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Mapping of the putative epitope domain of *Clonorchis sinensis* paramyosin (CsPmy) recognized by CsPmy-specific immunoglobulin G in sera of human clonorchiasis

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ABSTRACT

Paramyosin of *Clonorchis sinensis* (CsPmy) is a myofibrillar protein localized in subtegumental muscle, tegument, and the muscle layer surrounding the intestine of the parasite. Previously, we have identified that CsPmy reacted with sera of human clonorchiasis and this protein had a potential as a candidate antigen for serodiagnosis of clonorchiasis. However, we also found that CsPmy is able to bind to human immunoglobulin G (IgG) in non-specific manners, which can affect the diagnostic value of the protein. Here, we mapped CsPmy-specific IgG binding site on CsPmy to analyze the putative epitopes recognized by CsPmy-specific IgG in sera of human clonorchiasis. The fragmental expression of CsPmy followed by immunoblot analyses with sera from patients with clonorchiasis and non-specific human IgG revealed that the middle portion of CsPmy (CsPmyC: 301–600 amino acid residues) had epitopes responsible for CsPmy-specific IgG recognition. The precise CsPmy-specific IgG binding site was further narrowed down to a fragment (CsPmyC-2), which harbors 151 amino acid residues (375–525) of CsPmy. Specific antibodies for CsPmyC-2 were produced in rats after two-weeks of post-experimental infection. The CsPmyC-2 showed low levels of cross reactivity against the sera from patients with other helminth parasites. Our results suggested that CsPmyC-2 has real epitopes recognized by CsPmy-specific IgG in sera of human clonorchiasis and the fragment can be useful as a reliable serodiagnostic antigen to develop a serodiagnostic method for clonorchiasis.

1. Introduction

Clonorchiasis is a hepatic disease resulting from infection by *Clonorchis sinensis*, the Chinese liver fluke. It affects about 35 million people, mostly those residing in Far East Asian countries including China, Korea, Taiwan, and northern Vietnam [1]. Human infection occurs through consumption of raw or under cooked freshwater fish that is infected with *C. sinensis* metacercariae. Most human infections by the parasite are usually asymptomatic or manifest mild clinical symptoms, but heavy and chronic infections are complicated by hepatobiliary diseases such as cholangitis, cholelithiasis, and cholangiectasis. A close epidemiological relationship between clonorchiasis and the incidence of cholangiocarcinoma implicates the parasite as a group 1 biological carcinogen that can induce cholangiocarcinoma [2,3].

Paramyosin is a myofibrillar protein that forms the tick myofilaments of invertebrate muscle and plays an important role in specialized contractile activities [4–6]. Paramyosin has also been identified in diverse helminth parasites and its multifunctional roles as a structural protein in muscle layers as well as an immunoregulatory molecule interacting with the host immune system has been characterized [7–13]. Paramyosin of helminth parasites modulates the host's immune system by repressing the classical pathway of the complement cascade [7,9] and is also involved in the immunological defense mechanism of parasites by acting as Fc receptors [8,14]. Moreover, paramyosin of helminth parasites has been attracting attention as a potential vaccine candidate antigen due to its high immunogenic properties [15–21].

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Table 1
Oligonucleotide primers.

Fragment	Corresponding amino acids	Primers	Sequences
CsPmyA	1–300	CsPmyA F	5′-GTCGACATGAGTCACGAGTCGGAATCACAC-3′
		CsPmyA R	5′-AAGCTTTTATTCCTCAGTTTTTGACATGAGCTC-3′
CsPmyB	181–460	CsPmyB F	5′-GTCGACTTGCAGCGGCAACTGAATGACTTG-3′
		CsPmyB R	5′-AAGCTTTTAATGCAAAGCGGATGCCAAATTATC-3′
CsPmyC	301–600	CsPmyC F	5′-GTCGACTACGAAGAACTCAAGAGGAAGTTA-3′
		CsPmyC R	5′-AAGCTTTTACTCGCTCGATAGCTGAATTCGCTT-3′
CsPmyD	461–740	CsPmyD F	5′-GTCGACGATGCAGAAGAAGCCCTGCGGGAA-3′
		CsPmyD R	5′-AAGCTTTTAGGCTTCGAGCTCGCGAACACGAGT-3′
CsPmyE	601–864	CsPmyE F	5′-GTCGACGTTGAAGAGTTACGTGGTGCCTTA-3′
		CsPmyE R	5′-AAGCTTTTACATCATGCTCGTCGCGCGCGT-3′
CsPmyC-1	301–450	CsPmyC-1 F	5′-GTCGACTACGAAGAACTCAAGAGGAAGTTA-3′
		CsPmyC-1 R	5′-AAGCTTTTACGCCTCAAGTTGAGACCGTAGAGC-3′
CsPmyC-2	375–525	CsPmyC-2 F	5′-GTCGACGATGAGATGACCATTGAAATCAAC-3′
		CsPmyC-2 R	5′-AAGCTTTTATGACTTGTATTTCACCTCCATCTC-3′
CsPmyC-3	451–600	CsPmyC-3 F	5′-GTCGACGAACGGGATAATTTGGCATCCGCT-3′
		CsPmyC-3 R	5′-AAGCTTTTACTCGCTCGATAGCTGAATTCGCTT-3′

Restriction enzymes (Sal I or Hind III) recognition sequences are underlined.

Previously, we have identified a paramyosin of *C. sinensis* (CsPmy) and partially characterized its functional properties [22]. CsPmy is constitutively expressed in diverse developmental stages of the parasite and is localized in subtegumental muscle, tegument, and the muscle layer surrounding the intestine of the parasite. Immunization of CsPmy in rats evokes specific antibody production in the experimental animals and the protein shows antigenicity against the sera from patients with clonorchiasis, suggesting the potential of the protein as a reliable serodiagnostic antigen for clonorchiasis [22]. However, we also identified that the protein had non-specific immunoglobulin G (IgG) binding capacity, which may have resulted in false-positive responses in serodiagnoses based on the protein. We analyze herein the antigenic domain of CsPmy, which is specifically recognized by CsPmy-specific IgG. The middle part of CsPmy was finally pinpointed as the CsPmy-specific IgG recognition site, where non-specific IgGs are not bound. Our results suggest that this fragment of CsPmy can be a useful antigen in developing a reliable serodiagnostic method for clonorchiasis.

2. Materials and methods

2.1. Fragmentation of CsPmy

The CsPmy gene used in this study was cloned in our previous study [22]. The full length CsPmy was fragmented into 5 overlapped fragments, CsPmyA, CsPmyB, CsPmyC, CsPmyD, and CsPmyE, and the regions encoding each fragment were amplified by polymerase chain reaction (PCR) using primer pairs for each fragment (Table 1, Fig. 1A). The amplification profile was 94 °C for 4 min and 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min followed by a 72 °C extension for 10 min. The amplified PCR products were purified from gel, cloned into T&A cloning vector (Real Biotech Corporation, Banqiao City, Taiwan) and transformed into *Escherichia coli* DH5α competent cells. The nucleotide sequences of each cloned CsPmy fragment were confirmed by automatic DNA sequencing. To further narrow down the CsPmy-specific IgG recognition site, CsPmyC was further fragmented into 3 overlapped subfragments, CsPmyC-1, CsPmyC-2 and CsPmyC-3, and the region encoding each subfragment was amplified by PCR using primer pairs for each subfragment (Table 1, Fig. 3A). The amplification profile was 94 °C for 4 min and 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 30 s followed by a 72 °C extension for 10 min. The amplified PCR product was purified from gel, cloned into T&A cloning vector (Real Biotech Corporation) and transformed into *E. coli* DH5α competent cells. The nucleotide sequences of the cloned fragments were also confirmed by automatic DNA sequencing.

2.2. Expression and purification of recombinant CsPmy fragments

Each fragmented CsPmy gene was ligated into pQE-30 expression vector (Qiagen, Hilden, Germany). Each ligate was transformed into *E. coli* M15 [pREP4] competent cells (Qiagen) and spread onto Luria–Bertani agar plates containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin. Selected clones were grown and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The cells were harvested by centrifugation at 10,000 × *g* for 15 min at 4 °C, suspended in a native lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and sonicated on ice. The expressed protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen) containing Complete protease inhibitor cocktail (Roche, Mannheim, Germany) under native conditions. The purity of each CsPmy was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining.

2.3. Immunoblot analysis

To determine the antigenic characteristics of each recombinant CsPmy fragment, each CsPmy fragment (10 μg) was separated by 12.5% or 15% SDS-PAGE and the proteins were transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was cut into strips and the strips were blocked with 3% skim milk in phosphate buffered saline (PBS, pH 7.2) containing 0.05% Tween 20 (PBST) for 1 h at room temperature. The strips were incubated with 1:200 diluted sera either from patients with clonorchiasis ($n = 30$) or from normal healthy individuals ($n = 15$) at room temperature for 3 h. All patients were diagnosed for *C. sinensis* infection by stool examination using Kato–Katz method. The number of eggs per gram of feces (EPG) ranged from 100 to 250. Informed consent obtained from all individuals before admission was reviewed and approved by the Ethics Committee of the National Institute of Health, Korean Centers for Disease Control and Prevention, Osong, South Korea. After several washes with PBST, the

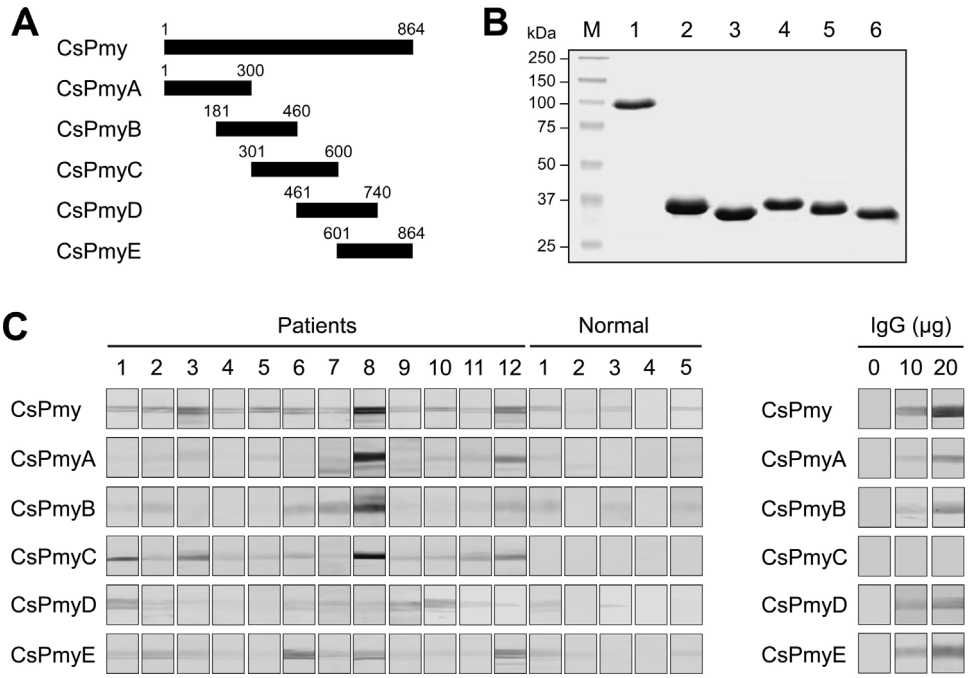

Fig. 1. Expression of recombinant CsPmy fragments and immunoblot analysis. To identify the epitopes recognized by CsPmy-specific IgG, CsPmy was fragmented into five overlapped fragments, expressed the recombinant proteins and analyzed by immunoblotting. (A) Schematic diagram of CsPmy fragments for expression. (B) Expression of recombinant CsPmy fragments. Each recombinant protein was expressed in *E. coli* and was purified with Ni-NTA affinity column. Lane M, size marker proteins; lane 1, Full-length CsPmy; lane 2, CsPmyA; lane 3, CsPmyB; lane 4, CsPmyC; lane 5, CsPmyD; lane 6, CsPmyE. (C) Immunoblot analysis of CsPmy fragments. Full-length CsPmy and its fragmented recombinant proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and performed immunoblot. Proved with sera from patients with clonorchiasis ($n = 30$) or normal healthy individuals ($n = 20$) or with different concentrations (0, 10, or 20 μg) of irrelevant human IgG. In the case of immunblot analysis with human sera, only representative results are presented.

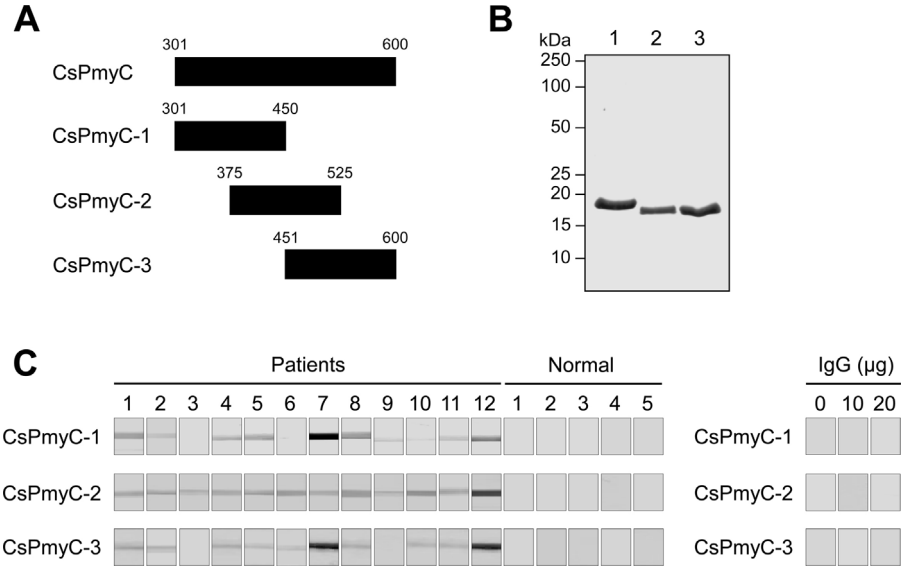

Fig. 2. Fragmental expression and immunoblot analysis of CsPmyC fragments. (A) Diagram of narrowed down CsPmy fragments for expression. CsPmyC was further fragmented into 3 overlapped fragments (CsPmyC-1, CsPmyC-2, and CsPmyC-3) for recombinant protein production. (B) Expression of recombinant proteins. The proteins were expressed in *E. coli*, purified by Ni-NTA affinity column, and analyzed by SDS-PAGE. (C) Immunoblot analysis. Recombinant proteins were blotted onto a nitrocellulose membrane and proved with sera from patients with clonorchiasis ($n = 30$) or normal healthy individuals ($n = 20$) or with different concentrations (0, 10, or 20 μg) of irrelevant human IgG. In the case of immunblot analysis with human sera, only representative results are presented.

strips were incubated with 1:1,000 diluted horseradish peroxidase (HRP)-conjugated anti-human immunoglobulin G (IgG; Sigma, St. Louis, MO, USA) at room temperature for 2 h. The strips were washed several times with PBST and developed with 4-chloro-1-naphthol (4CN; Sigma) for 10 min in the dark. The reaction was stopped by washing the strips with distilled water. To analyze non-specific binding of human IgG, the strips were also incubated with different concentrations (0, 10, or 20 μg) of purified human IgG (Sigma) at room temperature for 3 h. After several washes with PBST, the strips were probed with anti-human IgG (Sigma) diluted 1:1,000 for 2 h at room temperature and washed several times with PBST. The strips were further incubated in HRP-conjugated anti-

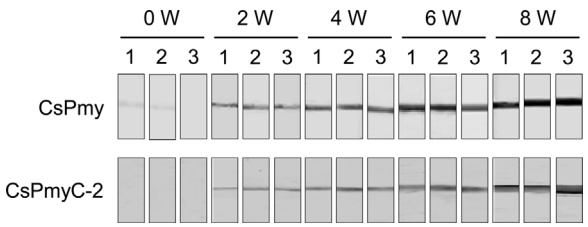

Fig. 3. Time course production of CsPmy-specific antibodies in rats experimentally infected with *C. sinensis*. The rat's sera were collected from each rat at 0, 2, 4, 6, and 8 weeks post-infection. CsPmy and CsPmyC-2 were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and performed immunoblot with the rat's sera. 0 W – 0 week; 2 W – 2 weeks; 4 W – 4 weeks; 6 W – 6 weeks; 8 W – 8 weeks.

human IgG (Sigma) diluted 1:1,000 for 2 h at room temperature, washed with PBST several times, and developed with 4CN.

2.4. Characterization of antigenic properties of CsPmyC-2

We further analyzed the production of antibodies specific for CsPmy in rats experimentally infected with *C. sinensis*. The sera of three rats, which were previously prepared from rats at 0, 2, 4, 6, and 8 weeks post-infection with 100 *C. sinensis* metacercariae [23–25], were used in this study. The purified CsPmy and CsPmyC-2 (each 10 μg) were separated on SDS-PAGE, transferred onto nitrocellulose membrane, and cut into strips. The strips were blocked and probed with the sera of rats followed by probing with HRP-conjugated anti-rat IgG (Sigma). The strips were developed with 4CN as described above.

2.5. Cross reactivity of CsPmyC-2

To analyze the cross reactivity of CsPmyC-2 with the sera from other trematode infection, immunoblot analysis was performed. CsPmyC-2 was separated on SDS-PAGE and was transferred to a nitrocellulose membrane. The strips were blocked and incubated with 1:200 diluted sera of patients with paragonimiasis ($n = 12$), schistosomiasis ($n = 5$), metagonimiasis ($n = 10$), gnathostomiasis ($n = 7$), or clonorchiasis ($n = 30$), respectively. The strips were further probed with 1:1,000 diluted HRP-conjugated human IgG (Sigma) and developed with 4CN. Sera from patients with schistosomiasis were kindly provided by Dr. Young-Ha Lee (Department of Infection Biology, Chungnam National University School of Medicine, Daejeon, Korea) and Dr. Sung-Tae Hong (Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine, Seoul, Korea).

3. Results and discussion

Diagnosis of *C. sinensis* infection has been classically performed by microscopic observation of the parasite's eggs in stool samples. However, the procedure is usually cumbersome and time- and labor-consuming, and requires well-trained microscopic experts. Moreover, the eggs are sometimes difficult to identify through microscopic examination, especially in patients with low worm burdens. As a result, various efforts to develop reliable immunodiagnostic assays for clonorchiasis using crude or recombinant antigens have been made to overcome these limitations [26–30], but these methods also have their own limitations such as low sensitivity and cross-reactivity with proteins from other closely related helminth parasites. Recently, molecular diagnostic methods based on specific amplifications of *C. sinensis* genes from eggs in stool samples have also developed [31–34], but they also have several drawbacks including limitation to apply in domestic or primary health care centers where molecular diagnosis system is lacking or not fully equipped. Therefore, development of a new simple method with high applicability, specificity and sensitivity is essentially required.

CsPmy is a potential candidate antigen for serodiagnosis of clonorchiasis [22], but its non-specific IgG binding capacity is a major concern in application of the protein for development of reliable CsPmy-based serodiagnostic method. Here, we mapped CsPmy-specific IgG binding sites in CsPmy by using overlapped recombinant CsPmy fragments followed by immunoblotting. To map the binding site of CsPmy-specific IgG on CsPmy, full length CsPmy was fragmented into the 5 overlapped fragments (CsPmyA–CsPmyE; Fig. 1A) and their recombinant proteins were expressed in *E. coli*. All recombinant CsPmy fragments were expressed as soluble proteins (Fig. 1B). To determine CsPmy-specific IgG binding site on CsPmy, antigenic property of each CsPmy fragment was analyzed by immunoblot with sera from patients with clonorchiasis and normal healthy individuals. As expected, full length CsPmy showed antigenicity against all the sera from patients with clonorchiasis, but positive responses were also detected against normal control sera (Fig. 1C). The five CsPmy fragments also showed positive responses against the sera from patients with clonorchiasis, albeit slightly different reactive patterns were observed in each other (Fig. 1C). The four CsPmy fragments (CsPmyA, CsPmyB, CsPmyD and CsPmyE) were also reacted with normal healthy control sera, but CsPmyC was not reactive for normal control sera. We further analyzed the non-specific human IgG binding on each CsPmy fragment to confirm no non-specific IgG binding occurred on CsPmyC. The CsPmyC fragment did not react with irrelevant human IgG (Fig. 1C). Meanwhile, full length CsPmy and the four CsPmy fragments except CsPmyC were recognized by human IgG. These results confirm that antigenic responses of CsPmy against sera from patients with clonorchiasis and normal healthy individuals might have resulted from non-specific binding of irrelevant human IgG. However, CsPmyC was not recognized by sera of normal healthy individuals and irrelevant IgG, but showed positive responses to only sera of patients with clonorchiasis suggesting that CsPmy-specific IgG recognition epitopes are located in CsPmyC.

To further narrow down the CsPmy region recognized by CsPmy-specific IgG, we further fragmented the CsPmyC fragment into three overlapped subfragments, CsPmyC-1, CsPmyC-2, and CsPmyC-3, and the recombinant proteins were produced in *E. coli* (Fig. 2A and B). The recombinant proteins were immunoblotted with sera from patients with clonorchiasis and normal healthy individuals. All three CsPmyC subfragments showed antigenic responses against the sera from patients with clonorchiasis, albeit the patterns were slightly different from each other (Fig. 2C). CsPmyC-2 reacted with all the tested sera of patients with clonorchiasis, but the other two fragments showed negative responses against some of the patients' sera. As expected, the three CsPmyC subfragments did not react with both the sera from normal healthy individuals and irrelevant human IgG (Fig. 2C). These collectively suggest that CsPmyC-2 region has common antigenic epitopes, which are recognized by CsPmy-specific antibodies produced by *C. sinensis* infections.

Early diagnosis and appropriate chemotherapy are important to prevent the disease progressing to a serious stage and to facilitate recovery from chronic pathologic changes of liver. We analyzed the time-course production of antibodies specific for CsPmy in rats, which are experimentally infected with *C. sinensis*. CsPmy-specific IgGs were starting to be detected in the sera of rats on 2 weeks of experimental *C. sinensis* infection (Fig. 3). Although non-specific IgG binding against full length CsPmy was detected in non-immune sera, no non-specific IgG binding to CsPmyC-2 was observed. Compared to most antigenic proteins studied so far as candidate antigens for serodiagnosis of clonorchiasis which are usually recognized by specific antibodies starting at least 3–4 weeks post-

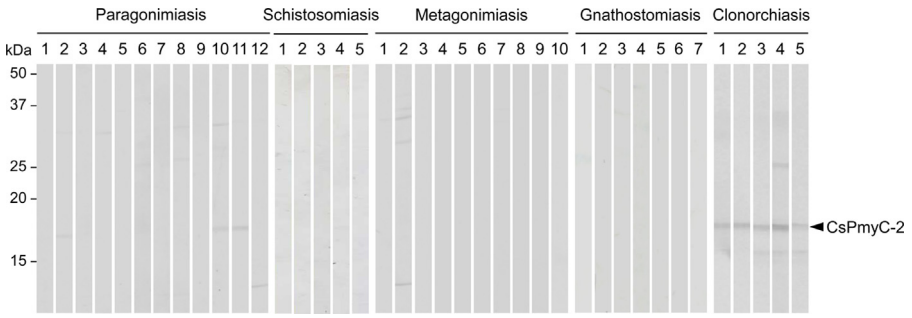

Fig. 4. Cross reactivity of CsPmyC-2. Cross reactivity of CsPmyC-2 was analyzed with the sera from patients with paragonimiasis ($n = 12$), schistosomiasis ($n = 5$), metagonimiasis ($n = 10$), gnathostomiasis ($n = 7$) or clonorchiasis ($n = 5$). In the case of clonorchiasis, only five representative strips among 30 tested sera were presented. Recombinant CsPmy-C2 was separated by SDS-PAGE, transferred onto nitrocellulose membrane, and proved each serum of patients, respectively.

infection [25,35], early detection of CsPmy-specific IgG may enable early diagnosis of clonorchiasis in an early phase infection. We also analyzed cross reactivity of CsPmyC-2 with the sera from patients with paragonimiasis ($n = 12$), schistosomiasis ($n = 5$), metagonimiasis ($n = 10$) and gnathostomiasis ($n = 7$). CsPmyC-2 did not react with the sera from schistosomiasis, metagonimiasis and gnathostomiasis, but weak cross reactivity was detected against the sera of paragonimiasis (2/12, 16.7%) (Fig. 4). Cross reactions have been one of the main obstacles in developing a reliable clonorchiasis-specific serodiagnostic method [26,28,29]. In this regard, the high specificity of CsPmyC-2 is very attractive for designing a more reliable serodiagnostic method, but a more comprehensive analysis using a larger number of sera from patients with diverse helminthiases would be required.

In conclusion, we mapped the CsPmy-specific IgG binding sites on CsPmy. The main epitopes of CsPmy recognized by CsPmy-specific IgGs were located in the middle portion of CsPmy (CsPmyC-2) corresponding to 150 amino acid residues. The high specificity of CsPmyC-2 and early detection of specific IgG for the fragment in experimental clonorchiasis animal model suggests that the fragment could be an attractive antigen applicable in the development of a reliable serodiagnostic method for clonorchiasis. Further comprehensive studies to evaluate CsPmyC and CsPmyC-based fragments or peptides as reliable serodiagnostic antigens for clonorchiasis would be necessary.

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