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Development of monoclonal antibodies against polar filaments and spore valves of *Myxobolus honghuensis* (Myxosporea: Bivalvulida)

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ABSTRACT: *Myxobolus honghuensis* infects the pharynx of allogynogenetic gibel carp *Carassius auratus gibelio* (Bloch) and can cause high mortality. Only morphology-based diagnostic methods are currently available for clinical samples, but these methods are laborious and have low efficiency of detection. To overcome this problem, we designed a more sensitive diagnostic method. Two monoclonal antibodies (MAbs 1C7 and 3B7) were prepared by immunizing mice with soluble protein from sonicated *M. honghuensis* spores. Immunofluorescence analysis revealed that MAb 1C7 specifically reacts with polar filaments from spores, whereas MAb 3B7 identified protein localized on the spore valves. The isotypes of MAb 1C7 and MAb 3B7 were IgM and IgG1, respectively. Results of Western blot analysis revealed that MAb 1C7 recognized 2 prominent protein bands with molecular weights of 130 and 180 kDa, while MAb 3B7 recognized a protein band of 28 kDa. Thus, in this study we have developed 2 MAbs that have the potential for efficient detection of *M. honghuensis*. Moreover, identification of MAb 1C7 and MAb 3B7 allows for further studies of the functions and biochemical composition of polar filament and spore surface antigens.

KEY WORDS: Parasite · *Carassius auritus gibelio* · Immunofluorescence · Diagnostic methods · Specificity

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INTRODUCTION

Gibel carp *Carassius auratus gibelio* (Bloch) is a triploid gynogenetic, fast-growing, and commercially important fish species in China. It has been cultured for over 30 yr in China, with an increasing annual production of more than 2 Mt (Gui & Zhou 2010, Wang et al. 2011). However, some aquacultural strains of gibel carp have been threatened by various pathogens (Gui & Zhu 2012), including myxosporean parasites (Liu et al. 2010, 2012, 2014c, Zhang et al. 2010a, Zhai et al. 2012), which cause diseases that often result in mortality. Around 20 myxosporean species have been isolated from different tissues and organs of gibel carp (Eiras et al. 2005, 2014, Zhang et al. 2010b, Ye et al. 2012, 2014, Liu et al. 2014a,b). Among these parasites, the newly identified species *Myxobolus honghuensis* is highly pathogenic in gibel carp, causing reduced productivity of fish, and is responsible for serious economic impacts on fish farmers (Liu et al. 2012).

A sensitive diagnostic tool will help detect parasitic infections in their early stages and will aid in designing strategies to control infections. This paper describes the development of monoclonal antibodies (MAbs) against *M. honghuensis* that can be used for identification of the parasite. In addition, effectiveness of these MAbs may be useful to

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identify critical *M. honghuensis* antigens for vaccine development.

Within the last 30 yr, MAbs have been developed to investigate various myxozoans. In the first report of a MAb produced against a myxozoan, Bartholomew et al. (1989) used MAbs to determine changes in the antigenic profile of *Ceratonova* (syn. *Ceratomyxa*) *shasta* over its life cycle and found that some *C. shasta* antigens were specific to certain life stages. Adams et al. (1992) used MAbs as diagnostic reagents in an immunohistochemistry test and as potential probes to study cell surface antigens of the causative agent of proliferative kidney disease (PKD), viz. PKX (later identified as *Tetracapsuloides bryosalmonae*). At the start of the 21st century, preliminary molecular characterization of *Kudoa thyrsites* and *M. rotundus* was achieved by using MAbs specific for antigens on spore surfaces, polar capsules, and polar filaments (Chase et al. 2001, Lu et al. 2002). Previous work on other myxozoan parasites has demonstrated that MAbs are useful tools for rapid immuno-diagnostics and antigen identification, and highlights the potential of controlling these diseases through immunological methods (Adams et al. 1995, Saulnier & de Kinkelin 1996). Our study was planned with the following objectives: (1) to develop a diagnostic tool, and (2) to understand the biology of *M. honghuensis*. Knowledge gained from application of these 2 stringent MAbs will help us to discover actinospore stages and will also aid in the identification of peptides, such that information generated from these MAbs together will elucidate the role of polar filaments and spore valves during invasion and pathogenesis of *M. honghuensis*.

MATERIALS AND METHODS

Spore collection and purification

Eighty allogynogenetic gibel carp ranging from 8.5 to 18.2 g in weight and 5.3 to 12.4 cm in length were harvested with a fine-meshed seine from Honghu Lake, Honghu City, Hubei Province, China, in June 2012. Fish were transported to the laboratory (Department of Aquatic Animal Medicine, College of Fisheries, Huazhong Agricultural University), where they were euthanized with 0.2 mg ml^{-1} tricaine methanesulfonate (MS-222, Sigma) prior to dissection. This work involving animals was conducted at the Laboratory of Fish Disease at Huazhong Agricultural University. The method used is approved by the American Fisheries Society (2004). Identification procedures were conducted using the protocol described by Liu et al. (2012). The cysts of *Myxobolus honghuensis* were dissected from the pharyngeal tract of infected allogynogenetic gibel carp and purified as previously described (Chase et al. 2001). Briefly, fish tissues were minced and sequentially sieved through 220 and 100 µm nylon mesh screens, and spores were suspended in phosphate-buffered saline (PBS). The suspension was layered onto a 12 to 75% (v/v) gradient of modified colloidal silica made in PBS (Percoll, stock density 1.13 g ml^{-1}; Sigma) obtained by centrifugation at 1500 × *g* for 35 min in a swinging bucket rotor (Sigma). The spore layer in the 75% Percoll band was removed and the spores were washed twice in PBS. Samples were pooled and stored at −20°C until used.

MAb production

The purified spores were sonicated to prepare parasite immunogens following methods devised for *Neoparamoeba* spp. (Villavedra et al. 2007). Spores were sonicated in PBS, pH 7.4, for 15 cycles of approximately 38 W for 15 s, with 45 s intervals between cycles on ice using a Misonix sonicator 3000. Soluble proteins were obtained after centrifugation at 10000 × *g* (10 min) and used to immunize BALB/c mice following the procedure described by Herbert (1978). Briefly, 4 wk old female SPF BALB/c mice (n = 3) were immunized subcutaneously with 50 µg of purified soluble antigens (equivalent to 1 × 10^7 parasites) emulsified in complete Freund's adjuvant (Sigma). After the first injection, 2 subcutaneous booster injections with the same antigen emulsion with incomplete Freund's adjuvant (Sigma) were administered at an interval of 2 wk. BALB/c mice were injected intraperitoneally with 150 µg antigens 7 d before splenectomy.

MAbs were produced following the method of Campbell (1984). Briefly, mouse splenocytes were harvested and fused with SP2/0 cells using 50% polyethylene glycol. All hybridoma culture supernatants were screened using ELISA (see the following section below). The positive hybridoma cells were cloned by a limiting dilution, and stable hybridoma clones were selected to inject into liquid paraffin-pretreated abdominal cavities of BALB/c mice. We could not adopt the usual *in vitro* method since we obtained a low titer of antibodies, even though we produced stable hybridoma clones under cell culture conditions. We therefore used *in vivo* procedures to obtain ascite fluid rich in antibodies.

Subsequently, the MAbs were harvested and purified from the seroperitoneum with an antibody purification kit (NAbTM Protein A/G Spin Kit, Thermo Scientific). Activities of MAbs were determined by an indirect fluorescent antibody test (IFAT) and Western blot analysis as described below.

Indirect enzyme-linked immunosorbent assay (ELISA)

To screen hybridoma culture supernatants in MAb production and to get the titers of purified MAbs, 96-well plates were coated overnight at 4°C with 100 µl of sonicated spore protein diluted in bicarbonate coating buffer (pH 9.6) and then blocked with 5% bovine serum albumin (BSA) in PBS (PBSA) for 1 h at 37°C. After discarding the blocking buffer, the plates were incubated with 100 µl well^{-1} of serial 2-fold dilutions of purified MAbs and negative mouse serum (preimmune serum) in PBSA (from 1:400 to 1:992000) for 30 min at 37°C. After incubation, plates were washed 3 times with PBS containing 0.05% Tween-20 (PBST). A 1:1000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (BOSTER) was added, and the plates were then incubated for 30 min at 37°C. After washing, 50 µl well^{-1} substrate solution A (0.1 M citrate/phosphate buffer, pH = 5.0) and 50 µl well^{-1} substrate solution B (0.04% o-phenylenediamine; 0.14% H_2O_2) were added, and the plates were incubated for 10 min at room temperature. Reactions were terminated by the addition of 50 µl well^{-1} 2 M H_2SO_4, and optical densities were measured at 450 nm using a microplate reader (TECAN M200). Absorbance values that were at least 2 times that of the background level reactivity were considered positive. Isotyping of positive and stable MAbs was performed using hybridoma culture supernatants and an antigen-capture ELISA kit (Rapid ELISA Mouse MAb Isotyping Kit, Sigma) according to instructions provided by the manufacturer.

IFAT and cross-reactivity analysis

The location of antigens on the parasite was determined with an IFAT technique using MAbs according to Villavedra et al. (2007). For each immunoreaction, a smear of spore suspension was air dried and permeabilized using ice-cold acetone for 10 min at −20°C and dried in air. A reaction with 10 µl of primary antibody (1:1000 dilution of ascites using BSA dilution buffer) was carried out at 37°C for 20 min in a humidified chamber. Slides were washed in PBS twice for 10 min and incubated for 30 min with fluorescein isothiocyanate-labeled goat-anti-mouse IgG/IgM (H + L) secondary antibody (BOSTER, China) diluted 1:50 in hybridoma culture medium containing 10% fetal bovine serum at 37°C. The slides were washed 3 times with PBS. Slowfade™ equilibration buffer and fluorescence fading inhibitor (BOSTER, China) were added. The slides were then sealed with a coverslip, examined microscopically, and photographed (Zeiss).

The cross-reactivity analysis of MAbs using spores of *M. nielii* (Nie et Li, 1973) Landsberg et Lom, 1991 from gibel carp gill, *M. wulii* (Wu et Li, 1986) Landsberg et Lom, 1991 from gibel carp hepatopancreas, *M. musseliusae* Yakovchuk, 1979 from common carp gill filament, *M. turpisrotundus* Zhang, 2010 from gibel carp subepidermal skin tissues, *M. tsangwuensis* Chen, 1954 from common carp gills, *Thelohanellus wuhanensis* Xiao & Chen, 1993 from gibel carp skin, and *T. testudineus* Liu, 2013 from gibel carp skin. All tested species were collected during a survey for myxozoan parasite diversity in gibel carp and common carp in Honghu Lake, Hubei Province, China. We wanted to test whether the MAbs cross react with these species, which parasitize the same or similar hosts as *M. honghuensis*. All tested spores were identified and deposited (in PBS at −20°C) in the Laboratory of Fish Diseases, College of Fisheries, Huazhong Agricultural University.

Western blot

SDS-PAGE was conducted using the standard protocol (Laemmli 1970), and proteins were transferred to Immun-Blot™ polyvinylidene membranes (0.45 µm pore size; Bio-Rad). After blocking overnight with 1% BSA and 5% skimmed milk powder in TBST buffer (0.01 M Tris–HCl pH 8.0; 150 mM NaCl, and 0.05% Tween-20), blots were reacted with a 1:1000 ascite dilution for 1 h. After washing 3 times with TBST buffer, the membranes were incubated with HRP-conjugated goat anti-mouse IgG (BOSTER, China) secondary antibody (dilution of 1:3000 in blocking solution) for 1 h at 37°C. After washing 3 times with TBST buffer and 2 times with TBS (TBST without 0.05% Tween-20), supersignal dura chemiluminescence substrate (Pierce Chemical) was used to detect protein bands recognized by MAbs and visualized on a MicroChemi instrument (DNR Bio-Imaging Systems).

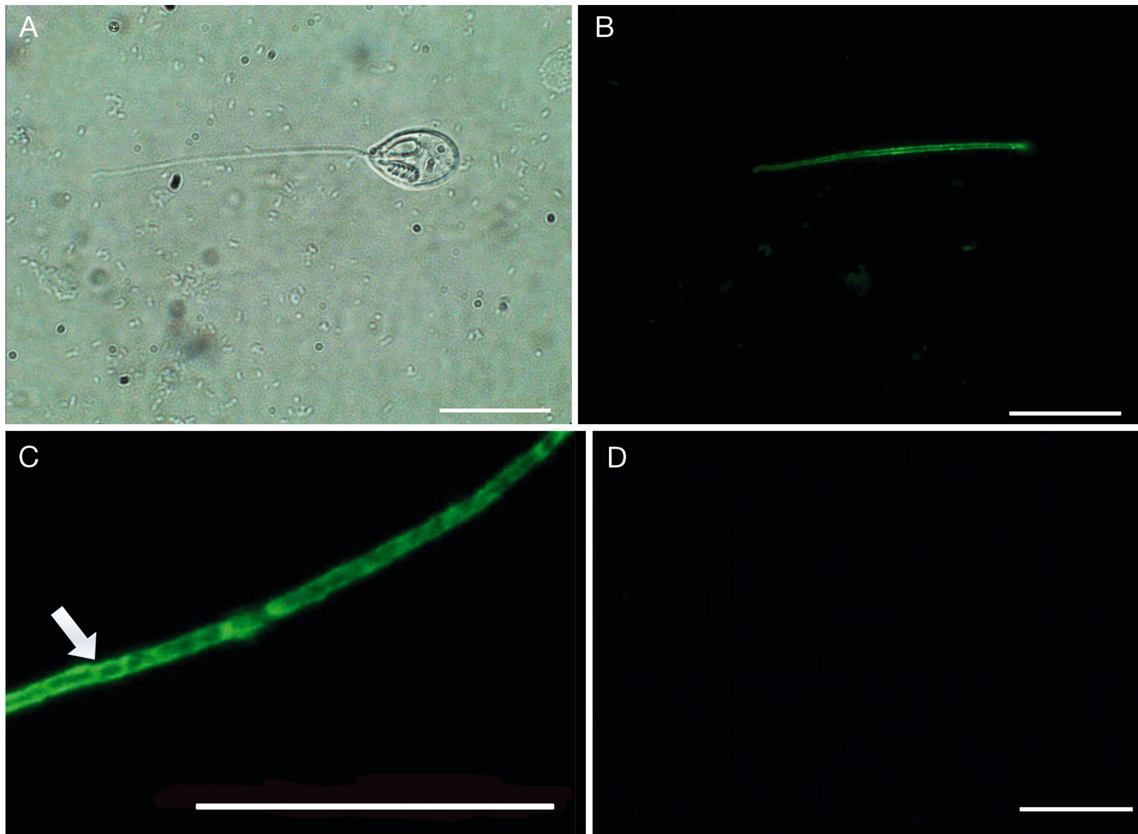

Fig. 1. (A) Light photomicrograph of *Myxobolus honghuensis.* (B) Extruded polar filament of *M. honghuensis* intensely stained with the monoclonal antibody MAb 1C7 following an indirect fluorescent antibody test. (C) High magnification of a polar filament showing banded appearance (arrow). (D) Control using *M. honghuensis* spore stain without primary antibody indicates that proteins on the filament are specifically recognized by MAb 1C7. Scale bars = 20 µm

RESULTS

Indirect ELISA and MAbs subtype

More than 100 hybridoma clones were obtained in the 96-well microplates 10 d post-fusion. All 9 positive hybridoma cells were cloned by a limiting dilution, and 2 stable hybridoma clones (1C7 and 3B7) were selected for isotyping, cryopreservation, and for further experiments. The isotypes of the selected MAbs (1C7 and 3B7) are IgM and IgG1, respectively. Effective ELISA titers of MAbs 1C7 and 3B7 were determined to be 1:124 000 and 1:248 000, respectively.

IFAT and cross-reactivity analysis

A bright field image of a *Myxobolus honghuensis* spore with a filament is shown in Fig. 1A. The same field by fluorescent microscopy using MAb 1C7 indicates that the antigen recognized is specifically localized to the polar filament of the spore (Fig. 1B). High magnification of the same parasite polar filament shows a braided-like appearance (Fig. 1C). Control staining without the primary antibodies (Fig. 1D) indicates that the MAb 1C7 specifically recognizes spore filament protein(s).

We further examined the interaction of MAb 3B7 with *M. honghuensis* spores. A bright field image of *M. honghuensis* spores is shown in Fig. 2A. Unlike MAb 1C7, MAb 3B7 recognized antigens across the entire spore surface, as indicated by prominently stained spores in Fig. 2B.

Reaction with other parasite species showed that MAb 1C7 is not reactive with other tested *Myxobolus* species, although it did react weakly with *Thelohanellus testudineus* (polar filament: Fig. 3A,B) and *T. wuhanensis* (top edge of spore: Fig. 3C,D), indicating that the location of identified antigen(s) is not the same among different parasites. Similarly, MAb 3B7 reacted weakly with the spore surface in *M. nielii*

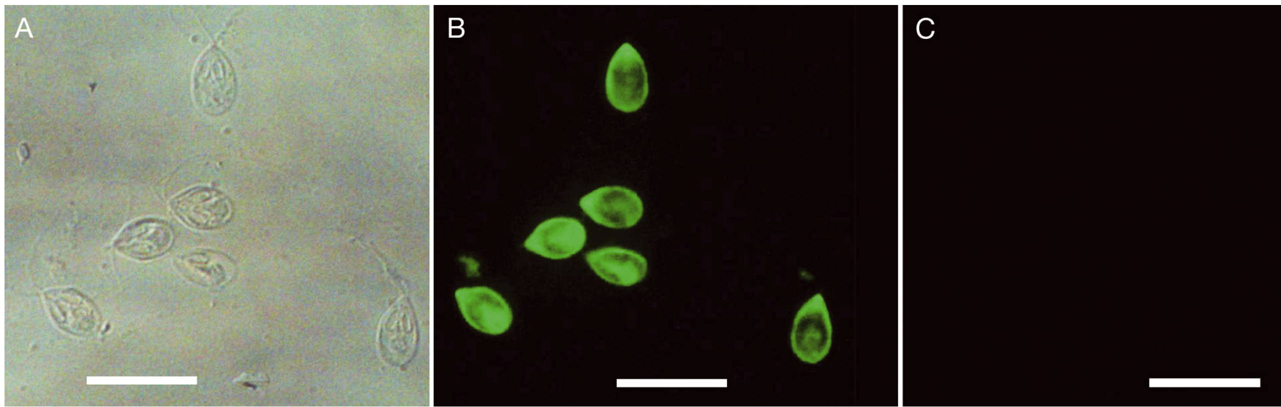

Fig. 2. (A) Light photomicrograph of *Myxobolus honghuensis*. (B) Antigen present in the spore valves reacts strongly with the monoclonal antibody MAb 3B7 following an indirect fluorescent antibody test. (C) Control using *M. honghuensis* spore stain without primary antibody indicates that spore proteins are specifically recognized by MAb 3B7. Scale bars = 20 µm

(Fig. 3I,J), *T. wuhanensis* (Fig. 3E,F), and *T. testudineus* (Fig. 3G,H). The reactivity of MAbs 1C7 and 3B7 with different parasite species is summarized in Table 1.

Western blot analysis

Immunoblotting of proteins of *M. honghuensis* resolved by SDS-PAGE was performed with 2 selected MAbs. MAb 1C7 recognized 2 protein bands with approximate molecular weights (MW) of 130 and 180 kDa, while MAb 3B7 recognized a protein of ca. 28 kDa (Fig. 4).

DISCUSSION

Two MAbs (1C7 and 3B7) against *Myxobolus honghuensis* were identified. MAb 1C7 specifically

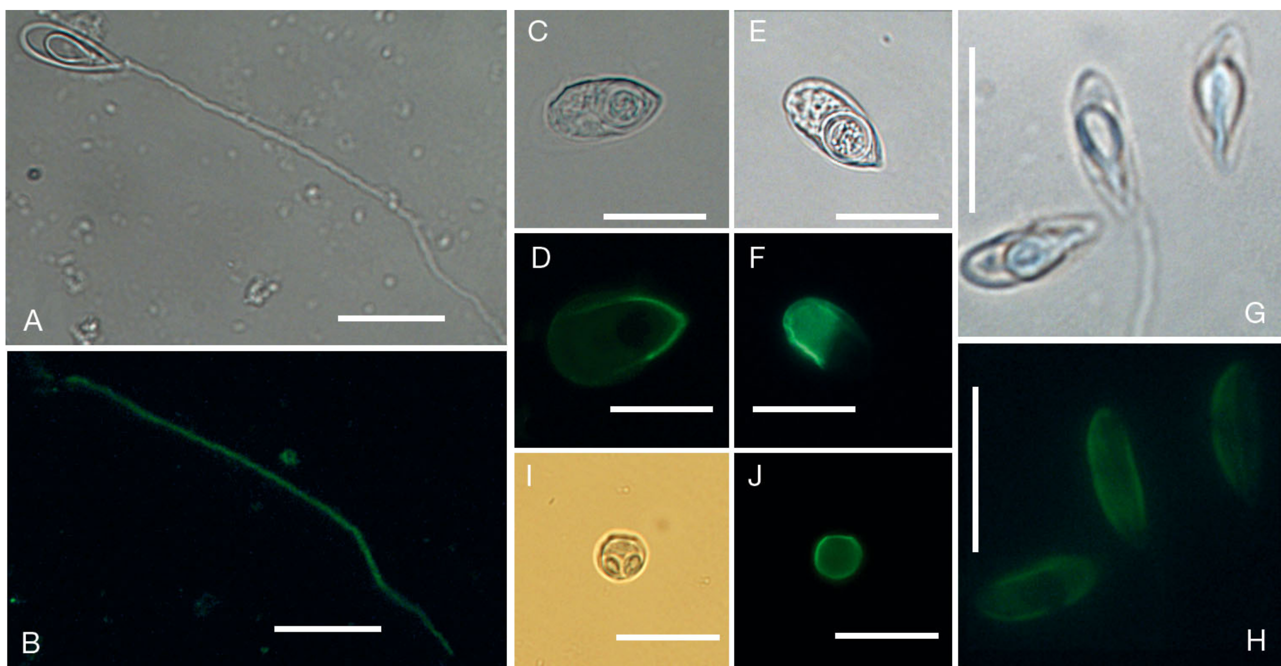

Fig. 3. Analysis of the cross-reactivity of monoclonal antibodies (MAbs 1C7 and 3B7) with different parasite species by an indirect fluorescent antibody test. Light photomicrographs of (A,G) *Thelohanellus testudineus*, (C,E) *T. wuhanensis*, and (I) *M. nielii*. Indirect fluorescent antibody tests indicated that MAb 1C7 reacted with (B) polar filaments of *T. testudineus* and (D) the top edge of *T. wuhanensis* spores. MAb 3B7 reacted with spore valves of (F) *T. wuhanensis*, (H) *T. testudineus*, and (J) *M. nielii*. Scale bars = 20 µm

Table 1. Cross-reactivity analysis of monoclonal antibodies (MAbs 1C7 and 3B7) against *Myxobolus honghuensis* determined by the indirect fluorescent antibody technique. Fluorescence intensity graded from strong (++) to weak (+) or negative (–)

Species	MAb 1C7	MAb 3B7
Myxobolus honghuensis	++	++
M. nielii	–	+
M. wulii	–	–
M. musseliusae	–	–
M. turpisrotundus	–	–
M. tsangwuensis	–	–
Thelohanellus wuhanensis	+	+
T. testudineus	+	+

reacted with polar filaments, which are the key structures that facilitate the binding of spores to the respective hosts and allow the sporoplasm to emerge and invade host tissues (El-Matbouli et al. 1995). At high magnification, polar filaments stained by MAb 1C7 revealed a banded pattern, similar to the filament morphology in *M. pendula*, *Nematostella vectensis*, and *Hydra vulgaris* presented by Ringuette et al. (2011), suggesting that polar filaments are composed of repeated subunits, such as observed with fibril-forming collagens. Although the function and specific structure of these repeating subunits is currently unknown, the existence of some conservative epitopes on filaments among different *Myxobolus* species have been confirmed (Ringuette et al. 2011). MAb 1C7 will facilitate future investigations of the role(s) played by myxozoan polar filaments during infection.

MAb 3B7 recognized spore proteins, which may have a role in host–parasite interactions (Chase et al. 2001, Estensoro et al. 2013). However, further studies need to determine the accurate recognition site on the spore surface and determine its contribution during infection. Markiw (1989) used an IFAT test to provide evidence for an antigenic relationship between the actinospore and the myxospore stages of *M. cerebralis*. MAbs also help in identifying the entry point of parasites and detecting early life stages in the fish host based on the observation of fluorescent stages in the skin, the base of the gills, or the buccal cavity (Belem & Pote 2001). In our future studies, we will use MAb 3B7 to help distinguish *M. honghuensis* actinospores and examine the role of the antigens it recognizes in the parasite life cycle.

The specificity of the MAb (1C7 and 3B7) interactions in our assays (Table 1) indicates their potential use in diagnostic testing even though some cross-

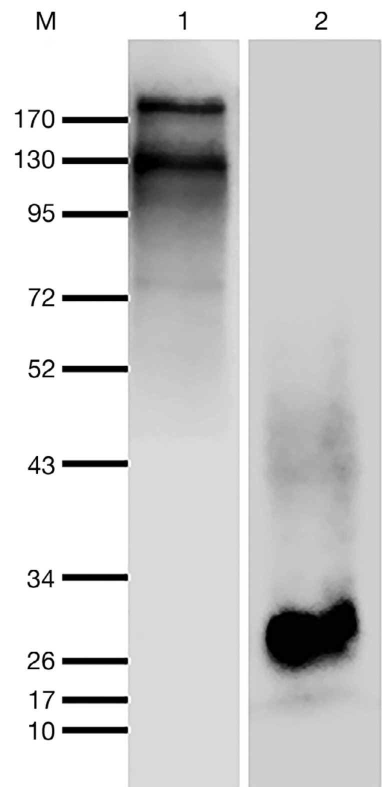

Fig. 4. Protein bands detected by Western-blot analysis using monoclonal antibodies (MAbs 1C7 and 3B7) against *Myxobolus honghuensis*. Lane representations are: (M) molecular weight (MW) ladder, with weight in kDa; (1) MAb 1C7 recognized antigens with MWs of 130 and 180 kDa; (2) MAb 3B7 identified a 28 kDa antigen

reaction occurs. Since many myxozoans have a close relationship with *M. honghuensis*, it is difficult to develop a MAb which does not react with other myxozoans. Fortunately, these 2 MAbs did not react with most *Myxobolus* species we tested here. We can therefore use these MAbs in combination with morphological characteristics (such as the number of capsules) for potential diagnostic tests. Our data also suggest the presence of some species-specific antigens of *M. honghuensis* on polar filaments and spore valves. However, the cross-reaction with 2 *Thelohanellus* species indicates the presence of common antigens between *Myxobolus* and *Thelohanellus.* This conservation of common epitopes lends some support to a shared molecular architecture between these 2 genera. Using a MAb, Morris et al. (2004) found that *M. cerebralis* shares antigens with *Tetracapsuloides bryosalmonae*. Similarly, Ringuette et al. (2011) indicated a conservation of polar filament epitopes between *M. pendula* and 2 related cnidarians using polyclonal antiserum. We intend to include

other parasitic species in our future studies to determine cross-reactions of MAbs and to use the data for phylogenetics. MAb 3B7 only cross-reacted with *M. nielii* within the tested *Myxobolus*, which suggests the presence of common antigenic epitopes on spores of different *Myxobolus* species. Similarly, MAbs from the *Kudoa* genus generated against *K. thyrsites* cross-reacted with several other *Kudoa* species (Chase et al. 2001), indicating the existence of shared antigenic epitopes, particularly carbohydrate moieties (Muñoz et al. 1998, 1999). In any case, these cross-reactive antigens are potential targets for multifunctional vaccines against various parasitic myxozoans.

In summary, MAbs have previously been used with some other diagnostic tools for myxozoans, such as ELISA, immunohistology, and flow cytometry, and they can also be useful in vaccination studies (Chase et al. 2001). Future studies should determine whether actinospore stages of *M. honghuensis* cross-react with these MAbs. Our stringent MAbs will be helpful in identifying peptides using mature *M. honghuensis* protein extracts with 2-dimensional gel electrophoresis, Western blotting, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry sequencing, and elucidating their role in polar filaments and spore valves during invasion and pathogenesis in the host.

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