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Diagnosis of bovine cryptosporidiosis by indirect immunofluorescence assay using monoclonal antibody

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Abstract: Two hybridoma cell lines against *Cryptosporidium parvum* oocysts (VRI-CN91) were produced. The isotype of these 2 monoclonal antibodies (mAbs) was IgG2b (1E7.2) and and IgM (C6). Enzyme immuno-transfer blotting analysis showed that 1E7.2 reacted specifically to 36 kDa protein and C6 reacted to 67 and 70 kDa proteins. *C. parvum* was bound specifically to the surface region of oocysts by these mAbs. No cross-reactivity was observed with tachyzoites of *Toxoplasma gondii* and oocysts of *Eimeria zuernii, E. bovis* and *E. canadensis* of bovine origin. The indirect immunofluorescence assay (IIF) using mAb C6 was successful with counterstain. With the IIF using mAb C6, oocysts appeared as 3 to 5 μm spherical objects fluorescing bright apple green against a reddish dark background. The IIF using mAb C6 was agreed in specificity and sensitivity with those of a commercial diagnostic kit. These results demonstrated that the produced mAbs were specific to *C. parvum* and that the mAb C6 could be used for diagnosis of cryptosporidiosis.

Key words: *Cryptosporidium parvum*, VRI-CN91, monoclonal antibody, diagnosis, Korea

INTRODUCTION

Cryptosporidium parvum (Tyzzer, 1912) is an opportunistic protozoan parasite of the intestinal tract. The cryptosporidiosis is self-limited in normal, healthy individuals of both men and animals. But when the immune status of the host is depressed, the infection becomes patent (Wagner and Prabhy Das, 1986; Chai *et al.*, 1990). Although the infected host discharges small number of oocysts into feces, it may also be a reservoir of infection. Many isolates of *Cryptosporidium* which infect mammalian species lack host specificity, therefore any animal, once infected, can act as a source of infection for others (Angus, 1987). Thus, it is important to investigate the infected hosts epidemiologically. Clinical diagnosis of cryptosporidiosis is primarily based on detection of oocysts from feces. The detection methods include flotation (Anderson, 1981), sedimentation (Zierdt, 1984; Baron *et al.*, 1989) and stain of fecal samples (Anderson, 1981; Garcia *et al.*, 1983; Bronsdon, 1984; Cross and Moorhead, 1984; Miller *et al.*, 1984; Pohjola, 1984; Smith *et al.*, 1989). Some fecal samples, however, may contain only a few oocysts, making it difficult for the diagnostician to decide whether one or two *Cryptosporidium*-like bodies seen in stained fecal smears warrant a positive diagnosis. Also, acid-fast stained fecal smears often contained nonstaining oocysts (oocyst ghosts) as well as unidentified acid-fast structures (Arrowood and Sterling, 1989). Recently, serological diagnostic methods using monoclonal antibody (mAb) are most often used (Arrowood and Sterling, 1989; Rusnak *et al.*, 1989; Smith *et*

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al., 1989; Anusz *et al.*, 1990; Robert *et al.*, 1990).

In the present study, mAbs were produced against *C. parvum* oocysts, which were isolated from a BALB/c mouse raised in Korea. The produced mAbs were characterized, and their diagnostic efficacy was evaluated.

MATERIALS AND METHODS

1. Oocyst isolation

The *C. parvum* oocysts were isolated from a BALB/c mouse raised in Korea. The isolate (VRI-CN91) was maintained by passage in specific pathogen free ICR mice (Wee *et al.*, 1992). Ether extraction followed by discontinuous sucrose gradients was applied to purify oocysts from feces of mice (Wee *et al.*, 1994). Purified oocysts were then washed three times by centrifugation in phosphate-buffered saline (PBS, pH 7.4) and incubated in PBS containing 0.1% (w/v) glucose, penicillin (5,000 IU/ml), streptomycin (5 mg/ml) and amphotericin-B (20 μg/ml) for 3 hours at 37°C to kill microbial contaminants (Current and Haynes, 1984). The number of oocysts was adjusted to 10^6 per 0.1 ml PBS, and then the suspension was treated with three cycles of freezing and thawing, and frozen at −20°C until used (Tilley *et al.*, 1991).

2. Production of monoclonal antibody

Adult BALB/c mice were immunized, intraperitoneally, four times at 2-week intervals with approximately 1×10^6 oocysts. The first immunization was done with Freund's complete adjuvant (FCA, Difco), whereas the second was done 2 weeks later with Freund's incomplete adjuvant (FIA, Difco). The third and fourth immunizations at week 4 and 6 were done without adjuvant. Final immunization was carried out intravenously on day 49. The spleens were removed from the immunized mice 3 days after final boosting and fused with mouse myeloma cells (SP_2/O) employing polyethylene glycol 1500 (Boehringer Mannheim). The resulting hybridoma cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) including hypoxanthine, aminopterin and thymidine (HAT, Boehringer Mannheim) supplemented with 10% fetal calf serum in 96-well culture plate for 2 weeks. Supernatants were screened for selecting specific antibodies using an indirect immunofluorescent assay (IIF) against *C. parvum* (VRI-CN91) oocysts. Hybridoma cells secreting antibodies were cloned by limiting dilution.

3. Characterization of monoclonal antibodies

Isotype: Isotypes of mAbs were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) isotyping kit including the isotype-specific rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA (Hyclone Inc.).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Oocysts were sonicated, and centrifuged at 10,000 rpm for 10 minutes. The supernatant was diluted in 2 × SDS sample buffer (200 mM Tris-HCl; pH 6.8, 10% 2-mercaptoethanol, 4% sodium dodecyl sulfate, 0.1% bromophenol blue, 20% glycerol), and denatured at 100°C for 3 minutes. Electrophoresis (Hoefer, SE 600) was done at 10 mA for 14 hours, based on the method of Laemmli (1970), using 4% stacking gels and 5-15% linear gradient separating gels (Peeters *et al.*, 1992). Gels were fixed and stained using the Coomassie brilliant blue G (CBB-G, Sigma) to enumerate the protein bands. Molecular weight standard markers (Gibco BRL) were used for calibration.

Enzyme immuno-transfer blot (EITB): Following electrophoresis, the oocyst proteins were blotted onto nitrocellulose paper (NC paper, Hoefer) by using electroblotter (Hoefer) in blotting buffer (20 mM Tris, 160 mM glycine, 20% methanol, pH 8.3). Transfer was done at 800 mA for 3 hours at 4°C. NC paper was then blocked at 4°C for overnight in 0.1 M Tris buffered saline containing 3% bovine serum albumin and 0.05% Tween 20 (TTBS, pH 7.4) and was incubated with hybridoma supernatant for 4 hours at room temperature. After being washed three times for 5 minutes in TTBS solution, NC papers were reacted for 1 hour with a 1:1,000 dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG or IgM (KPL). After being washed in TTBS solution, specific protein bands were visualized

following the addition of 0.022% 3-amino-9-ethyl carbazol (AEC, Sigma) in 0.1 M sodium citrate buffer (pH 5.2) with 0.03% H_2O_2.

4. Production of polyclonal antibody

Two rabbits were immunized intramuscularly, four times at biweekly intervals with approximately 1×10^7 oocysts. Oocysts with FCA (V/V) were inoculated at four sites in the back, and the first booster of oocysts in FIA was done 2 weeks later. The third and fourth booster was administered at week 4 and 6 with oocysts only. The titer of serum from these rabbits was determined by IIF. Preimmunization antibody-negative sera were also obtained from these rabbits and stored at –20 °C.

5. Cross-reactivity of mAbs with other protozoa

Cross-reactivity of mAbs was checked by IIF with tachyzoites of *Toxoplasma gondii* (RH strain) and oocysts of *Eimeria zuernii*, *E. bovis*, *E. canadensis* of bovine origin (Wee *et al.*, 1990).

6. Experimental infection of a calf and oocyst preparation

A 7-day-old calf was infected with *C. parvum* oocysts (7×10^7), and its feces containing *C. parvum* oocysts was subjected to ether extraction to remove some of fat from feces. The materials were washed by centrifugation ($1,500 \times g$ for 10 minutes, 3 times) in PBS and were sieved sequentially through stainless steel screens with a final mesh of 250 (61 μm porosity) to remove other debris. After sieving, oocysts were counted by using a hemocytometer chamber. The number of oocysts was adjusted to $< 10^3$, $10^3 - 10^4$, $10^4 - 10^5$, $10^5 - 10^6$ and $> 10^6$ oocysts per gram of feces (OPG), and 12 samples were prepared at each concentration.

7. Detection of oocysts from fecal samples

Procedure of DMSO-modified acid-fast stain (MAFS) method: Twenty μl of nonconcentrated fecal samples were smeared on the slide. All steps were performed as described by Bronsdon (1984).

Procedure of commercial Merifluor *Cryptosporidium* kit: Twenty μl of nonconcentrated fecal samples were applied to slides provided in the kit (Meridian Diagnostics Inc., Ohio). The slides were then processed and examined according to the manufacturer's instruction.

Procedure of IIF with mAb C6: For IIF testing, 20 μl of nonconcentrated fecal samples were smeared on the slide. Slides were allowed to dry completely at room temperature. Fourty μl of mAb C6 was dropped on the slides, and the slides were incubated in a humidity chamber for 30 minutes at room temperature. Slides were rinsed with PBS. Twenty μl of fluorescein-conjugated goat anti-mouse IgM (KPL, 1:100) was dropped and an equal volume of counterstain solution (Evans blue 1:250) was added on the slides. After incubation for 30 minutes at room temperature, and given a final rinse with PBS, IIF slides were examined with a fluorescence microscopy (Carl Zeiss, SH-250) at × 250 or × 500 magnification.

RESULTS

1. Electrophoretic analysis

SDS-PAGE analysis of solubilized extracts of *C. parvum* oocysts revealed more than 37 different bands ranging in molecular weight from 300 kDa to 10 kDa in CBB-G staining (Fig. 1). The majority of these bands was transferred to NC paper as demonstrated by EITB using immunized rabbit serum. More than 33 bands were detected; of these, 260, 225, 185, 165, 130, 78, 72, 67, 62, 41, 36, 31, 24 and 12 kDa were the major bands (Fig. 2).

2. Characterization of mAbs

Two hybridoma cell lines against *C. parvum* oocysts were produced. The isotypes of these two mAbs were IgG_{2b} (1E7.2) and IgM (C6). EITB analyses have shown that mAb 1E7.2 reacted specifically to 36 kDa protein (Fig. 2) and the mAb C6 reacted to 67 and 70 kDa proteins (Fig. 3). These mAbs were applied to IIF. These mAbs were bound specifically to the surface region of the oocysts (Fig. 4). No cross-reactivity was observed with tachyzoites of *Toxoplasma gondii* and oocysts of *Eimeria zuernii*, *E. bovis*, and *E. canadensis* of bovine

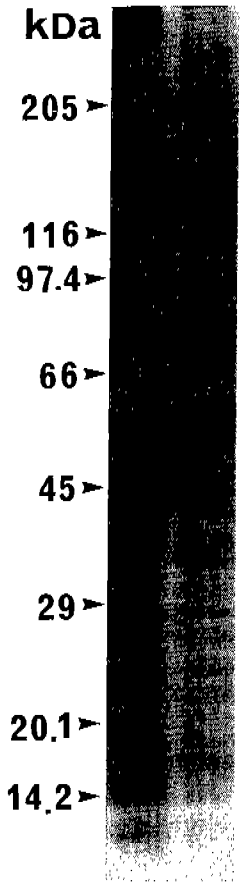

Fig. 1. The proteins of *Cryptosporidium parvum* shown on 5-15% gradient SDS-PAGE using Coomassie brilliant blue G staining technique; standard molecular weights are indicated on the left.

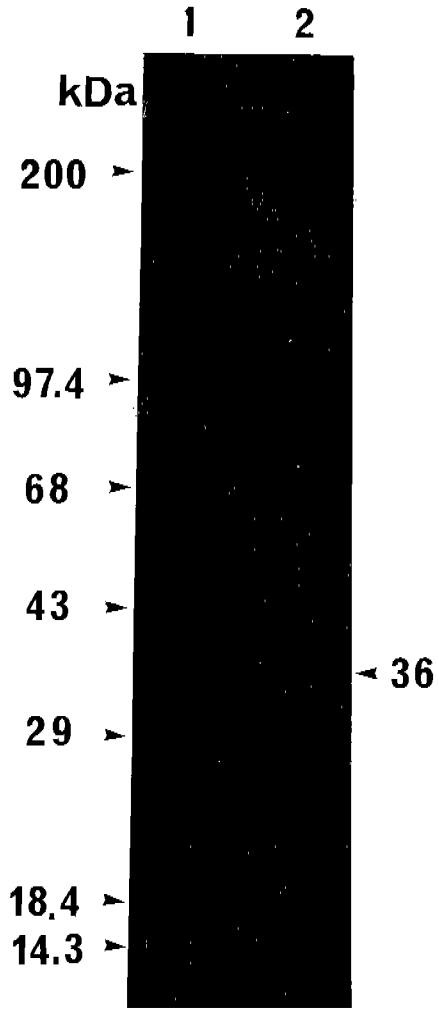

Fig. 2. Antigenic profiles of *Cryptosporidium parvum* oocysts detected by enzyme immunotransfer blotting. Lane 1: polyclonal anti-*C. parvum* rabbit serum. Lane 2: hybridoma supernatants containing monoclonal antibody 1E7.2 followed by a goat anti-mouse horseradish peroxidase-conjugated IgG; standard molecular weights are indicated on the left.

origin.

3. Detection of *Cryptosporidium* oocysts in bovine feces

IIF assays using mAb C6 were successful with counterstain. The oocysts appeared as 3 to 5 μm spherical objects fluorescing bright apple green color against a reddish dark background (Fig. 5).

A total of 60 fecal samples was examined for *Cryptosporidium* oocysts by MAFS, commercial kit and IIF using mAb C6. Results of applying three methods for oocyst detection in fecal smears are summarized in Table 1. Of the 60 fecal samples, 42 samples (70.0%) were identified by MAFS, 49 samples (81.6%) by the commercial kit, and 48 samples (80.0%) by the IIF using mAb C6. The commercial kit and the IIF using mAb C6 were able to detect the oocysts completely when the fecal sample contained 10^4 OPG or more. But it was possible to detect the oocysts completely in MASF when OPG was 10^5 or more. The IIF using mAb C6 was agreed to the specificity and sensitivity of the commercial diagnostic kit (Table 2).

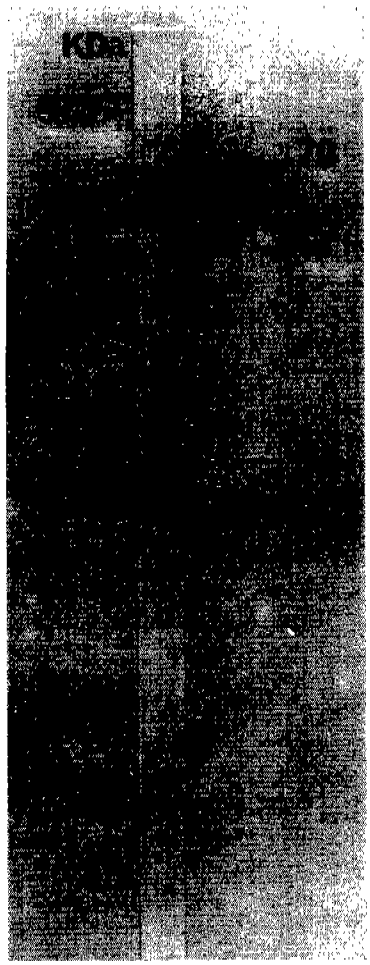

Fig. 3. Enzyme immuno-transfer blotting of 5-15% gradients SDS-PAGE of oocyst antigens detected with hybridoma supernatants containing monoclonal antibody C6 followed by a goat anti-mouse horseradish peroxidase-conjugated IgM; standard molecular weights are indicated on the left.

DISCUSSION

In the diagnosis of cryptosporidiosis, monoclonal antibody-based methods have provided enhanced sensitivity and specificity over those of the conventional stain methods especially when the number of oocysts in stool specimens is low (Arrowood and Sterling, 1989; Rusnak *et al.*, 1989; Smith *et al.*, 1989; Anusz *et al.*, 1990; Robert *et al.*, 1990). In the present study, mAbs were produced against *C.*

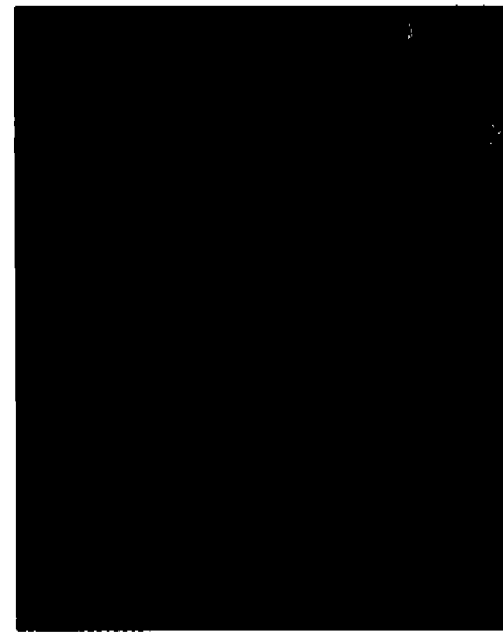

Fig. 4. *Cryptosporidium parvum* oocysts labeled by monoclonal antibody 1E7.2, showing the antibody reacted specifically with the surface region of the oocysts (× 500).

Fig. 5. *Cryptosporidium parvum* oocysts in calf diarrheal fecal specimens diagnosed by indirect immunofluorescent assay using monoclonal antibody C6 (× 250).

Table 1. Comparison of diagnostic efficacy between modified acid-fast stain and immunofluorescent assay for detection of *Cryptosporidium parvum* oocysts in diarrheal feces of an experimentally infected calf

OPG[a] in samples	No. of repetition	Diagnostic method		
		MAFS[b] (%)	Commercial IIF kit[c] (%)	IIF using mAb C6 (%)
$< 10^3$	12	4 (33.3)	5 (41.7)	4 (33.3)
10^3-10^4	12	5 (41.7)	8 (66.7)	8 (66.7)
10^4-10^5	12	9 (75.0)	12 (100.0)	12 (100.0)
10^5-10^6	12	12 (100.0)	12 (100.0)	12 (100.0)
$>10^6$	12	12 (100.0)	12 (100.0)	12 100.0)
Total	60	42 (70.0)	49 (81.6)	48 (80.0)

[a]Oocysts per gram of feces. [b]DMSO-modified acid-fast stain. [c]Merifluor *Cryptosporidium* kit (Meridian Diagnostics Inc., Ohio).

Table 2. Comparison of the relative specificity and sensitivity between modified acid-fast stain and immunofluorescent assay for detection of *Cryptosporidium parvum* oocysts in diarrheal feces of an experimentally infected calf

	MAFS[a]		Relative specificity	Relative sensitivity
	Positive (42)	Negative (18)		
Commercial IIF kit[b]				
Positive (49)[c]	41	8	55.6	97.6
Negative (11)	1	10		
IIF using mAb C6				
Positive (48)	41	7	61.1	97.6
Negative (12)	1	11		

[a]DMSO-modified acid-fast stain. [b]Merifluor *Cryptosporidium* kit (Meridian Diagnostics Inc., Ohio). [c] Explain number in parentheses.

parvum oocysts which were isolated from a BALB/c mouse (Wee *et al.*, 1992); the produced mAbs were characterized and their detection rates of *C. parvum* were evaluated.

Electrophoresis of the soluble fraction in polyacrylamide gels with sodium dodecyl sulfate and silver staining of *Cryptosporidium* sp. oocysts revealed the presence of 41 bands (Lazo *et al.*, 1986). Silver staining of SDS-PAGE gels of *Cryptosporidium* sp. sporozoite detected a total of 46 bands ranging in molecular weight from approximately 300 kDa to 3 kDa (Mead *et al.*, 1988). The reactivity of sera from naturally-infected humans, calves and horses to *Cryptosporidium* sporozoite antigens was investigated using a western blot technique. Immunoblot studies have resulted in the detection of small molecular weight (MW) antigen (20 to 23 kDa) in either intact oocysts or sporozoite life cycle stages which is frequently recognized by acute and convalescent sera from humans, goats, cattle and horses (Ungar and Nash, 1986; Mead *et al.*, 1988; Lumb *et al.*, 1989). Surface glycoproteins of approximately 11, 15, 23 and 44 kDa in MW also demonstrated intense reactivity (Tilley *et al.*, 1991; Whitmire and Harp, 1991; Peeters *et al.*, 1992). In the present study, SDS-PAGE analysis of solubilized extracts of *C. parvum* oocysts revealed more than 37 bands. Rabbit sera against *C. parvum* oocyst antigens reacted to a

total of 33 bands and demonstrated intense reactivity to 14 major bands including two bands at 36 kDa and 67 kDa, respectively. EITB analysis has shown that mAb C6 reacted to 67 kDa and 70 kDa oocyst proteins and mAb 1E7.2 reacted to 36 kDa, 45 kDa, and 190 kDa oocyst proteins, respectively. But the oocyst proteins of 45 and 190 kDa were found to react non-specifically because those reacted to the control.

Polyclonal antibodies raised against *C. parvum* oocysts were found to cross-react with *Eimeria* spp. oocysts in an IIF, but no cross-reaction was observed with cystozoites of *Toxoplasma* and *Sarcocystis* spp. These results show the existence of epitopes common to *C. parvum* and various *Eimeria* spp. (Ortega-Mora *et al.*, 1992). However, mAb-ELISA was specific for *Cryptosporidium* sp. oocysts and did not recognize oocysts of *Eimeria auburnensis, E. bovis, E. ellipsoidalis* or *E. zuernii* (Anusz *et al.*, 1990). In this study, two mAbs were bound specifically to the surface region of the *C. parvum* oocysts, but no cross-reactivity was demonstrated with tachyzoites of *Toxoplasma gondii* and oocysts of *Eimeria zuernii, E. bovis,* and *E. canadensis* of bovine origin. These results confirmed that the produced mAbs were specific to *C. parvum* though they were screened only 4 species of protozoa.

The mAb C6 was reacted more intensive and clear to *C. parvum* oocysts than mAb 1E7.2 by IIF assay. So, we decided to choose the mAb C6. When scanned at a magnification of × 250 or × 500 by the IIF using the mAb C6 without counterstain, the apple green fluorescence of the oocysts was not easily differentiated from the yellow fluorescence of the background debris. But by the IIF using mAb C6 with counterstain (Evans blue), oocysts were successfully differentiated from the background debris. Oocysts appeared as spherical objects 3 to 5 μm fluorescing bright apple green against a reddish dark background. The IIF using mAb C6 was more sensitive than MAFS in this study. Also, the IIF using mAb C6 was agreed in specificity and sensitivity with those of the commercial diagnostic kit. These results confirmed that the produced mAbs were specific to *C. parvum* and that the mAb C6 could be used for diagnosis of cryptosporidiosis.

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=초록=

단세포군항체를 이용한 간접형광항체법에 의한 송아지 작은와포자충증의 진단

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국내 마우스에서 분리된 작은와포자충(*Cryptosporidium parvum*)을 마우스에서 증식시킨 다음 오오시스트만을 순수 분리하여 BALB/c 마우스에 면역시켰다. 면역된 마우스의 비장에서 림프구를 분리하여 PEG 1500을 융합촉진제로 사용하여 Sp2/0 myeloma cell과 세포융합을 실시하였으며, 융합된 세포중 작은와포자충에 특이항체를 생산하는 hybridoma cell을 선발하였다. 한계희석법으로 제작된 2주의 단세포군항체는 IgG2b class(1E7.2)와 IgM class(C6)에 속했으며, SDS-PAGE와 Western blotting한 결과 1E7.2는 원충의 단백항원중 36 kDa과 반응하였고, C6는 67 kDa 및 70 kDa과 반응하였다. 생산된 단세포군항체를 간접 형광항체법으로 작은와포자충과 반응시킨 결과 오오시스트 외막에 특이적으로 반응하였던 반면, *Toxoplasma gondii*의 tachyzoite, *Eimeria zuernii, E. bovis, E. canadensis*의 오오시스트와는 반응을 나타내지 않았다. 단세포군항체 C6을 이용한 간접 형광항체법은 분변의 이물들의 대조염색을 위해 Evans blue를 사용하였으며, 관찰소견으로는 3-5 μm의 둥근 오오시스트가 밝은 형광을 띄고 있었고 그외의 주변이물들은 대조염색에 의하여 검붉게 염색되었다. 또한 그의 진단율은 현재 일반적으로 사용되고 있는 수입진단킷트(Merifluor, Meridian diagnostic Ins.)와 거의 일치하고 있었다. 이상의 결과를 종합해보면 이번에 생산된 단세포군항체들은 작은와포자충에 특이적으로 반응하고 있었으며, 이를 이용한 형광항체 진단법은 작은와포자충을 진단하는데 유용하게 사용될 수 있을 것으로 판단된다.

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