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Alteration of *Cryptosporidium parvum* (Apicomplexa: Eucoccidiorida) Oocyst Antigens Following Bleach Treatment

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Summary. Oocysts of the protozoan parasite *Cryptosporidium parvum* are passed in infected feces and subsequently ingested by susceptible hosts, thus perpetuating transmission of the infection in the natural environment. Detection of oocysts is important to the water industry, especially in treatment plants using sanitizing and disinfecting chemicals. Commercial bleach containing sodium hypochlorite is frequently used by researchers to decontaminate the surface of oocysts prior to inoculating cell cultures. The present study analyzed oocyst protein patterns and antigen profiles before and after bleach treatment. Oocysts isolated from mouse feces were treated with a 20% bleach solution at 4°C for 30 min. Treated and non-treated oocysts were frozen, thawed, and sonicated to produce a *C. parvum*-oocyst homogenate (CPOH), which was subjected to gel electrophoresis and immunoblotting. Coomassie blue-stained electrophoretic gels revealed 33 and 15 protein bands from non-treated and treated CPOH, respectively. On Western blotting, 15 protein bands from non-treated CPOH were identified by polyclonal antibodies (hyperimmune mouse serum), but only 10 bands could be observed following bleach treatment. Monoclonal antibodies (Mabs) 6B4 and 9D10, specific for epitopes on the intact oocyst wall, revealed different immunoblotting patterns before and after bleach treatment. Prior to treatment, Mab 6B4 reacted with 2 protein bands with molecular weights of 55 and 246 kD, while Mab 9D10 reacted with 2 bands with molecular weights of 168 and 230 kD. Following bleach treatment, both the 55 and 246 kD bands (Mab 6B4) were still visible, but bands at 168 and 230 kD (Mab 9D10) were not. The utility of polyclonal and monoclonal antibodies in detecting oocysts from different sources will depend upon the chemical sensitivities of their target epitopes.

Key words: antigens, bleach, cryptosporidiosis, *Cryptosporidium parvum*, epitopes, monoclonal antibodies, oocysts.

Abbreviations: CPOH - *C. parvum*-oocyst homogenate, FITC - fluroecsein isothiocyanate, HMS - hyperimmune mouse sera, IFA - indirect immunofluorescent assay, Mab(s) - monoclonal antibody(ies), PBS - phosphate buffered saline, PVDF - polyvinylidene difluoride, SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

Cryptosporidium parvum, a protozoan parasite having worldwide distribution, is an enteric pathogen causing diarrhea in humans and a variety of other animals. Despite some recent progress (Riggs *et al.* 1999), consistently effective therapy against this parasite remains an enigma. The high rate of infection among immunocompromised humans and young animals, in large part, is due to the tenacious resistance of oocysts in the natural environment (Campbell *et al.* 1982, Madore *et al.* 1987, Peeters *et al.* 1989, Korich *et al.* 1990). Consequently, oocysts and their associated antigens remains the subject of scientific investigations.

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Antigenic profiles of *C. parvum* oocysts have been summarized (Lumb *et al.* 1988, Current and Garcia 1991). The water industry relies heavily on treatment plants to make drinking water safe. Moreover, chemical treatment is routinely used to decontaminate the oocyst surface prior to cell culture inoculation in scientific investigations (Yang *et al.* 1996, Upton 1997, Zhang *et al.* 1998). Many standard disinfection procedures do not necessarily reduce *C. parvum* oocyst viability, but frequently alter oocyst wall antigens (Campbell *et al.* 1982, Korich *et al.* 1990). Consequently, any knowledge of antigen liability to oxidizing conditions and chemical disinfectants can be very useful.

Commercial bleach (Clorox®) containing sodium hypochlorite is the most common disinfectant used to decontaminate the surface of *C. parvum* oocysts. The present study analyzed oocyst protein patterns and antigen profiles before and after bleach treatment. Results indicated that certain oocyst antigens and epitopes were sensitive to 20% bleach treatment for 30 min while others were not. Therefore, polyclonal and Mab probes used to detect oocysts should be selected on the basis of their target epitopes and the sensitivity of these epitopes to known chemical exposure.

MATERIALS AND METHODS

Oocyst amplification and purification

Cryptosporidium parvum oocysts, originally isolated from Holstein calves (Iowa isolate), were amplified by using C57BL/6N mice (B&K Universal, Fremont, CA, USA) immunosuppressed with dexamethasone as described by Yang and Healey (1993). The mice (4 ~ 6 wk old) were inoculated *per os* with 10^6 oocysts/mouse, housed in wire-floored cages, and allowed to consume drinking water *ad libitum* containing 14 µg/ml dexamethasone phosphate throughout the experiment. Mouse feces were collected daily, beginning on day 3 postinoculation, from steel trays underlying the cage floor. The fecal pellets were collected in 2.5% potassium dichromate ($K_2Cr_2O_7$) solution and stored at 4°C prior to oocyst purification.

A salt flotation technique (Petry *et al.* 1995) and the cesium chloride gradient centrifugation technique (Kilani and Sekla 1987) were combined to purify the oocysts from feces. Briefly, fecal pellets were crushed and sieved sequentially through 3 screens with a final mesh of 400 (38 µm porosities), and then suspended in 20 volumes of water to sediment for 45 min. The supernatant was decanted and the sediment resuspended. This procedure was repeated 3 times. All the decanted supernatants were centrifuged at 1900 g for 10 min (unless otherwise specified all centrifugations were at 1900 g for 10 min). The oocyst-rich pellets were combined and suspended in 10 volumes of saturated NaCl solution with 1 volume of water overlaid and then centrifuged. Following centrifugation, the oocyst-containing interphase was removed and washed once with phosphate buffered saline (PBS, 25 mM, pH 7.2). The oocysts were washed and then diluted with 50 mM Tris-10 mM EDTA buffer to a suspension of 1 x 10^8 oocysts/ml. A CsCl gradient was prepared in 14 x 89 mm Ultra-Clear™ tubes (Beckman Instruments, Inc., Palo Alto, CA, USA). Gradient densities were 1.40, 1.10, and 1.05 g/ml in the bottom, middle, and top 3 ml, respectively. One ml of oocyst suspension was layered on the top of the gradient. Gradient tubes were ultra-centrifuged at 16000 g for 1 h at 4°C. Purified oocysts were harvested from the top band, washed with deionized water by centrifugation, resuspended in the desired amount of PBS or lysis buffer (formula provided below), and stored at -20°C.

Oocyst treatment and homogenate preparation

Approximately 2.5 x 10^9 purified oocysts in a 50 ml conical tube were centrifuged to produce an oocyst pellet. The supernatant was removed by aspiration. A commercial bleach preparation (Clorox®) containing 5.25% sodium hypochlorite was diluted with distilled deionized water (ddH_2O) to make a 20% working solution of 1.05% sodium hypochlorite. Ten ml of this solution was poured onto the oocyst pellet and vortexed. The tube was then incubated for 30 min at 4°C with limited agitation every 5 min. Oocysts were washed 4 times by centrifugation with PBS to remove residual bleach.

Bleach and non-bleach-treated oocysts were each suspended in a lysis buffer to a concentration of approximately 9.0 x 10^8 oocysts/ml. The lysis buffer consisted of 25 mM PBS, 50 mM Tris, 5 mM EDTA, 1% (w/v) octyl glucoside, 10 µg/ml α_1-antitrypsin, 100 µg/ml aprotinin, 0.5 mM diisopropylfluorophosphate, 5 mM iodoacetamide, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Nα-tosyl-L-lysylchoromethyl ketone, and 1 mM L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane (Luft *et al.* 1987, Riggs *et al.* 1997). The oocyst suspension was snap-frozen in liquid nitrogen and thawed in a 37°C water bath for 12 cycles. It was then sonicated on ice with a Virsonic 50 Cell Disrupter (VirTis Company, Gardiner, NY, USA) for 20 s/sonication x 15 sonications at 50% power output with 1-min intervals. Samples of the resultant *C. parvum*-oocyst homogenate (CPOH) were examined by indirect IFA to ensure that more than 95% of the oocysts were disrupted. The CPOH was then divided into 200 µl aliquots and stored at -80°C until used.

Antibody production and screening

Murine polyclonal antibodies. Hyperimmune mouse serum (HMS) against *C. parvum* was produced in 7 adult BALB/c mice. Each mouse received 5 intramuscular injections of CPOH in Freund's incomplete adjuvant at monthly intervals. Each injection contained about 5x10^6 oocysts in a 50µl suspension. Five days after the last injection, blood was collected from the orbital sinus. Antibody titers against CPOH were measured by the enzyme-linked immunosorbent assay (ELISA) reported by Forney *et al.* (1996). Serum samples were pooled and stored at -20°C.

Murine monoclonal antibodies: Splenic lymphocytes harvested from CPOH-hyperimmunized BALB/c mice were fused with SP2/0 myeloma cells in a 50% polyethylene glycol solution. The presence of Mabs produced by resultant hybridomas was confirmed by ELISA as before. Hybridomas determined to be positive were cloned by limiting dilution, isotyped with a sub-isotyping kit (HyClone® Laboratories, Inc., Logan, UT, USA), and cryopreserved in liquid nitrogen.

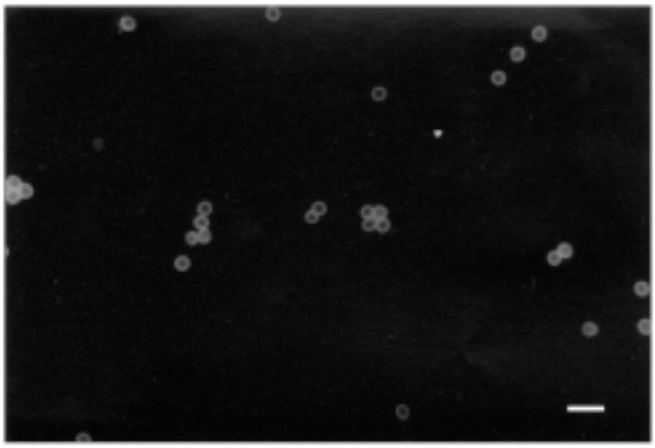

Fig. 1. Bleach-treated *Cryptosporidium parvum* oocysts examined by an indirect immunofluorescent assay using Mab 6B4. Oocysts appear brightly stained with hollow centers. Scale bar - 12 μm

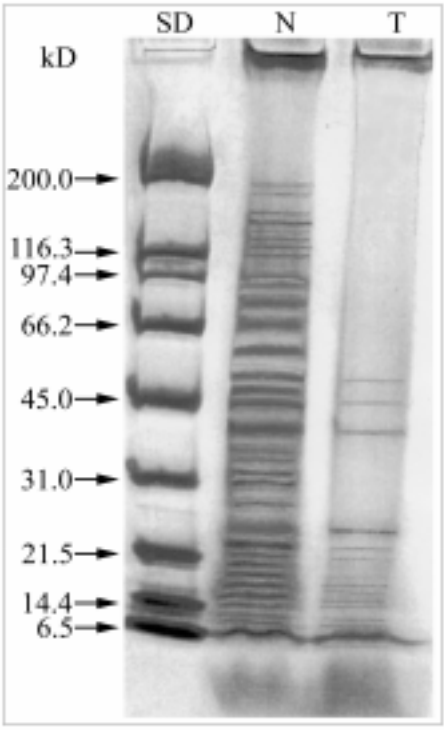

Fig. 2. Profile of *Cryptosporidium parvum*-oocyst homogenate (CPOH) proteins resolved by SDS-PAGE and stained by GelCode® blue reagent. Molecular weight standards are shown in lane SD and labeled in kilodaltons (kD) in the left-hand margin. Non-bleach-treated CPOH demonstrate at least 33 protein bands, with molecular weights ranging from 6.5 to 187 kD (Lane N). Beach-treated CPOH reduced the number of protein bands to 15, with molecular weights ranging from 6.5 to 55 kD (Lane T)

Indirect immunofluorescent assay (IFA)

A modified Mab-based IFA (Garcia *et al.* 1987, Arrowood and Sterling 1989) was used to examine oocysts and intermediate stages of *C. parvum* by employing different Mabs generated in our laboratory. Individual samples (either oocysts in feces, purified oocysts, or CPOH) were applied to the surface of pre-cleaned microscope slides, air-dried at 25°C for 30 min, and fixed by gentle flaming. Samples were incubated with one of the candidate Mabs (60 μl/application) for 30 min in a 100% humidified chamber at 37°C, followed by an additional 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Sigma Chemical Company, St. Louis, MO, USA) diluted at 1:100 (50 μl/application). Following incubation, samples were thoroughly washed with PBS, covered with 2 drops of non-drying mounting medium (50% glycerol in PBS), and examined under a Zeiss epifluorescence microscope equipped with an ultraviolet lamp. To evaluate Mabs against intermediate parasitic stages, *C. parvum*-infected cell cultures growing on coverslips were subjected to the same procedure as described above for oocysts.

Dot blot assay

Mabs and HMS reacting with respective native and denatured CPOH were discerned by a dot-blot microfiltration apparatus in accordance with the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA, USA). Denaturation of CPOH was done by the same procedure as described for SDS-PAGE (below). The intensity of reaction was scored as: - = negative, 1+ = light, 2+ = moderate, 3+ = strong, and 4+ = very strong. Mabs for use in Western blot analysis were selected by the results of this assay.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A commercially available 4 ~ 20% gradient gel and Mini-PROTEAN® II dual slab cell (Bio-Rad Laboratories) were employed in SDS-PAGE. One part of solubilized CPOH was diluted with 2 parts (v/v) of sample buffer [2% SDS, 5% (v/v) β-mercaptoethanol, 0.0125% bromophenol blue, and 0.25% (v/v) glycerol in 62.5 mM Tris-HCl, pH 6.8] and boiled for 5 min before loading 30 μl into each gel lane. Prestained and non-prestained molecular weight standards (broad range, Bio-Rad Laboratories) were incorporated into the control gel lanes to determine the relative molecular weights of the resolved proteins. Electrophoresis was performed with a discontinuous buffer system (Laemmli 1970) at 175 V until the dye front reached the bottom of the slab gel (approximately 50 min). Following electrophoresis, GelCode® blue stain reagent (Pierce Chemical Company, Rockford, IL, USA), which employs the colloidal properties of Coomassie® blue G-250 for protein staining, was used to visualize CPOH protein bands in the slab gels.

Western blot analysis

Following SDS-PAGE, CPOH protein bands were electrophoretically transferred (Towbin *et al.* 1979) to the polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot® Transfer Cell (Bio-Rad). Transfer was carried out overnight at 4°C at 30 V, followed by 60 V for 2 h. Transfer efficiency was monitored by observing the prestained molecular weight standards. Following transfer, the membrane sheets were rinsed with ddH_2O and dried with a cold air stream. Nonspecific sites on the sheets were blocked for 18 h with a solution

Table 1. Dot-blot assay scores for polyclonal and monoclonal antibodies against native and denatured *Cryptosporidium parvum*-oocyst homogenate (CPOH)

Antibody[1]	HMS	9D10	2C5	2F8	3F11	9G12	2B7	2C12	8B1	D8	6B4	8C6	9F11
Isotype[2]	ND[3]	IgM	ND	IgM	IgA	ND	IgG2b	IgA	ND	ND	IgM	ND	ND
Native[4]	4+	3+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	ND
Denatured[5]	4+	2+	3+	1+	1+	1+	1+	1+	1+	1+	2+	2+	1+

[1] Polyclonal (HMS - hyperimmune mouse serum) and monoclonal antibody designations; [2]Isotyping was done on only select Mabs; [3]ND - not done; [4]Native - score for non-denatured CPOH; [5]Denatured - score for denatured CPOH.

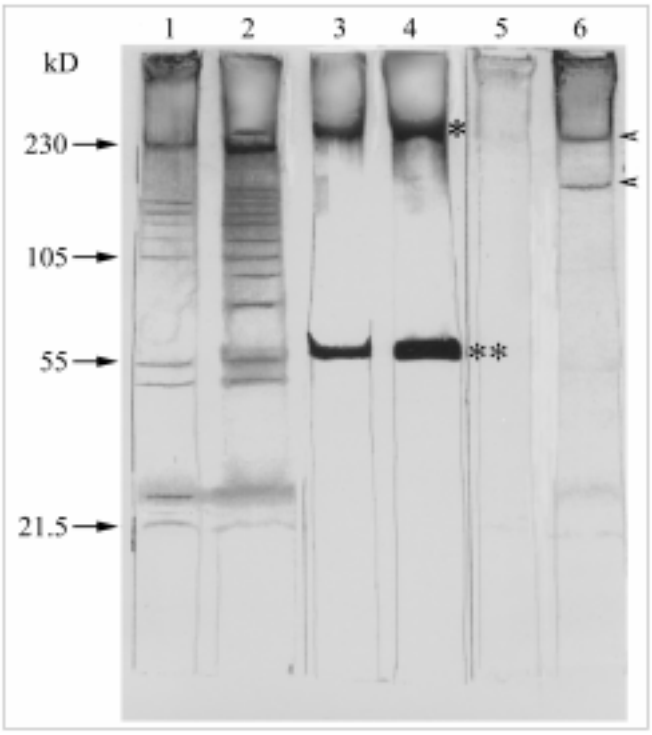

Fig. 3. Western blot analysis of bleach and non-bleach-treated *Cryptosporidium parvum*-oocyst homogenate (CPOH) using HMS and Mabs (6B4 and 9D10). Prior to bleach treatment, HMS recognized 15 protein bands with molecular weights ranging from 21.5 to 246 kD (Lane 2). Following bleach treatment, 10 protein bands remain with molecular weights ranging from 21.5 to 230 kD (Lane 1). Prior to and following bleach treatment, Mab 6B4 recognized 2 protein bands having molecular weights of 55 kD (**) and 246 kD (*) (Lanes 4 and 3, respectively). Similarly, Mab 9D10 recognized two protein bands before bleach treatment. The respective molecular weights are 168 kD and 230 kD (arrowheads) (Lane 6). Following bleach treatment, Mab 9D10 failed to recognize either band (Lane 5)

of 5% bovine serum albumin in 0.05 M Tris Buffered Saline (BSA-TBS, pH 7.4). The blocked sheet was cut into strips and incubated with either HMS (1:3000) or a Mab for 2 h, washed with PBS + 0.05% Tween 20 (PBS-T), and incubated for 1.5 h with biotinylated anti-mouse IgG goat serum (Sigma) diluted at 1:1000 in 1% BSA-TBS. The strips were further reacted with ExtrAvidin-Alkaline Phosphatase (Sigma) for 1.5 h after washing with PBS-T. Color development was accomplished by using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad) and stopped by washing with ddH_2O.

RESULTS

Antibody screening

ELISA showed that more than 70 hybridomas producing Mabs against *C. parvum* were generated. IFA determined that some Mabs (6B4 and 9D10) were against epitopes present on the oocyst stage (Fig. 1), while others (such as 2C5) were against epitopes on intermediate parasitic stages (data not shown).

Bleach treatment on oocysts

Prior to bleach treatment, oocysts reacted strongly with Mabs 6B4 and 9D10 as determined by IFA. Oocysts appeared brightly stained with hollow centers (Fig. 1). Following bleach treatment, oocysts lost their reaction with Mab 9D10, but retained their reaction with Mab 6B4 showing bright apple green fluorescence.

Dot blotting

Dot blot assay revealed that HMS had very strong reactions with both native and denatured CPOH (Table 1). The optimal concentration of HMS used in Western blotting was 1:3000, as determined by serial-dilution in dot blotting. Almost all Mabs reacted with native (non-denatured) CPOH antigens, with reaction intensity varying from 1+ to 4+. Following CPOH denaturation, only 12 Mabs continued to react with CPOH antigens (Table 1). Based on reaction intensity (2+ ~ 3+), 4 Mabs (6B4, 9D10, 2C5, and 8C6) were selected for further analysis in Western blotting.

SDS-PAGE of CPOH

The profile of CPOH proteins resolved by SDS-PAGE and stained by GelCode® blue reagent is shown in Fig. 2. Non-bleach-treated CPOH demonstrated at least 33 protein bands (Lane N). The molecular weights of these proteins ranged from 6.5 kD to 187 kD. Proteins with molecular weights below 6.5 kD

or over 200 kD could not be clearly discerned. Bleach treatment of oocysts reduced the number of protein bands to 15, with molecular weights ranging from 6.5 kD to 55 kD (Lane T).

Western blotting

The majority of CPOH protein bands in the gradient gels were transferred to PVDF membranes as noted by the disappearance of prestained standards in the gels and also by light staining of gels with GelCode® blue reagent following transfer. Western blot analysis of bleach- and non-bleach-treated CPOH using HMS and Mabs (6B4 and 9D10) are illustrated in Fig. 3. Prior to bleach treatment, HMS recognized 15 protein bands with molecular weights ranging from 21.5 kD to 246 kD (Lane 2). Following bleach treatment, 10 protein bands remained with molecular weights ranging from 21.5 kD to 230 kD (Lane 1). No bands were observed when normal mouse serum was used (data not shown).

Prior to and following bleach treatment of oocysts, Mab 6B4 recognized 2 protein bands from CPOH having molecular weights of 55 kD and 246 kD (Lanes 4 and 3, respectively). The band having the lowest molecular weight (55 kD) appeared much more intense (Lanes 4 and 3, respectively). Similarly, Mab 9D10 recognized two protein bands before bleach treatment. The respective molecular weights were 168 kD and 230 kD (Lane 6). However, following bleach treatment, Mab 9D10 failed to recognize either band (Lane 5). No protein bands were visible following incubation with Mab 2C5, Mab 8C6, and cell culture medium (negative control) (data not shown).

DISCUSSION

Cryptosporidium parvum is an undisputed waterborne pathogen of humans and other animals. This intestinal protozoan has been responsible for outbreaks of cryptosporidiosis traced back to surface and potable water sources (MacKenzie *et al.* 1994, Wallis *et al.* 1996). Because low numbers of oocysts present a potential risk for infection, the challenge has been to develop immunofluorescent and flow cytometric assays capable of detecting only a few oocysts (Vesey and Slade 1990, Vesey *et al.* 1993). A confounding factor is the effect that water disinfectants may have on oocyst detection. It has been reported that oocyst viability or infectivity is not affected by exposure of oocysts to 1.05 ~ 3% sodium hypochlorite (equivalent to 20 ~ 60% commercial bleach) for up to 18 h at 4 or 37°C, and only 70 ~ 100% commercial bleach can destroy oocyst infectivity (Campbell *et al.* 1982, Korich *et al.* 1990, Sterling 1990). Our results regarding the alteration of oocyst antigens following bleach treatment not only support the notion that the routine chlorination of drinking and recreation water has little or no effect on oocyst viability (Madore *et al.* 1987, Peeters *et al.* 1989, Current and Garcia 1991, Moore *et al.* 1998), but also suggest that routine chlorination may lead to a false-negative detection if an improper antibody is used in the assay. Moore *et al.* (1998) reported that 4 commercially available antibodies recognized a similar set of immunodominant epitopes on the oocyst wall. Unfortunately, these epitopes were labile to chlorine treatment and oxidizing conditions. Specifically, sodium hypochlorite and sodium meta-periodate reduced the ability of antibodies to detect the oocysts. This problem could have been circumvented by using *Cryptosporidium*-specific antibodies that recognize non-labile antigens on the oocyst wall. We report herein the generation of a panel of Mabs, the relative molecular weights of their target antigens, and the ability of at least one Mab to react with an epitope on the oocyst wall before and after treatment with 20% commercial bleach (1.05% sodium hypochlorite) for 30 min.

From 17 to 51 protein bands have been identified in electrophoretic profiles of freeze-thawed *C. parvum* oocysts, with molecular weights ranging from less than 6.5 to larger than 330 kD (Lazo *et al.* 1986, Luft *et al.* 1987, Lumb *et al.* 1988, Tilley and Upton 1990, Bonnin *et al.* 1991, Nina *et al.* 1992, Peeters *et al.* 1992). We demonstrated at least 33 protein bands in the electrophoretic analysis of non-bleach-treated CPOH, with molecular weights ranging from 6.5 to 187 kD (Fig. 2). Nine additional weak bands appeared in Western blotting, extending the band number to 42 and the molecular weight to 246 kD (Fig. 3). Our findings are, for the most part, in agreement with those reported by investigators cited above. Slight differences are likely due to the varying percentage of acrylamide gels used and the staining methods employed. In addition, the number of protein bands counted can be somewhat arbitrary. We enumerated protein bands by linear and exponential regression analysis. Bleach treatment reduced the number of protein bands in CPOH from 33 to 15, underscoring the effect that the sodium hypochlorite treatment has on oocyst wall proteins (Fig. 2).

Luft *et al.* (1987) demonstrated that antisera from orally infected mice consistently recognized 4 oocyst

antigens with molecular weights ranging from 72 kD to greater than 100 kD. Current and Garcia (1991) reported several Mabs specific for *C. parvum* oocyst/sporozoite antigens, with molecular weights ranging from approximately 48 kD (Nina *et al.* 1992) to more than 200 kD (Arrowood and Sterling 1989, Bonnin *et al.* 1991). However, the sensitivity of these antigens to bleach treatment was not evaluated. In the present study, HMS recognized 15 CPOH protein bands before and 10 bands after oocysts were treated with bleach, again signaling protein sensitivity to sodium hypochlorite exposure (Fig. 3). Not surprisingly, epitopes recognized by Mabs produced to CPOH showed variable susceptibility to bleach. Whereas Mabs 9D10, 2C5, and 8C6 reacted with labile epitopes, the epitope recognized by Mab 6B4 was clearly not sensitive to bleach treatment (Fig. 3).

Moore *et al.* (1998) predicted that the development of antibodies targeted to antigens that were not sensitive to sodium hypochlorite treatment was unlikely. Although we agree that commercially available antibodies specific for *Cryptosporidium* antigens either fail to react or react very weakly with oocysts following sodium hypochlorite treatment, Mab 6B4 from our panel recognizes an epitope that resists exposure to 20% bleach for at least 30 min, while Mab 9D10 recognizes an epitope that does not resist such an exposure. This is particularly relevant for investigators studying the life cycle of *C. parvum* in vitro because there is a measured advantage in discriminating between oocysts used to inoculate cell culture (decontaminated with bleach) and oocysts produced in cell culture as the parasite completes its life cycle (not exposed to bleach) (Healey *et al.* 1997, Upton 1997). Moreover, the water industry has a dedicated interest in antibodies capable of detecting *C. parvum* oocysts that have been exposed to various disinfection procedures (Campbell *et al.* 1982). Such procedures frequently reduce oocyst detection by antigenic alteration, but rarely reduce oocyst viability (Korich *et al.* 1990).

The importance of *C. parvum* oocyst antigenic liability to disinfectants, sanitizing treatments, and naturally occurring oxidizing conditions is uncontested from the vantage point of antibody-mediated oocyst detection. The next step will be to develop Mabs capable of specifically identifying *C. parvum* in samples containing different species of *Cryptosporidium*.

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