Cryptosporidium and Giardia in Tama River, Japan, 2nd Asia Conference on Ultraviolet Technologies for Environmental Applications (2004.10).

16) 真砂佳史、小熊久美子、片山浩之、大垣眞一郎：環境水中の病原性微生物の濃度変動測定手法の開発．平成16年度土木学会全国大会第59回年次学術講演会(2004.9、豊田)

17) 阿部欽章、渡部徹、大村達夫：日光による不活化に着目した河川中の病原ウイルスの挙動に関する研究、平成16年度土木学会東北支部技術研究発表会 (2005.3)

18) 三浦尚之，阿部欽章，渡部徹：GISを用いた阿武隈川流域における水中病原微生物の挙動評価モデルの開発，平成16年度土木学会東北支部技術研究発表会(2005.3)

19) 渡部徹、橋本剛志、阿部欽章、大村達夫：DALYを指標とした下水処理水再利用による健康リスク評価、平成16年度土木学会全国大会第59回土木学会年次学術講演会(2004.9、豊田)

20) T. Watanabe, K. Hashimoto, Y. Abe and T. Omura: Evaluation of Health Risks in the Wastewater Reclamation in the Abukuma Watershed, Japan, Proceedings of 8th IWA International Conference on Diffuse/Nonpoint Pollution (CD-ROM), 801-808(2004)

平成１５年度

1) Tokoro M, Iseki M : A novel one-step polymerase chain reaction method for identification of co-infection with different genotypes of Cryptosporidium 11th International Conference of Infectious Diseases, Mexico, Mar. 4-7, 2004.

2) 井関基弘、岡本雅子、阿部仁一郎：ペットショップの子犬等におけるクリプトスポリジウムおよびジアルジアの感染状況と分離株の遺伝子解析．第72回日本寄生虫学会大会（2003.3、久留米）

3) 木俣　勲、井関基弘：市販の糞便内クリプトスポリジウム抗原検出用キット3種の評価．第72回日本寄生虫学会大会（2003.3、久留米）

4) 木俣　勲、阿部仁一郎、井関基弘：遺伝子型の異なるCryptosporidium parvumオ-シストのHCT-8細胞への感染性と紫外線感受性．第59回目本寄生虫学会西日本支部大会(2003.11、金沢）

5) 所　正治、仲本賢太郎、野崎智義、阿部仁丁郎、木俣　勲、井関基弘：クリプトスポリジウムのアデノシルメチオニン合成酵素の解析．第59回日本寄生虫学会西日本支部大会（2003.11、金沢）

6) 井関基弘：クリプトスポリジウム症、サイクロスポーラ症、微胞子虫症一背景とこれからの課題．第59回日本寄生虫学会西日本支部大会・特別講演（2003.11、金沢）

7) 大川美奈子、今井邦典、宇根有美、黒木俊郎、遠藤卓郎、石橋　徹、鈴木哲也、野村靖夫：ヒョウモントカゲモドキ由来のクリプトスポリジウムの感染性と病原性、第136回日本獣医学会学術集会（2003、東京）

8) 森田重光、平田 強：紫外線の原虫不活化効果、第 6 回日本水環境学会シンポジウム

(2003.9、神戸)

9) 猪又明子、橋本　温、保坂三継、平田強：わが国の水道原水並びに浄水における原虫汚染の実態、第 6 回日本水環境学会シンポジウム(2003.9、神戸)

10) 貝森繁基，森田重光、平田強：紫外線照射した *Cryptosporidium parvum* オーシストにおける DNA の損傷と光・暗回復、第 38 回日本水環境学会年会（2004.3、札幌）

11) 杉本ひとみ，橋本　温，森田重光，平田強：下水から単離した *Cryptosporidium* オーシストの遺伝子型の解析，第 38 回日本水環境学会年会（2004.3、札幌）

12) 森田重光，平田　強（2004.3）電子線による微生物不活化に及ぼす汚泥の影響，第 38 回日本水環境学会年会（2004.3、札幌）

13) Kim H.-S., Akiba M.,and Kunikane S.: The effect of turbidity and coagulation dose on the removal *Cryptosporidium* oocysts in drinking water treatment, 3rd IWA-Asia Pacific Regional Conference (2003.10, Bangkok)

14) T. Watanabe, K. Hashimoto, Y. Abe and T. Omura: Risk Evaluation of Pathogens in Wastewater Reclamation as a Drinking Water Source. WEFTEC 2003 (the 76th Annual Water Environment Federation (WEF) Technical Exhibition and Conference,(2003)

15) T. Watanabe, K. Hashimoto, Y. Abe, K. Fukushi and T. Omura: Risk Evaluation of Viral Infectious Diseases through Drinking Water with Secondary Infection. Asian Waterqual 2003 (3rd International Water Association (IWA) Asia-Pacific Regional Conference), poster presentation(2003.10, Bangkok)

16) 藤倉雅浩、阿部欽章、渡部徹、大村達夫：河川における病原ウイルスの挙動評価のための基礎的実験、第 58 回年土木学会次学術講演会（2003.9）

17) 大村達夫、橋本剛志、渡部徹：下水処理水再利用に伴う健康リスクの評価，平成 15 年度土木学会東北支部技術研究発表会（2004.3）

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Cross-reactivities with *Cryptosporidium* spp. by chicken monoclonal antibodies that recognize avian *Eimeria* spp.

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Abstract

In a previous study, we have developed several chicken monoclonal antibodies (mAbs) against *Eimeria acervulina* (EA) in order to identify potential ligand molecules of *Eimeria*. One of these mAbs, 6D-12-G10, was found to recognize a conoid antigen of EA sporozoites and significantly inhibited the sporozoite invasions of host T lymphocytes in vitro. Furthermore, some of these chicken mAbs showed cross-reactivities with several different avian *Eimeria* spp. and the mAb 6D-12-G10 also demonstrated cross-reactivities with the tachyzoites of *Neospora caninum* and *Toxoplasma gondii*. *Cryptosporidium* spp. are coccidian parasites closely related to *Eimeria* spp., and especially *C. parvum* is an important cause of diarrhea in human and mammals. In the present study, to assess that the epitopes recognized by these chicken mAbs could exist on *Cryptosporidium* parasites, we examined the cross-reactivity of these mAbs with *Cryptosporidium* spp. using an indirect immunofluorescent assay (IFA) and Western blotting analyses. In IFA by chicken mAbs, the mAb 6D-12-G10 only showed a immunofluorescence staining at the apical end of sporozoites of *C. parvum* and *C. muris*, and merozoites of *C. parvum*. Western blotting analyses revealed that the mAb 6D-12-G10 reacted with the 48-kDa molecular weight band of *C. parvum* and *C. muris* oocyst antigens, 5D-11 reacted the 155 kDa of *C. muris*. Furthermore, these epitopes appeared to be periodate insensitive. These results indicate that the target

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antigen recognized by these chicken mAbs might have a shared epitope, which is present on the apical complex of apicomplexan parasites.

Keywords: *Cryptosporidium*; Chicken; Monoclonal antibodies; Zoite

1. Introduction

Cryptosporidium (Protozoa, Apicomplexa) causes cryptosporidiosis in man and economically important food animals throughout the world. Cryptosporidiosis is characterized by a self-limiting diarrhea in immunocompetent individuals but it can be chronic and life-threatening in immunocompromised patients (Colford et al., 1996; Pozio et al., 1997). Because specific immune responses can control cryptosporidiosis, passive immunization strategies have been investigated (Riggs, 1997; Crabb, 1998). However, to date, there are no approved vaccines, immunotherapy or effective pharmaceuticals for the prevention and treatment of cryptosporidiosis (Riggs, 1997; Jenkins et al., 1999; Perryman et al., 1999, Sagodira et al., 1999).

Cryptosporidium zoites including sporozoites and merozoites initiate infection by recognition, attaching to and invading host cells. Although the processes of invasion have not been well characterized, highly differentiated apical organelles of *Cryptosporidium* zoites are thought to play a key role in host-cell invasion (Bonnin et al., 1993). Therefore, new strategies for cryptosporidiosis control are being focused on understanding the nature of these organelles during the invasion process and elucidating whether some apical organelles could be candidate antigens.

Until now, monoclonal antibodies (mAbs) have been widely used for the identification of candidate antigens on *Cryptosporidium* zoites. Several mouse mAbs that recognize the apical regions or the surface molecules of invasive stages have been reported (Petersen et al., 1992; Riggs et al., 1997, 1999; Cevallos et al., 2000). However, in mouse immunization studies, the molecule was reported to be poorly immunogenic (Fayer et al., 1997), and optimal vaccine candidates that could completely prevent or terminate *Cryptosporidium* infection have not been identified.

In a previous study, we have developed several chicken monoclonal antibodies against *Eimeria acervulina* (EA) to identify potential ligand molecules. Most of these chicken mAbs recognized the apical region of *Eimeria* invasive stages (Sasai et al., 1996; Constantinoiu et al., 2003). One of these mAbs, 6D-12-G10, was found to recognize a conoid antigen of EA sporozoites by immunelectron microscopy and 21-kDa molecular weight of EA sporozoite antigen by Western blotting analyses, and significantly inhibited the sporozoite invasions of host T lymphocytes in vitro (Sasai et al., 1996). Thus, we reported that the antigen recognized by mAb 6D-12-G10 might be important for immunological therapy against EA infection. Furthermore, some of these chicken mAbs showed cross-reactivities with several different avian *Eimeria* spp. (Constantinoiu et al., 2003), and the mAb 6D-12-G10 also demonstrated a cross-reactivity with the tachyzoites of *Neospora caninum* and *Toxoplasma gondii* (Sasai et al., 1998). These results suggested that these chicken mAbs might recognize epitopes conserved in many species of cyst-forming coccidian. We hypothesized that epitopes recognized by these chicken mAbs might exist on *Cryptosporidium* zoites. To assess the hypothesis that *Cryptosporidium* antigens recognized by these chicken mAbs could be candidate vaccines for cryptosporidiosis, we tested cross-reactivities of these chicken mAbs with *Cryptosporidium* parasites.

2. Materials and methods

2.1. Parasites

C. parvum oocysts, strain HNJ-1, were originally obtained from the feces of a patient in Japan (Abe et al., 2002), and *C. muris* oocysts, strain RN 66, from mice in Japan (Iseki et al., 1989). These oocysts were passaged in severe combined immunodeficient (SCID) mice, purified by sugar flotation, and stored at 4 °C in a 2.5% potassium dichromate solution until 1 month before their use or freeze-dried for longer preservation.

2.2. Preparation of sporozoites

The *C. parvum* oocysts were washed three times with Hanks' balanced salt solution (HBSS) (Gibco BRL, Tokyo, Japan) by centrifugation at 1500 × *g* for 10 min and were excysted by the method previously reported (Kato et al., 2001). Briefly, a million (10^6) oocysts of *C. parvum* were pretreated in HBSS containing 0.01N HCl at 37 °C for 1 h. After washing two times with HBSS, the sporozoites were excysted in HBSS containing 0.1% bovine bile solution (Wako, Osaka, Japan) for 10 min. The *C. muris* oocysts were washed three times with HBSS by centrifugation at 1500 × *g* for 10 min and oocysts were resuspended in HBSS. Excystation of sporozoites was obtained by incubation of oocysts at 37 °C (Nina et al., 1992). In brief, 1 ml of HBSS containing 10^6 oocysts were incubated in flat-bottom 24-well tissue culture plates (Iwaki Grass, Tokyo, Japan) at 37 °C in an incubator with 5% CO_2 for 30 min. To conduct IFA, 50 μl mixture of excystated sporozoites and oocysts of *C. parvum* or *C. muris* was applied to clean siliconized slides (Dako Japan, Kyoto, Japan). Slide preparations were air-dried or fixed after drying using methanol or acetone, and stored at −80 °C until use.

2.3. Preparation of merozoites

For preparation of *C. parvum* merozoites, we used a modified method based on those previously reported (Slifko et al., 1997; Weir et al., 2001). Briefly, human colonic tumor (HCT-8) cells (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) were used for in vitro cultivation of *C. parvum*. HCT-8 cells were grown in a maintenance medium that consists of RPMI 1640 (Sigma Chemical Co., Tokyo, Japan) supplemented with 10% (v/v) fetal calf serum (FCS), 20 mM HEPES, 4 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained in 25 cm^2-bottom plastic tissue culture flasks at 37 °C with 5% CO_2 and passaged every 3–4 days. For use in experiments, HCT-8 cells were trypsinized by Trypsin–EDTA solution (0.5% trypsin, 0.195% EDTA-2Na in PBS) (Gibco BRL), washed in the maintenance medium, resuspended at 4×10^5/ml and cultured in perfusion chambers (Cover Well™, Grace Bio-Labs, Inc., Bend, Oregon) fixed on siliconized slide glasses (Matsunami, Osaka, Japan), containing 0.25 ml of maintenance medium per well for 72 h at 37 °C with 5% CO_2 to form a monolayer. To prepare merozoites, *C. parvum* oocysts were pretreated with 0.01N HCl for 30 min at 37 °C and washed with maintenance medium. HCT-8 cell monolayers were washed with maintenance medium before inoculation, and replaced with 0.25 ml of inoculation medium containing 0.1% bovine bile solution (Wako), which contains 2×10^5 ml^{-1} of oocysts. After oocysts inoculation, the HCT-8 cells were incubated at 37 °C with 5% CO_2 for 24 h. After incubation, the medium was aspirated and the perfusion chambers were removed from slide glasses. Then the infected monolayer on slide glasses was fixed in methanol or acetone for IFA, or 10% formaldehyde in PBS for confocal laser scanning microscopy (LSM510, Carl Zeiss Co., Tokyo, Japan), and stored at −80 °C until use.

2.4. Monoclonal antibodies

Six chicken monoclonal antibodies named 5D-11, 8D-2, 6D-12-G10, 8E-1, 8C-3 and HE-4 were produced as described (Sasai et al., 1996; Constantinoiu et al., 2003). The antibody isotypes were of the immunoglobulin G class and its reactivities were previously described (Sasai et al., 1996; Constantinoiu et al., 2003, 2004).

2.5. Indirect immunofluorescence assay

Prepared slides were incubated with 50 μl of culture supernatant from chicken hybridomas for 40 min at room temperature. The slides were washed three times with phosphate-buffered saline (PBS, pH 7.2) and incubated with 50 μl of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken IgG (1:1000, Sigma) for 30 min. As a control, purified normal chicken IgG (1:1000, Sigma) was used. After staining, the slides were mounted with 50% glycerol in PBS for examinations using fluorescent (Nikon, Tokyo, Japan) and confocal laser scanning microscope (LSM510, Carl Zeiss Co.). All steps were performed at room temperature.

2.6. Western blot analysis

Freeze-dried *C. parvum* or *C. muris* oocysts were resuspended in PBS, sonicated in ice bath with ultrasonic processor (Taitec Co., Saitama, Japan) and

centrifuged at 600 × *g* for 5 min. Supernatant was aliquoted and cryopreserved at −80 °C until use. The concentration of crude antigen was determined by BCA protein assay kit (Pierce, Lockford, Illinois). The size of the target *C. parvum* or *C. muris* oocysts antigens that were recognized by the chicken mAbs was determined by Western blotting analyses. Briefly, 20–40 μg of *Cryptosporidium* oocysts antigens in sample buffer (125 mM Tris, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.0025% bromophenol blue) were heated at 95 °C for 4 min. Molecular weight standard proteins (Bio-Rad, Tokyo, Japan) were also treated the same way as the parasite antigen. Sample was resolved on 4% stacking/12% resolving sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V constant voltages (Laemmli, 1970) and the separated proteins were blotted to PVDF membrane (Immobilon Transfer Membranes, Millipore, Bedford, Massachusetts) using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) in a buffer (50 mM Tris, 192 mM glycine, 20% methanol) for 1 h at 100 V constant voltages. After blotting, the membrane was air-dried. Individual lanes were stained for 1 h at room temperature with each of the chicken mAbs or a chicken-purified IgG as a control and washed three times with PBS containing 0.05% Tween 20 for 5 min. Bound antibodies were detected by staining with horseradish peroxidase-labeled rabbit anti-chicken IgG $F(ab)'_2$ fragment (1:1000, Cortex Biochem., California, USA) for 1 h and developed with Konica Immunostaining HRP-1000 (Konica, Tokyo, Japan). Molecular weights were estimated using molecular weight standard proteins after staining with 0.2% Coomassi Brilliant Blue R-250 (Wako).

2.7. Determination of carbohydrate epitopes

To determine whether the epitopes contain carbohydrate, we used the procedure developed by Woodward et al. (1985) for antigens on Western blots. Briefly, each PVDF strip transferred electrophoretically after SDS-PAGE was incubated with 10 or 50 mM sodium *m*-periodate in 50 mM sodium acetate buffer (pH 4.5) in a dark environment at room temperature for 1 h, and was then exposed to 50 mM sodium borohydride in PBS for 30 min. Controls were incubated in the same buffer in the absence of periodate. Following three washes with PBS, the PVDF strips were probed with mAbs as described above.

3. Results

3.1. Indirect immunofluorescence analysis

In IFA with six chicken mAbs, the mAb 6D-12-G10 only reacted at the apical portion of the methanol, acetone fixed and non-fixed sporozoites of *C. parvum* and *C. muris* (Figs. 1 and 2). The mAb 6D-12-G10 did not react with the oocyst wall. Other chicken mAbs and purified normal chicken IgG did not show any binding on *C. parvum* and *C. muris* sporozoites or oocysts (data not shown).

In HCT-8 cells that were infected with *C. parvum*, asexual stages were observed at 24 h after inoculation. In IFA of cultured *C. parvum* zoites fixed by methanol or acetone, only the mAb 6D-12-G10 showed reactivities. Fig. 3A and B shows the meronts during the early stage of development at 24 h after the inoculation into host cells. The mAb 6D-12-G10 showed reactivity with zoites, which were present in the outermost layer of the immature meront (Fig. 3B). Fig. 3C–F shows the mature meront stage including merozoites, which were fixed using methanol or acetone. The positive staining was observed at the apical region of the merozoites within mature meronts. Other chicken mAbs and purified normal chicken IgG did not show any reactions (data not shown). By confocal laser scanning microscopy, we could confirm cultivated type I meront because more than four positive merozoites in one meront were seen (Fig. 3G). After 24 h incubation, other stages of *C. parvum* were not observed.

3.2. SDS-PAGE and immunolot analysis of *C. parvum* and *C. muris* oocysts antigens with chicken mAbs

By Western blotting analyses of the solubilized *Cryptosporidium* oocysts antigens with chicken mAbs, only the mAb 6D-12-G10 recognized predominantly the 48-kDa antigens of *C. parvum* and *C. muris* (Fig. 4). The positive band of 155 kDa reactive with the mAb 5D-11 was only observed with *C. muris*.

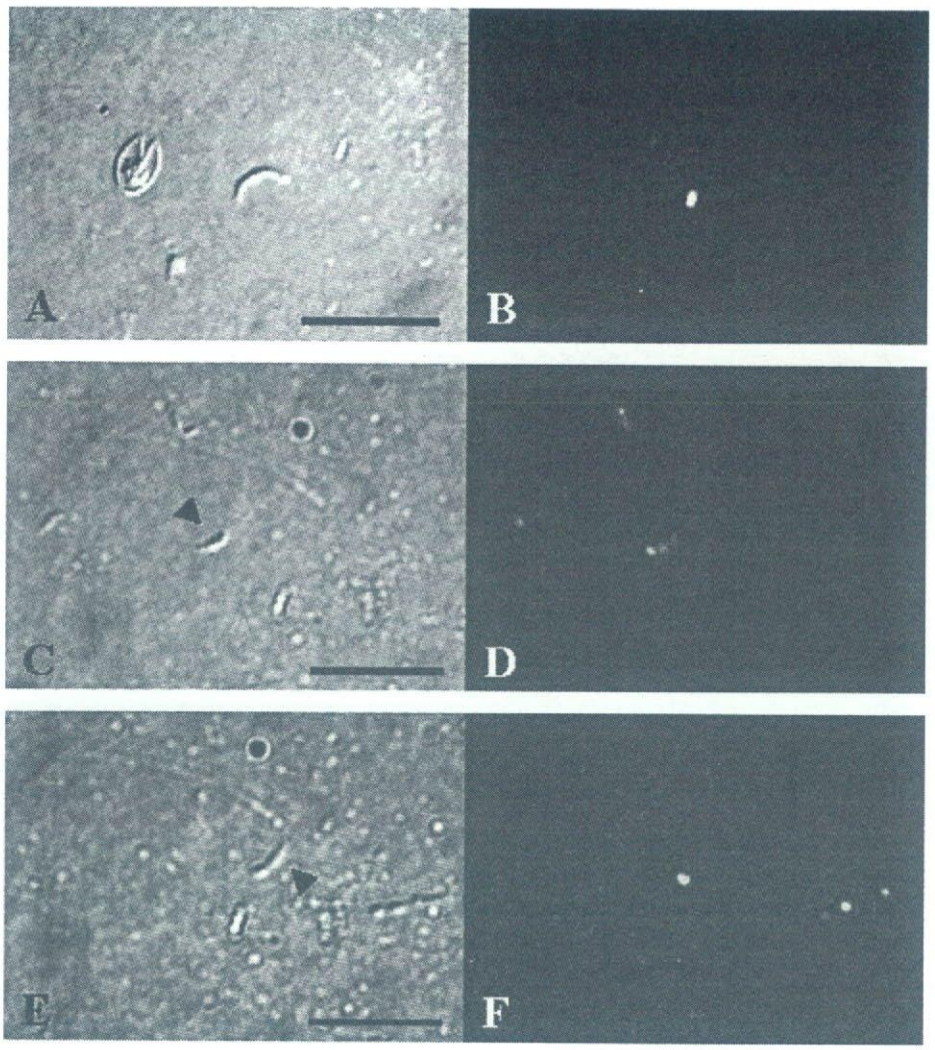

Fig. 1. Indirect immunofluorescence staining of *C. parvum* sporozoites with chicken mAb 6D-12-G10. Photomicrographs from bright field by interference contrast microscopy (A, C, E) and immunofluorescence microscopy (B, D, F) are shown. *C parvum* sporozoites were fixed by methanol (A, B), acetone (C, D) or non-fixed (E, F). Arrowheads indicate sporozoites of *C. parvum*. Bar = 10 μm.

None of the other mAbs and chicken-purified IgG showed any reactions. The left lane is molecular weight standard.

3.3. Periodate sensitivity

The reactivities of the mAbs 6D-12 and 5D11 to *C. parvum* and *C. muris* antigens treated with 10 or 50 mM sodium *m*-periodate are shown in Fig. 5. By peridodate treatment, the mAbs 6D-12 and 5D11 still recognized the 48-kDa antigens of *C. parvum* and *C. muris*, and the 155-kDa of *C. muris*, respectively. The left lane is molecular weight standard.

4. Discussion

In the present study, we showed the cross-reactivities of chicken mAbs with *Cryptosporidium* parasites. By IFA of six chicken mAbs, the mAb 6D-12-G10 only reacted with *C. parvum* and *C. muris* sporozoites, and *C. parvum* merozoites. To date, few

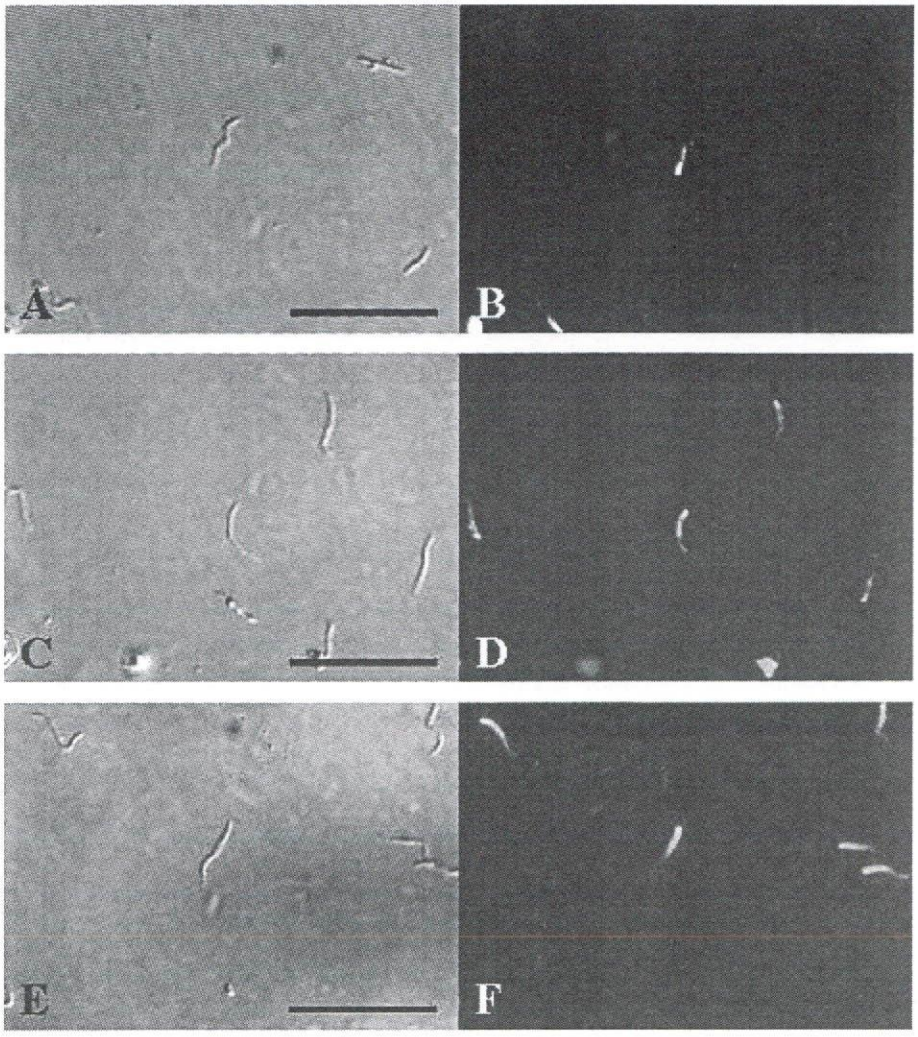

Fig. 2. Indirect immunofluorescence staining of *C. muris* sporozoites with the chicken mAb 6D-12-G10. Photomicrographs from bright field by interference contrast microscopy (A, C, E) and immunofluorescence microscopy (B, D, F) are shown. *C parvum* sporozoites were fixed by methanol (A, B), acetone (C, D) or non-fixed (E, F). Bar = 10 μm.

reports of mAbs which showed cross-reactivities with zoites of *Cryptosporidium* and other apicomplex parasites were available. Our results suggested that the antigen recognized by mAb 6D-12-G10 could highly share among the apicomplexa parasites, including *Eimeria*, *Toxoplasma*, *Neospora*, and *Cryptosporidium*.

In IFA, the positive reactions localized only to the anterior region of invasive stages. The mAb 6D-12-G10 recognized external antigen of zoites because the mAb 6D-12-G10 reacted with non-fixed as well as methanol- or acetone-fixed sporozoites. Although we have reported that the mAb 6D-12-G10 recognized the conoid antigens of EA sporozoites (Sasai et al., 1996), we could not determine the precise organelles on *Cryptosporidium* sporozoites and merozoites. At the anterior end of invasive forms in phylum Apicomplexa, a unique complex of organelles which are made of rhoptries, dense granules and micronemes is located (Sam-Yellowe, 1996), and the conoid, polar rings and subpellicular microtubules are believed to provide a cytoskeletal framework during host cell invasion

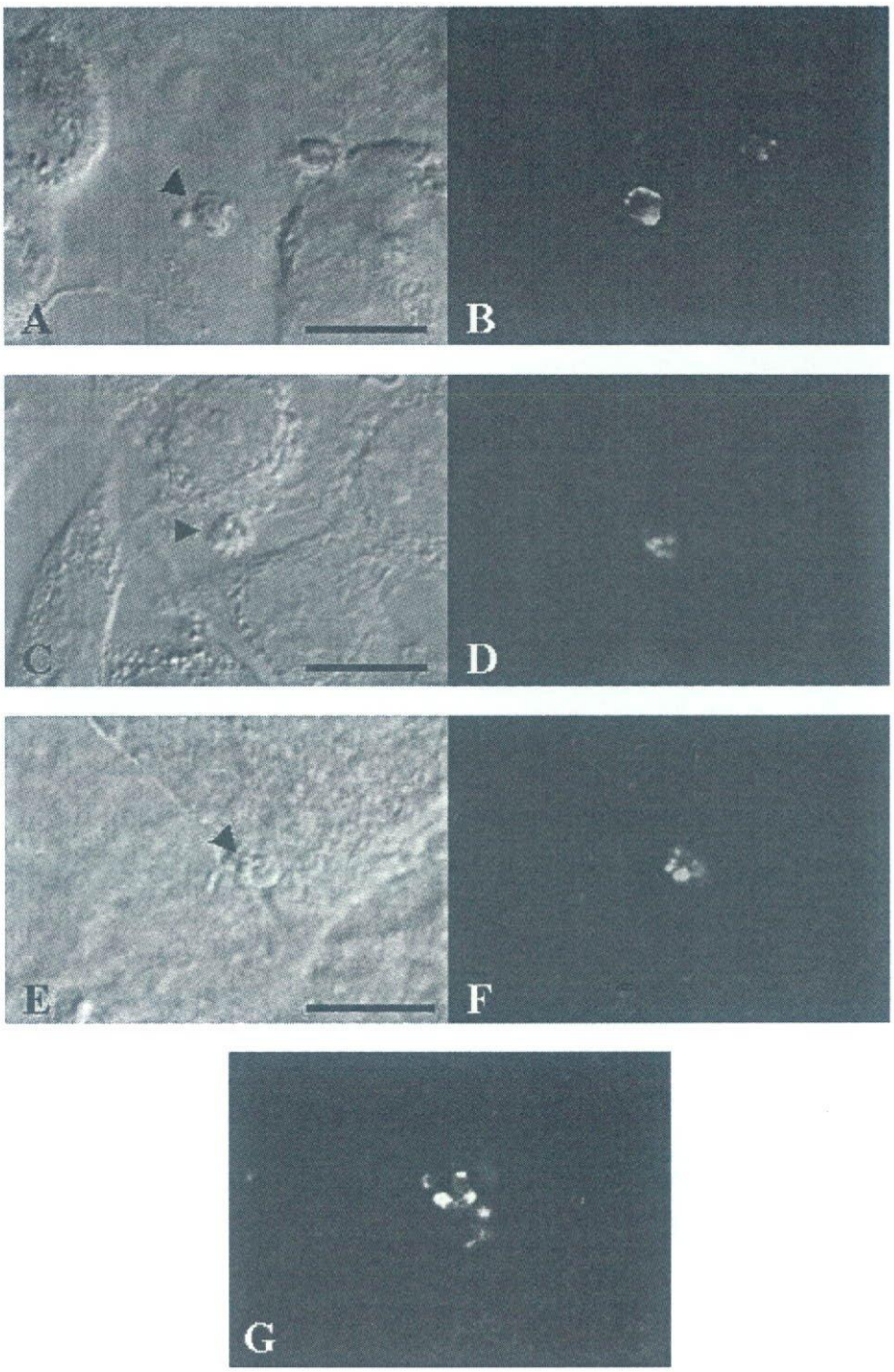

Fig. 3. Indirect immunofluorescence staining of in vitro cultivated *C. parvum* zoites with the chicken mAb 6D-12-G10. Photomicrographs from bright field by interference contrast microscopy (A, C, E), immunofluorescence microscopy (B, D, F) and confocal laser scanning microscopy (G) are shown. At 24 h after inoculation, cultivated *C. parvum* zoites were fixed by methanol (A–D), acetone (E, F) or formaldehyde (G). *C. parvum* immature meront on early stage (A, B) and *C. parvum* mature meront, merozoites (C–G) were shown. Bar = 10 μm.

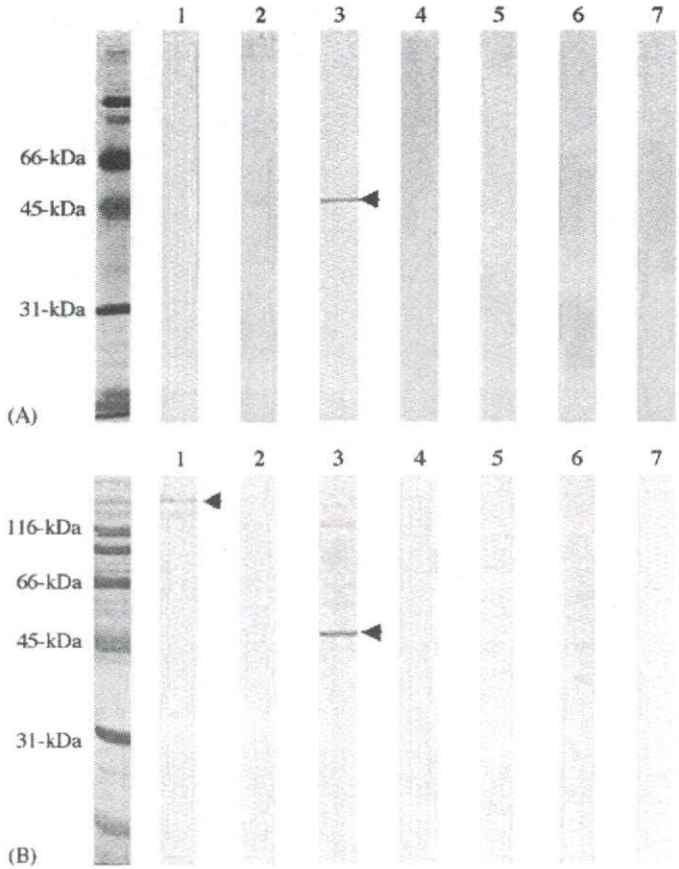

Fig. 4. Western blot analysis of *C. parvum* (A) and *C. muris* (B) antigens with six different chicken mAbs. Lanes: 1, mAb 5D-11; 2, mAb 8D-2; 3, 6D-12-G10; 4, 8E-1; 5, 8C-3; 6, HE-4; 7, normal chicken IgG. The left lane is molecular weight standard. Arrowheads indicate positive bands.

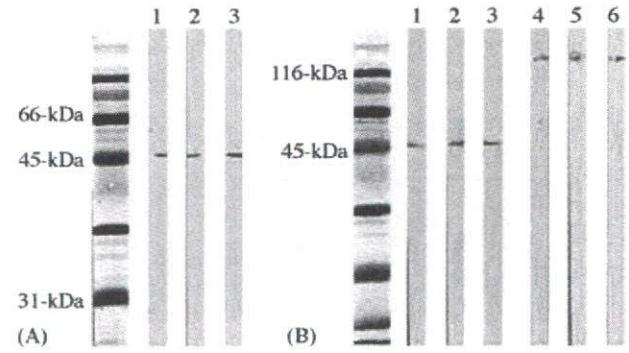

Fig. 5. Western blot analysis of *C. parvum* (A) and *C. muris* (B) after *m*-periodate treatment. Lanes 1 and 4 were incubated in buffer without *m*-periodate, lanes 2 and 5 were with 10 mM *m*-periodate, lanes 3 and 6 were with 50 mM *m*-periodate. 1–3 lanes were probed with chicken mAb 6D-12-G10 and 4–6 lanes were 5D-11, respectively. The left lane is molecular weight standard.

(Lindsay et al., 1991, 1993; Hu et al., 2002). Thus, the mAb 6D-12-G10 might recognize one or more of these organelles on *Cryptosporidium* zoites. However, there is little information about the ultrastructural details of *Cryptosporidium* sporozoites and merozoites (Uni et al., 1987; Lumb et al., 1988), or the role of these organelles in host–parasite interaction.

The mAb 6D-12-G10 showed cross-reactivity with the in vitro cultivated zoites of *C. parvum*. At 24 h after infection to host cells with *Cryptosporidium* oocysts, meronts including merozoites were localized on the surface of host cell (Fig. 3A, C and E). In IFA, positive reactions were seen in immature meronts and the apical region of merozoites within type I meronts. Thus, these results suggested that the apical antigen recognized by the mAb 6D-12-G10 was expressed on the zoites during the early stage of merogony, and was conserved among the zoites of the motile stages of *Cryptosporidium* parasites.

On Western blotting analyses, the mAb 6D-12-G10 recognized a 48-kDa band associated with *C. parvum* and *C. muris* oocysts antigens, and the mAb 5D-11 recognized 155-kDa of *C. muris*. In a previous study, the molecular weight of EA antigen recognized by the mAb 6D-12-G10 was 21 kDa. These results showed that the molecular mass including epitope recognized by mAb 6D-12-G10 was different between *Cryptosporidium* and *Eimeria*. Although the mAb 5D-11 showed no reactions with *Cryptosporidium* zoites or oocysts in IFA, the mAb 5D-11 recognized 155 kDa of only *C. muris* antigen. Until now, there are no reports about 155-kDa antigen on *C. muris*. Although the immunolocalization of *C. muris* recognized by the mAb 5D-11 remains unknown, the 5D-11 might recognize the internal oocyst or sporozoite antigen of *C. muris*, or only solubilized antigens of *C. muris*.

There are very few reports of mouse mAbs, which specifically recognize the apical complex of *Cryptosporidium*, compared to the number of mouse mAbs which recognize surface antigens of invasive stages or oocyst wall. Some papers have reported the development of mAbs, which recognized the apical organelles of *C. parvum* zoites, e.g., micronemes (Bonnin et al., 1991, 2001; Robert et al., 1994), and dense granules (Bonnin et al., 1995), but there are no papers about other organelles. On Western blotting analyses, these mAbs reacted with the high-molecular weight antigens of more than 100 kDa, which are much higher than that of the antigen recognized by mAb 6D-12-G10. Furthermore, most epitopes recognized by these mAbs were reported to be periodate sensitive (glycosylated). Of these mAbs previously reported, the epitope recognized only by the mAb HAD was periodate insensitive (Bonnin et al., 1991). The mAb HAD was thought to recognize *Cryptosporidium* microneme antigens. However, this mAb recognized 63–210 kDa antigens. Thus, the 48-kDa antigen recognized by the mAb 6D-12-G10 would be a new one.

In the present study, we first examined reactivities with *Cryptosporidium* parasites by chicken mAbs. Immune systems of chickens are different from those of mammals in mechanisms of B cell repertoire formation (Reynaud et al., 1994), and chicken IgGs are considered to be useful for immunochemical research and clinical application (Nishinaka et al., 1996). By comparing with *Cryptosporidium* antigens recognized by mouse mAbs, the antigens recognized by chicken mAbs in the present study were found to be novel. Furthermore, the epitope recognized by the mAb 6D-12-G10 was found to be highly conserved among apicomplexan, parasites including *Eimeria*, *Toxoplasma*, *Neospora* and *Cryptosporidium*. Although further studies are needed to characterize these antigens of *Cryptosporidium*, these chicken mAbs, in particular 6D-12-G10, may have use as analytical tools for research on cryptosporidiosis.

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References

Abe, N., Kimata, I., Iseki, M., 2002. Identification of genotypes of *Cryptosporidium parvum* isolates from a patient and a dog in Japan. J. Vet. Med. Sci. 64, 165–168.

Bonnin, A., Dubremetz, J.F., Camerlynck, P., 1991. Characterization of microneme antigens of *Cryptosporidium parvum* (Protozoa, Apicomplexa). Infect. Immun. 59, 1703–1708.

Bonnin, A., Dubremetz, J.F., Camerlynck, P., 1993. A new antigen of *Cryptosporidium parvum* micronemes possessing epitopes cross-reactive with macrogamete granules. Parasitol. Res. 79, 8–14.

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

Bonnin, A., Ojcius, D.M., Souque, P., Barnes, D.A., Doyle, P.S., Gut, J., Nelson, R.G., Petersen, C., Dubremetz, J.F., 2001. Characterization of a monoclonal antibody reacting with antigen-4 domain of gp900 in *Cryptosporidium parvum* invasive stages. Parasitol. Res. 87, 589–592.

Cevallos, A.M., Bhat, N., Verdon, R., Hamer, D.H., Stein, B., Tzipori, S., Pereira, M.E., Keusch, G.T., Ward, H.D., 2000. Mediation of *Cryptosporidium parvum* infection in vitro by mucin-like glycoproteins defined by a neutralizing monoclonal antibody. Infect. Immun. 68, 5167–5175.

Constantinoiu, C.C., Lillehoj, H.S., Matsubayashi, M., Hosoda, Y., Tani, H., Matsuda, H., Sasai, K., Baba, E., 2003. Analysis of cross-reactivity of five new chicken monoclonal antibodies which recognize the apical complex of *Eimeria* using confocal laser immunofluorescence assay. Vet. Parasitol. 118, 29–35.

Constantinoiu, C.C., Lillehoj, H.S., Matsubayashi, M., Tani, H., Matsuda, H., Sasai, K., Baba, E., 2004. Characterization of stage-specific and cross-reactive antigens from *Eimeria acervulina* by chicken monoclonal antibodies. J. Vet. Med. Sci. 66, 403–408.

Colford Jr., J.M., Tager, I.B., Hirozawa, A.M., Lemp, G.F., Aragon, T., Petersen, C., 1996. Cryptosporidiosis among patients infected with human immunodeficiency virus. Factors related to symptomatic infection and survival. Am. J. Epidemiol. 144, 807–816.

Crabb, J.H., 1998. Antibody-based immunotherapy of cryptosporidiosis. Adv. Parasitol. 40, 121–149.

Fayer, R., Speer, C.A., Dubey, J.P., 1997. General biology of *Cryptosporidium*. In: Fayer, R. (Ed.), *Cryptosporidium* and Cryptosporidiosis. CRC Press, Boca Raton, Florida, pp. 1–41.

Hu, K., Roos, D.S., Murray, J.M., 2002. A novel polymer of tubulin forms the conoid of *Toxoplasma gondii*. J. Cell. Biol. 156, 1039–1050.

Iseki, M., Maekawa, T., Moriya, K., Uni, S., Takada, S., 1989. Infectivity of *Cryptosporidium muris* (strain RN 66) in various laboratory animals. Parasitol. Res. 75, 218–222.

Jenkins, M.C., O'Brien, C., Trout, J., Guidry, A., Fayer, R., 1999. Hyperimmune bovine colostrum specific for recombinant *Cryptosporidium parvum* antigen confers partial protection against cryptosporidiosis in immunosuppressed adult mice. Vaccine 17, 2453–2460.

Kato, S., Jenkins, M.B., Ghiorse, W.C., Bowman, D.D., 2001. Chemical and physical factors affecting the excystation of *Cryptosporidium parvum* oocysts. J. Parasitol. 87, 575–581.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lindsay, D.S., Blagburn, B.L., Toivio-Kinnucan, M., 1991. Ultrastructure of developing *Isospora suis* in cultured cells. Am. J. Vet. Res. 52, 471–473.

Lindsay, D.S., Speer, C.A., Toivio-Kinnucan, M.A., Dubey, J.P., Blagburn, B.L., 1993. Use of infected cultured cells to compare ultrastructural features of *Neospora caninum* from dogs and *Toxoplasma gondii*. Am. J. Vet. Res. 54, 103–106.

Lumb, R., Smith, K., O'Donoghue, P.J., Lanser, J.A., 1988. Ultrastructure of the attachment of *Cryptosporidium* sporozoites to tissue culture cells. Parasitol. Res. 74, 531–536.

Nina, J.M., McDonald, V., Dyson, D.A., Catchpole, J., Uni, S., Iseki, M., Chiodini, P.L., McAdam, K.P., 1992. Analysis of oocyst wall and sporozoite antigens from three *Cryptosporidium* species. Infect. Immun. 60, 1509–1513.

Nishinaka, S., Akiba, H., Nakamura, M., Suzuki, K., Suzuki, T., Tsubokura, K., Horiuchi, H., Furusawa, S., Matsuda, H., 1996. Two chicken B cell lines resistant to ouabain for the production of chicken monoclonal antibodies. J. Vet. Med. Sci. 58, 1053–1056.

Perryman, L.E., Kapil, S.J., Jones, M.L., Hunt, E.L., 1999. Protection of calves against cryptosporidiosis with immune bovine colostrums induced by a *Cryptosporidium parvum* recombinant protein. Vaccine 17, 2142–2149.

Petersen, C., Gut, J., Leech, J.H., Nelson, R.G., 1992. Identification and initial characterization of five *Cryptosporidium parvum* sporozoite antigen genes. Infect. Immun. 60, 2343–2348.

Pozio, E., Rezza, G., Boschini, A., Pezzotti, P., Tamburrini, A., Rossi, P., Di Fine, M., Smacchia, C., Schiesari, A., Gattei, E., Zucconi, R., Ballarini, P., 1997. Clinical cryptosporidiosis and human immunodeficiency virus (HIV)-induced immunosuppression: findings from a longitudinal study of HIV-positive and HIV-negative former injection drug users. J. Infect. Dis. 176, 969–975.

Reynaud, C.A., Bertocci, B., Dahan, A., Weill, J.C., 1994. Formation of the chicken B-cell repertoire: ontogenesis, regulation of Ig gene rearrangement, and diversification by gene conversion. Adv. Immunol. 57, 353–378.

Riggs, M.W., 1997. Immunology: host response and development of passive immunotherapy and vaccines. In: Fayer, R. (Ed.), *Cryptosporidium* and Cryptosporidiosis. CRC Press, Boca Raton, Florida, pp. 129–161.

Riggs, M.W., Mcneil, M.R., Perryman, L.E., Stone, A.L., Scherman, M.S., O'Connor, R.M., 1999. *Cryptosporidium parvum* sporozoite pellicle antigen recognized by a neutralizing monoclonal antibody is a beta-mannosylated glycolipid. Infect. Immun. 67, 1317–1322.

Riggs, M.W., Stone, A.L., Yount, P.A., Langer, R.C., Arrowood, M.J., Bentley, D.L., 1997. Protective monoclonal antibody defines a circumsporozoite-like glycoprotein exoantigen of *Cryptosporidium parvum* sporozoites and merozoites. J. Immunol. 158, 1787–1795.

Robert, B., Antoine, H., Dreze, F., Coppe, P., Collard, A., 1994. Characterization of a high molecular weight antigen of *Cryptosporidium parvum* micronemes possessing epitopes that are cross-reactive with all parasitic life cycle stages. Vet. Res. 25, 384–398.

Sagodira, S., Buzoni-Gatel, D., Iochmann, S., Naciri, M., Bout, D., 1999. Protection of kids against *Cryptosporidium parvum* infection after immunization of dams with CP15-DNA. Vaccine 17, 2346–2355.

Sam-Yellowe, T.Y., 1996. Rhoptry organelles of the Apicomplexa: their role in host cell invasion and intracellular survival. Parasitol. Today 12, 308–316.

Sasai, K., Lillehoj, H.S., Hemphill, A., Matsuda, H., Hanioka, Y., Fukata, T., Baba, E., Arakawa, A., 1998. A chicken anti-conoid monoclonal antibody identifies a common epitope which is present on motile stages of *Eimeria*, *Neospora*, and *Toxoplasma*. J. Parasitol. 84, 654–656.

Sasai, K., Lillehoj, H.S., Matsuda, H., Wergin, W.P., 1996. Characterization of a chicken monoclonal antibody that recognizes the apical complex of *Eimeria acervulina* sporozoites and partially inhibits sporozoite invasion of $CD8^+$ T lymphocytes in vitro. J. Parasitol. 82, 82–87.

Slifko, T.R., Friedman, D., Rose, J.B., Jakubowski, W., 1997. An in vitro method for detecting infectious *Cryptosporidium* oocysts with cell culture. Appl. Environ. Microbiol. 63, 3669–3675.

Uni, S., Iseki, M., Maekawa, T., Moriya, K., Takada, S., 1987. Ultrastructure of *Cryptosporidium muris* (strain RN 66) parasitizing the murine stomach. Parasitol. Res. 74, 123–132.

Weir, S.C., Pokorny, N.J., Carreno, R.A., Trevors, J.T., Lee, H., 2001. Improving the rate of infectivity of *Cryptosporidium parvum* oocysts in cell culture using centrifugation. J. Parasitol. 87, 1502–1504.

Woodward, M.P., Young Jr., W.W., Bloodgood, R.A., 1985. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Methods 78, 143–153.

(65) Denaturing gradient gel electrophoresis (DGGE) 法による水中のクリプトスポリジウムの種・遺伝子型判別手法の開発

Genotyping of *Cryptosporidium* spp. in water by denaturing gradient gel electrophoresis (DGGE).

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ABSTRACT; A new genotyping method of *Cryptosporidium*, denaturing gradient gel electrophoresis (DGGE) followed by DNA sequencing, was developed to genotype *Cryptosporidium* spp. in river water. The DGGE method could successfully discriminate nine species of *Cryptosporidium*: *C. parvum*, *C. hominis*, *C. canis*, *C. meleagridis*, *C. felis*, *C.* sp. strain 938, *C. andersoni*, *C. serpentis* and *C. saurophilum*. The sequential combination of the QProbe PCR method for quantification; and the DGGE method and DNA sequencing for genotyping, enabled the simultaneous quantification and genotyping of *Cryptosporidium* spp. in the same water sample. This method was applied to analyze *Cryptosporidium* in the Koyama River, a tributary of the Tone River, both for the total concentration of organisms, and for their genotype. Seven *Cryptosporidium* genotypes (*C. andersoni, C.* sp. strain 938, *C. parvum*, *C. hominis*, *C.* sp. PG1-26, *C.* sp. t03, *C.* sp. t04) were found in 11 positive samples (positive ratio = 69%). A bovine specific species, *C. andersoni*, was found most frequently (7 samples). The genotypes infectious to human accounted for only 16% of the concentration of all genotypes. These results showed that this detection method could provide valuable information on *Cryptosporidium* in river water both in the quantity and in the genotypes, which is essential for the precise assessment of waterborne risk to human health.

KEYWORDS; *Cryptosporidium*, genotyping, denaturing gradient gel electrophoresis (DGGE)

1. 研究の背景

1984 年に、テキサス州で最初のクリプトスポリジウムによる感染症患者が報告されて [1]以来、クリプトスポリジウムによる大規模な感染症集団発生が世界各地で発生している。これまでに発生した中で最も大きな被害を出したのは、1993 年 3 月にアメリカ合衆国ウィスコンシン州ミルウォーキー市で発生した事例である [2,3]。この事例では、人口 161 万人のうち 403,000 人が発症したと報告されている [2,3]。この集団発生による経済的な損失は総計 9,620 万ドルに達したと試算されている [4]。後の調査により、この集団発生の原因となったのは、ヒトに特異的に感染する *C. hominis* (*C. parvum* genotype I) であったことが示されている [5]。国内でも、1996 年に埼玉県越生町全町民 13,800 人中 8,800 人以上が感染したという事例が報告されている [6]。本事例も、ミルウォーキー市の事例と同様に、水道水が *C. hominis* により汚染されたことが原因となって発生したことが確認されている [7]。

クリプトスポリジウムの分類に関しては、2004 年の報告に限っても、その分類方法や総数がまちまちである [8-11]が、例えば Xiao ら [8]の分類法にしたがうと、現在クリプトスポリジウム属には 13 の種が存在して

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おり、その宿主は哺乳類、鳥類、爬虫類、魚類と多岐にわたる。この中で、ヒトへの感染のほとんどは、*C. parvum*（*C. parvum* genotype II）または *C. hominis* によるものであるとされている。これ以外の種では、*C. meleagridis*[7,12-17]、*C. felis*[15,18]、*C. canis*[15,18]、*C. muris*[19]、*C.* sp. pig genotype[20]、*C.* sp. deer genotype[21]、*C.* sp. monkey genotype[22]がヒトに対して感染した例が報告されている。このように、一口にクリプトスポリジウムといっても、その中には多くの種が存在し、ヒトに対して感染力を持つ種はそのうちごく一部である。したがって、環境中、例えば水中のクリプトスポリジウムの動態を評価するにあたり、その種・遺伝子型について調査することが重要である。

これまでにも、水中のクリプトスポリジウムの種・遺伝子型の判別のための手法が開発されてきた（例えば restriction fragment length polymorphism（RFLP）法[23,24]、single strand conformational polymorphism（SSCP）法[25-27]、クローニングーシーケンシング法[23,24,28,29]）。しかし、これらの手法には、技術的、あるいは実用的な面で限界があることが指摘されている。例えば RFLP 法では、その識別能力が増幅領域内部に存在する制限酵素が切断する部位の数に依存しているため、識別可能な種・遺伝子型に限界が存在する[30-33]。SSCP 法は、1 本鎖の状態で電気泳動を行うという特質上、単一の種が複数のバンドを形成する[25]ため、多数の種を対象とした判別手法の確立には至っていない。クローニングーシーケンシング法は、1 つの試料から得られた多数のクローン DNA についてシーケンシングを行う必要があるため、多数の検体からの検出には非常に費用がかかる。現在、これらの問題を解決し得る新しい種・遺伝子型の判別手法の確立が望まれている。

以上の背景を受けて、本研究では、クリプトスポリジウムの種・遺伝子型の類別に Denaturing Gradient Gel Electrophoresis（DGGE）法を適用し、シーケンシング法と組み合わせることで、水中のクリプトスポリジウムの種・遺伝子型を判別する手法を開発した。そして、その手法を実河川水に対して適用することで、開発した手法の有用性を評価すると共に、国内の河川水中に存在するクリプトスポリジウムの種・遺伝子型を調査した。

2．実験手法

2．1．DGGE 法による種・遺伝子型の類別力調査

（1）供試したクリプトスポリジウムの種・遺伝子型

国立感染症研究所 寄生動物部の遠藤 卓郎氏より分与していただいた、*C. parvum*（Accession No.: AF161856）、*C. hominis*（同 AF093491）、*C. canis*（同 AF112576）、*C. meleagridis*（同 AF112574）、*C. felis*（同 AF112575）、*C.* sp. strain 938（同 AY120913）、*C. andersoni*（同 AB089285）、*C. serpentis*（同 AF093502）、*C. saurophilum*（同 AF112573）のゲノム DNA を用いた。分与されたゲノム DNA を、QProbe PCR 法で使用するプライマーで増幅し、DGGE 法に供する試料とした。PCR 条件は既報[33]にしたがった。DGGE 法によりこれら 9 種類のゲノム DNA を類別できるかどうかを調査するため、試験には、これら 9 種類のゲノム DNA を単独で使用した試料と、全てのゲノム DNA を混合した試料の両方を用いた。

（2）DGGE 法による種・遺伝子型の類別手法

DGGE 法で解析対象とする遺伝子増幅領域は、クリプトスポリジウムに対して特異的であるのと同時に、種・遺伝子型の差による遺伝子配列の違いが大きい領域である必要がある。本研究では、クリプトスポリジウムの 18S rRNA 遺伝子のうち 295bp（*C. parvum* IOWA 株の場合）を増幅するプライマー[28]を使用した（表 1）。この増幅領域は、シーケンシング法による種・遺伝子型の判別に適用例があり[28]、また QProbe PCR 法で増幅する領域の内部に位置するため、QProbe PCR 法で得られた増幅産物に対して適用することが可能である。

GC クランプは、Muyzer ら[34]のもの（表 1）を使用した。この GC クランプを、フォワードプライマーにつけたものと、リバースプライマーにつけたものをそれぞれ用意し、予備実験を行った。その結果、フォワードプライマーに GC クランプをつけた試料は、DGGE において明瞭なバンドを生成したのに対し、リバ

表1 DGGEに用いたプライマーおよびGCクランプの塩基配列

Name	Sequence	Position in 18S rRNA gene of *C. parvum* (AF164102)	Reference
フォワード	AGT GAC AAG AAA TAA CAA TAC AGG	445－468	28
リバース	CCT GCT TTA AGC ACT CTA ATT TTC	716－739	
GCクランプ	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G		34

ースプライマーにGCクランプをつけた試料は、バンドを生成しなかった。そこで、以下の実験では、フォワードプライマーの5'末端にGCクランプをつけたものを用いた。

DNAの増幅にはT3 Thermocycler（Whatman Biometra, Goettingen, Germany）またはGeneAmp9600（Applied Biosystems, 東京）を用いた。PCR反応液（50μL）の組成は、2.5[U] AmpliTaq Gold（Applied Biosystems, 東京）、10×PCRバッファー5[μL]、dNTP各200[nM]、$MgCl_2$ 2[mM]、テンプレートDNA（QProbe PCR産物）1 [μL]とし、各プライマー濃度は500[nM]とした。温度条件は、94℃5分の熱変性の後、94℃30秒、60℃30秒、72℃30秒のサイクルを30回行い、最後に72℃5分で伸長を行った。

ゲルの作成および電気泳動は、Dcodeシステム（Bio-Rad, 東京）を用いて行った。変性剤（ホルムアミド、尿素）濃度範囲は5%～25%とし（変性剤濃度100%とは、ホルムアミド20[mL/50mL gel sol.]、尿素7[M]に相当する）、泳動条件は、130[V]、60[℃]で10[hour]とした。泳動後、ゲルをVistra Green（GE healthcare, 東京）で染色し、蛍光イメージアナライザーFluorImager595（GE healthcare, 東京）で画像を取り込み、付属のソフトウェアで画像解析を行った。

2．2．小山川における現地調査

（1）調査地点の概要

測定試料は、小山川中流部の高橋（埼玉県深谷市大字矢島）にて採取した。調査は、2004年8月から10月までの3ヶ月間に合計4回行った。各測定日に1時間半間隔で連続的に4つの試料を採取することで、合計16の試料を得た。小山川は、利根川の支流のひとつで、武蔵水路の取水口である利根大堰の上流約15kmの地点で利根川右岸に流入している。試料採取地点の上流側に位置する市町村（埼玉県本庄市、上里町、美里町、児玉町、神川町、岡部町）の市町村の人口は合計156,000人であった（平成15年6月）。

（2）河川水の濃縮および夾雑物の除去

試料水を、ポリエチレン製MF中空糸膜モジュール（孔径0.1[μm]、有効濾過面積4,000[cm^2]、三菱レイヨン、東京）で濾過した。濾過時間は約1時間とし、濾過速度は、U.S.EPA[35,36]が推奨している2 [L/min]という上限を超えていないことを確認した。これにより、おおよそ30～70[L]の河川水を膜に通水し、その中に含まれている懸濁物質を回収することができた。

得られた懸濁液を遠心分離（1,050×g、10 [min]）により濃縮し、高密度Percoll-ショ糖溶液を用いた密度勾配遠心法と免疫磁気ビーズ法を組み合わせた手法（PS-IMS法）[37]により精製した。本手法は、八木ら[38]の手法を改変したもので、大容量の水試料に対して適用しても、高いオーシスト／シスト回収率を得ることができるとされている。免疫磁気ビーズを用いたクリプトスポリジウムの精製には、U.S.EPAにより定められた手法[36]に従い、Dynabeads CG-Combo Kit（Dynal Biotech, Oslo, Norway）を用いた。

（3）DNA 抽出および濃縮

25%（w/w）の Chelex 100 樹脂（Bio-Rad, California, U.S.A.）懸濁液 100 [μL]を、精製済みの試料 100[μL]に添加し、凍結融解（-80℃、95℃、5 回）によりオーシストからゲノム DNA を抽出した。次に、抽出液全量を、遠心式 MF 濾過ユニット（GHP Nanosep MF centrifugal device, Pall, 東京）に移し、10,000×g で 5 分間遠心分離して Chelex 100 樹脂および夾雑物を取り除いた。濾液を DNA 精製キット（QIAamp DNA Mini Kit, QIAGEN, 東京）で精製したのち、液全量を遠心式フィルターユニット（Microcon YM-100, Millipore,東京）に移し、500×g で 25 分間遠心分離した。最後に、フィルター部分に 10[μL]の超純水を加えてから上下逆向きにセットし、500×g で 3 分間遠心分離して濃縮 DNA を得た。最終液量は約 10[μL]となった。

（4）クリプトスポリジウムの検出、種・遺伝子型の判別

QProbe PCR 法により、試料中のクリプトスポリジウム濃度を定量した。実験条件は既報[33]にしたがった。ただし、プライマー濃度をフォワードプライマー濃度 600[nM]、リバースプライマー濃度 200[nM]に変更した。その結果、検出下限は 0.83[oocyst/tube]、定量下限は 2.5[oocyst/tube]にそれぞれ改善された。

DGGE 法は、2．1．（2）で示した手法で行った。必要に応じて PCR と DGGE を繰り返し、DGGE で単一のバンドが観察されるまで、DNA の精製を行った。得られた DNA 断片を、Montage PCR m96 プレート（Millipore, 東京）で精製した後、BigDye Terminator v3.1 Cycle Sequencing Kit（Applied Biosystems, 東京）を用いたシーケンシング反応を行った。反応後の液を Montage SEQ プレート（Millipore, 東京）で精製してシーケンシングに供した。シーケンシングは、ABI 3100（Applied Biosystems, 東京）で行った。

（5）ポアソン分布を基にした最尤法（MPN 法）による種・遺伝子型ごとの濃度推定

（4）で示した手法は、ある試料中に存在する全ての種・遺伝子型を合わせたの濃度と、その試料中に存在する種・遺伝子型を検出できるが、個々の種・遺伝子型の濃度を知ることができない。そこで、以下の手法で、河川水中のそれぞれの種・遺伝子型の存在濃度を推定した。

ある種・遺伝子型の河川水中の存在濃度は、時間によらず一定で、x[oocyst/100L]であるとする。また、濃縮および精製操作における回収率は考慮せず、また検出下限は 1[oocyst/sample] であるとする。このとき、ある試料（検査水量 V[L]）中から、その種・遺伝子型が検出される確率 $P_{positive}$、および検出されない確率 $P_{negative}$ は、ポアソン分布の確率分布関数より、それぞれ以下の式（1）で計算できる。

$$P_{positive}(x,V)=1-\exp\left(-\frac{x\times V}{100}\right),\quad P_{negative}(x,V)=\exp\left(-\frac{x\times V}{100}\right) \qquad \text{式（1）}$$

ただし、x：河川水中のクリプトスポリジウム濃度 [oocyst/100L]、V：試料水量 [L]

最尤法の原理により、河川水中に存在する、ある種・遺伝子型の濃度の推定値は、下式（2）の$-LN$ の値を最小にする x に等しい。

$$-LN=-\sum_{i=1}^{n}\ln\left(F(x,V_i)\right) \qquad \text{式（2）}$$

ただし、V_i：i 番目の試料の試料水量 [L]、n：試料数（ここでは n=16）

$$F(x,V_i)=\begin{cases}P_{positive}(x,V_i) & \text{when the } i \text{ th sample was positive for } \textit{Cryptosporidium}\\ P_{negative}(x,V_i) & \text{when the } i \text{ th sample was negative for } \textit{Cryptosporidium}\end{cases}$$

この操作を、小山川の試料から検出された全ての種・遺伝子型に対してそれぞれ行い、個々の存在濃度の推定値を算出した。

3．実験結果

3．1．DGGE 法による種・遺伝子型の類別能力の評価

図１に、DGGE により得られたゲルの画像を示す。全ての配列が、変性剤濃度がおおよそ 8%～20%のところにバンドを生成した。全てのバンドが作成した変成剤濃度域にあったことと、バンドの生成位置が濃度域全体に分散していたことから、この実験条件が、本研究で評価対象とした DNA 領域に対する DGGE 法として適していることが確認された。

ほとんどの種は、明瞭な１本のバンドを生成したが、*C. andersoni* は、明瞭な１本のバンドのみが見られた試料（レーン７）と、１本の濃いバンドと、２本の薄いバンドの計３本を生成した試料（レーン８）が見られた。小山川で得た試料においても同様の傾向が見られたことから、この種については、同じ塩基配列ではあるが、なんらかの要因で３本のバンドを生じることがあることが示された。

それぞれの種・遺伝子型のバンドの位置を見ると、いくつかの種が近い位置（変性剤濃度）にバンドを生成したことがわかる。たとえば、*C. parvum*、*C. hominis*、*C. canis* が非常に近い位置にバンドを生成した。しかし、9 種類を混合した試料（レーン１１）を見ると、それぞれが違う位置にバンドを生成していることから、仮に同一試料にこれらの種・遺伝子型が混在していても、それを類別することが可能であるといえる。

以上より、本研究で開発した DGGE 法により、今回使用した 9 種類の種・遺伝子型をそれぞれ他の種由来のバンドと識別することが可能であるといえる。

3．2．小山川における調査結果

表２に、各試料の検査水量、QProbe PCR 法によるクリプトスポリジウムの検出結果、および検出された

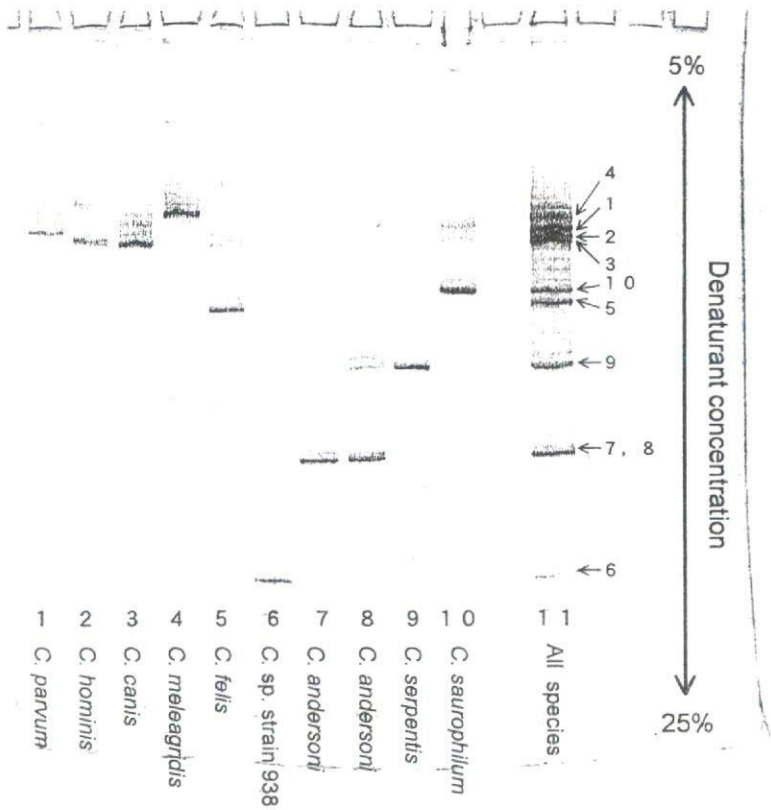

図１ DGGE 法による種・遺伝子型の類別（DGGE ゲル写真）
（種名の上の数字は本文中のレーン番号を示す。
レーン１１右側の数字は、それぞれのレーン番号の試料と同じであることを示す。）

表２ 小山川でのクリプトスポリジウム調査結果

採水開始時間	検査水量 [L]	検出結果[a]	検出されたクリプトスポリジウムの種・遺伝子型[b]			
8/3 11:00	45.2	−				
8/3 12:30	32.0	−				
8/3 14:00	43.3	+	*C. hominis*[c]	*C.* sp. 938		
8/3 15:30	33.5	+	*C.* sp. 938			
8/26 11:00	56.3	−				
8/26 12:30	43.1	−				
8/26 14:00	37.8	−				
8/26 15:30	65.1	+	*C. andersoni*			
9/16 10:30	41.6	+	*C. andersoni*			
9/16 12:00	47.4	+	*C. parvum*[c]	*C.* sp. 938		
9/16 13:30	47.4	+	*C. andersoni*			
9/16 15:00	66.1	+	*C.* sp. PG1-26			
10/27 10:45	30.9	59	*C. andersoni*	*C.* sp. 938	*C.* sp. t03	*C.* sp. t04
10/27 12:15	53.4	+	*C. andersoni*			
10/27 13:45	23.8	+	*C. andersoni*	*C. parvum*[c]		
10/27 15:15	44.9	14	*C. andersoni*			

a −：非検出、＋：検出、ただし濃度は定量下限（2.5[oocysts/sample]）以下のため不明、数値：検出、定量下限以上（値は濃度 [oocysts/100L]）

b それぞれの種の遺伝子配列：

C. parvum（AF164102）、*C. hominis*（AY204231）、*C. andersoni*（AB089285）、*C.* sp. 938（AY120913）、*C.* sp. PG1-26（AY271721）、*C.* sp. t03（AB231612）、*C.* sp. t04（AB231613）

c 網掛け部は、ヒトへの感染が報告されている種を示す。

クリプトスポリジウムの種・遺伝子型を示す。クリプトスポリジウムは、16 試料中 11 試料から検出された（試料陽性率 69%）。また DGGE-シーケンシング法により、合計 7 種類の遺伝子配列が検出された。そのうち 3 種類は既存の 3 つの種（*C. parvum*（AF164102）、*C. hominis*（AY204231）、*C. andersoni*（AB089285））の遺伝子配列と一致した。2 種類（*C.* sp. 938（AY120913）、*C.* sp. PG1-26（AY271721））は、まだどの種にも分類されていない遺伝子型であり、残りの 2 種類（*C.* sp. t03（AB231612）、*C.* sp. t04（AB231613））は、*C.* sp. 1665 と相同性の高い（共に 97%）未知の配列であった。同一試料に複数の種・遺伝子型が含まれていたのは陽性 11 試料中 4 試料で、そのうち 3 試料は 2 種類の遺伝子配列、残りの 1 試料では 3 種類の遺伝子配列が検出された。最も多く検出されたのは、ウシに特異的に感染するとされる *C. andersoni*（7 試料、44%）で、以下 *C.* sp. 938（4 試料、25%）、*C. parvum*（2 試料、13%）の順となった。*C. hominis*、*C.* sp. PG1-26、*C.* sp. t03、*C.* sp. t04 の近縁種はそれぞれ 1 試料ずつからのみ検出された。ヒトへの感染が報告されている種・遺伝子型では、*C. parvum* と *C. hominis* の 2 種類が、計 3 試料（19%）から検出された。

図２に、ポアソン分布に基づいた最尤法（MPN 法）により推定した、個々の種・遺伝子型の濃度を示す。最も濃度が高かったのは、検出数が最も多かった *C. andersoni*（16 試料中 7 試料から検出）で、1.3 [oocysts/100L] であった。次いで、*C.* sp. 938（同 4 試料）の 0.63 [oocysts/100L]、*C. parvum*（同 2 試料）の 0.30 [oocysts/100L] の順となった。全ての種を合わせたクリプトスポリジウム濃度は 2.8[ooysts/100L]であったのに対し、ヒトに対する感染力のある種のみの濃度は 0.44[ooysts/100L]であり、全体の約 16%であった。