

Metagonimus yokogawai: a 100-kDa Somatic Antigen Commonly Reacting with Other Trematodes

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Abstract: This study was undertaken to characterize the properties of a 100 kDa somatic antigen from *Metagonimus yokogawai*. Monoclonal antibodies (mAbs) were produced against this 100 kDa antigen, and their immunoreactivity was assessed by western blot analysis with patients' sera. The mAbs against the 100 kDa antigen commonly reacted with various kinds of trematode antigens, including intestinal (*Gymnophalloides seoi*), lung (*Paragonimus westermani*), and liver flukes (*Clonorchis sinensis* and *Fasciola hepatica*). However, this mAb showed no cross-reactions with other helminth parasites, including nematodes and cestodes. To determine the topographic distribution of the 100 kDa antigen in worm sections, indirect immunoperoxidase staining was performed. A strong positive reaction was observed in the tegumental and subtegumental layers of adult *M. yokogawai* and *C. sinensis*. The results showed that the 100 kDa somatic protein of *M. yokogawai* is a common antigen which recognizes a target epitope present over the tegumental layer of different trematode species.

Key words: *Metagonimus yokogawai*, 100 kDa protein, common trematode antigen, tegument, immunoblot, immunohistochemistry

The intestinal fluke, *Metagonimus yokogawai*, is one of the important heterophyid flukes causing fishborne helminthic zoonoses in Eastern Asia, including the Republic of Korea, China, Taiwan, Japan, and Russia [1-3]. The infection is caused by eating raw sweetfish, *Plecoglossus altivelis*, and prevalent along riverside areas, in accordance with the distribution of snail and fish intermediate hosts [3,4]. This fluke infection can cause severe gastrointestinal troubles and chronic diarrhea, particularly in heavily infected cases [3,5].

For diagnosis of metagonimiasis, fecal examination to detect eggs is useful, and at times immunodiagnosis such as ELISA can be applied [3,6]. However, in immunodiagnosis, cross-reactivity is one of the most adverse obstacles [7]. Two types of antigens have been used; one is the metabolic antigen released from the excretory-secretory (ES) proteins of adult flukes, and the other is the somatic antigen which includes their tegumental proteins [8-10]. ES proteins, such as cysteine proteinases,

have been reported to be universal enzymes found in almost all parasite species and may be non-specific for each parasite species [11-14]. Similarly, a proteolytic enzyme, probably a hemoglobinase, from the ES product of *Fasciola hepatica* showed high cross reactivity with *Schistosoma mansoni* [15]. Tegumental proteins were also demonstrated to be either universal or species-specific in *M. yokogawai*, *Opisthorchis viverrini*, *F. hepatica*, and *Fasciola gigantica* infections [10,16-18].

In this study, we discovered a 100 kDa somatic protein of *M. yokogawai* and characterized its antigenic properties. Monoclonal antibodies (mAbs) were produced against this protein and reacted with various kinds of trematode, nematode, and cestode antigens. Immunohistochemistry was performed to determine the topography of this antigen in worm sections.

M. yokogawai-infected human serum samples were collected from 21 patients from an endemic area in Samchok-shi (city), Korea [19], who were egg positive for *M. yokogawai*. Healthy individual sera were collected from a non-endemic area in Korea. Metacercariae of *M. yokogawai* were collected through artificial digestion of naturally infected sweetfish (*Plecoglossus altivelis*), which were caught in the same endemic area. Two hundred metacercariae each were fed orally to 10 Sprague-Dawley rats. The rats were killed at week 2 post-infection and adult flukes

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were recovered.

The crude antigens of *M. yokogawai* were prepared by homogenization of adult flukes in the presence of E-64 (20 μ M) (Sigma-Aldrich Co., St. Louis, Missouri, USA), a cysteine proteinase specific inhibitor, followed by centrifugation at 15,000 g for 1 hr. Protein concentrations were determined by the method of Lowry et al. [20]. The supernatant was stored at -70°C until used as the source of crude antigen. Fresh live worms, about 200 in number, were incubated overnight aseptically at 37°C in sterile physiological saline containing penicillin/streptomycin (100 units/ml and 100 $\mu\text{g}/\text{ml}$) (Invitrogen, Carlsbad, California, USA). The supernatant was used as the excretory-secretory (ES) antigen after centrifugation at 15,000 g for 1 hr. Worms and eggs were harvested, homogenized, and centrifuged.

To check the cross reactivity of the mAb, crude antigens were prepared from various helminths, which included trematodes such as *Gymnophalloides seoi* (adults from experimentally infected mice and metacercariae from oysters), *Clonorchis sinensis* (adults from experimentally infected rabbits), *Paragonimus westermani* (adults from experimentally infected dogs), and *F. hepatica* (adults from naturally infected cattle), nematodes including *Anisakis* sp. (third-stage larvae from naturally infected fish *Anago anago*) and *Trichinella spiralis* (third-stage larvae from experimentally infected rats), and cestodes such as *Taenia solium* metacestode (cystic fluid from naturally infected swine), *Echinococcus granulosus* (cystic fluid from a naturally infected human), and *Taenia asiatica* (gravid proglottid from a naturally infected human).

For production of mAbs, female BALB/c mice (6-week-old) were immunized intraperitoneally with 100 μg crude antigen of *M. yokogawai* adult worms in 0.2 ml normal saline (0.85% NaCl in H_2O) emulsified in complete Freund's adjuvant. The second injection was given 3 weeks later. The final boost was given 2 weeks later by intravenous injection with 60 μg of this antigen in 0.1 ml. To produce hybridoma, spleen cells from BALB/c mice immunized with the antigen were fused with mouse myeloma cells, P3X63-Ag8.653. MAb-secreting wells were screened by ELISA and immunoblot analysis, and positive wells were expanded in RPMI 1640 and Dulbecco's Modified Eagle's media (DMEM) (Invitrogen).

The antigenic proteins were separated by SDS-PAGE under reducing conditions. The separated proteins were transferred onto 0.45 μm PVDF membranes (Millipore, Billerica, Massachusetts, USA) in a transfer buffer (50 mM Tris, 190 mM glycine, 3.5 mM SDS, 20% methanol) at a constant 400 mA for

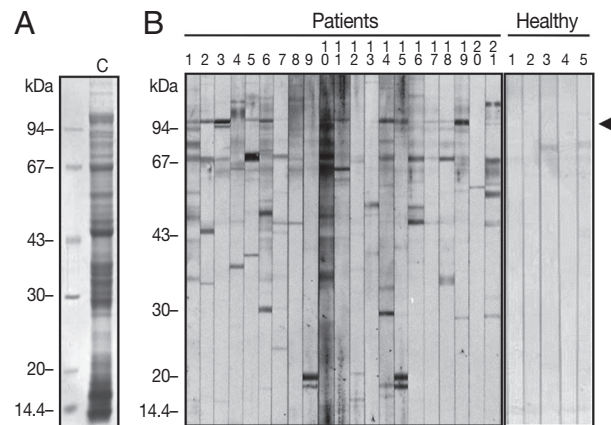


Fig. 1. SDS-PAGE of *Metagonimus yokogawai* crude antigen (A) and immunoblot analysis with sera of *M. yokogawai*-infected patients and healthy individuals (B). The crude antigen (marked 'C' in [A]) consisted of a large number of soluble antigens of different size. Several antigenic bands, including 100 kDa and 67 kDa (B), were reactive against the parasite-infected patients' sera.

40 min. After blocking with 5% skim milk in PBS containing 0.2% Tween 20 (PBS/T), metagonimiasis patient's sera were reacted with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Cappel, Cochranville, Pennsylvania, USA) diluted in 1:1,000 and HRP-conjugated goat anti-mouse IgM (Cappel) diluted in 1:2,000. The final reactions were developed with 4-chloro-1-naphthol (4C1N) and H_2O_2 .

Immunolocalization of the 100 kDa antigen was carried out in paraffin-embedded worm sections. Adult flukes (*M. yokogawai* and *C. sinensis*) recovered from experimental animals were processed for immunohistochemistry using *M. yokogawai* 100 kDa-specific mAb (only RPMI medium for controls) as the primary antibody, and HRP-conjugated goat anti-mouse IgG (Cappel) as the secondary antibody. Color was developed by diaminobenzidine.

The crude antigen of *M. yokogawai* adults consisted of major 100, 67, 46, 28-35, and 17 kDa bands (Fig. 1A). In western blot analysis, several antigenic proteins reacted with the patients' sera at 100, 67, 46, 30, and 20 kDa, whereas no special reactive bands were visible in healthy controls (Fig. 1B). Among these, 100 kDa and 67 kDa proteins showed most frequently strong reactions with the patients' sera (Fig. 1B).

Five hybridoma clones (7B11-1E, 5C10-2B, 8D3-5E, 2A7-2C, and 4G3-6D) were identified to produce specific antibodies against the 100 kDa antigen. Among these, the mAb 5C10-2B (Fig. 2A) had the highest titer and used in subsequent experiments. In immunoblots with crude adult and egg antigens of *M. yokogawai*, the mAb 5C10-2B reacted strongly with the adult

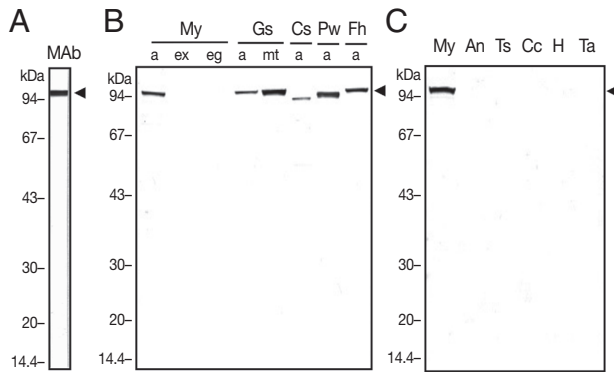


Fig. 2. (A) Immunoblot patterns of the 100 kDa-specific monoclonal antibodies (mAbs). The mAbs were reacted with the 100 kDa antigen of *M. yokogawai* adults. (B, C) Reactivity of the mAbs to crude antigens of several species of trematodes (B), nematodes, and cestodes (C). MABs were reactive against similar-sized antigens (around 100 kDa) of various trematodes but not nematode and cestode antigens. My, *M. yokogawai*; Gs, *Gymnophalloides seoi*; Cs, *Clonorchis sinensis*; Pw, *Paragonimus westermani*; Fh, *Fasciola hepatica*; An, anisakid larvae; Ts, *Trichinella spiralis*; Cc, cysticercus cystic fluid; H, hydatid cystic fluid; Ta, *Taenia asiatica* gravid proglottid; a, adult; eg, egg; mt, metacercariae.

antigen but not with the egg antigen (Fig. 2B). In immunoblots with crude antigens from adults and metacercariae of *G. seoi*, adult *C. sinensis*, adult *P. westermani*, and adult *F. hepatica* using the *M. yokogawai*-specific mAb, antigenic proteins of about 100 kDa were detected in all of the flukes examined (Fig. 2B). By contrast, in immunoblots with crude antigens of nematodes (*Anisakis* sp. and *T. spiralis* third-stage larvae) and cestodes (*T. solium* metacestode cystic fluid, *E. granulosus* cystic fluid, and *T. asiatica* gravid proglottid) using the *M. yokogawai*-specific mAb, this antigen, that appeared to be common to trematodes, was not detected (Fig. 2C).

In indirect immunohistochemical staining of sections of adult *M. yokogawai* using the mAb as the primary antibody, a high intensity of brown color was observed in the tegument, particularly around subtegumental cells (data not shown). Similar patterns and intensity of immunohistochemical staining were observed non-specifically in the tegument of *C. sinensis* also (data not shown).

In our study, we produced 5 mAbs against the 100 kDa tegumental protein of *M. yokogawai* adult. Among them, the clone 5C10-2B was the most potent one. Western blot analyses strongly suggested that the 100 kDa protein of *M. yokogawai* is a common antigen among different trematode species, including *G. seoi*, *C. sinensis*, *P. westermani*, and *F. hepatica*. This protein was localized in the tegument and subtegument of adult

M. yokogawai but not present in their eggs.

In a previous ultrastructural study, the antigenic localization in *M. yokogawai* adult parasite was determined using the serum of cats experimentally infected with the metacercariae [16]. It was suggested that the major antigenic protein was specifically concentrated at the tegumental syncytium as well as in the cytoplasm of tegumental cells and epithelial lamella of the cecum. In our study, a 100 kDa antigen was detected in the tegument of the adult *M. yokogawai*. This antigen may be a protein released from the tegument, together with other kinds of proteins and metabolic enzymes, such as proteases, which are released from the cecum of the worm. This discharge of antigens from the tegument may arise as a consequence of the continual renewal of the surface membrane and linked glycocalyx, as was reported to occur generally in trematode parasites that dwell in the bile duct, such as the sheep liver fluke, *F. hepatica* [21].

In a previous western blot analysis of juvenile *M. yokogawai* and serum samples of infected patients, major antigens were detected at around 200, 100, 70, 66, 48, 40, 38, 28-31, 22, 16, 10, and 8 kDa areas [22]. Among them, 66 and 22 kDa antigens showed the most strong reactions. However, viewing from their immunoblot figures, thick and strong reaction bands also occurred between 90 and 120 kDa, among which the 100 kDa protein identified in our study may have been included. Some heterologous sera of clonorchiasis, paragonimiasis, and fascioliasis patients showed positive reactions at around 100 kDa of *M. yokogawai* metacercarial antigen [22], as observed in our study.

In conclusion, we discovered a 100 kDa tegumental antigen from *M. yokogawai* adults and produced 5 mAbs against this antigen. One of these mAbs, the clone 5C10-2B, was found to have a low specificity for *M. yokogawai* and cross-reacted with other trematode antigens but not with nematodes and cestodes.

CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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