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Diagnosis of *Fasciola gigantica* infection using a monoclonal antibody-based sandwich ELISA for detection of circulating cathepsin B3 protease

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ABSTRACT

A reliable monoclonal antibody (MoAb)-based sandwich enzyme-linked immunosorbent assay (sandwich ELISA) was developed for the detection of circulating cathepsin B3 protease (CatB3) in the sera from mice experimentally infected with *Fasciola gigantica* and cattle naturally infected with the same parasite. The MoAb 2F9 and biotinylated rabbit polyclonal anti-recombinant CatB3 antibody were selected due to their high reactivities and specificities to *F. gigantica* CatB3 antigen based on indirect ELISA and immunoblotting. The lower detection limit of the sandwich ELISA assay was 10, 100 and 400 pg/ml, when applied for the detection of rCatB3 antigen and CatB3 in whole body (WB) of newly excysted juveniles (NEJ) and metacercariae (Met) of *F. gigantica*, respectively. This sandwich ELISA assay could detect *F. gigantica* infection from day 1 to 35 post infection and revealed that circulating level of CatB3 peaked at day 1 post infection. In contrast, the antibody detection by indirect ELISA could only demonstrate the antibody level from 35 days post infection. The reliability of the assay method was evaluated using serum samples from mice infected with *F. gigantica* or *Schistosoma mansoni*, and hamsters infected with *Opisthorchis viverrini*, as well as normal mice and hamsters. In addition, sera from cattle infected with *Paramphistomum cervi*, Strongylid, *Trichuris* sp. and *Strongyloides* sp., as well as sera from normal cattle were also assessed. In experimental mice, the diagnostic sensitivity, specificity, positive predictive value, negative predictive value, false positive rate, false negative rate and accuracy of ELISA were 95%, 100%, 100%, 97.9%, 0%, 5.3% and 98.5%, while in natural cattle they were 96.7%, 100%, 100%, 98.5%, 0%, 3.4% and 98.9%, respectively. Hence, this assay method showed high efficient and precision for early diagnosis of fasciolosis by *F. gigantica*.

1. Introduction

Fasciola gigantica caused fasciolosis in ruminants in the tropical parts of Asia and Africa, which causes a substantial economic loss to the livestock industry in developing and underdeveloped countries of the world (Sobhon et al., 1998; Spithill et al., 1999). Moreover, fasciolosis is a zoonotic disease recognized by the World Health Organization as an emerging human health problem (Spithill et al., 1999; Mas-Coma et al., 2005). The current diagnosis of fasciolosis is based on the microscopic detection of the fluke's eggs in the feces. However, this can only be detected at 13–14 weeks after infection when the major damage to the host's hepatic system has already occurred (Guobadia and Fagbemi, 1997). Immunodiagnosis of fasciolosis in animals by antibody detection has been developed (Zimmerman et al., 1985; Swarup et al., 1987; Fagbemi and Obarisiagbon, 1990; Guobadia and Fagbemi, 1995; Sriveny et al., 2006); however, the presence of antibodies is not a direct indicator of active infection, and cross-reactivity with other parasites' antigens is often difficult to differentiate. Hence, antigen detection is considered to be a more reliable method for identifying animals with pre-patent or occult infection, which could not be detected by the usual parasitological test. Moreover, the antigen detection can give a more accurate current rather than past infection (Langley and Hillyer, 1989; Fagbemi et al., 1995; Viyanant et al., 1997; Velusamy et al., 2004; Anuracpreeda et al., 2009a,b).

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In liver flukes, cathepsin B (CatB) is a major protease, but it is less studied in terms of characteristics and functions in comparison to other proteases. In *F. gigantica* (FG), Meemon et al. (2004) had cloned cathepsin B cDNAs from three different stages (adult, newly excysted juveniles-NEJ, and metacercariae). FG cat-B1 transcripts were detected in all stages, while FG cat-B2 and FG cat-B3 transcripts were expressed in metacercariae, NEJ, and juvenile parasites only. The switching off of FG cat-B2 and FG cat-B3 genes during the maturation of the parasites indicates that these enzymes may be used by the juvenile stages to digest host tissues during their penetration and migration to the liver, while FG cat-B1, which is present in all stages, may perform general digestive function. In the present study, we produced a monoclonal antibody (MoAb) against recombinant *F. gigantica* cathepsin B3 (rFgCatB3), and use it in a sandwich enzyme-linked immunosorbent assay (sandwich ELISA) for detection of circulating cathepsin B3 antigen of *F. gigantica* in the sera of experimentally and naturally infected animals. The use of this MoAb provides the immunodiagnosis of fasciolosis with high sensitivity and specificity.

2. Materials and methods

2.1. Collection of parasites

2.1.1. F. gigantica metacercariae (Met)

To obtain metacercariae, the *Lymnaea ollula* snails were infected with miracidiae, hatched from the mature eggs, and allowed to develop into sporocysts and cercariae. After 8 weeks, the cercariae were shed from the snails and attached to the 5 cm × 5 cm cellophane papers to form metacercariae. The metacercariae were collected from cellophane papers and washed several times with 0.85% NaCl solution and used immediately (Anuracpreeda et al., 2009a).

2.1.2. F. gigantica newly excysted juveniles (NEJ)

The method described by Anuracpreeda et al. (2009a) was used to obtain NEJ of *F. gigantica*. Briefly, metacercariae were excysted by incubation in distilled water containing 1% (w/v) pepsin (pepsin A from porcine gastric mucosa, P-7000, Sigma–Aldrich Co.) and 0.4% (v/v) HCl at 37 °C for 45 min, and washed with distilled water. Thereafter, they were resuspened in 0.02 M sodium dithionite (Fluka Biochemika), 0.2% (w/v) taurocholic acid (T-4009, Sigma–Aldrich Co.), 1% (w/v) $NaHCO_3$ and 0.8% (w/v) NaCl in water. Subsequently, 0.005% (v/v) HCl was added to the tube to generate CO_2 gas. Specimens were incubated at 37 °C for 45 min and washed with distilled water. The activated metacercariae were transferred to fresh RPMI-1640 medium containing 10 μg/ml gentamycin, and 10% fetal calf serum. Then, the contents were transferred to an excystment tower fitted with 100 μm meshes within a 24-well plate and incubated at 37 °C overnight. On the following day, the newly excysted juveniles (NEJ) migrated across the mesh were collected and washed several times with 0.85% NaCl solution and used immediately.

2.1.3. Other parasites

For the cross-reactivity study, other adult trematodes including *Fasciola hepatica*, *Eurytrema pancreaticum*, *Gigantocotyle explanatum*, *Cotylophoron cotylophorum*, *Paramphistomum cervi*, *Fischoederius cobboldi*, *Gastrothylax crumenifer* and *Schistosoma spindale*, and adult nematode parasites including *Haemonchus placei* and *Setaria labiato-papillosa* were collected from the infected cattle or water buffaloes killed at local abattoirs. Adult *Opisthorchis viverrini* were collected from hamsters infected orally with metacercariae obtained from the muscles of naturally infected cyprinoid fishes. After 4 weeks of infection, the adult *O. viverrini* were collected by teasing the liver apart. Adult *Schistosoma* sp. including *Schistosoma mansoni*, *Schistosoma japonicum* and *Schistosoma mekongi* were obtained from mice infected 8 weeks previously with the cercariae by perfusion. All parasite samples were washed several times in 0.85% NaCl solution before being processed for further experiments.

2.2. Antigen preparations

2.2.1. Whole body (WB) antigens of the parasites

WB antigens of the parasites were carried out according to the method of Anuracpreeda et al. (2008, 2009b). The WB antigens were obtained by extracting Met and NEJ of *F. gigantica* and other parasites in lysis buffer containing 10 mM Tris–HCl, pH 7.2, 150 mM NaCl, 0.5% (v/v) Triton X-100, 1 mM EDTA and 1 mM PMSF (P-7626, Sigma–Aldrich Co.). The parasite samples were homogenized, and then sonicated for 5 min with 15-s pulse and pause cycles in an ice bath. After rotation at 4 °C for 1 h, the suspensions were centrifuged at 5000 × *g* at 4 °C for 20 min to get rid of the eggs, and the supernatants were collected.

2.2.2. Protein determinations

Protein concentrations in the parasites' extracts were determined by Lowry's method (Lowry et al., 1951) using bovine serum albumin as a standard. These extracts were stored at −70 °C until use.

2.3. Preparation of recombinant F. gigantica cathepsin B3 protease (rFgCatB3)

The method described by Sethadavit et al. (2009) and Anuracpreeda et al. (2009a) was used to obtain rFgCatB3. Briefly, the cDNA of FgCatB3 was cloned by PCR screening of an NEJ cDNA library. The PCR products from tertiary screen were subcloned into pGEM®-T Easy vector (Promega) and the sequence confirmed by DNA sequence analysis (Macrogen, South Korea). The proFgCatB3 cDNA was generated and subcloned into the pPICZαA vector using the primer pairs 5′-GCTGAAGCT*GAATTC*AAGCCAAACTAC-3′ and 5′-ATCTTGGTAGAC*GCGGCCGC*AGGTAATCCGGC-3′ containing the EcoRI and NotI restriction endonuclease sites (italics), respectively, using Platinum® *Pfx* DNA polymerase (Invitrogen) with the optimal conditions: 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 90 s, and finally at 68 °C for 10 min. The sequence was confirmed by DNA sequence analysis. The pPICZαA containing proFgCatB3 gene was linearized with SacI then electroporated into *Pichia pastoris* GS115. Genomic integration was confirmed by screening genomic DNA by direct PCR using 5_*AOX1* and 3_*AOX1* primers (Linder et al., 1996). Recombinant FgCatB3 (rFgCatB3) was expressed in *Pichia pastoris* and purified by Ni^{2+}-NTA affinity chromatography (QIAGEN).

2.4. Production of monoclonal antibodies (MoAbs) against rFgCatB3

Inbred BALB/c mice, 6–8-weeks-old, were immunized according to the method described by Anuracpreeda et al. (2006). The hybridoma clones expressing MoAb against rFgCatB3 were produced by fusing the splenocytes of immunized mice with non-secreting mouse myeloma cells (P3x63-Ag8.653) using polyethylene glycol (Sigma–Aldrich Inc., St. Louis, MO, USA). The hybridoma cells that grew successfully in culture were screened for anti-rFgCatB3 antibodies by indirect enzyme-linked immunosorbent assay (indirect ELISA) (Engvall and Perlman, 1971), and the highly reactive hybrids were cloned by limiting dilution methods using a feeder layer of spleen cells (Anuracpreeda et al., 2011). The antibody isotypes were determined by ELISA using