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Enzyme-linked immunosorbent assay (ELISA) using recombinant *Fasciola* cathepsin L1 for the diagnosis of human fasciolosis caused by *Fasciola hepatica/gigantica* hybrid type

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ABSTRACT

Recombinant *Fasciola* cathepsin L-1 (rCatL1) was evaluated in enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of human fasciolosis in Japan. Quality characteristics of the test were accessed by receiver operating characteristic (ROC) analysis, with sera from fasciolosis patients (n = 10), patients with no evidence of parasitic infections (n = 29), and patients with other helminth infections (n = 119). Both the sensitivity and specificity of the test achieved 100% with the control samples. To test the performance of the assay in an authentic situation, 311 serum samples, which had been sent to our laboratory for the diagnosis of parasitic infections from January 2018 to February 2019, were re-assessed using the rCatL1 ELISA. In this case, the sensitivity of the rCatL1 ELISA was 100%, giving positive results to all fasciolosis sera (n = 7), and the specificity was 99.0%, in which three of the 304 non-fasciolosis samples were judged positive. Careful re-examination of the laboratory data and medical imaging of these three patients revealed that one of the patients, who had been diagnosed as having larva migrans syndrome, was judged to be infected with *Fasciola*, in addition to ascarid nematodes. Thus the true specificity of the assay in the authentic reached 99.3% (302/304). As the rCatL1 ELISA exhibited a highly significant positive likelihood ratio (152.0) and negative likelihood ratio (0.0), calculated from the 311 sample data, this rCatL1 ELISA can be used for routine screening and definitive diagnosis test for fasciolosis in reference laboratories.

1. Introduction

Fasciolosis is one of the most neglected zoonotic diseases. It is caused by an infection of trematodes belonging to the genus *Fasciola*, in which two distinct species, *F. hepatica* and *F. gigantica*, have been recognized [1,2]. *Fasciola hepatica* is found on all continents but Antarctica, particularly where sheep or cattle are raised. *Fasciola gigantica* is mainly found in tropical and subtropical regions in Asia and Africa [3]. In addition to these two species, it has been known that *F. hepatica/gigantica* hybrid type individuals also exist, which exhibit intermediate phenotypes of the two species [4–7]. Hybrid types occur in areas where the distributions of the two species overlap [6,7]. In Japan, only hybrid *Fasciola* are distributed, which were presumably introduced in around the second century from China through Korean peninsula along with the host animals [8–11].

As sheep and cattle are the major definitive hosts of *Fasciola* liver flukes, the livestock industry suffers from a significant economic loss caused by the disease, regardless of the fluke species. Fasciolosis in these animals can result in sudden death, decreased milk yield, and reduced body weight and carcass quality [12–14]. At the same time, people are at risk of infection as well, especially in countries in South America, The Middle East, and South-East Asia. Recent studies estimate that from 2.4

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million to up to 17 million people are affected [1,15], and up to 91 million people are considered at risk worldwide [16]. According to the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD 2010 study) and a WHO initiative, in 2015 more than 2.6 million people were infected globally, in which approximately 300 thousand were serious infections [17].

When definitive hosts, including humans, ingest metacercariae (infective larvae) of *Fasciola* liver flukes, these excyst in the duodenum, and the newly excysted juveniles (NEJs) quickly penetrate the intestinal wall. NEJs then attach themselves to the surface of the liver, and burrow their way into the liver parenchyma before settling in the bile ducts. In the bile ducts, they reach maturity and produce eggs six to eight weeks after the infection [18]. Because of this migratory behavior, symptoms of human fasciolosis are non-specific, e.g., fever, appetite loss, general fatigue, epigastralgia, and the clinical presentation in most cases is hepatic nodules/abscess with peripheral blood eosinophilia and elevated total IgE [19].

As for the diagnosis of human fasciolosis, demonstration of eggs in the stool or bile drainage is not always possible, since eggs are only intermittently released [20], and the worms often remain immature in humans [21]. Because of the low sensitivity of fecal egg examination, an antibody test has been recognized as the choice of laboratory test for the diagnosis [21]. Enzyme-linked immunosorbent assay (ELISA) has been tested vigorously using various antigen preparations. It is known that cathepsin-like cysteine proteases, the major components of excretory-secretory products of adult worms, are excellent antigens for ELISA [22–24]. In particular, recombinant cathepsin L1 (rCatL1) has been shown to be superior to other recombinant proteins [25], and recent studies have demonstrated that rCatL1-ELISA is suited for the standardized diagnostic test for human fasciolosis [26–28], not only for *F. hepatica* but for *F. gigantica* infections as well [29].

Here, we present that rCatL1-ELISA is a reliable method to detect *Fasciola* infections and can even uncover overlooked cases. We also demonstrate that rCatL1 can be used in a multiple dot-ELISA, suggesting that rCatL1-ELISA cab be employed at reference clinical laboratories for the routine screening and definitive diagnosis tests for human fasciolosis.

2. Material and methods

2.1. Ethics statement

All human sera used in the present study were residual samples, sent for diagnosis purposes to the Division of Parasitology, Department of Infectious Diseases, Faculty of Medicine, University of Miyazaki. The use of the residual sera was approved by the Research Ethics Review Board of the Faculty of Medicine, University of Miyazaki, under the title of 'Development of comprehensive test and diagnosis system for parasitic diseases-a retrospective study with clinical information and residual specimens' (permission # O-0359). Our study strictly adhered to the Ethical Guideline for Clinical Study released by the Ministry of Health, Labour and Welfare (MHLW), Japan.

2.2. Patient sera

All serum samples were stored at −80 °C at the Division of Parasitology, Department of Infectious Diseases, Faculty of Medicine, University of Miyazaki. Sera used for the present study were those primarily obtained after October 2006, when one of the authors (HM) became responsible for testing samples and diagnosis.

For the positive control, serum samples received between December 2006 and December 2017 were selected (n = 10). Inclusion criteria for fasciolosis positive cases were; i) hepatic abscess or nodules demonstrated in medical imaging, such as computer tomography (CT) and ultrasonography (US), ii) peripheral blood eosinophilia (>500/μL) or local eosinophilic infiltration demonstrated in biopsy specimen, iii) positive binding of the serum to crude *Fasciola* antigen in ELISA, and iv) a favorable response to triclabendazole (Egaten®, Novartis Pharmaceuticals) treatment. None of the patients during this period were positive for *Fasciola* eggs or worms.

Sera used for assessing cross-reactions due to other helminth infections were obtained from 119 patients, including clonorchiasis (n = 9), paragonimiasis (n = 22), schistosomiasis (n = 10), sparganosis (n = 19), gnathostomiasis (n = 28), and larva migrans syndrome due to toxocariasis/ascariasis (n = 31). Parasitologically confirmed cases (positive for worms or eggs) were preferentially selected, though a substantial fraction of clonorchiasis, paragonimiasis, schistosomiasis, gnathostomiasis, and larva migrans syndrome patients, were egg/worm-negative. Therefore, these were selected based on clinical signs and strong antibody binding to antigens of the respective parasites in ELISA [30,31]. A one egg-positive clonorchiasis sample in 2003 was added to the positive control group to increase the number of samples. Infection negative sera (n = 29) were randomly selected from patients with no sign of parasitic infections, i.e. no antibodies of any parasite antigen, no peripheral blood eosinophilia, no suspected clinical history of parasite infections.

To test the performance of the assay in an authentic situation, all serum samples which had been sent to our laboratory for the diagnosis of parasitic infections from January 2018 to February 2019 were tested in ELISA (n = 311). These samples were sent to us because attending physicians suspected either some kind of parasitic infection, or they intended ruling-out parasitic diseases (diagnosis of exclusion). We diagnosed these 311 patients by examining sera in conventional ELISA with crude antigens. Our previous diagnosis included fasciolosis (n = 7), toxocariasis/ascariasis (n = 36), paragonimiasis (n = 21), schistosomiasis (n = 6), gnathostomiasis (n = 5), strongyloidiasis (n = 4), anisakiasis (n = 3), and sparganosis (n = 1). The remaining 228 cases were judged as having no parasitic infection.

2.3. Clinical information of the patients

Clinical information of individual patients was provided to us by the attending physicians, in the form of a consultation sheet, which contained the patients' age, sex, address (city or county), ethnic origin, chief complaint, brief summary of the present history, dietary history, overseas traveling history, medical imaging findings and laboratory data including fecal egg examination, blood cell count, and blood chemistry. When necessary data were missing, a direct inquiry was made to the patient's attending physician in order to obtain the relevant information.

2.4. Crude Fasciola antigen

Somatic crude antigen of *Fasciola* sp. was prepared as follows. Adult worms collected from infected cattle were homogenized in 0.15 M phosphate-buffered saline (PBS), pH 7.2, containing Protease Inhibitor Cocktail (Mini Protease Inhibitor Cocktail, Roche Diagnostics, Mannheim, Germany). Soluble components were extracted by magnetic stirring at 4 °C overnight and insoluble debris was spun down by centrifugation at 10,000 ×*g* for 10 min at 4 °C. The protein concentrations were measured using a Quant-iT™ Protein Assay Kit and a Qubit fluorometer (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Antigen preparations were stored at −80 °C until used.

2.5. Recombinant cathepsin L-1 (rCatL1)

Total RNA was isolated using TRI reagent (Sigma, St. Louis, MO) from an adult worm of *Fasciola* strain wuh2-1 (*F. hepatica/gigantica* hybrid type), which was isolated from a cow in Wuhan, China, in 2007, and has been maintained in Wistar rats and *Lymnaea ollula* at the Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University. RNA was then reverse transcribed using ReverTra Ace®

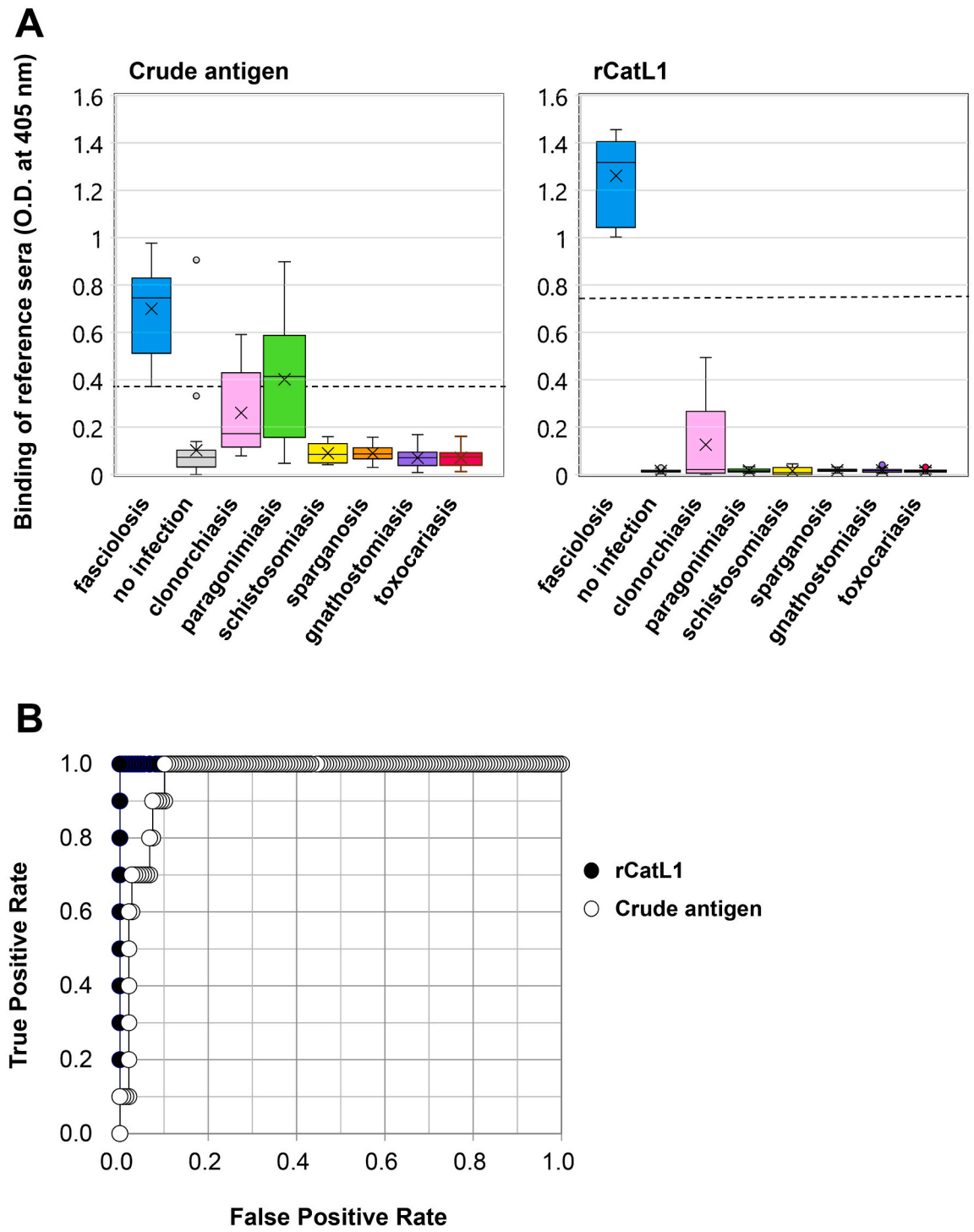

Fig. 1. Binding of reference sera to *Fasciola* crude antigen and rCatL1.
[A] ELISA of sera from infection positive and negative patients, shown in box-and-whisker diagrams (dashed lines: cut-off values). Samples were from patients with fasciolosis (n = 10), clonorchiasis (n = 9), paragonimiasis (n = 22), schistosomiasis (n = 10), sparganosis (n = 19), gnathostomiasis (n = 28), larva migrans syndrome due to toxocariasis/ascariasis (n = 31), and from patients with no evidence for parasitic infections (n = 29). [B] Receiver-operating characteristics (ROC) curves generated from the same data set. Area under curve (AUC) for rCatL1 ELISA was significantly larger than that for crude *Fasciola* antigen ELISA ($p = 0.0169$). Cut-off values for crude antigen ELISA and rCatL1 ELISA were calculated as 0.37 and 0.75, respectively.

(Toyobo, Tokyo, Japan), according to the manufacturer's instructions.

DNA sequence for pro-cathepsin L1 (37 kDa) was amplified by polymerase chain reaction (PCR) with forward (5'-GATGACAAAGTCGACCGATTATTCGTATTAGCCG-3′) and reverse (5'-ACGGCCAGTGAATTCTCACGGAAATCGTGCCAC-3′) primers. The PCR was performed in a total volume of 50 μL, containing 1 μL of cDNA as a template, 200 μM of dNTPs, 0.2 μM of each forward and reverse primer, with 1.25 U of Prime STAR HS DNA polymerase in 1× PCR buffer (TaKaRa Bio Inc., Otsu, Japan). The amplification was carried out by 30 cycles of denaturation at 98 °C for 10s, annealing at 55 °C for 15 s, and an extension at 72 °C for 1 min.

The PCR product was purified and cloned into pHAT10 vector by

using In-fusion HD cloning kit (Clontech, Palo Alto, CA), and *Escherichia coli* BL21 cells were transformed with the recombinant plasmid using a heat-shock method. The transformants were incubated on Luria-Bertani (LB) agar plates containing 100 μg/mL of ampicillin at 37 °C overnight. Colonies containing the insert were identified by colony PCR, and a positive clone was grown overnight in LB broth containing ampicillin (100 μg/mL). Plasmid DNA was isolated using NucleoSpin Plasmid Quick Pure (Macherey-Nagel, Diiren, Germany), according to the manufacturer's instructions. The construct was sequenced to ensure in-frame position of the reading frame. The sequence for cathepsin L1 used in this study was deposited in GenBank under accession number LC557123.

Recombinant CatL1 (rCatL1) was expressed as a fusion protein with histidine affinity tag (HAT) in BL21 at 37 °C for 6 h with 1 mM isopropyl β-D-1-thiogalactopyranoside (Wako Inc., Osaka, Japan), purified under denaturing conditions (6 M Guanidine, Wako Inc., Osaka, Japan) using packed columns (TALON Metal Affinity Resin, Clontech). rCatL1 was appropriately refolded by decreasing concentrations of urea (Wako Inc), and diluted by 150 mM imidazole (nacalai tesque, Kyoto, Japan). The eluate was passed through PD-10 desalting columns (GE Healthcare, Uppsala, Sweden) and dialyzed against Tris-buffered saline (TBS), pH 7.5. The purity and quantity of the proteins were checked by using sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Coomassie brilliant blue R250 staining (MP Biomedicals Inc., Santa Ana, CA, USA) as well as a western blotting with anti-HAT antibody (Funakoshi, Tokyo, Japan). The protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.6. Enzyme-linked immunosorbent assay (ELISA)

In microtiter plate ELISA, wells of microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 1 μg/mL of purified rCatL1 or crude somatic antigen in 0.05 M carbonate/bicarbonate buffer, pH 9.6. The wells were washed with PBS containing 0.05% Tween 20 (PBST), blocked with 1% casein (nacalai tesque, Kyoto, Japan) in TBS for 2 h at room temperature.

Patient sera, diluted 1 to 1000, were added to the wells and incubated for 1 h at 37 °C. After washing with PBST, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark) was added and incubated for 1 h at 37 °C. For color development, ABTS (KPL, Gaithersburg, MD, USA) was added and incubated at room temperature for 30 min. Optical densities at 405 nm were read in a Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). All serum samples were tested in duplicate.

For multiple-dot ELISA, affinity-purified human IgG (50 ng/spot for positive control, FUJIFILM Wako Pure Chemical Co., Osaka, Japan), bovine serum albumin (BSA, 50 ng/spot for negative control, New England Biolabs, Ipswich, MA, USA), and various amounts of rCatL1 (5, 1, 50, 500 ng/spot), were dotted on pieces of nitrocellulose (NC) membrane (ADVANTEC, Tokyo, Japan). The NC membrane was blocked with 1% casein in TBS at 4 °C overnight, and dried on a paper towel (CRECIA, Tokyo, Japan) for 20 min, followed by silica gel at 4 °C for 3 h. Patient sera, diluted 1 to 200, were added to the NC membrane and incubated for 30 min at 37 °C, followed by washing and incubation with HRP-conjugated rabbit anti-human IgG, diluted at 1:1000, at 37 °C for 30 min. After washing, the membrane was immersed in substrate solution (2.2 mM 4-chloro 1-naphthol, 0.015% H2O2, 0.32% Et-OH in PBS) for 8 min, for color development.

Images of dot-blot spots on NC membrane were imported to a Dual Lens System GT-X900 imaging system (EPSON, Japan), and the color intensities of the spots were converted to pixel luminance by using ImageJ v. 1.53a (National Institutes of Health, Bethesda, MD, USA). For each dot sheet, the cumulative pixel luminance was measured across the human IgG (positive control), BSA (negative control), and rCatL1 spots. The color densities for rCatL1 blots on each sheet were normalized in relative to human IgG spot (positive control) as 1.0, and BSA spot (negative control) as 0.0.

Table 1
Comparison between crude somatic antigen and rCatL1 in ELISA with reference sera and actual clinical samples.

		Fasciolosis (+)	Fasciolosis (−)	Total
		Reference sera		
crude somatic antigen	test (+)	10	15	25
	test (−)	0	133	133
	total	10	148	158
rCatL1	test (+)	10	0	10
	test (−)	0	148	148
	total	10	148	158
		Actual clinical samples		
crude somatic antigen	test (+)	7	18	25
	test (−)	1	285	286
	total	8	303	311
rCatL1	test (+)	8	2	10
	test (−)	0	301	301
	total	8	303	311

2.7. Statistical analyses

For receiver operator characteristic (ROC) curve analysis, samples from the 10 fasciolosis patients represented the positive status, whereas samples from the 29 patients with no signs of parasitic infection and 119 patients infected with parasites other than Fasciola, represented the negative status. The ROC curve and the 95% confidence intervals were calculated by Prism v. 7.05 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Binding of reference sera to rCatL1

Recombinant CatL1 was expressed in *E. coli* cells and successfully purified. The deduced amino acid sequence of rCatL1 was 99.4% identical to cathepsin L1 of *F. gigantica* (AAF44675). Sera from fasciolosis patients, patients with no parasitic infections, and patients infected with various parasitic diseases, were tested in ELISA for the binding to crude adult worm antigen and rCatL1. In contrast to the ELISA with crude somatic antigen, in which optical densities of positive and negative sera were overlapping, optical densities of positive samples did not overlap those of negative samples in CatL1-ELISA (Fig. 1A).

In order to determine the cut-off values for infection-positive or -negative, ROC curves were created by plotting the true positive rate against the false positive rate (Fig. 1B). The area under the curve (AUC) for rCatL1 was significantly larger than that for crude *Fasciola* antigen. AUC for crude *Fasciola* antigen and rCatL1 were 0.97 (95% confidence interval (CI): 0.94%–0.99%) and 1.0 (CI: 1.0%–1.0%), respectively. The cut-off values (O.D. at 405 nm) inferred from the ROC curves, were 0.37 and 0.75 for crude antigen- and rCatL1-ELISA, respectively. Based on these cut-off values, the sensitivity and specificity of rCatL1 ELISA were 100% (CI: 88.8%–100%) and 100% (CI: 97.6%–100%), whereas those of crude antigen-EISA were 100% (CI: 88.8%–100%) and 86% (CI: 79.4%–91.1%), respectively (Table 1).

3.2. Re-examination of patient sera in rCatL1 ELISA

Having determined cut-off values for crude antigen- and rCatL1-ELISA, all 311 serum samples which had been sent to our laboratory from January 2018 to February 2019 for the diagnosis of parasitic infections, were tested to evaluate the performance of the ELISAs in an authentic situation. In these patients, seven had been diagnosed as having fasciolosis.

Binding of the 311 individual samples to crude somatic antigen and rCatL1 is shown in Fig. 2A. Samples that bound strongly to rCatL1 bound to crude antigen too, but the reverse was not true. All seven samples

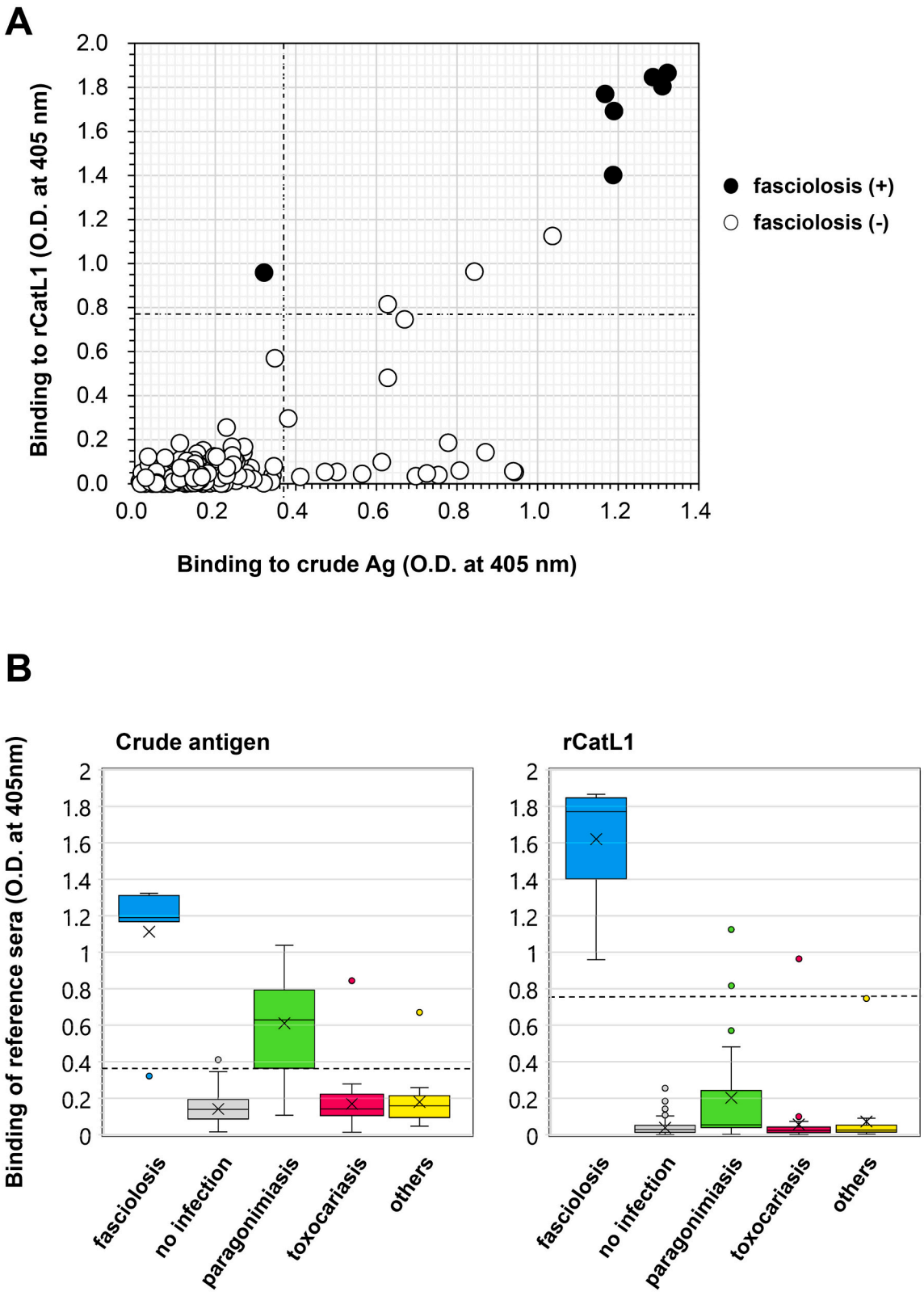

Fig. 2. Re-examination of 311 serum samples.
[A] Binding of 311 serum samples to crude antigen (x-axis) and rCatL1 (y-axis). Dashed lines represent the cut-off values for corresponding antigens. Samples which had been diagnosed as fasciolosis were shown in closed circle (●), and the rest in open circle (○). [B] Box-and-whisker plots of ELISA showing binding to crude antigen and rCatL1. Our previous diagnosis included fasciolosis (n = 7), toxocariasis/ascariasis (n = 36), paragonimiasis (n = 21), schistosomiasis (n = 6), gnathostomiasis (n = 5), strongyloidiasis (n = 4), anisakiasis (n = 3), and sparganosis (n = 1). The remaining 228 cases were judged as having no parasitic infections. Two outliers in paragonimiasis patients and one outlier in larva migrans syndrome patient were above the cut-off value of rCatL1.

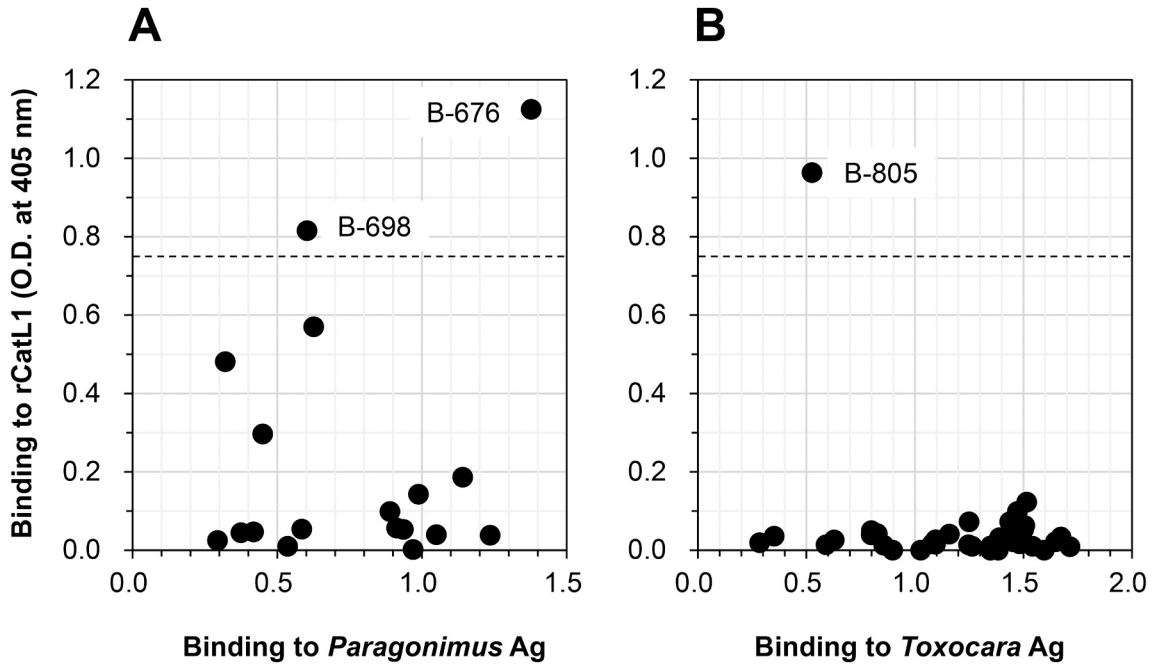

Fig. 3. Binding of sera from paragonimiasis and larva migrans syndrome patients to rCatL1.
Relationship of the binding to rCatL1 and *Paragonimus* or *Toxocara* antigens, in patients with paragonimiasis [A] or larva migrans syndrome [B]. Fraction of paragonimiasis patient sera bound to rCatL1, while only a single sample in larva migrans syndrome patients (B-805) bound to rCatL1.

which had been previously diagnosed as fasciolosis (closed circle) were positive in rCatL1 ELISA, but one was below the cut-off in crude antigen ELISA. Based on these results, the sensitivity of rCatL1 ELISA and crude antigen ELISA were 100% and 85.7%, respectively. As for the specificity, a substantial portion of samples which were not from fasciolosis patients (open circle) were above the cut-off values not only in crude antigen ELISA, but rCatL1 ELISA as well. In crude antigen ELISA, 19 fasciolosis-negative samples were binding-positive, whereas three were positive in rCatL1 ELISA. Thus the specificity of rCatL1 ELISA and crude antigen ELISA were 99.0% and 93.8%, respectively.

In order to find a clue to the nature of these positive binding of non-fasciolosis samples, the 311 samples were divided into groups according to their previous diagnosis. In crude antigen ELISA, most of the positive samples were from paragonimiasis patients, as with reference sera shown in Fig. 1A. In rCatL1 ELISA, two outliers from paragonimiasis patients and one outlier from larva migrans syndrome were judged positive (Fig. 2B).

We then examined the correlation between the binding to rCatL1 and the corresponding antigen, *Paragonimus* antigen in paragonimiasis patients and *Toxocara* antigen in larva migrans syndrome, to see if the binding resulted from cross-reactivity. In samples from paragonimiasis patients, those that bound strongly to rCatL1 showed relatively high binding to *Paragonimus* antigen, but the reverse was not true. Two samples, B-676 and B-698 were above the cut-off for rCatL1 (Fig. 3A). On the contrary, in larva migrans syndrome, there was only one outlier sample that bound strongly to rCatL1 above the cut-off (B-805), and the rest did not bind to rCatL1 at all, regardless of the binding intensity to *Toxocara* antigen (Fig. 3B). These findings suggested that the binding to rCatL1 of the samples from larva migrans syndrome was not a simple cross-reactivity.

3.3. Re-evaluation of clinical pictures of positive cases

To assess the possibility that these 'false positive' samples were actually true positive, we took a closer look at the clinical information on the consultation sheet and attached data of these patients. We also directly contacted the attending physicians for supplementary information. In patients B-676 and B-698, we could not find any sign of hepatic lesion, e.g. abnormal findings in abdominal imaging, or elevated liver enzymes. It appeared that patient B-676 had an active paragonimiasis, and patient B-698 was in the chronic phase of extra-pulmonary paragonimiasis.

In patient B-805, hepatic mass lesion was the only finding that made the responsible radiologist and the attending physician suspect parasitic diseases. The description on the consultation sheet of the hepatic lesion was 'low density in CT and hypoechoic in US', which can be seen both in larva migrans syndrome and fasciolosis. However, the actual images of the nodular lesion in the abdominal CT, downloaded directly from the patient's electronic chart, matched those of fasciolosis much better than those of larva migrans syndrome (Fig. 4). The lesion was a cluster consisting of round or oval low density areas, frequently seen in fasciolosis patients. Based on the CT images, this patient B-805 was likely to have been infected with *Fasciola*, in addition to *Toxocara* or *Ascaris* larvae. Therefore, this sample was considered true-positive, not false-positive in ELISA.

Given that the sample B-805 was true-positive, the number of fasciolosis patients in the 311 patients was eight. Thus the re-calculated specificity of rCatL1 and crude antigen ELISA were 99.3% and 94.1%, respectively (Table 1). With these revised data, positive likelihood ratio (LR+) and negative likelihood ratio (LR-) of rCatL1 ELISA for fasciolosis in actual samples were calculated as 151.5 and 0.0, respectively.

3.4. Multiple dot ELISA using rCatL1

Because of the excellent LR+ and LR- values, rCatL1 ELISA was considered suited for a routine screening test for human fasciolosis. Therefore, we tested rCatL1 in multiple dot-ELISA, because multiple dot-ELISA is more convenient than conventional microtiter plate ELISA [32,33]. We tested 30 samples, containing eight fasciolosis, five paragonimiasis, one gnathostomiasis, and 16 non-infected human sera, which were randomly selected from the above assayed 311 samples. Sera were incubated with dot- ELISA blot sheets, spotted with human IgG (positive control), BSA (negative control), and rCatL1.

As shown in Fig. 5A, sera from fasciolosis patients produced clear

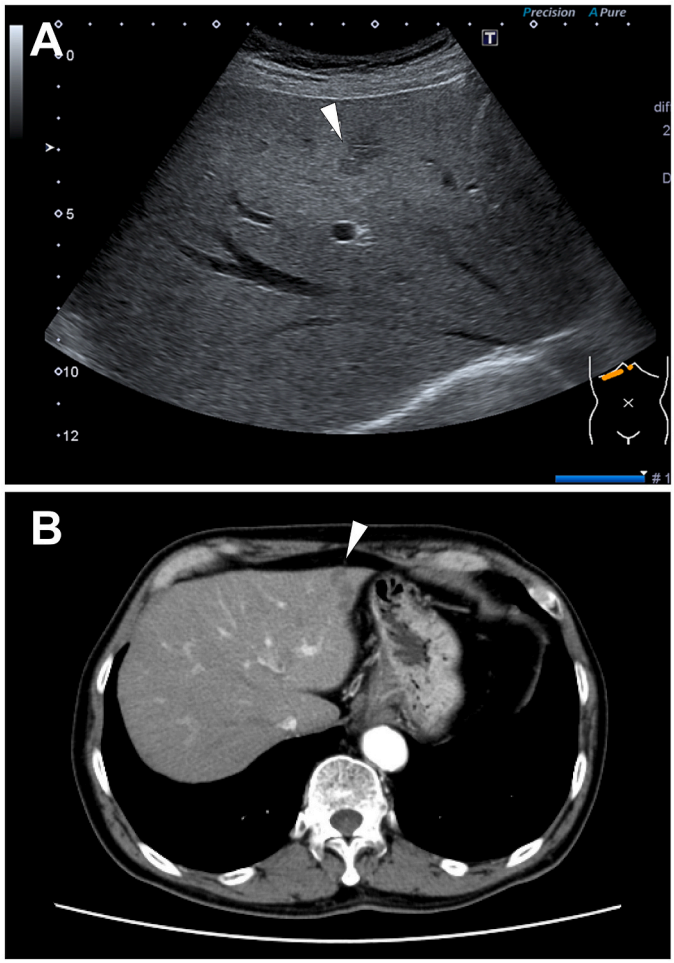

Fig. 4. Abdominal images of the patient B-805.
Ultrasonography [A], and enhanced CT [B] of the patient's hepatic lesion. Note areas which are hypoechoic in US and low density in CT.

positive spots, whereas no color development was observed with non-infected serum samples. Among the 30 samples tested, six were visually judged positive, all of which were fasciolosis samples, and the rest negative. Samples which showed optical densities less than 1.2 in microtiter plate ELISA were completely negative in multiple dot-ELISA (Fig. 5B). No paragonimiasis sera bound to rCatL1 in dot-ELISA. Thus the sensitivity and the specificity of the rCatL1 dot-ELISA were 75% (6/8) and 100% (22/22).

4. Discussion

For the detection of anti-parasite antibodies in ELISA, the most widely-used antigens are excretory-secretory (ES) products of parasites, or recombinant proteins which are major components of ES products. Proteases of different classes, e.g., metalloproteases and cysteine proteases, in ES products have been well characterized [34–36], and have been shown to elicit potent antibody responses to various parasitic infections [37–41]. In the case of *Fasciola*, cysteine proteases belonging to the cathepsin-like family are the dominating components of ES products [42–44]. They are stored as precursor forms in vesicles in the gut epithelial cells, released, and activated in the gut lumen of the flukes [45]. These enzymes are recognized by the host immune system, because they are voided regularly with gut contents, as the digestive tract of flukes is blind-ended [46]. Proteomics analysis of bile taken from infected animals harboring adult *Fasciola* confirmed that these enzymes also represent the majority of protein in situ [47].

In the present study, we confirmed the highly effective performance of rCatL1-ELISA for the diagnosis of human fasciolosis. All quality parameters, including AUC in ROC curve, sensitivity, specificity, and likelihood ratios, were excellent and significantly superior to those of crude somatic antigen. These quality values accorded well with previous studies [23–29]. Cross-reactivity was seen only in two of the paragonimiasis samples. In addition, what was remarkable in the present study was that the re-examination of past patient sera with the rCatL1-ELISA revealed one previously overlooked fasciolosis case (Case B-805). This patient had been diagnosed as having larva migrans syndrome, but the serum sample of this patient actually contained antibodies specific to *Toxocara* antigen as well as rCatL1. This patient had certainly been exposed to both parasites, but we did not previously notice the *Fasciola* infection.

The consultation sheet for Case B-805 filled by the attending physician noted that this patient had a mass in the liver without peripheral blood eosinophilia, and that the serum sample of this patient was positive for the binding to *Toxocara* and *Clonorchis* antigens, which was conducted by a clinical test company in multiple dot-ELISA. Because clonorchiasis was almost extinct in Japan, and we were aware that false positives were sometimes seen with the *Clonorchis* antigen in this company's assay, we examined the serum sample for anti-*Toxocara* and anti-*Ascaris* antibodies only in microtiter plate ELISA.

In retrospect, we should have paid much more attention to the abdominal CT and US images, which were attached to the consultation sheet. We were not careful enough to suspect this disease, because of the rarity of human fasciolosis in Japan. Since the early 1990's, our laboratory has been one of the antibody test centers for parasitic diseases in

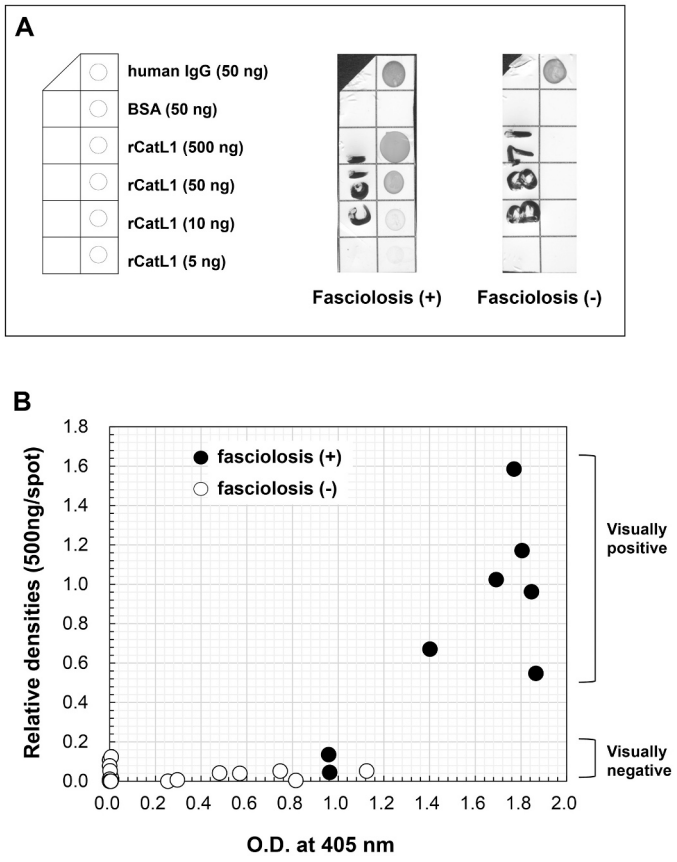

Fig. 5. Multiple dot-ELISA with recombinant CatL1.
[A] Sheet arrangement and representative results. [B] Relationship between dot intensity and optical density of microplate ELISA. Relative intensities of 500 ng spots were plotted against ELISA O.D. Samples which had been diagnosed as fasciolosis were shown in closed circle (●), and the rest in open circle (○).

Japan, testing hundreds of sera and body fluids for anti-parasite antibodies, to help physicians make diagnosis [30,31,48,49]. Although we assist in the diagnoses of approximately one hundred patients with parasitic infections every year, the annual number of fasciolosis cases has been three at most in recent years (data not shown). This is the reason we could find only ten samples that could be used for the positive control in the present study (see Materials and Methods).

One practical measure to avoid this kind of 'misdiagnosis' is to introduce a screening test, covering possible parasite species. In fact the clinical test company, to which the attending physician of Case B-805 had ordered an antibody test, employs a screening test in multiple dot-ELISA with a panel of parasite antigens including *Fasciola*. Unfortunately, it did not work very well. Our rCatL1-ELISA exhibited very high positive likelihood ratio (LR+, 151.5) and low negative likelihood ratio (LR-, 0.0), suggesting the rCatL1-ELISA is useful for both confirming and ruling-out the diagnosis [50]. In order to improve the performance of the anti-parasite screening test, replacement of the present antigen with rCatL1 would have to be considered. However, there is one additional problem to be solved. As shown in Fig. 5B, two out of eight positive samples were judged negative in the present protocol of multiple dot-ELISA. Higher sensitivity has to be achieved by utilizing solid phase capable of binding more antigen molecules, such as CAP system [51], and/or brand new detection technology [52].

One of the limitations of the present study is that all serum samples were derived from a single laboratory in Japan, with a relatively small number of positive reference sera. No samples from developing countries were included, where multiple parasitic infections occur more frequently. Re-evaluation of the assay would be required, with a larger number of positive samples, preferably confirmed by egg detection, and various negative samples. Sera from patients with other infectious diseases, hematological malignancy, or liver cancer will have to be tested.

In conclusion, we propose rCatL1-ELISA as the first choice clinical test for the diagnosis of human fasciolosis, along with the direct identification of eggs/worms. At the same time, introducing this assay to routine screening at reference clinical laboratories is desirable.

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References

[1] S. Mas-Coma, M.A. Valero, M.D. Bargues, Fascioliasis, Adv. Exp. Med. Biol. 1154 (2019) 71–103, https://doi.org/10.1007/978-3-030-18616-6_4.

[2] T.N. Petney, P. Sithithaworn, R.H. Andrews, J.P. Webster, Foodborne trematodes: a diverse and challenging group of neglected parasites, Trans. R. Soc. Trop. Med. Hyg. 110 (1) (2016 Jan) 1–3, https://doi.org/10.1093/trstmh/trv112.

[3] D. Harrington, P.H.L. Lamberton, A. McGregor, Human liver flukes, Lancet Gastroenterol. Hepatol. 2 (9) (2017 Sep) 680–689, https://doi.org/10.1016/S2468-1253(17)30111-5.

[4] T. Agatsuma, Y. Arakawa, M. Iwagami, Y. Honzako, U. Cahyaningsih, S.Y. Kang, S. J. Hong, Molecular evidence of natural hybridization between *Fasciola hepatica* and *F. gigantica*, Parasitol. Int. 49 (3) (2000 Sep) 231–238, https://doi.org/10.1016/s1383-5769(00)00051-9.

[5] M. Ichikawa-Seki, M. Peng, K. Hayashi, T. Shoriki, U.K. Mohanta, T. Shibahara, T. Itagaki, Nuclear and mitochondrial DNA analysis reveals that hybridization between *Fasciola hepatica* and *Fasciola gigantica* occurred in China, Parasitology. 144 (2) (2017 Feb) 206–213, https://doi.org/10.1017/S003118201600161X.

[6] M.V. Periago, M.A. Valero, M. Panova, S. Mas-Coma, Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS), Parasitol. Res. 99 (4) (2006 Sep) 368–378, https://doi.org/10.1007/s00436-006-0174-3.

[7] M.V. Periago, M.A. Valero, M. El Sayed, K. Ashrafi, A. El Wakeel, M.Y. Mohamed, M. Desquesnes, F. Curtale, S. Mas-Coma, First phenotypic description of *Fasciola hepatica/Fasciola gigantica* intermediate forms from the human endemic area of the Nile Delta, Egypt, Infect. Genet. Evol. 8 (1) (2008 Jan) 51–58, https://doi.org/10.1016/j.meegid.2007.10.001.

[8] T. Itagaki, K. Tsutsumi, Triploid form of *Fasciola* in Japan: genetic relationships between *Fasciola hepatica* and *Fasciola gigantica* determined by ITS-2 sequence of nuclear rDNA, Int. J. Parasitol. 28 (1998) 777–781, https://doi.org/10.1016/s0020-7519(98)00037-x.

[9] T. Itagaki, K.I. Tsutsumi, K. Ito, Y. Tsutsumi, Taxonomic status of the Japanese triploid forms of *Fasciola*: comparison of mitochondrial ND1 and COI sequences with *F. hepatica* and *F. gigantica*, J. Parasitol. (1998) 445–448.

[10] M. Ichikawa, N. Iwata, T. Itagaki, DNA types of aspermic *Fasciola* species in Japan, J. Vet. Med. Sci. 72 (2010) 1371–1374, https://doi.org/10.1292/jvms.10-0104.

[11] M. Ichikawa, T. Itagaki, Molecular analysis of aspermic *Fasciola* flukes from Korea on the basis of the nuclear ITS1 region and mitochondrial DNA markers and comparison with Japanese aspermic *Fasciola* flukes, J. Vet. Med. Sci. 74 (2012) 899–904, https://doi.org/10.1292/jvms.11-0523.

[12] A. Howell, M. Baylis, R. Smith, G. Pinchbeck, D. Williams, Epidemiology and impact of *Fasciola hepatica* exposure in high-yielding dairy herds, Prev. Vet. Med. 121 (1–2) (2015 Sep 1) 41–48, https://doi.org/10.1016/j.prevetmed.2015.05.013.

[13] K. Köstenberger, A. Tichy, K. Bauer, P. Pless, T. Wittek, Associations between fasciolosis and milk production, and the impact of anthelmintic treatment in dairy herds, Parasitol. Res. 116 (7) (2017 Jul) 1981–1987, https://doi.org/10.1007/s00436-017-5481-3.

[14] R.A. da Costa, L.G. Corbellini, E. Castro-Janer, F. Riet-Correa, Evaluation of losses in carcasses of cattle naturally infected with *Fasciola hepatica*: effects on weight by age range and on carcass quality parameters, Int. J. Parasitol. 49 (11) (2019 Oct) 867–872, https://doi.org/10.1016/j.ijpara.2019.06.005.

[15] K. Cwiklinski, S.M. O'Neill, S. Donnelly, J.P. Dalton, A prospective view of animal and human Fasciolosis, Parasite Immunol. 38 (9) (2016 Sep) 558–568, https://doi.org/10.1111/pim.12343.

[16] J. Keiser, J. Utzinger, Food-borne trematodiases, Clin. Microbiol. Rev. 22 (2009) 466–483, https://doi.org/10.1128/cmr.00012-09.

[17] T. Fürst, J. Keiser, J. Utzinger, Global burden of human food-borne trematodiasis: a systematic review and meta-analysis, Lancet Infect. Dis. 12 (3) (2012 Mar) 210–221, https://doi.org/10.1016/S1473-3099(11)70294-8.

[18] S.J. Andrews, The life cycle of *Fasciola hepatica*, in: P.J. Dalton (Ed.), Fasciolosis, CAB International, Wallingford, UK, 1999, pp. 1–20.

[19] S. Perrodin, L. Walti, B. Gottstein, C. Kim-Fuchs, D. Candinas, V. Banz, *Fasciola hepatica* in a country of low incidence: a tricky diagnosis, Hepatobiliary Surg. Nutr. 8 (2019) 597, https://doi.org/10.21037/hbsn.2019.04.02.

[20] M.A. Valero, M. Panova, A.M. Comes, R. Fons, S. Mas-Coma, Patterns in size and shedding of *Fasciola hepatica* eggs by naturally and experimentally infected murid rodents, J. Parasitol. 88 (2002) 308–313, https://doi.org/10.1645/0022-3395(2002)088[0308:pisaso]2.0.co;2.

[21] S. Mas-Coma, M. Bargues, M. Valero, Diagnosis of human fascioliasis by stool and blood techniques: update for the present global scenario, Parasitology 141 (2014) 1918–1946, https://doi.org/10.1017/s0031182014000869.

[22] S.M. O'Neill, M. Parkinson, W. Strauss, R. Angles, J.P. Dalton, Immunodiagnosis of *Fasciola hepatica* infection (fascioliasis) in a human population in the Bolivian Altiplano using purified cathepsin L cysteine proteinase, Am. J. Trop. Med. Hyg. 58 (1998) 417–423, https://doi.org/10.4269/ajtmh.1998.58.417.

[23] M.B. Rokni, J. Massoud, S.M. O'Neill, M. Parkinson, J.P. Dalton, Diagnosis of human fasciolosis in the Gilan province of northern Iran: application of cathepsin L-ELISA, Diagn. Microbiol. Infect. Dis. 44 (2002) 175–179, https://doi.org/10.1016/s0732-8893(02)00431-5.

[24] M. Adela Valero, M. Victoria Periago, I. Pérez-Crespo, E. Rodríguez, M. Jesús Perteguer, T. Gárate, E.M. González-Barberá, S. Mas-Coma, Assessing the validity of an ELISA test for the serological diagnosis of human fascioliasis in different epidemiological situations, Tropical Med. Int. Health 17 (2012) 630–636, https://doi.org/10.1111/j.1365-3156.2012.02964.x.

[25] K. Mokhtarian, L. Akhlaghi, M. Mohammadi, A.R. Meamar, E. Razmjou, M. Khoshmirsafa, R. Falak, Evaluation of anti-Cathepsin L1: a more reliable method for serodiagnosis of human fasciolosis, Trans. R. Soc. Trop. Med. Hyg. 110 (2016) 542–550, https://doi.org/10.1093/trstmh/trw063.

[26] S.M. O'Neill, M. Parkinson, A.J. Dowd, W. Strauss, R. Angles, J.P. Dalton, Immunodiagnosis of human fascioliasis using recombinant *Fasciola hepatica* cathepsin L1 cysteine proteinase, Am. J. Trop. Med. Hyg. 60 (1999) 749–751, https://doi.org/10.4269/ajtmh.1999.60.749.

[27] S. Carnevale, M.I. Rodríguez, E.A. Guarnera, C. Carmona, T. Tanos, S.O. Angel, Immunodiagnosis of fasciolosis using recombinant procathepsin L cystein proteinase, Diagn. Microbiol. Infect. Dis. 41 (2001) 43–49, https://doi.org/10.1016/s0732-8893(01)00288-7.

[28] B.G. Santana, J.P. Dalton, F.V. Camargo, M. Parkinson, M. Ndao, The diagnosis of human fascioliasis by enzyme-linked immunosorbent assay (ELISA) using recombinant cathepsin L protease, PLoS Negl. Trop. Dis. 7 (2013), e2414, https://doi.org/10.1371/journal.pntd.0002414.

[29] C. Wongkham, C. Tantrawatpan, P.M. Intapan, W. Maleewong, S. Wongkham, K. Nakashima, Evaluation of immunoglobulin G subclass antibodies against recombinant *Fasciola gigantica* cathepsin L1 in an enzyme-linked immunosorbent assay for serodiagnosis of human fasciolosis, Clin. Diagn. Lab. Immunol. 12 (2005) 1152–1156, https://doi.org/10.1128/cdli.12.10.1152-1156.2005.

[30] E. Nagayasu, A. Yoshida, A. Hombu, Y. Horii, H. Maruyama, Paragonimiasis in Japan: a twelve-year retrospective case review (2001−2012), Intern. Med. 54 (2015) 179–186, https://doi.org/10.2169/internalmedicine.54.1733.

[31] A. Yoshida, A. Hombu, Z. Wang, H. Maruyama, Larva migrans syndrome caused by *Toxocara* and *Ascaris* roundworm infections in Japanese patients, Eur. J. Clin. Microbiol. Infect. Dis. 35 (2016) 1521–1529, https://doi.org/10.1007/s10096-016-2693-x.

[32] H.I. Shaheen, K.A. Kamal, Z. Farid, N. Mansour, F.N. Boctor, J.N. Woody, Dot-enzyme-linked immunosorbent assay (dot-ELISA) for the rapid diagnosis of human fascioliasis, J. Parasitol. (1989) 549–552.

[33] M. Itoh, S. Sato, Multi-dot enzyme-linked immunosorbent assay for serodiagnosis of trematodiasis, Southeast Asian J. Trop Med. Public Health 21 (1990) 471–474.

[34] J.H. McKerrow, The diverse roles of cysteine proteases in parasites and their suitability as drug targets, PLoS Negl. Trop. Dis. 12 (8) (2018 Aug 23), e0005639, https://doi.org/10.1371/journal.pntd.0005639.

[35] J. Feng, B. Zhan, Y. Liu, S. Liu, A. Williamson, G. Goud, A. Loukas, P. Hotez, Molecular cloning and characterization of ac-MTP-2, an astacin-like metalloprotease released by adult *Ancylostoma caninum*, Mol. Biochem. Parasitol. 152 (2) (2007 Apr) 132–138, https://doi.org/10.1016/j.molbiopara.2007.01.001.

[36] V.L. Hunt, A. Hino, A. Yoshida, T. Kikuchi, Comparative transcriptomics gives insights into the evolution of parasitism in *Strongyloides* nematodes at the genus, subclade and species level, Sci. Rep. 8 (1) (2018 Mar 26) 5192, https://doi.org/10.1038/s41598-018-23514-z.

[37] D.H. Yun, J.Y. Chung, Y.B. Chung, Y.Y. Bahk, S.Y. Kang, Y. Kong, S.Y. Cho, Structural and immunological characteristics of a 28-kilodalton cruzipain-like cysteine protease of *Paragonimus westermani* expressed in the definitive host stage, Clin. Diagn. Lab. Immunol. 7 (2000) 932–939, https://doi.org/10.1128/cdli.7.6.932-939.2000.

[38] H. Park, S.I. Kim, K.M. Hong, M.J. Kim, C.H. Shin, J.S. Ryu, D.Y. Min, J.B. Lee, U. W. Hwang, Characterization and classification of five cysteine proteinases expressed by *Paragonimus westermani* adult worm, Exp. Parasitol. 102 (2002) 143–149, https://doi.org/10.1016/s0014-4894(03)00036-5.

[39] B.-K. Na, H.-J. Lee, S.-H. Cho, H.-W. Lee, J.-H. Cho, W.-G. Kho, J.-S. Lee, J.-S. Lee, K.-J. Song, P.-H. Park, C.-Y. Song, T.-S. Kim, Expression of cysteine proteinase of *Clonorchis sinensis* and its use in serodiagnosis of clonorchiasis, J. Parasitol. 88 (2002) 1000–1006, 7, https://doi.org/10.1645/0022-3395(2002)088[1000:eocpoc]2.0.co;2.

[40] L.N. Liu, Z.Q. Wang, X. Zhang, P. Jiang, X. Qi, R.D. Liu, Z.F. Zhang, J. Cui, Characterization of *Spirometra erinaceieuropaei* plerocercoid cysteine protease and potential application for serodiagnosis of sparganosis, PLoS Negl. Trop. Dis. 9 (2015), e0003807, https://doi.org/10.1371/journal.pntd.0003807.

[41] A. Loukas, A.J. Dowd, P. Prociv, P.J. Brindley, Purification of a diagnostic, secreted cysteine protease-like protein from the hookworm *Ancylostoma caninum*, Parasitol. Int. 49 (2000) 327–333, https://doi.org/10.1016/s1383-5769(00)00054-4.

[42] J.P. Dalton, S.O. Neill, C. Stack, P. Collins, A. Walshe, M. Sekiya, S. Doyle, G. Mulcahy, D. Hoyle, E. Khaznadji, N. Moiré, G. Brennan, A. Mousley, N. Kreshchenko, A.G. Maule, S.M. Donnelly, *Fasciola hepatica* cathepsin L-like proteases: biology, function, and potential in the development of first generation liver fluke vaccines, Int. J. Parasitol. 33 (11) (2003 Sep 30) 1173–1181, https://doi.org/10.1016/s0020-7519(03)00171-1.

[43] M.W. Robinson, J.F. Tort, J. Lowther, S.M. Donnelly, E. Wong, W. Xu, C.M. Stack, M. Padula, B. Herbert, J.P. Dalton, Proteomics and phylogenetic analysis of the cathepsin L protease family of the helminth pathogen *Fasciola hepatica*: expansion of a repertoire of virulence-associated factors, Mol. Cell. Proteomics 7 (2008) 1111–1123, https://doi.org/10.1074/mcp.m700560-mcp200.

[44] M.W. Robinson, R. Menon, S.M. Donnelly, J.P. Dalton, S. Ranganathan, An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: proteins associated with invasion and infection of the mammalian host, Mol. Cell. Proteomics 8 (2009) 1891–1907, https://doi.org/10.1074/mcp.m900045-mcp200.

[45] P.R. Collins, C.M. Stack, S.M. O'Neill, S. Doyle, T. Ryan, G.P. Brennan, A. Mousley, M. Stewart, A.G. Maule, J.P. Dalton, Cathepsin L1, the major protease involved in liver fluke (*Fasciola hepatica*) virulence propeptide cleavage sites and autoactivation of the zymogen secreted from gastrodermal cells, J. Biol. Chem. 279 (2004) 17038–17046.

[46] D.W. Halton, Nutritional adaptations to parasitism within the platyhelminthes, Int. J. Parasitol. 27 (1997) 693–704, https://doi.org/10.1016/s0020-7519(97)00011-8.

[47] R.M. Morphew, H.A. Wright, E.J. LaCourse, D.J. Woods, P.M. Brophy, Comparative proteomics of excretory-secretory proteins released by the liver fluke *Fasciola*

hepatica in sheep host bile and during *in vitro* culture ex host, Mol. Cell. Proteomics 6 (6) (2007 Jun) 963–972, https://doi.org/10.1074/mcp.M600375-MCP200.

[48] A. Yoshida, K. Matsuo, J. Moribe, R. Tanaka, T. Kikuchi, E. Nagayasu, N. Misawa, H. Maruyama, Venison, another source of *Paragonimus westermani* infection, Parasitol. Int. 65 (6 Pt A) (2016 Dec) 607–612, https://doi.org/10.1016/j.parint.2016.09.009.

[49] A. Hombu, A. Yoshida, T. Kikuchi, E. Nagayasu, M. Kuroki, H. Maruyama, Treatment of larva migrans syndrome with long-term administration of albendazole, J. Microbiol. Immunol. Infect. 52 (1) (2019 Feb) 100–105, https://doi.org/10.1016/j.jmii.2017.07.002.

[50] A.-M. Šimundić, Measures of diagnostic accuracy: basic definitions, EJIFCC 19 (2009) 203.

[51] Y.H. Nam, S.K. Lee, Comparison between skin prick test and serum immunoglobulin E by CAP system to inhalant allergens, Ann. Allergy Asthma Immunol. 118 (5) (2017 May) 608–613, https://doi.org/10.1016/j.anai.2017.03.005.

[52] Q. Zhao, D. Lu, G. Zhang, D. Zhang, X. Shi, Recent improvements in enzyme-linked immunosorbent assays based on nanomaterials, Talanta 223 (Pt 1) (2021 Feb 1) 121722, https://doi.org/10.1016/j.talanta.2020.121722.