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ORIGINAL PAPER

Multiple recombinant antigens of *Clonorchis sinensis* for serodiagnosis of human clonorchiasis

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Abstract Antigenic proteins from *Clonorchis sinensis* have been previously purified and evaluated for their antigenicity to enable the serodiagnosis of clonorchiasis. Though they were of high specificity, molecularly defined proteins were reported to be less sensitive as single antigens than crude antigen. To resolve this issue, 11 clones were selected by immunoscreening an adult *C. sinensis* cDNA library using infected human sera. Mixed antigens were prepared using recombinant proteins of positive clones and investigated for antigenicity by immunoblotting against *C. sinensis*- and helminth-infected patient sera. A mixed antigen of recombinant 28 and 26 kDa glutathion *S*-transferases (Cs28GST and Cs26GST) produced 76% sensitivity and 95% specificity. Furthermore, a triple mix of recombinant Cs26GST and Cs28GST with vitelline precursor protein pushed up the sensitivity to 87% and maintained specificity at 95%. It is proposed that multiple antigen mixes should be further studied to develop rapid serodiagnostic test kits for the serodiagnosis of human clonorchiasis.

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Introduction

Clonorchiasis is a fish-borne trematode infection which is endemic in China, Korea, and Viet Nam. About 35 million people are estimated to be infected with *Clonorchis sinensis* in this region (Lun et al. 2005). In South Korea, clonorchiasis is currently the most prevalent parasitic infection, and an estimated 1.17 million people are believed to be infected with *C. sinensis* (Kim et al. 2009). People in endemic areas become infected by eating raw or undercooked freshwater fish containing *C. sinensis* metacercariae. The adult fluke inhabits the biliary passages and provokes epithelial hyperplasia, periductal fibrosis, obstructive jaundice, dyspepsia, and liver cirrhosis in man (Rim 2005). Furthermore, chronic *C. sinensis* infections can cause cholangiocarcinoma in man and animals (Bouvard et al. 2009; Lee et al. 1993; Lim et al. 2006).

Early diagnosis and chemotherapy are crucial to prevent the disease progressing to a serious level and to facilitate recovery from chronic pathologic changes in liver. Parasitological microscopic stool examination remains the standard method for diagnosing clonorchiasis. However, a more suitable diagnostic method is required for individual diagnosis and epidemiological surveys because it is difficult to collect stools due to low patient and subject compliance. Additionally, stool examinations are less sensitive at detecting early stage infections, because *C. sinensis* eggs appear in human feces 4 weeks after initial infection (Rim 1986).

Several studies reported that the 7-, 8-, 17-, 26-, 28-, 34-, 37-, 43-, 70-, and 100-kDa protein bands of *C. sinensis* are

candidate antigenic proteins for the serodiagnosis of clonorchiasis (Hong et al. 1997; Li et al. 2004). Antigenic preparations of *C. sinensis* adult worm extracts have been reported to be sensitive and specific, but cross-reactions hinder the use of crude antigens clinically and in the field (Hong 1988). Accordingly, more sensitive and specific antigenic proteins are required for the serodiagnosis of clonorchiasis. The excretory–secretary antigen has been reported to prove a sensitive and specific ELISA substrate for the serodiagnosis of human clonorchiasis (Choi et al. 2003), but this antigen has several practical holdbacks, which are: it is not easy to obtain sufficient amounts, standardization is required for every preparation, testing is costly, and animals are required for testing. Recombinant proteins offer a practical alternative and have been examined in terms of serodiagnostic antigenicity toward *C. sinensis*-infected sera.

In characterization of biological functions of the proteins molecular biologically defined from parasites, antigenicity of the proteins was assayed against sera of human infected with the respective or other parasites. Recombinant antigenic proteins such as cysteine proteases and 7-kDa protein of *C. sinensis* have been proposed as potential serodiagnostic reagents (Song et al. 1990; Na et al. 2002; Nagano et al. 2004; Zhang et al. 2008). In ELISA and immunoblotting studies, the recombinant 7 kDa protein showed relatively high sensitivities (81.2% and 71.9%) but also high cross-reactivities (35.3% and 47.1%) to paragonimiasis sera (Zhao et al. 2004). In other studies, recombinant 28 and 26 kDa glutathione *S*-transferases (Cs28GST and Cs26GST) showed 30–33% sensitivity and 100% specificity for trematode infected human sera (Kang et al. 2001; Hong et al. 2002). As an excretory–secretory protein of *C. sinensis*, lysophosphatidic acid phosphatase was proposed to be valuable diagnostic antigen for human clonorchiasis (Hu et al. 2007). Antigenic proteins, 7-kDa protein, 28-kDa cysteine protease, 26 and 28 kDa GSTs of *C. sinensis*, were produced using wheat germ cell-free protein synthesis system. In ELISA, these antigenic recombinant proteins, as a single antigen, revealed moderate to low sensitivities and high specificities for serodiagnosis of clonorchiasis. To improve low sensitivity of the molecularly defined single antigens, cocktail or chimeric antigens were suggested (Shen et al. 2009).

When used as single antigen for the serodiagnosis of human clonorchiasis, the defined antigenic proteins were found to have high specificity but low sensitivity. However, this low sensitivity of single recombinant antigens could be improved by employing multivalent cocktail antigens, which could be prepared by mixing recombinant antigenic proteins. However, to achieve this, antigenic proteins of high sensitivity and specificity are a prerequisite.

This study was undertaken to produce recombinant proteins of high antigenic specificity and to prepare multivalent antigenic cocktails by combining these single recombinant proteins.

Materials and methods

cDNA cloning

A previously described cDNA expression library of adult *C. sinensis* was used (Hong et al. 2000). The cDNA library (6×10^6 pfu) was mixed with *Escherichia coli* XL1-Blue and cultured on LB agar plates. The plates were then overlaid with nitrocellulose membranes (previously soaked in 10 mM isopropyl-D-thiogalactoside (IPTG)) and incubated at 37°C for 4 h. Membranes were then incubated for 3 h within a *C. sinensis*-infected human serum at a dilution of 1:100 at room temperature. After incubating membranes with secondary antibody, they were treated with alkaline phosphatase-conjugated anti-human IgG at a dilution of 1:2,000. Color signals were developed in buffer containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT, Sigma Chemical Co., St. Louis, MO, USA). Positive clones were purified by secondary and tertiary screening using the same serum and then in vivo excised to single-stranded phagemids by employing a helper phage. They were then converted to double-stranded plasmids according to the manufacturer's instructions (λ ZAP cDNA library, Stratagene, La Jolla, CA, USA).

cDNA sequencing and data base searching

cDNA sequences of the clones were determined using BigDye™ Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA). Putative peptide sequences of cDNA were analyzed using the DNASIS program (Hitachi Software Engineering Co., Yokohama, Japan). DNA and conceptual polypeptide sequences were searched for homologs in GenBank database using BLAST algorithms (Altschul et al. 1997). Multiple alignments of peptide sequences were generated using CLUSTAL W (Thompson et al. 1994) and in part were optimized manually.

Production of recombinant proteins

Coding regions of the cloned cDNA clones were cut out by restriction enzyme digestion or PCR amplified using specific primers and *Taq* polymerase. Target cDNA fragments were subcloned in-frame into pRSET or pGEX-4T, pET-23 expression plasmid vectors, and transformed into *E. coli* BL21[DE3]pLysS (Novagen, Madison,

WI). The recombinant proteins were induced by adding 1 mM of IPTG to the culture medium and were later purified by affinity column chromatography according to the manufacturer's instructions. Recombinant protein production was confirmed by SDS–PAGE and immunoblotting with specific antibody reacting with the respective tag peptide.

Parasites and sera

Metacercariae of *C. sinensis* were collected from a naturally infected freshwater fish (*Pseudorasbora parva*) caught at Shenyang (Liaoning Province, China). Metacercariae were fed orally to New Zealand White rabbits, and adult flukes were recovered alive from bile duct 3 months after infection and washed in cold phosphate-buffered saline (PBS).

The human sera used in this study were obtained from patients with clonorchiasis, opisthorchiasis, paragonimiasis, schistosomiasis, or fascioliasis. Parasite infections were confirmed by microscopic stool examination. Cysticercosis sera were obtained from patients diagnosed by CT/MRI imaging. The sera of uninfected healthy individuals were included as controls.

Assays of the antigenicities of recombinant proteins

Multivalent cocktail antigens were prepared by combining equal amounts of single recombinant antigens. To prepare nitrocellulose membrane stick striped with the *C. sinensis* recombinant proteins, the Miniblotter™ (Immunetics, Cambridge, MA, USA) was used. First, the nitrocellulose membrane (NC, Hybond TM-C, Amersham Pharmacia Biotech, Uppsala, Sweden) was put on a base plate, and the MiniSlot Unit was assembled. Recombinant antigens and cocktail antigen mixes were pipetted into the slots of the unit, and air suction was applied to immobilize proteins onto the NC membrane. After disassembling MiniSlot unit, the NC membrane was cut vertically through the slots using a razor blade and then immunoblotted with *C. sinensis*-infected sera.

After blocking for 2 h in PBS containing 0.05% Tween-20 and 5% skim milk, the NC strips were incubated in helminth-infected or uninfected human sera at a dilution of 1:100, washed, and incubated in alkaline phosphatase-conjugated anti-human IgG antibody at 1:2,000. Color was developed within BCIP/NBT substrate.

Crude extract of adult *C. sinensis* was included as a positive control antigen. These extracts were prepared by homogenizing adult *C. sinensis* on ice in PBS containing protease inhibitor and spun at 5,000×*g* for 10 min. Supernatants were saved and their protein concentrations determined.

Results

Antigenic clones

By immunoscreening an adult *C. sinensis* cDNA library with *C. sinensis*-infected sera, 45 positive clones were purified. Of these positive clones, 10 were selected, which encoded the full open reading frame of a polypeptide.

Clone CsHA-5 (CsEF-1) cDNA was 1.6 kb and the deduced polypeptide showed 80–90% homology with elongation factor-1 of invertebrate animals and was identical to that of *C. sinensis* (Kim et al. 2007). Clone CsHA-8 (CsMRLC) contained a 1.6-kb insert cDNA, and its deduced polypeptide was found to be homologous (59–62%) with the myosin regulatory light chain of invertebrate animals and almost identical to that of *C. sinensis* (Kwon et al. 2005).

Clone CsHA-9 (CsVpB1) contained an 838 bp long cDNA and encoded a polypeptide of 245 amino acids that was homologous with vitelline precursor protein of invertebrates and *C. sinensis*.

Clone CsHA-15 (CsMXL) contained an insert cDNA of 1.8 kb encoding a putative polypeptide homologous with methylcrotonyl CoA carboxylase of invertebrates. Clone CsHA-16 (Cs28GST) cDNA was 799 bp long and encoded a polypeptide of 212 amino acids showing sequence identity (25–43%) with the 28 kDa glutathione *S*-transferase of vertebrates and invertebrates, and which was identical to that of *C. sinensis* (Kang et al. 2001). Clone CsHA-19 (CsRP) cDNA was 844 bp long and encoded a full open reading frame that shared homology with the repetitive proteins of invertebrates, including *C. sinensis* (Kim et al. 2001). Clone CsHA-24 (CsMCP) harbored a 1,525-bp long cDNA encoding a polypeptide that showed a low degree of homology (~27%) with migratory cell-specific protein. Clone CsHA-25 (Cs26GST) contained an insert cDNA of length 788 bp encoding a putative peptide homologous (45–65%) with 26 kDa glutathione *S*-transferase of vertebrates and invertebrates, and which was identical to that of *C. sinensis* (Hong et al. 2001).

Clone CsHA-26 (CsFtn) contained a 571-bp cDNA encoding a putative polypeptide homologous (46–59%) with ferritin of helminth parasites and identical to that of *C. sinensis* (Tang et al. 2006). Clone CsHA-32 (CsTLD) cDNAs was 1.2 kb long and encoded a polypeptide showing high homology with tolloid-like proteins.

Clone CsHA-33 (CsPGK) cDNA was 1.5 kb long and its deduced polypeptide sequence showed high homology (60–75%) with the phosphoglycerate kinase of vertebrates and invertebrates and which was also similar to that of *C. sinensis* (Hong et al. (2000).

Recombinant proteins

CsEF-1 cDNA encoded an open reading frame of 461 amino acids with a starting methionine and had a calculated molecular mass of 50.5 kDa. The *Bam*H1/*Kpn*1 double-digested fragment of CsEF-1 cDNA was subcloned into pRSET expression vector. Recombinant CsEF-1 protein was produced as a fusion protein with a His-tag peptide derived from the expression vector and had a molecular mass approximately 55 kDa. The fusion protein was purified by Ni-NTA column chromatography and appeared to be homogeneous in Coomassie-stained SDS–PAGE gel.

CsMRLC cDNA encoded a polypeptide of 204 amino acid residues of molecular mass 23.6 kDa. CsMRLC protein was produced using pRSET expression vector as a recombinant protein of approximately 29 kDa, which included a His-tag peptide, and was purified to homogeneity by Ni-NTA affinity chromatography.

The CsTLD cDNA was double-cut with *Bam*H1/*Xho*1 enzymes and the cDNA fragment obtained was subcloned into pRSET expression vector. Its encoding protein was produced as recombinant protein and purified by Ni-NTA affinity column chromatography. It had a molecular mass of 34 kDa.

The CsVpB1 cDNA encoded a polypeptide of 245 amino acids with an estimated molecular mass of 27.6 kDa. The coding region of CsVpB1 cDNA was double-cut with *Bam*HI and *Kpn*I enzymes and subcloned into pREST expression vector. CsVpB1 recombinant protein was induced by adding 1 mM IPTG and purified to homogeneity by Ni-NTA affinity column chromatography.

CsPGK cDNA encoded a polypeptide of 415 amino acids with a molecular mass of 45.5 kDa. It was double-cut with *Eco*RI and *Xho*I and cloned into pGEX-4T-3 expression vector. The recombinant CsPGK protein was overexpressed in *E. coli* JM 803 by 1 mM IPTG induction and purified to homogeneity by a single round of reduced-glutathione agarose affinity chromatography.

28CsGST cDNA encoded a putative polypeptide of 212 amino acids with a predicted molecular mass of 24.7 kDa. The cDNA was double-cut with *Eco*RI and *Xho*I enzymes and subcloned into pET-23c expression vector. The 26CsGST cDNA coding a putative peptide of 218 amino acids (estimated molecular mass 25.1 kDa) was PCR-amplified using specific forward and reverse primers. The amplified cDNA fragment was double-digested with *Nde*I and *Xho*I and subcloned into pET-23b expression vector. Recombinant Cs28GST and Cs26GST proteins were produced in *E. coli* BL21[DE3]pLysS and purified to homogeneity by reduced-glutathione agarose affinity chromatography.

CsFtn cDNA encoded a polypeptide of 168 amino acids with a molecular mass of 19.4 kDa. The cDNA was excised by double digestion with *Bam*HI and *Kpn*I and subcloned into pREST expression vector. CsFtn recombinant protein was induced in *E. coli* BL21[DE3]pLysS strain and purified to homogeneity by Ni-NTA affinity chromatography.

CsMCP cDNA (1,525 bp) encoded a putative polypeptide of 470 amino acids of predicted molecular mass 50.9 kDa, which showed moderate homology with migratory cell protein of vertebrate animals. CsRP cDNA encoded a putative polypeptide containing a repetitive oligopeptide (15 repeats of GPDAPVPKSG) The coding regions of CsMCP and CsRP cDNAs were PCR-amplified sing forward and reverse promoters specific for each cDNA clone, double-digested with restriction enzymes, and subcloned directionally into plasmid expression vector pRSET. The two recombinant proteins expressed in *E. coli* BL21[DE3]pLysS strain were insoluble and, thus, were purified under denaturing condition. *E. coli* transformed and induced for the respective recombinant proteins were homogenized in 6 M urea and spun at 4°C. Supernatants were loaded on Ni-NTA affinity columns, and recombinant proteins were eluted with elution buffer at pH 5.8 and 4.5. Both CsMCP and CsRP recombinant proteins were of high purity as revealed by Coomassie blue-stained SDS–PAGE gels.

Antigenic reactivity of single antigens

In immunoblots, Cs26GST and Cs28GST reacted with 33% and 30% clonorchiasis sera but did not cross-react with the sera of paragonimiasis, schistosomasis, or cysticercosis, suggesting that recombinant Cs26GST and Cs28GST proteins are specific serodiagnostic antigens for human clonorchiasis. CsMCP reacted with 15% of clonorchiasis sera, but not with any other test sera. Recombinant CsRP-1 protein displayed an antigenic reaction with 80% of clonorchiasis sera but cross-reacted with 21% of paragonimiasis and 18% of cysticercosis sera.

Recombinant CsMRLC protein reacted with 35% of clonorchiasis sera and cross-reacted with 40% of the sera of other helminth infections. Recombinant CsTLD protein reacted with 65% of clonorchiasis sera and cross-reacted at similar levels with the sera of other helminth infections. To reduce previously reported cross-reactivity (Tang et al. 2005), a smaller amount of CsVpB1 protein was applied to NC membranes. CsVpB1 protein detected specific antibody in 15% clonorchiasis sera and did not cross-react with paragonimiasis, schistosomiasis, or cysticercosis sera.

Recombinant CsPGK protein produced positive reactions with clonorchiasis sera, all helminths-infected sera, and even with the sera of healthy controls. Recombinant CsEF and CsMXL proteins did not produce any positive reaction with any test serum sample and crude *C. sinensis* antigen reacted non-specifically with all serum samples (Table 1).

Table 1 Sero-reactivity of *Clonorchis sinensis* recombinant proteins evaluated toward helminth-infected human sera using Minislot™

Antigen	Positive rate (%) toward sera of				
	Cs	Pw	Sj	Cc	Control
Lysate	100	100	100	100	100
CsPGK	100	100	100	100	100
Cs26GST	30	0	0	0	0
Cs28GST	65	0	0	0	0
CsMCP	15	0	0	0	0
CsRP	95	50	100	60	70
CsMRLC	65	60	80	60	0
CsTLD	65	80	60	80	20
CsFtn	100	70	100	100	60
CsVpB1	15	0	0	0	0
CsEF	0	0	0	0	0
CsMXL	0	0	0	0	0

Cs, clonorchiasis sinensis (n=20); Pw, paragonimiasis westermani (n=10); Sj, schistosomiasis japonicum (n=5); Cc, cysticercosis celluloase (n=5); Cnt, Control human sera (n=5).

As single antigens, the tested recombinant proteins could be categorized based on their antigenic reactivities as follows: (1) antigenic proteins with specificity but low sensitivity (Cs26GST, Cs28GST, CsMCP), (2) high sensitivity and cross-reactivity (CsRP, CsFtn), (3) moderate sensitivity and low specificity (CsTLD, CsMRLC), (4) pan reactive antigen (CsPGK), and (5) of little antigenicity (CsVpB1, CsEF, CsMXL) (Table 1).

Antigenic reactivities of multiple cocktail antigens

Multiple cocktail antigens were prepared by mixing equal amounts of the recombinant Cs28GST and Cs26GST proteins with high antigenic specificity with lower amounts of the CsVpB1 or CsFtn proteins with less specific antigenicity. A cocktail of Cs26GST and Cs28GST proteins in equal amounts had a sensitivity of 75% and a specificity of 95% toward sera of helminth-infected patients. A triple cocktail of Cs26GST, Cs28GST, and CsFtn recombinant proteins showed a sensitivity of 80% but cross-reacted with the sera of other helminth-infected patients. Another triple cocktail of Cs26GST, Cs28GST, and CsVpB1 recombinant proteins had a sensitivity of 80% and a specificity of 95% (Table 2 and Fig. 1).

To extend the applicability of the *C. sinensis* cocktail antigens, human sera of opisthorchiasis and fascioliasis patients were included in the evaluation and cases of number of sera used were increased. Sensitivity of the two cocktail antigens, Cs26GST+Cs28GST cocktail, and Cs26GST+Cs28GST+CsVpB1cocktail enhanced more but cross-reacted with two and one sera of opisthorciasis and pargonimiasis patients (Table 3).

Discussion

Although many antigenic proteins have been reported for the detection of clonorchiasis, it is still difficult to diagnose helminth-infected patients using serological tests because of antigen cross-reactions. However, recently, antigenic proteins with high purity have been produced in recombinant forms from the cloned cDNAs of *C. sinensis*. In this study, Cs28GST and Cs26GST recombinant proteins were found to have high antigenic specificity for clonorchiasis in patient serum samples. Furthermore, the former showed had twice the sensitivity, which is consistent with previous reports (Hong et al. 2001, 2002; Kang et al. 2001; Shen et al. 2009). These two GSTs are cytosolic enzymes, which are distributing in the tegument and subtegument, and released by turn over of the tegument (Hong et al. 2000).

Ratio of Cs28GST to Cs26GST in the worm is 14:1, indicating Cs28GST is a major GST isoenzyme in adult *C. sinensis* (Kang et al. 2001). Given its abundance, antigen-presenting cells have a better chance of taking up and presenting Cs28GST antigen; thus, *C. sinensis*-infected individuals may more efficiently produce specific antibodies. It has been reported that the 28 kDa GST of *Schistosoma mansoni* is antigenic but cross-reactive to that of the closely related *Schistosoma* spp. (Trottein et al. 1992). The Cs28GST and Cs26GST proteins possess key conserved amino acid residues in their catalytic domains and conserved secondary structures, rendering their enzymatic function and substrate specificity. Furthermore, the linear sequences of Cs28GST and Cs26GST share low to moderate homology with those of the helminthes and vertebrates (Hong et al. 2001; Kang et al. 2001). This low sequence homology of the CsGSTs with other animals

Table 2 Sero-reactivity of cocktail antigens of *Clonorchis sinensis* toward helminth-infected human sera

Antigen cocktail	Positive rate (%) to sera of				
	Cs	Pw	Sj	Cc	Cnt
CsPGK	100	100	100	100	100
Cs26GST+28GST	75	0	0	0	0
Cs26GST+28GST+CsRP	100	80	100	80	0
Cs26GST+28GST+CsFtn	80	80	80	80	80
Cs26GST+28GST+CsVpB1	80	0	0	0	0

Cs Clonorchiasis sinensis (n=20); Pw Paragonimiasis westermani (n=10); Sj Schistosomiasis japonicum (n=5); Cc Cysticercosis celluloase (n=5); Cnt control human sera (n=5)

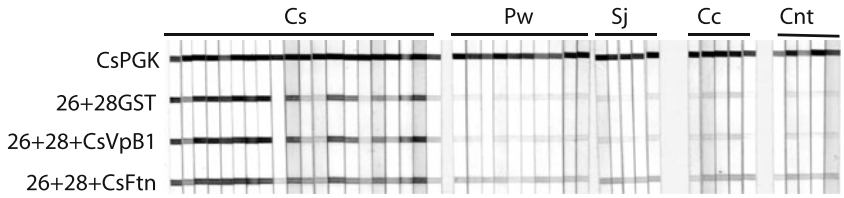

Fig. 1 Immunoblot of multiple cocktail antigens. The cocktail was prepared by combining the recombinant Cs26GST, Cs28GST, CsVpB1, and CsFtn proteins and loaded onto an NC membrane using the Minislot™. Helminth-infected human sera were used at a dilution of 1:100. Cs sera of Clonorchiasis sinensis patients; Pw Paragonimiasis westermani; Sj Schistosomiasis japonicum; Cc Cysticercosis cellulosae; Cnt sera of healthy humans

accounts for their antigenic specificities. In the present study, the antigenic specificities of Cs28GST and Cs26GST suggested that they are good candidates for multiple antigen cocktails.

PGK is an important enzyme that catalyzes the production of ATP in the glycolytic pathway and has been identified to be highly conserved among animals (Lee et al. 1995a). *S. mansoni* PGK was proposed as an antigenic protein (Lee et al. 1995b). CsPGK shows high similarity to the PGK of *S. mansoni* and of other animals and revealed antigenicity toward the sera of rabbits experimentally infected with *C. sinensis* (Hong et al. 2000). In the present study, recombinant CsPGK protein reacted nonspecifically with almost all sera of other helminth-infected patients and even with the sera of normal controls. PGK is highly conserved, and its secondary and tertiary structures show high homology among invertebrates and vertebrates. Furthermore, the high homology of CsPGK may render to the production of antigenic epitopes conserved between parasites and hosts. Therefore, the nonspecific cross-reactivity of CsPGK is considered to be derived from those of conserved antigenic epitopes. This result suggested that CsPGK could be used as a positive control to develop a serodiagnostic system for clonorchiasis.

Parasite proteins containing repetitive peptides of various lengths and repeat numbers have been reported to be antigens that are specific and sensitive for the serodiagnosis of the respective parasitic infections (Marín et al. 1992; Charest and Matlashewski 1994; Imboden et al. 1995). A glycin-rich *C. sinensis* protein (GRCSP) containing a peptide with a 10 amino acid repeat showed a sensitivity of 55% and high specificity by ELISA for the sera of clonorchiasis patients (Yong et al. 1998). Another repetitive protein of *C. sinensis* containing 15 repeats of a 10 amino acid sequence (CsPR) revealed low overall sequence homology with GRCSP but high sequence homology in its N- and C-terminal regions. Furthermore, recombinant CsPR protein showed high sensitivity and moderate specificity toward the sera of clonorchiasis and other helminth-infected patients (Kim et al. 2001). In the present study, in which we employed the MiniSlot™ format, the CsRP, CsMRLC, CsFtn, and CsTLD recombinant proteins also showed high sensitivity but unexpectedly exhibited low specificity. Accordingly, these proteins were considered less useful for preparing *C. sinensis* antigen cocktails.

Table 3 Sero-reactivity of cocktail antigens of *Clonorchis sinensis* toward trematode-infected human sera

Antigen cocktail	Positive rate (%) to sera of					
	Cs	Ov	Pw	Sj	Fh	Control
CsPGK	100	100	100	100	100	100
Cs26GST+28GST	77	22	6	0	0	0
Cs26GST+28GST+CsVpB1	87	22	6	0	0	0

Cs Clonorchiasis sinensis (n=30); Ov Opisthorchiasis vinerrini (n=9); Pw Paragonimiasis westermani (n=18); Sj Schistosomiasis japonicum (n=10); Fh Fascioliasis hepatica (n=10); Cnt Control human sera (n=17)

From the serodiagnostic perspective, recombinant antigens of *C. sinensis* appeared to offer high specificity but only moderate sensitivity for human clonorchiasis. We propose that a cocktail prepared with recombinant antigenic proteins may offer a solution to the problems encountered by those developing serodiagnostic reagents for clonorchiasis. The use of a cocktail of antigens of molecularly defined proteins has been previously proposed to improve sensitivity and to retain specificity (Kim et al. 2001). In the present study, the Cs26GST+Cs28GST cocktail antigen enhanced the positive rate by 10% as compared to Cs28GST antigen, indicating that the Cs26 GST antigen reacted with several sera not detected by Cs28GST and, thus, supplemented the antigenicity of the cocktail.

Cs28GST protein shows high sequential homology with an equivalent protein of *O. viverrini* (Ov28GST), which is not surprising as *O. viverrini* is its closest relative in trematode phylogenetic system. Nevertheless, recombinant Ov28GST was recently reported not detecting antibodies from sera of opisthorchiasis viverrini patients (Eursitthichai et al. 2010), suggesting that recombinant Cs28GST nonspecifically cross-react with other antibodies in the patients' sera.

Vitelline precursor protein B1 of *C. sinensis* (CsVpB1) has been reported to be moderately sensitive and highly

cross-reactive (Tang et al. 2006). When we applied less CsVpB1 protein to the MiniSlot™ in present study, even though sensitivity plummeted, the cross-reactivity issue was resolved. The Cs26GST+Cs28GST cocktail antigen supplemented with CsVpB1 antigen showed enhanced sensitivity over the Cs26GST+Cs28GST cocktail without damaging specificity, whereas supplementation with CsFtn antigen cause a serious reduction in specificity. Accordingly, we propose that the multiple antigen cocktail Cs26GST+Cs28GST+CsVpB1 be considered an useful serodiagnostic reagent for human clonorchiasis.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Cogliano V, WHO International Agency for Research on Cancer Monograph Working Group (2009) A review of human carcinogens-Part B: biological agents. Lancet Oncol 10:321–322

Charest H, Matlashewski G (1994) Developmental gene expression in *Leishmania donovani*: differential cloning and analysis of an amastigote-stage-specific gene. Mol Cell Biol 14:2975–2984

Choi MH, Park IC, Li S, Hong ST (2003) Excretory-secretory antigen is better than crude antigen for the serodiagnosis of clonorchiasis by ELISA. Korean J Parasitol 41:35–39

Eursitthichai V, Viyanant V, Tesana S, Sithithawon P, Kosa N, Grams R (2010) *Oipsthorchis viverrini*: Evaluation of 28 kDa glutathione *S*-transferase as diagnostic tool in human opisthorchiasis. Acta Trop 114:76–80

Hong ST (1988) Changes of anti-*Clonorchis sinensis* IgG antibody in serum after praziquantel treatment in human clonorchiasis. Korean J Parasitol 26:1–8

Hong ST, Kho WG, Lee M, Lee JS, Lee SH (1997) Immunoblot patterns of clonorchiasis. Korean J Parasitol 35:87–93

Hong SJ, Seong KY, Sohn WM, Song KY (2000) Molecular cloning and immunological characterization of phosphoglycerate kinase from *Clonorchis sinensis*. Mol Biochem Parasitol 108:207–216

Hong SJ, Lee JY, Lee DH, Sohn WM, Cho SY (2001) Molecular cloning and characterization of a mu-class glutathione *S*-transferase from *Clonorchis sinensis*. Mol Biochem Parasitol 115:69–75

Hong SJ, Yun Kim T, Gan XX, Shen LY, Sukontason K, Sukontason K, Kang SY (2002) *Clonorchis sinensis*: glutathione *S*-transferase as a serodiagnostic antigen for detecting IgG and IgE antibodies. Exp Parasitol 101:231–233

Hu F, Yu X, Ma C, Zhou H, Zhou Z, Li Y, Lu F, Xu J, Wu Z, Hu X (2007) *Clonorchis sinensis*: Expression, characterization, immunolocalization and serological reactivity of one excretory/secretory antigen-LPAP homologue. Exp Parasitol 117:157–164

Imboden M, Müller N, Hemphill A, Mattioli R, Seebeck T (1995) Repetitive proteins from the flagellar cytoskeleton of African trypanosomes are diagnostically useful antigens. Parasitology 110:249–258

Kang SY, Ahn IY, Park CY, Chung YB, Hong ST, Kong Y, Cho SY, Hong SJ (2001) *Clonorchis sinensis*: molecular cloning and characterization of 28-kDa glutathione *S*-transferase. Exp Parasitol 97:186–195

Kim TY, Kang SY, Ahn IY, Cho SY, Hong SJ (2001) Molecular cloning and characterization of an antigenic protein with a repeating region from *Clonorchis sinensis*. Korean J Parasitol 39:57–66

Kim TY, Cho PY, Na JW, Hong SJ (2007) Molecular cloning and phylogenetic analysis of *Clonorchis sinensis* elongation factor-1α. Parasitol Res 101:1557–1562

Kim TS, Cho SH, Huh S, Kong Y, Sohn WM, Hwang SS, Chai JY et al (2009) A nationwide survey on the prevalence of intestinal parasitic infections in the Republic of Korea, 2004. Korean J Parasitol 47:37–47

Kwon YD, Cho PY, Hong SJ (2005) *Clonorchis sinensis*: molecular cloning and localization of myosin regulatory light chain. Parasitol Res 97:21–26

Lee JH, Rim HJ, Bak UB (1993) Effect of *Clonorchis sinensis* infection and dimethylnitrosamine administration on the induction of cholangiocarcinoma in Syrian golden hamsters. Korean J Parasitol 31:21–30

Lee KW, Shalaby KA, Thakur A, Medhat AM, Karim AM, LoVerde PT (1995a) Cloning of the gene for phosphoglycerate kinase from *Schistosoma mansoni* and characterization of its gene product. Mol Biochem Parasitol 71:221–231

Lee KW, Thakur A, Karim AM (1995b) LoVerde PT (1995b) Immune response to *Schistosoma mansoni* phosphoglycerate kinase during natural and experimental infection: identification of a schistosome-specific B-cell epitope. Infect Immun 63:4307–4311

Li S, Chung BS, Choi MH, Hong ST (2004) Organ-specific antigens of *Clonorchis sinensis*. Korean J Parasitol 42:169–174

Lim MK, Ju YH, Franceschi S, Oh JK, Kong HJ, Hwang SS, Park SK, Cho SI, Sohn WM, Kim DI, Yoo KY, Hong ST, Shin HR (2006) *Clonorchis sinensis* infection and increasing risk of cholangiocarcinoma in the Republic of Korea. Am J Trop Med Hyg 75:93–96

Lun ZR, Gasser RB, Lai DH, Li AX, Zhu XQ, Yu XB, Fang YY (2005) Clonorchiasis: a key food-borne zoonosis in China. Lancet Infect Dis 5:31–41

Marín MS, Prieto M, Martín JM, Casais R, Boga JA, Parra F (1992) Identification and expression of a *Fasciola hepatica* gene encoding a gut antigen protein bearing repetitive sequences. Mol Biochem Parasitol 55:155–165

Na BK, Lee HJ, Cho SH, Lee HW, Cho JH, Kho WG, Lee JS, Lee JS, Song KJ, Park PH, Song CY, Kim TS (2002) Expression of cysteine proteinase of *Clonorchis sinensis* and its use in serodiagnosis of clonorchiasis. J Parasitol 88:1000–1006

Nagano I, Pei F, Wu Z, Wu J, Cui H, Boonmars T, Takahashi Y (2004) Molecular expression of a cysteine proteinase of *Clonorchis sinensis* and its application to an enzyme-linked immunosorbent assay for immunodiagnosis of clonorchiasis. Clin Diagn Lab Immunol 11:411–416

Rim HJ (1986) The current pathobiology and chemotherapy of clonorchiasis. Korean J Parasitol 24:1–141

Rim HJ (2005) Clonorchiasis: an update. J Helminthol 79:269–281

Shen C, Lee JA, Allam SRA, Bae YM, Han ET, Takeo S, Tsuboi T, Hong ST, Choi MH (2009) Serodiagnostic applicability of recombinant antigens of *Clonorchis sinensis* expressed by wheat germ cell-free protein synthesis system. Diagn Microbiol Inf Dis 64:334–339

Song CY, Dresden MH, Rege AA (1990) *Clonorchis sinensis*: purification and characterization of a cysteine proteinase from adult worms. Comp Biochem Physiol B 97:825–829

Tang Y, Cho PY, Kim BS, Hong SJ (2005) Molecular cloning and characterization of vitelline precursor protein B from *Clonorchis sinensis*. J Parasitol 91:1374–1378

Tang Y, Cho PY, Kim TI, Hong SJ (2006) *Clonorchis sinensis*: molecular cloning, enzymatic activity, and localization of yolk ferritin. J Parasitol 92:1275–1280

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Trottein F, Godin C, Pierce RJ, Sellin B, Taylor MG, Gorillot I, Silva MS, Lecocq JP, Capron A (1992) Inter-species variation of schistosome 28-kDa glutathione *S*-transferases. Mol Biochem Parasitol 54:63–72

Yong TS, Yang HJ, Park SJ, Kim YK, Lee DH, Lee SM (1998) Immunodiagnosis of clonorchiasis using a recombinant antigen. Korean J Parasitol 36:183–190

Zhang X, Jin Z, Da R, Dong Y, Song W, Chen X, Huang Q, Ling H, Che Y, Li Y, Zhang F (2008) Fas/FasL-dependent apoptosis of hepatocytes induced in rat and patients with *Clonorchis sinensis* infection. Parasitol Res 103:393–399

Zhao QP, Moon SU, Lee HW, Na BK, Cho SY, Kong Y, Jiang MS, Li AH, Kim TS (2004) Evaluation of *Clonorchis sinensis* recombinant 7-kilodalton antigen for serodiagnosis of clonorchiasis. Clin Diagn Lab Immunol 11:814–817