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Original article

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Antigen detection of *Fasciola gigantica eggs* using Indirect ELISA and Western blot techniques

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Abstract

Fasciolosis are foodborne zoonotic trematode parasites and major pathogens of cattle and sheep. Fasciolosis causes an economic loss of livestock industry, meat and milk from the cattle, and the disease in humans. The mature worm produces eggs, the egg are released with the feces in natural water resources and hatching into the parasites. Various genes and proteins are involved in different processes of parasite. The aim of this study was to detect specific protein expression for hatching of *F. gigantica* egg using Indirect ELISA and Western blot techniques. The results showed that Indirect ELISA could detect FgTRP14, FgSOD, FgTrx, FgCatL3, FgCatL1H, FgLAP and FgTGR proteins in Fasciola egg on days 0, 9 and 14. In addition, immunoblots of whole body of egg protein on day 0, 9 and 14 were positively reacted with rabbit anti-rFgTrx, rFgCatL1H and rFgSOD. The identification of proteins actively secreted by live *F. gigantica* eggs provides an important new information for understanding immune modulation and it may be a good effective target for the diagnosis development

Keywords: *Fasciola gigantica*, Hatching, Egg secreted protein

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Introduction

Fasciolosis in domestic ruminants are caused by infection with the parasites *F. gigantica* and *F. Hepatica.* Fasciolosis is a disease of ruminants, especially cattle and buffalos that leads to remarkable in both productivity and reproduction (Kenyon et al., 2009). It causes liver damage, anemia, and death in heavily infected animals, thus causing severe economic loss in sheep, cattle, and goat. (Yamasaki et al., 2002). Quantifying egg production through faecal egg count reduction tests is the most widely used diagnostic tests to measure drug efficacy (Hanna et al., 2006). Each parasite species has its own particular egg shape and the length and width of the eggs are generally within a specific range (WHO, 1991). To complete the life cycle, the egg' parasites must migrate to the lumen of the intestines so that it can be passed to the outside environmental along with the feces. Hatching of Fasciola eggs occur when the feces are diluted by natural water and developed to miracidia. Currently, the control of fasciolosis based on the anthelmintic drugs include albendazole, triclabendazole and halogenated hydrocarbons. Long-term use of these anthelmintic drugs does not prevent reinfection. (Overend and Bowen, 1995)

Diagnosis of fasciolosis usually use is to detect parasite eggs in the stool sample as coprological analyses. Although this method is completed and often unreliable during acute infection. (Mas-Coma et al., 2005). Due to the limitation in the diagnosis of fasciolosis, need to investigate proteins to test their ability to diagnosis and prevent disease. Proteins are complex organic compounds and plays role in various processes in the body such as growth, enzymes and immunity. Therefore, in this study, we had detected protein expression for hatching of *F. gigantica* egg that have not been reported at each incubation period. This information will be used for further studies that will exploit this protein as a drug target and possibly in the diagnosis in early stages of infection.

Material and Methods

Collection of the eggs of F. gigantica

The eggs of *F. gigantica* were collected from gallbladder of the naturally infected cattle. They were washed with 0.85% NaCl solution and several times with PBS, pH 7.4.

Development of F. gigantica eggs

The eggs were kept in distilled water and put in Petri dish under normal laboratory conditions of temperature (25-27 ° C) for hatching within a period of 13-16 days. Eggs were collected on day 0, 9 and 14 before the miracidia hatched from the eggs.

Antigen preparation

The eggs of *F. gigantica* were washed several times with 0.85% NaCl solution and then the eggs were mixed with lysis buffer (50mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) The parasite eggs were subsequently homogenized and sonicated on ice bath. The suspensions were centrifuged at 12000g at 4° C for 30 minutes. The supernatants were collected and used as egg antigen. The concentrations of the proteins were determined by Lowry's method.

Detection of egg antigens by indirect ELISA

A 96-well plate was coated with 1 µg/ml of egg antigen in coating buffer (15 mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and incubated at 4°C for overnight. The coated plate was washed three times with phosphate buffer saline with Tween 20 (PBST) (0.05% Tween 20) and nonspecific binding was blocked with 1% bovine serum albumin at room temperature for 1 h. Then, the coated plate was washed three times with PBST. The primary antibody (rabbit anti-rFgTRP14, rabbit anti-rFgSOD, rabbit anti-rFgTrx, rabbit anti-rFgCatL3, rabbit anti-rFgCatL1H, rabbit anti-rFgLAP, rabbit anti-rFgTGR) diluted at 1:500 to 1:1,024,000 with PBS, were added and incubated at room temperature for 1 h. the plated was washed three times with PBST and incubated with HRP-conjugated goat anti-rabbit diluted at 1:5,000 at room temperature for 1 h. Then, the plate was washed with 3,3',5,5'-tetramethylbenzidine (KPL, Gaithersburg, USA) at room temperature for 10 minutes.

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Finally, enzymatic reaction was stopped by adding 1 N HCl at 100 µl per well. The optical densities (OD450) were measured at 450 nm in the automatic Titertek Multiscan Spectrophotometer (Flow Laboratories, VA, USA). The experiments were performed in triplicate.

Immunoblot analysis

The 5 µg/well of each antigen (on day 0, 9, 14) fractions were separated on a 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were block with 10% skim milk in PBS containing 0.1% Tween-20 (PBST) at room temperature (RT) for 1 h, then incubated with rabbit anti-rFgTrx, rabbit anti-rFgCatL1H and rabbit anti-rFgSOD serum diluted 1:5,000 with PBS at RT for 1 h. The positive bands were visualized using AP-conjugated goat anti-rabbit IgG (Invitrogen-Life Technologies, Carlsbad, CA, USA) diluted 1:5,000 with PBS at room temperature for 1 h and the color developed with nitro-blue tetrazolium chloride 5-bromo-4-chloro-3-indodyl phosphate (NBT/BCIP) substrates (Roche, Mannheim, Germany). Finally the reaction was stopped by adding a buffer containing 10 mM Tris-HCl and 1 mM EDTA.

Results

Expression of F. gigantica eggs protein

Eggs protein of *F. gigantica* on days 0, 9 and 14 were analyses by 12.5% SDS-PAGE

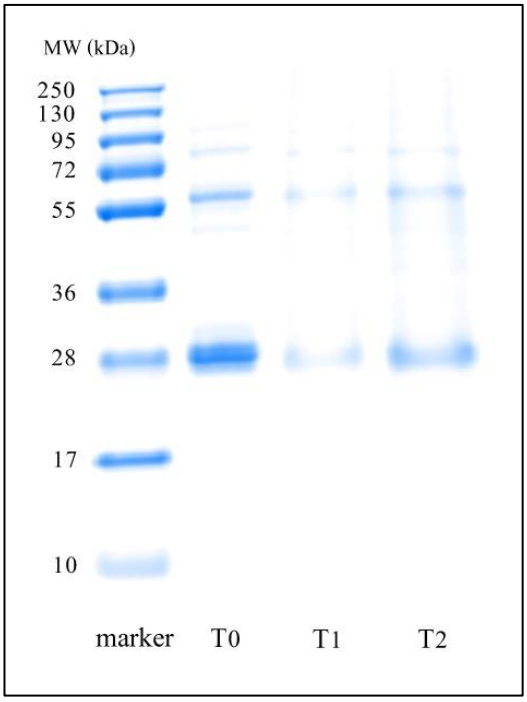

Figure 1 SDS-PAGE of *F. gigantica* eggs protein expression analysis (Lane M: The standards marker of PageRuler TM Plus Prestained Protein Ladder (Fermantas), Lane T0: *F. gigantica* eggs on days 0, Lane T1: *F. gigantica* eggs on days 9, Lane T2 *F. gigantica* eggs on days 14)

ISSN 2673-0200 (print) ISSN 2673-0251 (online)

Photomicrograph of F.gigantica eggs

The morphological analysis of the eggs shows that regularity on the shell surface is very common. The eggs are large, yellowish, oval body with a thin and flat opercula.

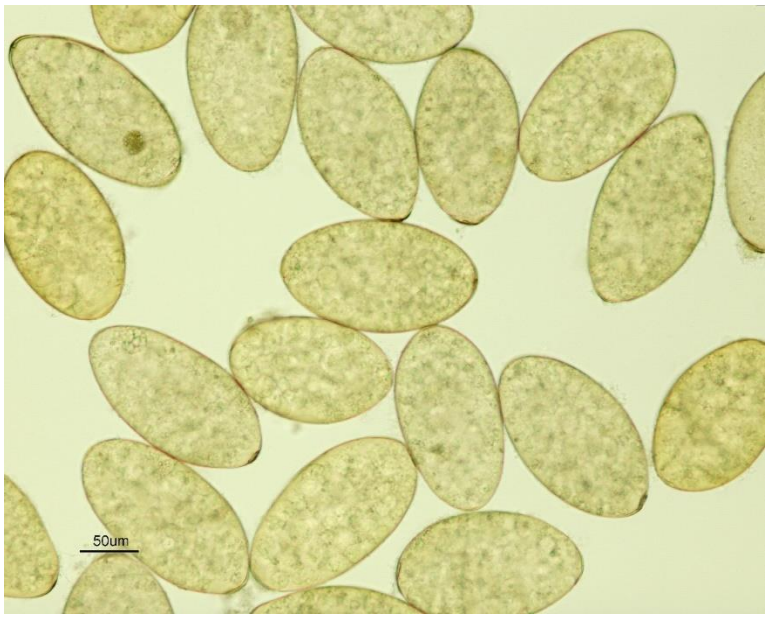

Figure 2 *F. gigantica* eggs are large yellowish

Expression of egg specific proteins to F.gigantica by Indirect ELISA

The Polyclonal antibody against rFgTRP14, rFgSOD, rFgTrx, rFgCatL3, rFgCatL1H, rFgLAP and rFgTGR was produced by immunization in each rabbits. The serum was first immunized by subcutaneous route with 125 µg of rFgTRP14, rFgSOD, rFgTrx, rFgCatL3, rFgCatL1H, rFgLAP and rFgTGR protein with Complete Freund's adjuvant (CFA). The second, third and fourth immunizations were done with 250 µg of the same recombinant protein in Incomplete Freund's adjuvant (IFA).

The egg specific protein expression of *F. gigantica* by Indirect ELISA revealed that antibody specificity to egg proteins at days 0, 9 and 14. The results showed that the lowest concentrations of antibody in the range 1:500 to 1:8,000 (Figure 3).

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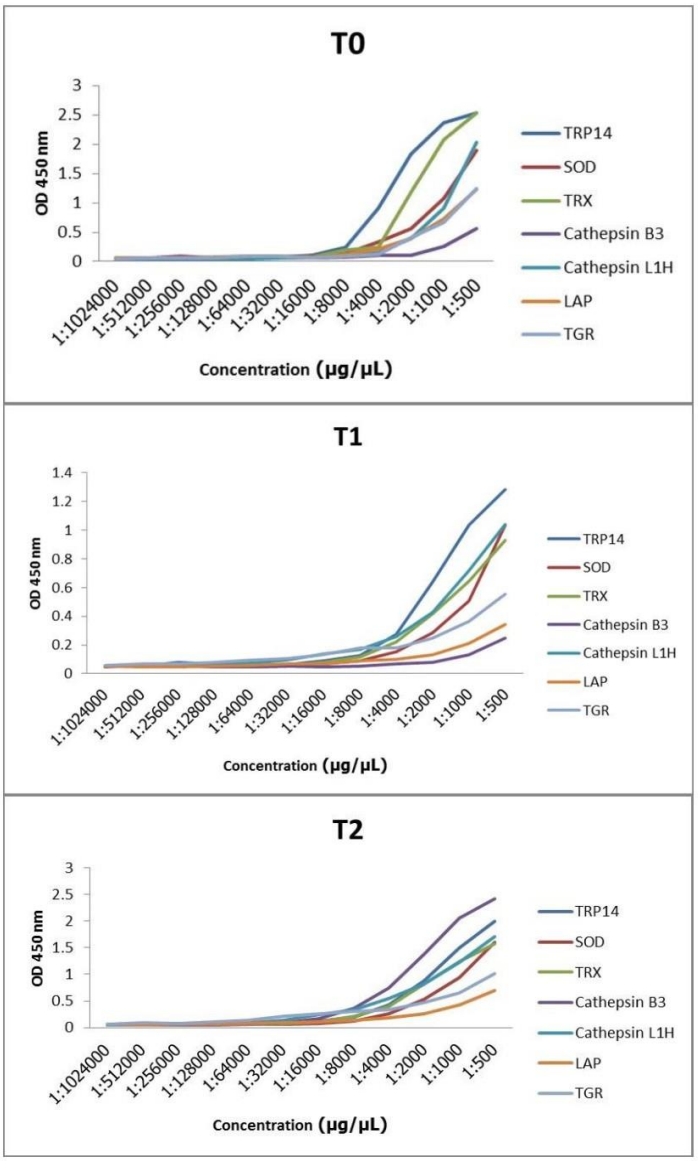

Figure 3 The expression of each egg specific proteins of *F. gigantica* by Indirect ELISA. The ELISA cut-off value was at 0.07. (T0: *F. gigantica* eggs on days 0, T1: *F. gigantica* eggs on days 9, T2: *F. gigantica* eggs on days 14)

The detection of egg specific protein expression by immunoblotting

The polyclonal antibody against *F. gigantica* eggs protein from days 0, 9 and 14 was determined using immunoblotting technique. The *F. gigantica* eggs protein were separated by 12.5% SDS-PAGE and electrotransferred onto a nitrocellulose membrane, and probed with primary antibody (rabbit anti-rFgCatL1H, rabbit anti-rFgTRX and rabbit anti-rFgSOD protein) which showed strong reaction at molecular weight 48, 11.68 and 17.5 kDa, respectively (Figure 4-6)

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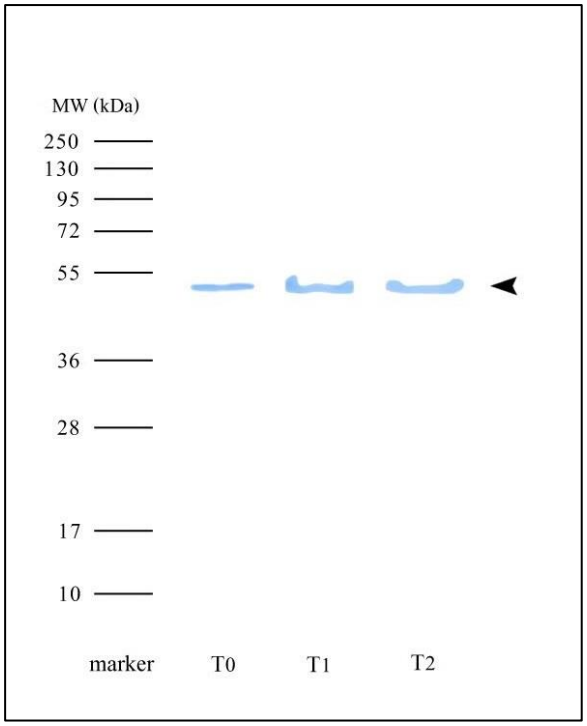

Figure 4 Western blot analysis of *F.gigantica* egg protein on days 0, 9 and 14 reacted with rabbit polyclonal antibody against rFgCatL1H (Lane1: F.gigantica egg on days 0, Lane2: *F. gigantica* eggs on days 9, Lane3: *F. gigantica* eggs on days 14)

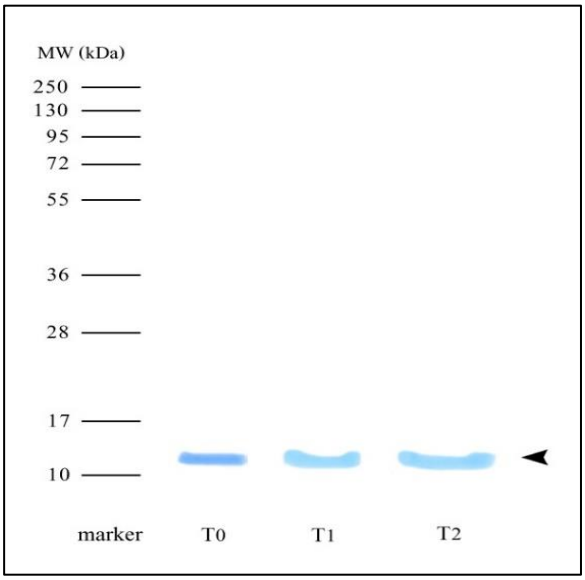

Figure 5 Western blot analysis of *F.gigantica* egg protein on days 0, 9 and 14 reacted with rabbit polyclonal antibody against rFgTrx (Lane1: F.gigantica egg on days 0, Lane2: *F. gigantica* eggs on days 9, Lane3: *F. gigantica* eggs on days 14)

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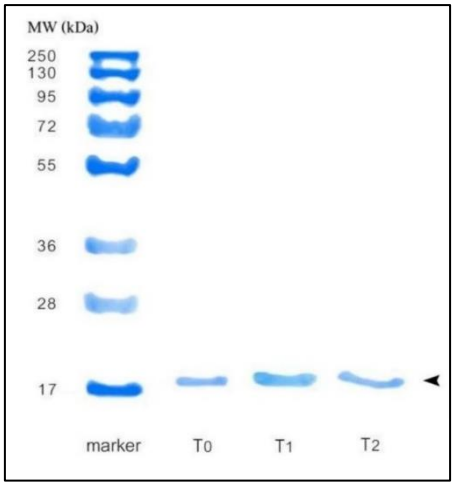

Figure 6 Western blot analysis of *F.gigantica* egg protein on days 0, 9 and 14 reacted with rabbit polyclonal antibody against rFgSOD (Lane1: F.gigantica egg on days 0, Lane2: *F. gigantica* eggs on days 9, Lane3: *F. gigantica* eggs on days 14)

Discussion

In the present study, the expression of egg specific proteins to *F. gigantica* on days 0, 9 and 14 were using Indirect ELISA and Immunoblotting technique. *F.gigantica* egg proteins of three stages (Days 0, 9 and 14) which specific binding to rFgCatL1H, rFgTrx and rFgSOD were found using the immunoblotting technique.

The Immunoblotting assay showed the expression of FgTrx in egg, 2-,4-week old juveniles and adult stages of *F.gigantica* (Changklungmoa et al., 2014) with similar pattern as detected by rabbit PoAb anti-rFgTrx on three stages of eggs.

FgTrx protein was highly expression in reproductive organs in order to neutralize Reactive oxygen species (ROS) because of their high metabolic activity and cell proliferation. (Alger et al., 2002). FgCatL1H is the isotype expressed in the early stages for migration and invasion. rFgCatL1H reacted with monoclonal antibody at a molecular weight 38-48 kDa in the extract of whole body (WB) of metacercaria and newly excysted juvenile (NEJ). (Wongwairot et al., 2015). FgCatL1H expression was detected by RT-PCR, in metacercariae and NEJ, and the expression gradually decreased in advanced developmental stages. (Sansri et al., 2013).

Jaikua et al. (2016) reported that native SOD protein expressed in all developmental stages of *F. gigantica* including egg, metacercaria, 2-,4- week old juveniles and adult consisted of a single band with MW at 17.5 kDa. It was confirmed that the native SOD protein expressed in all stages of egg specific proteins to *F. gigantica* on days 0, 9 and 14. During infection, the parasites was exposed to ROS generated from the host's immune cell. One mechanism that the parasites use to detoxify the free radicals is the antioxidant enzymes.

The identification of proteins secreted by live eggs is of fundamental interest. The list of egg protein secreted protein is available, these could be important factor for specific role in the immunodiagnosis

Acknowledgements

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