RESEARCH ARTICLE

Prevalence of *Fasciola gigantica* Infestation in Beef Cattle at Nongduang Slaughterhouse in Vientiane Capital of Lao People's Democratic Republic Using an iELISA Approach

Souk Phomhaksa[1], Chaiyapas Thamrongyoswittayakul[1*], Aran Chanlun[1], Suthida Chanlun[2], Noppadon Somphol[1], Chittraporn Yeanpet[1]

Abstract

Objective—To determine the prevalence of *F. gigantica* in beef cattle at Nongduang slaughterhouse, Vientiane capital in Lao PDR by using iELISA approach as compared to quantitative fecal examination.

Materials and Methods—One hundred seventy two each of fecal and blood samples were randomly collected from slaughtered native beef cattle to determine fecal egg counts (sedimentation and modified formalin-ether concentration method) and serum IgG antibodies against *F. gigantica*-excretory-secretory antigens (Indirect enzyme-linked immunosorbent assay and immunoblot). Information regarding age, sex, body condition score, and fecal score of each cattle was recorded. All related data were normalized and analyzed by Chi-square tests, Independent t-test and Kappa statistic using SPSS version 17.

Results—Prevalences of *F. gigantica* infestation in beef cattle were 11.6% (20/172) and 94.7% in relation to the FEC and effective iELISA, respectively, which was insignificantly associated with sex, age, hair and skin score, BCS and fecal score. Most cattle were harbored by single and multi-parasitic infestation by *F. gigantica*, rumen fluke and nematodes (77.9%, 134/172), whereas negative cattle was 22.1% (38/172). Four main *Fg*-ES antigens (14, 22, 25, and 34 kDa molecular weight) were recognized by pooled serum IgG antibodies according to the immunoblotting approach.

Conclusion—The abattoir survey demonstrated a high iELISA-based prevalence of *F. gigantica* infestation in the beef cattle at Nongduang slaughterhouse, Lao PDR. The iELISA, an alternatively serological test could overcome the false negative results according to a traditional fecal examination and investigate *F. gigantica*-infected beef cattle during prepatent period. Moreover, 14- and 22-kDa excretory-secretory proteins could be alternative protein markers for detection of *F. gigantica* infestation in beef cattle.

[1]Department of Medicine, [2]Department of Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, Thailand 40002

[*]Corresponding author: E-mail: chaiyapas@kku.ac.th

ความชุกของการติดพยาธิ *Fasciola gigantica* ในโคเนื้อที่โรงฆ่าสัตว์หนองด้วง นครหลวงเวียงจันทน์ ประเทศสาธารณรัฐประชาธิปไตยประชาชนลาวโดยวิธี iELISA

Souk Phomhaksa[1], ไชยพัศร์ ธำรงยศวิทยากุล[1*], อรัญ จันทร์ลุน[1], สุธิดา จันทร์ลุน[2], นพดล สมผล[1], จิตราภรณ์ เยียนเพชร[1]

บทคัดย่อ

วัตถุประสงค์   เพื่อสำรวจความชุกของ *F. gigantica* ในโคพันธุ์พื้นเมือง ที่โรงฆ่าสัตว์หนองด้วง นครหลวงเวียงจันทน์ ประเทศสาธารณรัฐประชาธิปไตยประชาชนลาวโดยวิธีการ iELISA เปรียบเทียบกับการตรวจนับไข่พยาธิเชิงปริมาณ

วัสดุ อุปกรณ์ และวิธีการ การเก็บตัวอย่างเลือดและมูลโคแบบสุ่มจากโคเนื้อทั้งหมด 172 ตัวอย่างจากโรงฆ่าสัตว์เพื่อประเมินความชุกด้วยวิธีการตรวจนับไข่พยาธิจากตัวอย่างมูลโคด้วยวิธีทำให้ตกตะกอนและวิธีเข้มข้นฟอร์มาลิน-อีเธอร์ และวิธี Indirect enzyme-linked immunosorbent assay (iELISA) และการตรวจวัดระดับอิมมูโนโกลบูลิน จี ในตัวอย่างซีรั่ม ที่ตอบสนองต่อสารคัดหลั่งจาก *F. gigantica* และ Western blot พร้อมบันทึกข้อมูลที่สำคัญอายุ เพศ คะแนนความสมบูรณ์ของร่างกายและคะแนนมูลโค วิเคราะห์ข้อมูลด้วย Chi-square, Independent t-test และ Kappa โดยใช้ SPSS รุ่น17

ผลการศึกษา   จากการตรวจด้วยวิธี iELISA พบว่ามีความชุกของการติดพยาธิ *F. gigantica* สูงคิดเป็นร้อยละ 94.7 (163/172) และจากการตรวจนับไข่พยาธิพบความชุกในระดับปานกลาง คิดเป็นร้อยละ 11.6 (20/172) โดยไม่พบความแตกต่างกันระหว่างเพศ อายุ ความสมบูรณ์ของร่างกายและลักษณะมูลโค นอกจากนี้ยังพบว่าโคเนื้อจำนวนมาก (ร้อยละ 77.9, 134/172) มีการติดพยาธิใบไม้ตับและ/หรือพยาธิในกระเพาะหมักและพยาธิตัวกลม และมีโคที่ไม่พบไข่พยาธิเลยคิดเป็นร้อยละ 22.1 (38/172) นอกจากนี้ยังพบ *Fg*-ES แอนติเจนน้ำหนักโมเลกุล 14, 22, 25, และ 34 kDa ที่ทำปฏิกิริยาต่อแอนติบอดี IgG จากซีรัมโครวมที่ติดพยาธิใบไม้ตับจากการตรวจโดยวิธี western blot

ข้อสรุป ผลการสำรวจโรงฆ่าสัตว์หนองด้วง ประเทศสาธารณรัฐประชาธิปไตยประชาชนลาวสามารถประเมินความชุกของการติดพยาธิใบไม้ตับที่เกิดจาก *F. gigantica* ในระดับสูงโดยวิธี iELISA ซึ่งเป็นวิธีการทางซีรัมวิทยาที่สามารถใช้เป็นทางเลือกในการตรวจวินิจฉัยการติดพยาธิใบไม้ตับได้ ที่สามารถลดปัญหาผลลบเทียมจากการตรวจนับไข่พยาธิในมูลโคเนื้อแบบเดิมและช่วยค้นหาโคเนื้อที่มีการติดพยาธิใบไม้ตับแบบแฝง นอกจากนี้สารคัดหลั่งโปรตีน *Fg*-ES ขนาด 14 และ 22 kDa อาจใช้เป็นโปรตีนทางเลือกในการตรวจหาการติดพยาธิใบไม้ตับที่เกิดจาก *F. gigantica* ในโคเนื้อ

[1]ภาควิชาอายุรศาสตร์ คณะสัตวแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น 40002

[2]ภาควิชาพยาธิชีววิทยา คณะสัตวแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น 40002

[*]*ผู้เขียนที่ให้การติดต่อ* E-mail: chaiyapas@kku.ac.th

Introduction

Fasciolosis is now considered as an important parasitic disease that causes economic losses in the beef production worldwide. Normally, this health problem is caused by two liver fluke species, i.e. *Fasciola hepatica* and *F. gigantica* (trematodes). In particular, *F. gigantica* is known as an extremely important cause of fasciolosis in livestock in a tropical region of Africa and Asia [1]. It has involved a considerable agricultural and economic losses resulting from infection estimated at US\$ 2,000–3,000 million annually [2]. The disease has undergone a sharp rise and has emerged as a major zoonotic disease in many countries [3], with an estimation of 17 million infected people and up to 180 million at risk [4]. The human form of the disease has also become a major public health problem in several parts of the world [5]. In cattle, sheep and goat, the disease also causes high economic losses from anemia, reduced production, poor performance, condemnation of liver and an increased mortality [6-8].

The typical climate of Lao PDR is a monsoon type with hot, wet summer and warm dry winter [9]; therefore, the cattle are at risk of *F. gigantica* infestation as in other tropical countries [1,10]. Abattoir survey has been widely used to recruit many epidemiological information regarding animal health and public health concern [11-14] especially *F. gigantica*-causing fasciolosis in slaughtered beef cattle. However, fasciolosis-related information in beef cattle in Lao PDR has been extremely limited. Previous reports are mainly from other international sources and unpublished information, which do not reflect the current status of the fasciolosis in beef cattle in this country. Moreover, the understanding about this liver fluke in each area is of great importance for management and control. Therefore, this study was conducted to determine the prevalence of *F. gigantica* infestation in beef cattle in Nongduang slaughterhouse, Lao PDR using serological approaches (iELISA and immunoblot) and quantitative fecal examination in the slaughterhouse.

Materials and Methods

Animal and study design

This cross-sectional study was implemented from September 2010 to February 2011 at Nongduang slaughterhouse, Vientiane capital of Lao PDR, in which 20-50 native beef cattle from Vientiane capital and vicinity were daily slaughtered. One hundred and seventy two beef cattle total were randomly selected during 20 visits to collect fecal, blood samples and adult *F. gigantica* from liver.

Information of each cattle was recorded including age, sex, body condition score (BCS) [15] and fecal scores [16].

Fecal examination

The formalin-preserved samples were proceeded in order to detect helminthes eggs in feces from beef cattle according to a modified quantitative formalin/ethyl acetate sedimentation [17]. Briefly, samples were sieved by a mesh, transferred into 15-ml conical tubes, and centrifuged at 4,000 rpm for 5 min. After discarding the supernatant, the pellets were re-constituted in 7 ml of 5% formalin plus 3 ml of ethyl acetate, shaken vigorously for 1 min, and re-centrifuged at 4,000 rpm for 5 minutes. After centrifugation, the detritus rings and the supernatants were discarded, whereas the *Fasciola* egg-bearing pellets were re-suspended in 5% formalin to yield a 2.5-ml mixed volume and transferred into screw top tubes. To determine egg counts, 300 µl of the resuspended sediments were placed on microslides for egg counting. The total number of eggs per gram (EPG) was calculated according to the method modified by Elkins et al. [17].

Indirect enzyme-linked immunosorbent assay (iELISA)

1. Preparation of *F. gigantica* excretory/secretory antigen (*Fg*-ES Ag)

The *Fg*-ES Ag, a metabolic antigen, was prepared according to a previous method [18]. Briefly, *F. gigantica* worms were collected from the liver of cattle, washed several times by 0.01 M PBS, pH 7.4 in order to remove all traces of blood and bile. The worms were incubated in PBS (5 worms/10 ml) at 37 °C for 6 hours and removed by sieving. The sieved E/S products-containing PBS were subsequently centrifuged at 9,660 ×g (Dynamica, Velocity 18R Versatile Centrifuge, Switzerland) for 30 minutes at 4 °C. The supernatant was filtered through a 0.2-µ Millipore filter (Sartorius Stedim, Germany), followed by a dialysis against sterile distilled water for 24 hours using 10,000 molecular weight cut off (MWCO) SnakeSkin[TM], Pleated Dialysis Tubing (Thermo Scientific, USA), aliquoted and stored at -20 °C until analysis. The dialyzed proteins were then determined by spectrophotometry at the 260- and 280-nm wavelengths (Life Science uv/vis spectrophotometer, Beckman Coulter[TM], DU[Ò]530 model) and expressed as µg/µl.

2. Indirect Enzyme-linked Immunosorbent Assay (iELISA)

The iELISA was carried out according to the method modified from Chaiyotwittayakun *et al.* [19] in 96-well microtiter plates (Nunc-Immuno Plate MaxiSorp, Thermo Fischer Scientific, Denmark). Briefly, the wells were coated by 100 µl of Fg-ES Ag in 0.1 M carbonate buffer, pH 9.6 (10 µg/ml) and incubated overnight at 4 °C. The plates were washed twice with PBS/Tween 20 (0.01%, PBST) and then blocked with 100 µl/well of 0.01% bovine serum albumin (BSA) in 0.01 M PBS (pH 7.4) for 1½ hours at 37 °C. After blocking, the plates were washed three times with PBST, added 100 µl of each tested serum diluted 1:100 ratios in PBS, pH 7.4 to the wells in duplicate, and incubated for 1½ hours at 37 °C. The plates were then washed five times, followed by adding 100µl/well of anti-bovine alkaline phosphatase-conjugated rabbit IgG (Bethyl Laboratories, USA) diluted in PBS, pH 7.4 at 1:6,000 ratios, and incubated at 37 °C for 2 hours. After other five washes, as a substrate, 50 µl of p-nitrophenyl phosphate disodium

salt (1-Step[TM] PNPP, Thermo Scientific, USA) was added to each well and incubated in the dark for 15 minutes at 37 °C. The enzymatic reaction was subsequently stopped by 50 µl/well of 2 N NaOH. The absorbance of each well was read at a 405-nm wavelength using a microplate reader (BioRad, Model 3550, USA) and expressed as optical density (OD).

       In each iELISA plate, negative and positive control diluted 1:100 in PBS, pH 7.4 were included in duplicate. Commercially available fetal bovine serum (FBS, Biochrom AG, Germany) was used as a negative control. A positive control was assigned from a tested serum with a positive gross plus fecal examination, relatively compared to the OD greater than the cut off value, which was obtained from the average OD from negative control plus three standard deviations as described in the methods [14, 19] with some modifications. The intra-assay and inter-assay coefficients of variability (CV) as a percentage of the assay were determined for precision of both within and between assays, respectively.

SDS-PAGE and Immunoblotting

       The dialyzed proteins were then characterized and analyzed by SDS-PAGE according to the manufacturing instruction and modified from a protocol [19]. Briefly, *Fg*-ES Ag was prepared using a 1:1 ratio in Laemmli sample buffer (BioRad, USA) plus 5% (v/v) of β-mercaptoethanol (AppliChem, Germany). The mixtures were heat-denatured at 95°C for 5 min prior to loading (23 µg of total protein in 25 µl/well) on pre-casted Tris-HCl, acrylamide-bis mini gels (12%, Amersham[TM] ELC[TM], GE Healthcare, Israel). The electrophoresis was carried out in the Amersham[TM] ELC[TM] Gel Box (GE Healthcare, Israel) using electrophoresis buffer (0.025 M Tris, 0.192 M Glycine and 0.1% SDS, Vivantis, Malaysia) under 160 volts, 45 mA, for 1 hour (Power supply, Model: PowerPac 3000, Bio-Rad, USA). The protein bands were then visualized by silver staining (Silver Stain Plus, Bio-Rad, USA) according to a manufacturing instruction and compared with the molecular weight markers (Precision Plus Protein[TM] Standards 10-250 kDa; Bio-Rad, USA).

       Western blotting was performed to characterize interaction between bovine IgG antibody and *Fg*-ES antigens by following the manufacturing instruction and a modified protocol [19]. The electrophoresed proteins were transferred from gel to polyvinylidene difluoride (PVDF) mini membranes (0.2µ, invitrogen[TM], USA) in dry blotting system under 20 volts for 7 minutes using the iBlot[TM] transfer device (invitrogen[TM], USA). The transferred PVDF membranes were rinsed by Tris buffered saline (TBS, 50 mM Tris, 150 NaCl, pH 7.4) and submerged in a blocking buffer containing 3% BSA in TBS/Tween 20 (0.1%, TBST) at room temperature for 1 hour to ensure the specific binding. One membrane was probed by primary antibody prepared from 5 pooled *Fg*-positive sera, the other was probed by FBS at a 1:1,000 optimized dilution in TBST. Secondary antibody (anti-bovine alkaline phosphatase-conjugated rabbit IgG, Bethyl Laboratories, USA) was subsequently used at a 1:6,000 dilution in TBST. Each probing step was carried out for 1 hour at a room temperature. Moreover, between each individual step, the PVDF membranes were immersed twice in TBS/Tween 20 (0.05%) for 10 minutes to get rid of excess BSA and antibodies. The enzyme substrate reaction was then visualized by reacting the BCIP/ NBT phosphate substrate (0.21 mg/ml of 5-bromo-4-chloro-3-indoxyl-phisphate (BCIP) and 0.42 mg/ml

of nitroblue tetrazolium (NBT) in an organic base/Tris buffer, KPL, USA) and stopped by imeering in reagent quality water for 20 minutes. The results were analyzed by ChemiDoc™ XRS Plus system with Image Lab™ software (Bio-Rad Laboratories, Inc., USA).

Data Analysis

The descriptive statistic was used to analysis and explained a data frequency, percentile, mean, and standard deviation for data as regards sex, age, BCS, and fecal score. Pearson Chi-square test was used to individually determine the difference of sex, age, BCS, and fecal score. Difference serum IgG between cattle with shedding and non-shedding *Fasciola* eggs was tested using an independent t-test. McNemar Chi-square test was applied to analyze consistency of the results of serum IgG and FEC grouping. Kappa statistic was also used to determine the agreement of iELISA and FEC results. All statistical analyses were performed using SPSS version 17. Significant difference was indicated when $P < 0.05$.

Results

There were 30 male and 142 female slaughtered cattle. FEC-based results found that *F. gigantica* infestation in older cattle (>2 years old) was higher 11% (19/172) than in young cattle (<2 years old) 0.6% (1/172). Most cattle (67.4%, 116/172) were poor to fair condition (BCS$\leq$2) and 8.1% (14/172) positive. In addition, age, BCS and fecal scores were not different between the infested and non-infested cattle (**Table 1**).

A sedimentation method using formalin-fixed fecal samples resulted in 11.6% of the *F. gigantica* positive cattle (20/172) including only 5% (1/20) of single *F. gigantica* infestation and 95% (19/20) of multi-parasitic infestation (mainly liver fluke, rumen fluke and round worm) as shown in Table 2. Based on the simple sedimentation technique, most cattle were positive for major parasites, while others were negative shedding for any parasitic eggs, accounted for 77.9% (134/172) and 22.1% (38/172), respectively.

iELISA yielded 94.7% of positive sera (163/172) using the OD at a cut-off point of 0.17. Both methods shared similar result of the 20 infected cattle. In addition, iELISA could detect 83.1% false negative (143/172) and 5.2% of true negative (9/172) sera from the cattle without *Fasciola* egg shedding in feces (**Table 2**). Average serum IgG absorbance (±SE) in cattle with FEC-based positive and negative were significantly different ($P<0.01$) accounted for 0.48±0.03 and 0.14±0.01, respectively.

Results indicated that Fasciola positive cattle using fecal examination were related with the IgG absorbance of infected cattle detected by iELISA. According to Independent t-test, serum IgG against *Fg*-ES antigens was significant different between the cattle shedding and non-shedding liver fluke eggs ($P<0.05$). However, serum IgG and log-transformed FEC was insignificantly associated as regard a Linear regression (data not shown). Results of serum IgG grouping test were inconsistent with results of the egg grouping test ($P > 0.05$), based on McNemar Chi-square test.

Six main proteins (14, 15, 22, 24, 52 and 53 kDa) were found according to SDS-PAGE followed by silver staining (**Figure 1**). Moreover, western blotting analysis revealed a detection of *Fg*-ES antigens with molecular weights of 14, 22, 25, and 34 kDa (**Figure 2**) after probing by pooled serum IgG antibodies, whereas none was found by FBS IgG (data not shown).

Table 1. *F. gigantica* Infestation in 172 Slaughtered Beef Cattle Categorized by Sex, Age, BCS, and Fecal Scores Based on Fecal Egg Counts

Items	Category	n	Infected cattle	%
Sex	Male	30	2	6.7
	Female	142	18	12.7
Age	<2 years old	4	1	25.0
	≥2 years old	168	19	11.3
BCS[a]	1	52	8	15.4
[16]	2	64	6	9.4
	3	49	6	12.2
	4	7	0	0
	5	0	0	0
Fecal score	1	23	3	13.0
[17]	2	35	2	5.7
	3	61	9	14.8
	4	37	2	5.4
	5	16	4	25.0

[a]BCS = body condition score

Table 2. Results of Fecal Examination and iELISA (Using Sera and Fg-ES Antigens) in 172 Slaughtered Beef Cattle from Nongduang Slaughterhouse, Lao PDR

		Fecal examination		
		Negative	Positive	Total
	Negative	9	0	9
iELISA	Positive	143	20	163
	Total	152	20	172

Figure 1. SDS-PAGE & Silver Staining of Fg-ES Antigens[a]
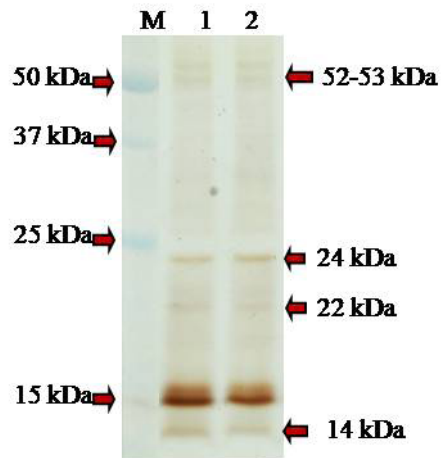
[a]Using 12% Tris-Glycine mini gel, 160 volts, 45 mA, 1 hour ; M = Protein marker, Lane 1-2 = Fg-ES antigens

Figure 2. Western Blot and Colorimetric Analysis Fg-ES Antigens[a]
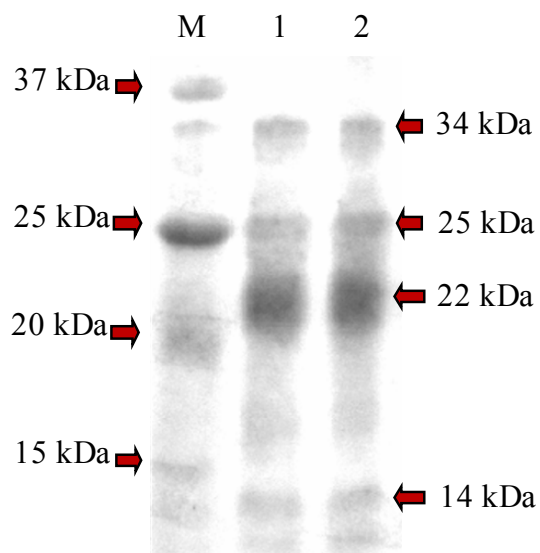
[a]Probed by pooled Fg-positive sera followed by anti-bovine alkaline phosphatase-conjugated rabbit IgG

Discussion

Abattoir survey was widely accepted for determination of parasitic infection especially liver fluke in being slaughtered beef cattle [11-14]. Because it was useful for determination the prevalence, risk assessment and prevention and control strategic planning of parasitic infection [13]. Fasciolosis survey at the Nongduang slaughterhouse, Lao PDR also provided relevant information as in previous studies [13,20-22]. In the north of India, epidemiological studies on *F. gigantica* infection found 10.8% and 13.9% in cattle and buffaloes, respectively [20]. Sukhapesna et al. [23] reported 13.9% and 8.9% in cattle and buffalo, respectively. Neamjui et al. [24] also found higher prevalent in buffaloes (13.9%, 25/180). Overall while *F. gigantica*-causing fasciolosis in buffalo and cattle were 8.6% [24] and 42.1% [25]. Besides, high prevalences of *Fasciola* infestation was reported (30-90%) [14]. In contrast, a low prevalence rate was also reported as minimal as 1.5% in Nigeria [13], 2.4% in Central Iran [12], and 3.33% (6/180) in Thailand [24].

However, the prevalence of liver fluke infection in beef cattle could be varied by many factors, including the animal's age, numbers of infected cattle, stages of infection maturation status of the fluke, locations, differences in landscape (such as swampy areas, agricultural irrigation practices), season, density of the animal in particular area, management, preventive control strategies, poor meat inspection facilities, and uncooperative attitudes of butchers [13,21,22,24]. In accordance with previous studies, more prevalent fasciolosis was observed in the older cattle [14,22,24,25].

Although, fecal examination has been routinely used for diagnosis of fasciolosis for several decades, certain limitations of this method were clearly mentioned including time consuming, suitable amount of feces, its low sensitivity, and false negative [18]. Theoretically, the eggs can be found in the feces 3½ month after a primary infection [22]. For the cattle with false negative sera, it could be explained that the cattle could be in prepatent period (early infection and maturation of the fluke), low infection, chronically infected, having adult flukes laying no or less number of eggs, intermittent shedding of *Fasciola* eggs in bovine feces, or ectopic fasciolosis, as previously discussed [22]. Furthermore, in chronic fasciolosis, a pathological change of the common bile duct can inhibit the egg release into the duodenal lumen and mix with feces even in the heavily infected animal harboring by adult flukes in the liver [22].

Among those techniques, ELISA technique has become one of the most widely used tests and effective tools for diagnosis of fasciolosis in ruminants with its simplicity and reliability [14,18,26,27], including iELISA. At least, the infestation can be diagnosed 2 week as early as after infection by ELISA [28]. Demonstrated the iELISA using crude adult antigen for diagnosis of experimental or naturally occurring *F. gigantica* infestation in cattle, was superior to a fecal egg examination. Therefore, it could overcome the limitation of the conventional methods especially the fecal egg count [29]. In this study, intra-assay and inter-assay CVs of iELISA were 7.3% and 2.4%, respectively, which were within the general acceptable levels (below 10% and 15%) [30].

Generally, Kappa statistic is used to determine the agreement of results from each paired test. As

a rule of thumb values of Kappa from 0.40 to 0.59 are considered moderate, 0.60 to 0.79 substantial, and 0.80 outstanding. The Kappa of the present result was 0.014, which was lower than previous reports of k=0.74 [31] and k=0.53 [32]. However, in this study, Kappa statistic may not be precise because of some factors affecting the results of each test as regards, sensitivity (iELISA versus FEC) [14,18,26,27], stage of infection, egg shedding rate, animal's age, and a pathological change in bile duct [22]. Descriptive analyses could be then more appropriate. As a result, the study revealed that iELISA is still effective diagnostic tool for bovine fasciolosis by using *Fg*-ES antigens.

SDS-PAGE followed by silver stain of *Fg*-ES Ag provided predominant 14, 15, 22, 24, 52 and 53 kDa protein bands (Figure 1) and yielded high quality of protein bands than Coomassie Blue staining used in previous reports [19,26]. Although studies suggested that a 28-kDa cysteine proteinase, an ES product was used to immunodiagnose for fasciola infestation in ruminant [26,28,33], such protein was not observed in this study. However, the current study demonstrated the interaction between 4 main *Fg*-ES antigens with molecular weight of 14, 22, 25, and 34 kDa and bovine IgG antibodies. Only 14- and 22-kDa *Fg*-ES antigens were detected in both silver staining and immunoblotting procedures. In particular, the detection of 14-kDa [33] and 34-kDa [34] antigens were corresponded to previous studies. Therefore, these two antigens (14 and 22 kDa) could be considered as alternative protein markers for fasciolosis diagnosis in beef cattle. Further research is warranted to understand the interaction between *F. gigantica* and a host response in beef cattle.

The abattoir survey demonstrated a high iELISA-based prevalence of *F. gigantica* infestation in the beef cattle at Nongduang slaughterhouse, Lao PDR. The iELISA, an alternatively serological test could overcome the false negative results according to a traditional fecal examination and investigate *F. gigantica*-infected beef cattle during prepatent period. Moreover, 14- and 22-kDa excretory-secretory proteins could be alternative markers for detection of *F. gigantica* infestation.

Acknowledgements

References

1. Mas-Coma S, Bargues MD. Human liver flukes. *Res Rev Parasitol*. 1997; 57: 145–218.

2. Boray JC. *Flukes of domestic animals*. In Gaafar SM, Howard WE, Marsh RE. Parasites, Pests and Predators: B2 (World Animal Science). Eds. New York: Elsevier Science Ltd; 1985. p. 179–218.

3. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. *Int J Parasitol*. 2005;35:1255–1278.

4. World Health Organization. Report of the WHO Informal Meeting on Use of Triclabendazole in Fascioliasis

Control. Geneva: The Organization. 2007.

5.   Fairweather I. An advancement of learning. *Triclabendazole Progress Report*, 2005–2009. 2009;83:139–150.

6.   Kendall SB. Fascioliasis in Pakistan. *Ann Trop Med Parasitol*. 1954;48:307-313.

7.   Hammond JA, Sewell MMH. The pathogenic effect experimental infection with *Fasciola gigantica* in cattle. *Br Vet J*. 1974;130(5):453-465.

8.   Fabiyi JP, Adeleye GA. Bovine fasciolosis on the job plateau, Nigeria with particular referent to economic importance. *Bull Anim Health Prod Afr*. 1982;30(1):41-43.

9.   Ministry of Agriculture and Forestry, Lao PDR. Forestry Strategy to the Year 2020 of the Lao PDR; 2005 [Cited 2012 May 15]. Available from: http://www.theredddesk.org/sites/default/files/forestry-strategy-to-the-year-2020-of-the-lao-pdr.pdf.

10.   Dalton JP. *Fasciolosis*. Ed. London, UK: University Press. 1999.

11.   Nega M, Bogale B, Chanie M, Melaku A, Fentahun T. Comparison of coprological and postmoretem examinations techniques for the determination of prevalence and economic significance of bovine fasciolosis. *J Adv Vet Res*. 2012;2(1):18-23.

12.   Ali TS, Zarichehr V, Reza TM, Amroallah B, Hossin T, Amir M, Akbar T, Hossin H, Tourag R, Hassan E. Prevalence of liver flukes infections in slaughtered animals in Kashan, Isfahan province, central Iran. *IIOAB J*. 2011;2(5):14-18.

13.   Bala AN, Garba AE, Yazah AJ. Bacterial and parasitic zoonoses encountered at slaughter in Maiduguri abattoir, Northeastern Nigeria. *Vet World*. 2011;4(10):437-443.

14.   Anderson N, Luong TT, Vo NG, Bui KL, Smooker PM, Spithill TW. The sensitivity and specificity of two methods for detecting Fasciola infections in cattle. *Vet Parasitol*. 1999;83(1):15–24.

15.   Brand A, Noordhuizen JPTM, Schukken YH. *Herd health and production management in dairy practice.* 2nd ed. [n.p.]: Wageningen Academic Publisher. 1996.

16.   Hughes J. A system for assessing cow cleanliness. *In Practice*. 2001;23(9):517-524.

17.   Elkins D, Haswell-Elkins M, and Anderson R. The epidemiology and control of intestinal helminthes in the Pulicot Lake of Southern India: I. Study design and pre-and post-treatment observations on *Ascaris lumbricoides* infection. *Trans R Soc Trop Med Hyg.* 1986;80(5):774-792.

18.   Awad WS, Ibrahim AK, Salib FA. Using indirect ELISA to assess different antigens for the serodiagnosis of *Fasciola gigantica* infection in cattle, sheep and donkeys. *Res Vet Sci*. 2009;86(3):466-471.

19.   Chaiyotwittayakun A, Erskine RJ, Bartlett PC, Herdt T, Sears P, Harmon RJ. The effect of ascorbic acid and L-Histidine therapy on acute mammary inflammation in dairy cattle. *J Dairy Sci*. 2002;85(1):60-67.

20.   Garg R. The epidemiology of fasciolosis in ruminants in different geo-climatic regions of north India. *Trop Anim Health Prod*. 2009;41,1695-1700.

21.   Ahmed E, Markvichitr K, Tumwasorn S, Koonawootrittriron S, Choothesa A, Jittapalapong S.. Prevalence of Fasciola spp. infections of sheep in the middle awash river basin, Ethiopia. *Southeast Asian J Trop Med Pub Health*. 2007;38:51-57.

22.   Tuntasuvan D, Kitikoon V. Bovine fasciolosis in Thailand: A review. *J Trop Med Partasitol*. (1996);19(1):1-12.

23. Sukhapesna V, Tuntasuvan D, Sarataphan N, Sangiumluksana S. A study on eqidemiology of liver fluke infection in buffaloes. *Thai J Vet M.* 1990;20:527-534.

24. Neamjui P, Pimpukdee K, Nachom C. Prevalence and risk factors of liver fluke (*Fasciola* spp.) infestation in cattle and buffaloes in Buriram province. *KKU Vet J.* 2012;22(1):10-17.

25. Bhutto B, Arijo A, Phullan MS, Rind R. Prevalence of fasciolosis in buffaloes under different agro-climate areas of Sindh Porvince of Pakistan. *Int J Agri Biol.* 2012;14(2): 241-245.

26. Gupta A, Dixit AK, Dixit P, Mahajan C, Sharma RL. Evaluation of dipstick-ELISA using 28 KDa *Fasciola gigantica* cathepsin L cysteine proteinase (FgCL3) for serodiagnosis of fasciolosis in naturally infected goats. *Vet Parasitol.* 2011;176(2-3): 165-169.

27. Estuninisih E, Spithill T, Raadsma H, Law, Adiwinata R, Meeusen E, et al. Development and application of a fecal antigen diagnostic sandwich ELISA for estimating prevalence of Fasciola gigantiga in cattle in central Java, Indonesia. *J Parasitol.* 2009;95(2):450-455.

28. Fagbemi BO, Guobadia EE. Immunodiagnosis of fasciolosis in ruminants using a 28-kDa cysteine protease of Fasciola gigantica adult worms. *Vet Parasitol.* 1995;57(4): 309-318.

29. Itagaki T, Ohto N, Hosaka Y, Iso H, Konishi M, Chinone S, et al. *JPN J Vet Sci.* 1989; 51(4):757-764.

30. Salimetrics. (2012). Inter- and Intra-assay coefficients of variability. [Cited 2012 June 13]. Available from: http://www.salimetrics.com/assets/documents/spit-tips/publications/Inter%20and%20Intra%20Assay%20 Coefficients%20of%20Variability.pdf

31. Abebe, R., Abunna,F., Berhane, M., Mekuria, S., Megersa, B., and Regassa, A., (2010). Fasciolosis: Prevalence, financial losses due to liver condemnation and evaluation of a simple sedimentation diagnostic technique in cattle slaughtered at Hawassa Municipal abattoir, southern Ethiopia. *Ethiopean Vet J.* 2010;14(1):39-51.

32. Ibrahim N, Wasihun P, Tolosa T. Prevalence of Bovine Fasciolosis and Economic Importance due to Liver Condemnation at Kombolcha Industrial Abattoir, Ethiopia. *Internet J Vet Med.* 2010 [Cited 2012 June 12];8(2). Available from: http://www.ispub.com/journal/the-internet-journal-of-veterinary-medicine/volume-8-number-2/prevalence-of-bovine-fasciolosis-and-economic-importance-due-to-liver-condemnation-at-kombolcha-industrial-abattoir-ethiopia.html.

33. Ghosh S, Rawat P, Velusamy R, Joseph D, Gupta SC, Singh BP. 27 kDa *Fasciola gigantica* Glycoprotein for diagnosis of prepatent Fasciolosis in cattle. *Vet Res Commun.* 2005;29(2):123-135.

34. Yokananth S, Ghosh S, Gupta SC, Suresh MG, Saravanan D. Characterization of specific and cross-reacting antigens of *Fasciola gigantica* by immunoblotting. *Parasitol Res.* 2005;97(1):41–48.