/g body weight of methylprednisolone acetate (Depomedrol, Pharmacia and Upjohn Ltd, Hwaseong, Korea) once a week (Yu and Chai 1995). Mouse feces were examined by modified Ziehl–Neelsen staining to confirm oocyst shedding (Casemore et al. 1985) and collected in 2.5% potassium dichromate at 4 °C. The oocysts were purified as described by Petry et al. (1995). For the antigen preparation, the oocysts were excysted in 0.04% taurocholic acid in phosphate-buffered saline (PBS) at 37 °C for 1 h. After washing once in PBS, excysted oocysts were homogenized by sonication for 20 min at 4 °C.

Production of mAbs

Eight-week-old female BALB/c mice were immunized intraperitoneally by the injection of 1.5×10^7 excysted and homogenized oocyst antigen emulsified in an equal volume of TiterMax (CytRx Corporation, Norcross, Ga.). Three additional intraperitoneal injections, each with 1×10^7 oocysts, were given at 3-week intervals. At 63 days, mice were bled from the tail and the sera obtained were tested for anti-*Cryptosporidium* antibody by indirect immunofluorescence. A final boosting (5×10^6 oocysts) was given intravenously 4 days before fusion. Spleen cells were isolated and fused with mouse myeloma NSI as described by Lin et al. (1985). The cultured medium of hybridomas was screened by enzyme-linked immunosorbent assay (ELISA), according to the method of Tijssen (1985). The cloning of positive hybridomas was performed as described previously (Lin et al. 1985). Two hybridoma clones, CMYL3 and CYML30, were used in this study. The antibody CMYL3 was an IgM, whereas the antibody CMYL30 was an IgG1 subclass, as determined by a Sigma ImmunoType kit (Sigma, St. Louis, Mo.).

Immunofluorescence microscopy

Purified oocysts were smeared and air-dried on glass slides. For immunofluorescence, the slides were then incubated with primary antibody and subsequently with fluorochrome-conjugated secondary antibodies, as described below. Mouse stomach and small intestine were dissected from *Cryptosporidium*-infected mice and immediately frozen with liquid nitrogen in OCT compound (Miles, Elkhart, Ind.). Tissues were cryo-sectioned at a thickness of 4 μm and stored at –70 °C until use. Upon use, tissue sections were fixed in 3.7% formaldehyde and permeabilized in cold acetone. After a PBS wash, the slides were incubated with cell supernatants or ascites diluted appropriately with 3% bovine serum albumin in PBS, washed in PBS and incubated with a 1:400 dilution of fluorescein isothiocyanate (FITC) – conjugated goat anti-mouse IgG whole molecule antibody (Cappel, West Chester, Pa.) in PBS. Slides were washed, mounted in polyvinyl alcohol (Gelvatol), and observed with a Zeiss epifluorescence microscope or a Leica scanning-laser confocal microscope.

SDS-PAGE and Western blotting

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970), with a low concentration of bisacrylamide as described previously (Lin et al. 1985). Total protein extracts were prepared by directly dissolving 1×10^8 oocysts in 1 ml of gel sample buffer, as described by Bonnin et al. (1991) and extracts from about $7.5\times10^5\sim2\times10^6$ oocysts were loaded per lane. Oocyst walls were further purified from both *C. parvum* and *C. muris* oocysts according to Harris and Petry (1999) and extracts from $2.5–5\times10^5$ oocyst walls were loaded per lane. After electrophoresis, the proteins in the gel were electrophoretically transferred to PVDF membranes. The membranes were blocked in 5% skim milk in Tris-buffered saline (TBS, 50 mM Tris pH 7.5, 150 mM NaCl) overnight at 4 °C. CMYL3 and CMYL30 ascites were diluted 4000-fold and 700-fold, respectively, in 3% bovine serum albumin in TBS. Each was administered to respective blots for 1 h at room temperature in a humidified chamber. After three changes of TBS containing 0.2% Tween 20 over 45 min, the membranes were incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (Ig) diluted 2,000-fold in TBS. After washing as above, bound antibodies were detected with ECL Western blotting analysis system (Amersham, UK).

Periodate treatment

To determine whether the antibody epitopes contain carbohydrate, Western blot analyses were performed on the antigen before and after periodate treatment, as described by Woodward et al. (1985). Briefly, after blocking with 5% skim milk in TBS, the nitrocellulose membrane was incubated with 10 mM sodium orthoperiodate in 50 mM sodium acetate buffer (pH 4.5) for 1 h in the dark at room temperature, and then exposed to 50 mM sodium borohydride in PBS for 30 min. Following three washes with TBS-Tween 20, the membranes were incubated in CMYL30 as described above. A control membrane was incubated in 50 mM sodium acetate buffer without sodium orthoperiodate.

Immunogold labeling

The stomach and small intestine of *Cryptosporidium*-infected mice were dissected out and fixed in 2% paraformaldehyde and 0.4% glutaraldehyde for approximately 2 h at room temperature. The fixed tissues were washed with 0.1 M phosphate buffer (pH 7.2), and dehydrated through an alcohol series from 30% to 95%. Dehydrated tissues were embedded in LR gold resin (London Resin Company, UK) and polymerized at –20 °C for 72 h under UV illumination. The ultrathin sectioning was performed at 90-nm thickness and sections were mounted onto nickel grids. The immunogold labeling procedure followed the method of Yu and Chai (1995). Briefly, tissue sections were incubated in PBS-milk-Tween (PMT, 3% skim milk and 0.01% Tween 20 in PBS) for 10 min and exposed to ascites solution of mAb CMYL3 or CMYL30 diluted with PMT for 2 h at room temperature. The sections were washed thoroughly with PBS-BSA-Tween (1% bovine serum albumin and 0.01% Tween 20 in PBS) and incubated with 5 nm gold conjugated goat anti-mouse IgG or IgM (Sigma) overnight at 4 °C. Silver enhancement was done with a commercial kit (Amersham)

followed by staining with uranyl acetate and lead citrate. The stained sections were examined under a transmission electron microscope, Jeol 1200 EXII.

Results

A common antigen CM250 was recognized by both CMYL3 and CMYL30

Although total protein profiles, analyzed by SDS-PAGE, of oocysts prepared from *C. muris* and *C. parvum* were very similar, species-enriched protein bands could be also detected (Fig. 1). Whether these species-enriched proteins may have roles in determining the different localization of infection for different species remains to be determined. In this study, we have generated two mAbs, CMYL3 (IgM) and CMYL30 (IgG1), by using total proteins from *C. muris* oocysts as immunogens. Western blot analysis showed that both CMYL3 and CMYL30 antibodies cross-reacted with a protein band of 250 kDa (called CM250) from *C. parvum* oocysts (lane 1 in Fig. 2) and oocyst walls (lane 2 in Fig. 2). Repeat Western blot analysis with CMYL3 antibody on the same oocyst wall sample after a week's storage at –20 °C revealed a size-shift in the antigen molecule (lane 3 in Fig. 2), suggesting that this CM250 antigen was very vulnerable to proteolytic cleavages. The proteolysis was more apparent when Western blot analyses were performed on the total proteins prepared from both oocysts and oocyst walls of *C. muris* (lanes 1 and 2 of Fig. 3). However, both antibodies recognized a protein band (CM250), which appeared to have the same molecular size as that from *C. parvum*. In addition, several bands with lower molecular sizes (such as 235, 150, 110, 92, 72, and 45 kDa) were detected, presumably representing proteolytic fragments of CM250. The inclusion of a mixture of protease inhibitors (complete protease inhibitor cocktail tablet, cat. no. 1607498, Boehringer Mannheim, Indianapolis, Ind.) in the sample preparation only slightly inhibited proteolysis (data not shown). Therefore, based on these studies, we concluded that a common band of 250 kDa (CM250) in *C. muris* and *C. parvum* was recognized by both CMYL3 and CMYL30. The CM250 appeared to be found in the oocyst walls of both *C. parvum* and *C. muris*. The next question was whether carbohydrates were involved in the antigenic determinants of CM250 for both antibodies. The oocyst-wall proteins, after membrane transfer, were treated with periodate as described above (Materials and methods) and then used for Western blot analysis. The reactivity of CMYL30 to periodate-treated CM250 and/or its fragments (such as 235, 110, 92, and 45 kDa) was drastically reduced (Fig. 4B), compared with that in the control blot without periodate treatment (Fig. 4A). Thus, the CM250 appeared to be a glycosylated antigen on the oocyst wall of *Cryptosporidium*.

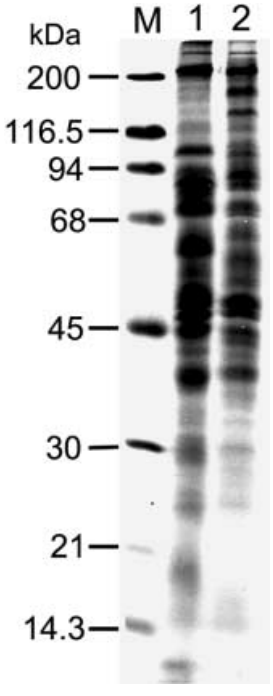

Fig. 1 Protein profiles of *Cryptosporidium* oocyst antigens as separated on a 12.5% SDS-polyacrylamide gel. *M* Molecular weight marker proteins; *lane 1* oocyst antigens of *C. muris*; *lane 2* oocyst antigens of *C. parvum*

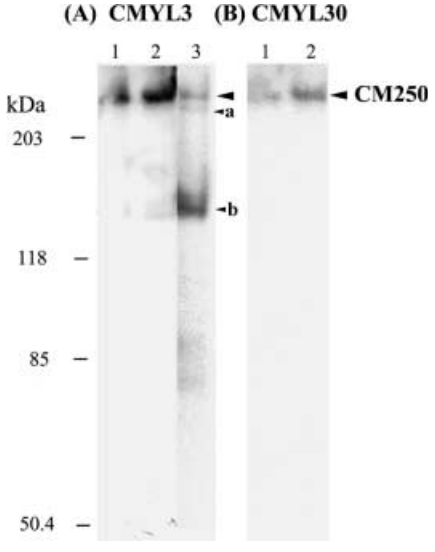

Fig. 2 Western blot detection of *C. parvum* antigens probed with **A** CMYL3 and **B** CMYL30. Antigens reacting to these antibodies were visualized by ECL method after labeling with sheep anti-mouse Ig conjugated to horseradish peroxidase. *Lane 1 C. parvum* whole oocyst antigens; *lane 2 C. parvum* oocyst wall antigens; *lane 3* same sample as that in lane 1 except the sample was stored for a week at –20 °C. Both antibodies recognized the antigen CM250 with molecular mass of 250 kDa. Upon storage of the same sample as that in lane 1, the antibody reacted strongly with a 150 kDa band (indicated by *b*) and weakly with a 235 kDa band (indicated by *a*). Consequently, the reactivity to the CM250 band became greatly diminished (*lane 3*). The result suggested that smaller size antigens were derived from the proteolytic cleavage of CM250

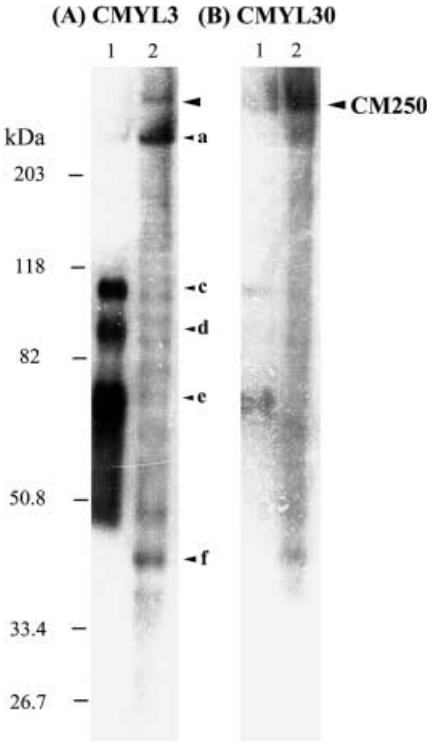

Fig. 3 Western blot detection of *C. muris* antigens probed with mAbs **A** CMYL3 and **B** CMYL30. Antigens reacting to these antibodies were visualized by the ECL method after labeling with sheep anti-mouse Ig conjugated to horseradish peroxidase. *Lane 1 C. muris* whole oocyst antigens; *lane 2 C. muris* oocyst-wall antigens. Both antibodies clearly recognized the CM250 band from oocyst-wall antigens (lane 2). In addition to the CM250, its proteolytic fragments including a 235 kDa band (*a*) and a 45 kDa band (*f*) in the wall antigens were also recognized by both antibodies. The proteolytic fragments recognized by mAbs were more prominent in the oocyst antigens (lane 1). These included 235 kDa (*a*), 110 kDa (*c*), 87 kDa (*d*), and 70 kDa (*e*)

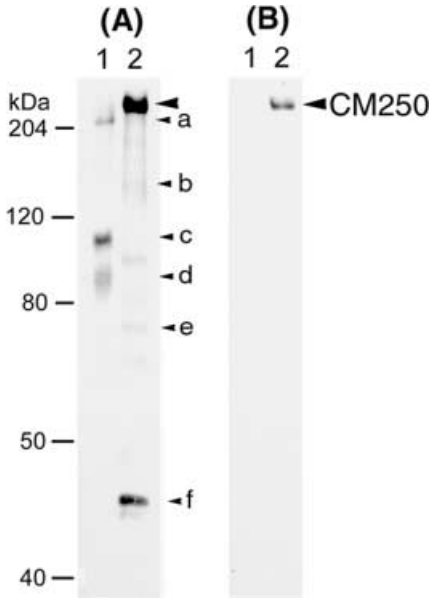

Fig. 4A, B Effects of periodate oxidation on the binding of mAb CMYL30 to *Cryptosporidium* oocyst antigens. Proteins of *C. muris* (*lane 1*) and *C. parvum* (*lane 2*) oocysts were separated by SDS-PAGE gel and electrophoretically transferred to duplicated membranes. **A** The membrane was incubated in 50 mM acetate buffer pH 4.5 only, washed before immunostaining, and used as a control membrane. **B** The other membrane was treated with 10 mM sodium orthoperiodate in 50 mM sodium acetate buffer pH 4.5, washed before immunostaining, and used as a periodate-treated membrane. The antibody reactivity to the CM250 antigen, as well as its proteolytic fragments (indicated by *a, b, c, d, e,* and *f*) was greatly reduced or absent

CM250 antigens were found in wall-forming vesicles of macrogametocytes and predominantly located in the wall of mature oocysts

CMYL3 and CMYL30 reacted with the oocyst walls of *C. muris* and *C. parvum*, as observed by indirect immunofluorescence (Fig. 5 A, D and Fig. 6 A, D, respectively). The cryosections of mouse stomach and small intestine infected with *Cryptosporidium* were immunofluorescently stained with these two antibodies. The *C. muris* oocysts (indicated by *o* in Fig. 5B, E) recognized by both antibodies were almost completely packed in the stomach glands (indicated by *sg* in Fig. 5C, F). Similarly, both antibodies strongly reacted with the *C. parvum* oocysts in small intestinal epithelia of infected mice (Fig. 6B, E).

To further demonstrate that the CM250 was localized in the oocyst walls, confocal microscopy was performed on antibody-labeled oocysts. Figure 7 B–H shows an optically sectioned CMYL30 labeled *C. muris* oocyst from the bottom to the top of oocyst. A uniform staining on the oocyst wall was particularly obvious in sections C–F. An infolded suture of the oocyst wall was also clearly labeled (arrow in Fig. 7E). This antibody did not recognize sporozoites or other residual bodies inside of the oocyst (Fig. 7). The CMYL3 antibody also specifically stained the oocyst wall of *C. muris* as observed by confocal microscopy (data not shown). Similar wall-staining patterns were observed with these two antibodies on the *C. parvum* oocysts by confocal microscopy (data not shown).

To further confirm the CM250 antigen as a wall antigen, and to follow the fate of the antigen during the life cycle of the parasites, immunoelectron microscopy was performed on *Cryptosporidium*-infected mouse tissues. Again, both CMYL3 and CMYL30 recognized an antigen (as revealed by 5-nm gold particles) predominantly located in the oocyst-wall structures of the parasites, *C. muris* (Fig. 8 A, B) and *C. parvum* (Fig. 8D, E). No gold particles were associated with the parasitophorous vacuole membrane (*pvm* in Fig. 8), which is of host origin. A broad band of the gold particles was occasionally seen at the wall region near the attachment zone to the host cell (*az* in Fig. 8E). Other developmental stages were not labeled by gold particles at all (data not shown), with the exception of the macrogametocyte (Fig. 8C, F).

In macrogametocytes, electron-dense vesicles near the periphery were heavily labeled by gold particles (*arrowheads* in Fig. 8C, F). These vesicles closely

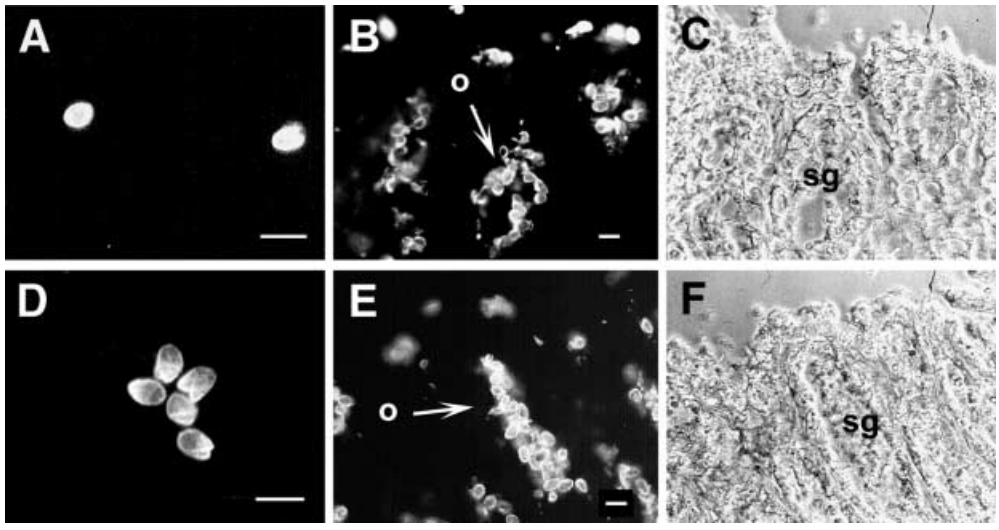

Fig. 5A–F Indirect immunofluorescence with mAb CMYL3 and CMYL30 on purified *C. muris oocysts* and on *C. muris*-infected mouse stomach. **A** CMYL3 labeled *C. muris* oocysts; **B** CMYL3 labeled *C. muris* oocysts (indicated by *o*) in mouse stomach gland; **C** phase-contrast image of **B**; **D** CMYL30 labeled *C. muris* oocysts; **E** CMYL30 labeled *C. muris* oocysts (indicated by *o*) in mouse stomach gland; **F** phase-contrast image of **E**. *Bar* 10 μm, *sg* stomach gland

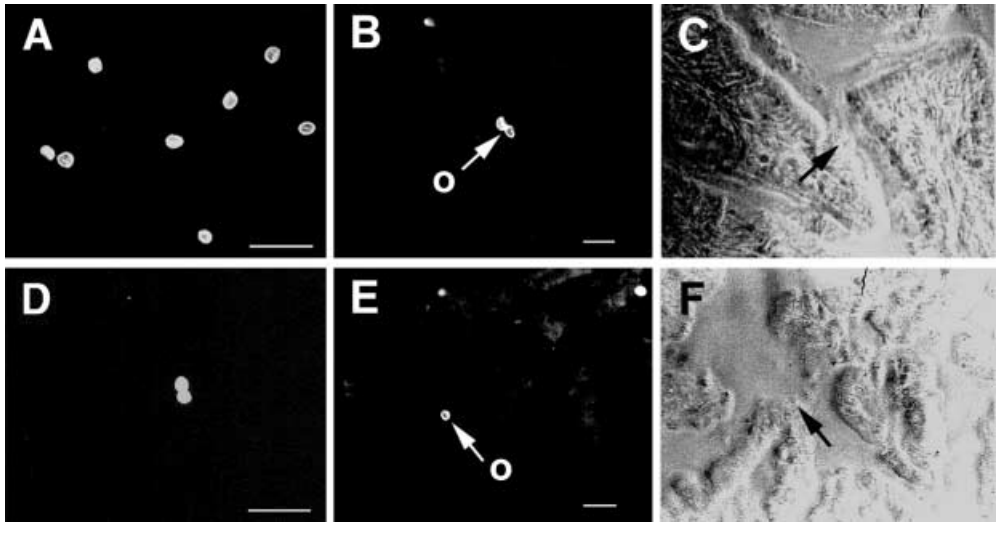

Fig. 6A–F Indirect immunofluorescence with mAb CMYL3 and CMYL30 on *C. parvum*. **A** CMYL3 labeled *C. parvum* oocysts; **B** CMYL3 labeled *C. parvum* oocysts (indicated by *o*) in small intestinal villi of mouse; **C** phase-contrast image of **B**; **D** CMYL30 labeled *C. parvum* oocysts; **E** CMYL30 labeled *C. parvum* oocysts (indicated by *o*) in small intestinal villi of mouse; **F** phase-contrast image of **E**. *Bar* 20 μm

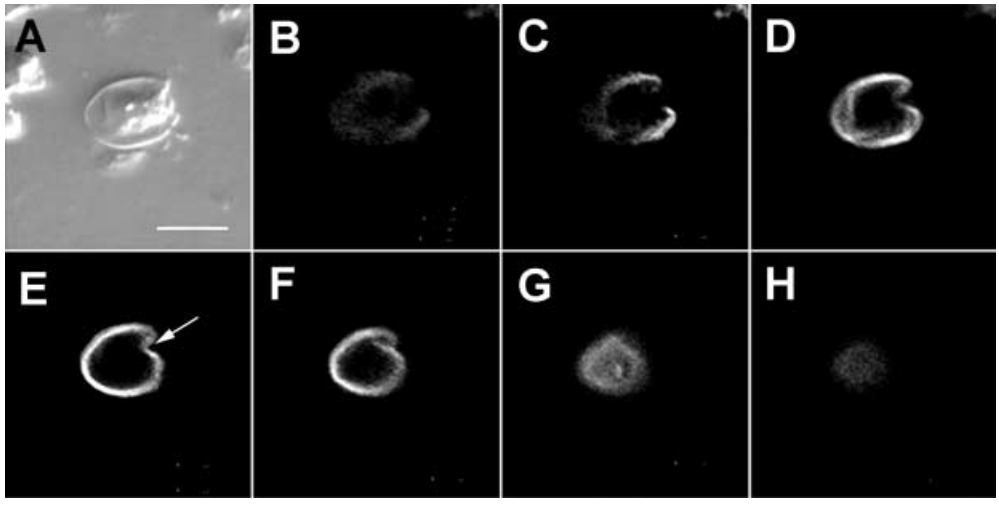

Fig. 7A–H Confocal micrographs of a *C. muris* oocyst showing the CM250 antigen localized in the oocyst wall. Purified *C. muris* oocysts after labeling with CMYL30 were observed and photographed using confocal microscope. **A** Differential interference-contrast image of a *C. muris* oocyst; **B–H** confocal micrographs depict a Z-series of the same oocyst in 0.5-μm sections. The photograph in **B** represents the bottom of the oocyst. The *arrow* in **E** indicates the infolded suture of the oocyst. *Bar* 5 μm

resembled so-called wall-forming bodies. In the *C. muris* macrogametocyte, many gold particles were also seen clustered around the amylopectin granules in the cytoplasm (arrow in Fig. 8C). No gold particles were detected at the pellicles of macrogametocytes in the infected mouse stomach and intestine (*p* in Fig. 8C, F).

CMYL antibodies cross-reacted with oocysts of all *Cryptosporidium* species tested but not with oocysts of *Giardia* and *Eimeria*

When both CMYL antibodies were used to stain a stomach mucosal smear from a *C. serpentis*-infected snake, clear cross-reactivity to the oocysts was observed (Fig. 9). Confocal microscopy revealed that the stained structures were the walls of *C. serpentis* oocysts (data not shown). It is known that *Giardia* infection in humans causes similar chronic diarrhea and produces slightly larger cysts than *Cryptosporidium* oocysts (Roberts and Janovy 1996). For diagnosis and detection of oocyst contamination in water supplies, it would be of interest to determine whether our CMYL antibodies can cross-react with *Giardia* cysts. Furthermore, it is known that *Eimeria* and *Cryptosporidium* belong to the same suborder *Eimeriina* and both can infect domestic animals (Roberts and Janovy 1996). It would also be of interest to determine whether our CMYL antibodies recognize oocysts from this protozoan. Therefore, we carried out immunofluorescence microscopy on purified cysts from *Giardia muris,* and purified oocysts from *Eimeria nieschulzi* and *E. falciformis*. There were no detectable stains on these cysts and oocysts (Fig. 10). These CMYL mAbs therefore specifically reacted with oocysts from *Cryptosporidium* only, and may be most useful in clinical diagnosis to distinguish different protozoan infections.

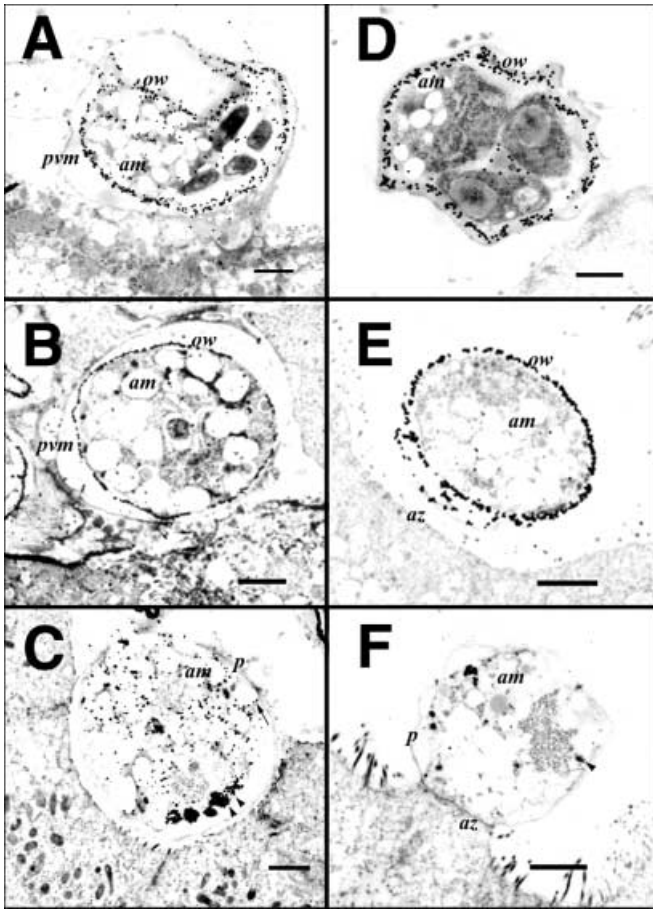

Fig. 8A–F Immunogold electron microscopy of ultrathin sections of *Cryptosporidium*-infected mouse tissues with mAb CMYL3 and CMYL30. *C. muris*-infected mouse stomach (**A–C**) and *C. parvum*-infected mouse small intestine (**D–F**) were fixed in 2% paraformaldehyde and 0.4% glutaraldehyde and embedded in LR gold. The 90-nm sections were prepared and processed for immunogold labeling. Five-nanometer gold-conjugated goat anti-mouse Ig was used as a secondary antibody. **A** Oocyst of *C. muris* labeled with CMYL3; **B** immature oocyst of *C. muris* labeled with CMYL30; numerous 5-nm gold particles are present on the oocyst wall (*ow*) but not on the parasitophorous vacuolar membrane (*pvm*). **C** Macrogametocyte of *C. muris* labeled with CMYL30. The peripheral electron-dense vesicles (*arrowheads*) are heavily labeled with gold particles. Some of the electron-lucent amylopectin granules (*am*) are also labeled with gold particles. Gold particles are also found in the cytoplasm around amylopectin granules (indicated by *arrow*). Note that the pellicle (*p*) of the macrogametocyte is not labeled. **D** Oocyst of *C. parvum* labeled with CMYL3; **E** immature oocyst of *C. parvum* labeled with CMYL30; both antibodies uniformly labeled the oocyst wall and a broader layer of gold particles can be occasionally observed at the attachment zone (*az*). **F** Macrogametocyte of *C. parvum* labeled with CMYL30. The electron-dense wall-forming bodies are labeled with gold particles (*arrowhead*), whereas the pellicle is not labeled (*p*). *Bar* 1 μm

Discussion

Many kinds of mAbs against *C. parvum* have been reported (Anusz et al. 1990; Bonnin et al. 1991, 1995; McDonald et al. 1991). However, no mAbs generated against *C. muris* have been described to date. Although most cryptosporidiosis cases reported are from *C. parvum* infections, a recent study suggested that *C. muris* could be infective in humans as well (Katsumata et al. 2000). Our mAbs against *C. muris* have the potential to be as useful as those against *C. parvum* for clinical diagnosis of cryptosporidiosis, yet could be immediately useful for the analysis of oocyst-wall formation and composition. While mAbs against sporozoite antigens could be useful for inhibition of parasite invasion, and as markers to study host–parasite interaction, mAbs against oocyst-wall antigens are valuable tools for the diagnosis of *Cryptosporidium* in fecal material and for the determination of the extent of oocyst contamination

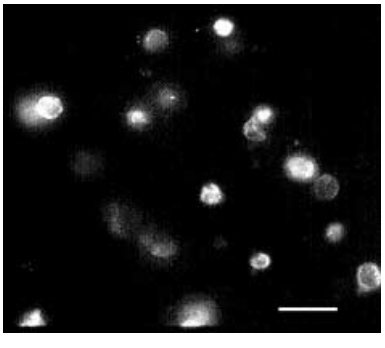

Fig. 9 Indirect immunofluorescence with mAb CMYL30 on a mucosal smear from a *C. serpentis*-infected snake stomach. *Bar* 10 μm

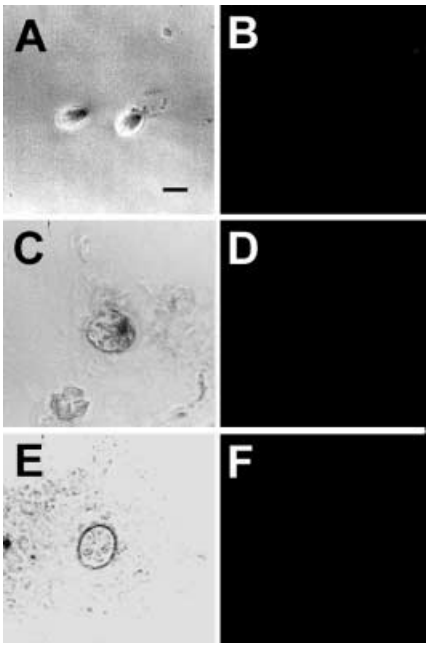

Fig. 10 Indirect immunofluorescence with mAb CMYL30 on purified cysts of *Giardia muris* (**A, B**), and purified oocysts of *Eimeria nieschulzi* (**C, D**), and *E. falciformis* (**E, F**). **A, C, E** Phase-contrast images; **B, D, F** fluorescent images in **A, C,** and **E,** respectively. *Bar* 10 μm

in water supplies (McLauchlin et al. 1987; Smith and Rose 1990; Sterling and Arrowood 1986).

In the present study, we describe two mAbs, CMYL30 (IgG1) and CMYL3 (IgM), generated against *C. muris* oocysts. Both recognize an oocyst-wall antigen with the molecular mass of 250 kDa (CM250) from both *C. muris* and *C. parvum* and also cross-react with *C. serpentis*. Occasionally, additional bands at 235, 150, 110, 92, 72, and 45 kDa in *C. parvum* can be also recognized by CMYL3. We believe that these smaller size antigens are likely derived from the proteolysis of the CM250 antigen, based on the fact that storage of the same protein sample has resulted in a shift in prominence from the 250- to the 150-kDa antigen (lane 3 of Fig. 2A). This interpretation is further supported by the detection of at least two types of proteases in sporozoites/oocysts (Nesterenko et al. 1995; Okhuysen et al. 1994), which have been proposed to play important roles in enhancing oocyst excystation during parasite infection (Forney et al. 1996).

Most oocyst-wall antigens reported so far, including CM250 in the present study, are glycosylated with the exception of an antigen recognized by the 2D7 antibody reported by Ward and Cevallos (1998). Bonnin et al. (1995) describe a mAb, OW-IGO, that also recognizes a 250 kDa antigen in *C. parvum*, as well as multiple lower molecular mass antigens. However, degradation of this 250 kDa antigen was not observed. Thus, there was no suggestion that degradation was a possible reason for the appearance of multiple bands. Furthermore, whether the OW-IGO antibody also recognizes the same antigen in *C. muris* remains unknown. The antigens recognized by OW-IGO localized to the parasitophorous vacuole membrane as well as the oocyst wall (Bonnin et al. 1991). In contrast, the CM250 recognized by CMYL3 and CMYL30 was only found in the structures originating from the parasite. These differences strongly suggest that our antibodies recognize a different wall antigen from that recognized by the OW-IGO antibody. Several mAbs against *C. parvum* oocyst antigens, with a molecular mass of approximately 40 kDa, have been previously reported. Anusz et al. (1990) described mAb 18.280.2, which recognized a 40-kDa molecule located in the exterior surface of *C. parvum* oocysts. Additionally, mAb 181B5 reacted with 41- and 44-kDa molecules of *C. parvum* (McDonald et al. 1991; Nina et al. 1992). MAb OW-IGO also recognized a 40-kDa antigen of the *C. parvum* oocyst wall (Bonnin et al. 1991). Nina et al. (1992) suggested that the 40-kDa antigen of *C. parvum* described by Anusz et al. (1990) may correspond to the 41-kDa band recognized by mAb 181B5. Furthermore, these two mAbs, 18.280.2 and 181B5, exhibited cross-reactivity to the *C. muris* oocyst wall. Thus, it appears that more than one type of glycoprotein exists on the *Cryptosporidium* oocyst wall. This is in contrast to the composition of *Eimeria* oocyst walls, in which only one glycoprotein has been described (Karim et al. 1996; Stotish et al. 1978). The purpose and function of these oocyst antigens are not known and should be evaluated further.

The origin of oocyst-wall proteins is not completely understood, although several papers have attempted to

Table 1 Characteristics of monoclonal antibodies produced against *Cryptosporidium muris*. – Negative, *n.d.* not done

	CMYL3 (IgM)			CMYL30 (IgG1)		
	C. muris	*C. parvum*	*C. serpentis*	*C. muris*	*C. parvum*	*C. serpentis*
Immunofluorescence	Oocyst wall	Oocyst wall	Oocyst wall	Oocyst wall	Oocyst wall	Oocyst wall
Immunogold						
Oocyst	Wall	Wall	n.d.	Wall	Wall	n.d.
Macrogametocyte	Vesicles and cytoplasm	Vesicles and cytoplasm	n.d.	Vesicles and cytoplasm	Vesicles and cytoplasm	n.d.
Meront	–	–	n.d.	–	–	n.d.
Sporozoite	–	–	n.d.	–	–	n.d.
Antigen size[a]	250 kDa	250 kDa	n.d.	250 kDa	250 kDa	n.d.

[a]As determined by Western blot analysis

address this question. Current and Reese (1986) reported that the *Cryptosporidium* wall is derived from two types of electron-dense (i.e., loose and tight electron-dense) wall-forming bodies present in the macrogametocytes. On the other hand, Bonnin et al. (1991) suggested that oocyst-wall antigens of *C. parvum*, recognized by mAb OW-IGO, are released from the macrogametocyte electron-lucent vesicles into the vacuolar space and incorporated into the oocyst wall. In the present study, the CM250 antigens recognized by CMYL3 and CMYL30 were detected in electron-dense vesicles in the cytoplasm around electron-lucent amylopectin granules of macrogametocytes. The electron-dense vesicles heavily labeled by these mAbs were located along the periphery of the macrogametocyte cytoplasm. Thus, there is high possibility that the component of the oocyst wall originates from these electron-dense vesicles of the macrogametocyte.

MAbs CMYL3 and CMYL30 against *C. muris* oocysts show cross-reactivity with the oocyst wall of *C. parvum* as well as *C. serpentis* but do not cross-react with *Eimeria* oocysts, even though both *Eimeria* and *Cryptosporidium* belong to the same suborder. Thus, the antigen CM250 is *Cryptosporidium* oocyst-specific. Cysts from *Giardia* are known to cause similar diarrhea symptoms in humans as cryptosporidiosis (Roberts and Janovy 1996). The present study also showed that our CMYL antibodies did not recognize *Giardia* cysts. In addition, these mAbs work well on indirect immunofluorescence, ELISA, and Western blot analysis. Table 1 summarizes such analyses. These antibodies, therefore, could be very useful for the diagnostic detection of *Cryptosporidium* oocysts from various sources and for the biological study of the oocyst wall of *Cryptosporidium*.

Acknowledgements We would like to thank Dr. Donald W. Duszynski for the generous gift of the oocysts of *Eimeria falciformis* and *E. nieschulzi*, and Dr. Stanley L. Erlandsen for the cysts of *Giardia muris*. We also thank Rebecca Reiter for critical reading of the manuscript. This study was partly supported by grant no. 2000-1-20200-003-1 from the Basic Research Program of the Korean Science and Engineering Foundation (to J.R.Y.) and by grant HD18577 from the National Institutes of Health (to J.J.C.L.). The experiments comply with the current laws of the United States in which the experiments were performed.

References

Anusz KZ, Manson PH, Riggs MW, Perryman LE (1990) Detection of *Cryptosporidium parvum* oocysts in bovine feces by monoclonal antibody capture enzyme-linked immunosorbent assay. J Clin Microbiol 28:2770–2774

Bonnin A, Dubremetz JF, Camerlynck P (1991) Characterization and immunolocalization of an oocyst wall antigen of *Cryptosporidium parvum* (Protozoa: Apicomplexa). Parasitology 103:171–177

Bonnin A, Gut J, Dubremetz JF, Nelson RG, Camerlynck P (1995) Monoclonal antibodies identify a subset of dense granules in *Cryptosporidium parvum* zoites and gamonts. J Eukaryot Microbiol 42:395–401

Casemore DP, Annstrong M, Sands RL (1985) Laboratory diagnosis of Cryptosporidiosis. J Clin Pathol 38:1337–1341

Current WL, Reese NC (1986) A comparison of endogenous development of three isolates of *Cryptosporidium* in suckling mice. J Protozool 33:98–108

Ditrich O, Palkovie L, Sterba J, Prokopic J, Loudova J, Giboda M (1991) The first finding of *Cryptosporidium baileyi* in man. Parasitol Res 77:44–47

Fayer R, Speer C, Dubey J (1997) The general biology of *Cryptosporidium*. In: Fayer R (ed) *Cryptosporidium* and cryptosporidiosis. CRC Press, Boca Raton, pp 1–41

Forney JR, Yang S, Healey MC (1996) Protease activity associated with excystation of *Cryptosporidium parvum* oocysts. J Parasitol 82:889–892

Harris JR, Petry F (1999) *Cryptosporidium parvum*: structural components of oocyst wall. J Parasitol 85:839–849

Karim MJ, Basak SC, Trees AJ (1996) Characterization and immunoprotective properties of a monoclonal antibody against the major oocyst wall protein of *Eimeria tenella*. Infect Immun 64:1227–1232

Katsumata T, Hosea D, Ranuh IG, Uga S, Yanagi T, Kohno S (2000) Short report: possible *Cryptosporidium muris* infection in humans. Am J Trop Med Hyg 62:70–72

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Langer R, Riggs M (1996) Neutralizing monoclonal antibody protects against *Cryptosporidium parvum* infection by inhibiting sporozoite attachment and invasion. J Eukaryot Microbiol 43:76S–77S

Lin JJ-C, Chou CS, Lin JL-C (1985) Monoclonal antibodies against chicken tropomyosin isoforms: Production, characterization, and application. Hybridoma 4:223–241

McDonald V, Deer RMA, Nina JM, Wright S, Chiodini PL, McAdam KPWJ (1991) Characteristics and specificity of hybridoma antibodies against oocyst antigens of *Cryptosporidium parvum* from man. Parasitol Immunol 13:251–259

McLauchlin J, Casemore DP, Harrison TG, Gerson PJ, Samuel D, Taylor AG (1987) Identification of *Cryptosporidium* oocysts by monoclonal antibody (letter). Lancet 1(8523):51

Nesterenko MV, Tiley M, Upton SJ (1995) A metallo-dependent cysteine protease of *Cryptosporidium parvum* associated with the surface of sporozoites. Microbios 83:77–88

Nina JMS, McDonald V, Dyson DA, Catchpole J, Uni S, Iseki M, Chiodini PL, McAdam KPWJ (1992) Analysis of oocyst wall and sporozoite antigens from three *Cryptosporidium* species. Infect Immun 60:1509–1513

O'Donoghue PJ (1995) *Cryptosporidium* and cryptosporidiosis in man and animals. Int J Parasitol 25:139–195

Okhuysen PC, DuPont HL, Sterling CR, Chappell CL (1994) Arginine aminopeptidase, an integral membrane protein of the *Cryptosporidium parvum* sporozoite. Infect Immun 62:4667–4670

Perryman LE, Riggs MW, Mason PH, Fayer R (1990) Kinetics of *Cryptosporidium parvum* sporozoite neutralization by monoclonal antibodies, immune bovine serum, and immune bovine colostrum. Infect Immun 58:257–259

Petry F, Robinson HA, McDonald V (1995) Murine infection model for maintenance and amplification of *Cryptosporidium parvum* oocysts. J Clin Microbiol 33:1922–1924

Roberts LS, Janovy JJ (1996) Gerald D. Schmidt & Larry S. Roberts' Foundations of parasitology, 5th edn. Brown, Dubuque, Iowa

Smith HV, Rose JB (1990) Waterborne cryptosporidiosis. Parasitol Today 6:8

Sterling CR, Arrowood MJ (1986) Detection of *Cryptosporidium* sp. infections using a direct immunofluorescent assay. Pediatr Infect Dis 5:S139–142

Stotish RL, Wang CC, Meyenhofer M (1978) Structure and composition of the oocyst wall of *Eimeria tenella*. J Parasitol 64:1074–1081

Taylor MA, Marshall RN, Green JA, Catchpole J (1999) The pathogenesis of experimental infections of *Cryptosporidium muris* (strain RN66) in outbred nude mice. Vet Parasitol 86:41–48

Tijssen P (1985) Practice and theory of enzyme immunoassays. In: Burdon RH, van Knippenberg RH (eds) Laboratory techniques in biochemistry and molecular biology, vol 15. Elsevier, Amsterdam, pp 361–365

Ward H, Cevallos AM (1998) *Cryptosporidium*: molecular basis of host–parasite interaction. Adv Parasitol 40:152–187

Woodward MP, Young JWW, Bloodgood RA (1985) Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J Immunol Methods 78:143–153

Xiao L, Morgan UM, Limor J, Escalante A, Arrowood M, Shulaw W, Thompson RC, Fayer R, Lal AA (1999) Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. Appl Environ Microbiol 65:3386–3391

Yu JR (1998) Distribution of actin and tropomyosin in *Cryptosporidium muris*. Korean J Parasitol 36:227–234

Yu JR, Chai JY (1995) Localization of actin and myosin in *Cryptosporidium parvum* using immunogold staining. Korean J Parasitol 33:155–164