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Comparison of protein profile of co-existing *Fasciola hepatica* and *Fasciola gigantica* parasite in *Bos taurus* (cattle) and *Bubalus bubalis* (Philippine water buffalo)

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ABSTRACT

Fasciola hepatica and *Fasciola gigantica* habitually co-exist as parasites of cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*), and despite variation in their morphometry, their species status is suspicious. Liver flukes isolated from cattle and water buffalo were initially sorted as *F. gigantica* or *F. hepatica*, and crude proteins were extracted and subjected to SDS-PAGE. Cattle had the preponderance of *F. gigantica*, while *F. hepatica* was the dominant species in water buffaloes. Co-existing cattle and bubaline fasciolids revealed similar protein profile suggestive of a close genetic relationship. The distinct heavy bands shared by co-existing bubaline *F. hepatica* and *F. gigantica* relative to those detected in cattle fasciolids suggests a host species-related influence. Between cattle and bubaline *F. hepatica*, six bands (220kDa, 150kDa, 115kDa, 67kDa, 34-37kDa, 30kDa) were bubaline-specific; between cattle and bubaline *F. gigantica*, four bands (212kDa, 150kDa, 70kDa, 30kDa) were bubaline-specific, while only three bands were shared (150kDa, 67-70kDa, 30kDa) by bubaline *F. hepatica* and *F. gigantica*. Current molecular findings represent the first in the country, where fascioliasia is prevalent. Confirmation of these results entails protein profiling of extracts of freshly-collected individual worms alongside extracts of infected and non-infected liver tissue samples, to mark out host-derived proteins. Its surveillance in susceptible host species in farms around the country, jointly with analysis of morphological and morphometric data of co-existing fasciolid species is highly recommended.

Keywords: cattle & carabao *Fasciola* spp.; protein profile; SDS-PAGE; Philippines

INTRODUCTION

Fasciola hepatica and *Fasciola gigantica* are two very important species of liver flukes widespread in cattle and water buffaloes (Marques and Scroferneker, 2003; Anderson *et al.*, 1999; Sharma *et al.*, 1989**;** Mahdi and Al-Baldawi, 1987). De Leon and Juplo (1966) recorded 68.0% infection of water buffaloes with intestinal worms, including *F. hepatica*. In southern Mindanao, Philippines, the high prevalence of fascioliasis called for an immediate implementation of a program to reduce parasite transmission (Intong *et al.*, 2003).

Species identification is traditionally based on differences in size and shape of adults (Periago *et al.*, 2006) and/or larval stages (Dar *et al.*, 2003), and intermediate snail host species (Ashrafi *et al.*, 2006; Hosseini *et al.*, 2004; Arfaa *et al.*, 1969). The existence of intermediate forms and variation in snail host species render parasite identification extremely difficult and inconsistent (McGarry *et al*., 2007; Ashrafi *et al.*, 2006; Lotfy *et al*., 2002). Analysis of data on gene sequences of the 28s rRNA genes (Marcilla *et al*., 2002), rDNA genes (Alasaad *et al*., 2007; Marcilla *et al*., 2002; Blair and McManus, 1989), and whole mtDNA (Itagaki *et al*., 2001, 1998; Hashimoto *et al*., 1997), and variations in protein peak points of *Fasciola* spp.

(Lee and Zimmerman, 1993; Lee *et al*., 1992) suggest the existence of intermediate forms either showing more identities to *F. gigantica* or to *F. hepatica* or to both species. Polymorphism in rDNA gene sequences likewise points to interspecific cross-hybridization between co-existing species *F. hepatica* and *F. gigantica* (Lin *et al*., 2007; Huang *et al*., 2004; Agatsuma *et al*., 2000).

Cattle and bubaline fascioliasis is established as highly prevalent in the country. Other than the work of Kimura *et al*. (1984) on liver flukes infecting Philippine carabaos which they found to possess morphological variations that complicate and impede species identification, studies to clarify species variation are essential non-existent. In this paper, we report our preliminary findings on protein profile of co-existing *Fasciola* spp. obtained from cattle and water buffalo (local name: carabao) host species.

MATERIALS AND METHODS

Collection, storage and measurement of fasciolid worms

Infected livers were obtained from one slaughtered cattle and one slaughtered water buffalo past midnight from an abattoir in Tondo, Manila, Philippines. Slices of liver placed inside properly labeled plastic bags were transported in a cooler to the laboratory and were kept in the -20^{o}C freezer prior to the isolation of flukes. Cattle and bubaline liver tissue slices were each weighed before and after the flukes were isolated. Cattle and bubaline liver tissues weighed 904 g and 769 g, respectively. Tissues were then defrosted and the worms were dissected out and transferred to a crystalline disk containing sterilized phosphate buffered saline (PBS) pH 7.4, for initial washing. The length and width of each fluke were measured. Based on the standard body length ranges of adult fasciolids: *F. hepatica*: 25-30 mm; *F. gigantica*: 25-75 mm (WHO, 2007; Lotfy *et al*., 2002; CUSRG, 1998; Kimura *et al*., 1984), and general morphology (Roberts and Janovy, 2000), they were segregated into *F. hepatica* and *F. gigantica* (Table 1). Each of the flukes was washed twice with sterile PBS, then placed in properly marked individual microcentrifuge tube and stored in a -20ºC freezer, prior to homogenization. To prevent tissue degradation, throughout this procedure, the flukes were always kept on a cooler with crushed ice.

Fluke homogenization and protein extraction and Bradford assay

Homogenization and protein extraction followed Maizels *et al*. (1991) protocol with modification. All the instruments, glasswares, homogenizers, dissecting sets and micropipettes and tips, among others, and dH_20 and solutions needed for worm homogenization, protein extraction and assay were sterilized (Hirayama Hiclave HV-85), prior to use. PBS and lysis buffer were stored in sterilized 100 ml reagent bottles at 4°C. Depending on the size of flukes, each worm was homogenized in either 500 µl or one ml lysis buffer. To prevent protein denaturation, homogenization was done on a cooler with crushed ice. The homogenates were then transferred into properly-marked microcentrifuge tubes, spun at 10,000 rpm for 5 min at 4°C, and stored in a -20°C freezer prior to use for protein assay.

The homogenate was centrifuged and the supernatant was assayed for protein concentration using the Bradford method (Maizels *et al*., 1991). The standards were prepared using a mixture of bovine serum albumin (PROMEGA, USA), sterilized dH_20 and Bradford reagent (SIGMA, Coomassie Brilliant Blue G C.I. 42655). Prior to protein quantification, the supernatants were thawed on a cooler with crushed ice. A mixture of 5 µl protein sample (=supernatant), 95 µl dH_2O and 900 µl Bradford reagent to equal to 1,000 µl solution was subjected to gentle vortexing. Protein concentration and absorbance of each sample was determined at 595 nm using a UV/Vis Spectrophotometer (Daigger Genesys Spectrophotometer, GENESYS 10 UV). The protein concentration of each individual cattle and bubaline fluke was recorded.

Table 1. Liver flukes segregated according to body length into *F. hepatica* and *F. gigantica* (WHO, 2007; CUSRG, 1998) indicating the number of worms obtained.

Size range (mm)	A: Bovine host	
	F. hepatica	*F. gigantica*
29	1	
34-39		9
40-43		7
44-48		5
50-52		2
Total	1	23

Size range (mm)	B: Bubaline host	
	F. hepatica	*F. gigantica*
18-21	2	
22-25	10	
26-30	19	
31-32		1
33-35		2
43		1
Total	31	4

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining

The supernatants in microcentrifuge tubes were thawed on a cooler containing crushed ice and kept in an -80°C temperature freezer (SANYO, MDF-U52V), for 10 min, prior to use. The protein extracts used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were selected based on the size range of flukes which were predetermined based on body morphometrics and general morphology. Small amounts of supernatants from flukes of a defined size range were pooled and then centrifuged in a thermomixer (Microcentrifuge 5415D Eppendorf) for 5 sec. From each individual or pooled supernatant, 7 µl sample was mixed with 7 µl treatment dye, covered with parafilm® and centrifuged for 15 sec. For bubaline protein extracts which during initial running showed clustered protein bands making it almost impossible to detect separate bands, the ratio of supernatant and treatment dye was reduced to 3 µl supernatant: 7 µl treatment dye. The SDS-PAGE was carried out according to Maizels *et al*. (1991). Silver staining (BIO-RAD, USA) was used to visualize the protein bands. The stained gels were photographed and analyzed for similarities and differences in protein bands with the aid of SynGene computer program.

RESULTS

Cattle fasciolids (n=24) measured 29-52 mm; one fluke measured 29 mm and the rest were 34-52 mm long (Fig. 1). Although the 29 mm cattle fluke labeled as *F. hepatica* according to size range looked like an intermediate form (Fig. 1C), it may really have been *F. gigantica.* The 35 bubaline liver flukes measured 18-43 mm long; 31 of the flukes measured ≤30 mm long (Fig. 2). An overlap in body length between bubaline *F. hepatica* and *F. gigantica* was apparent. In the 904 g of cattle liver, there was dominance of *F.*

gigantica in contrast to the preponderance of *F. hepatica* in a 769 g of bubaline liver tissue.

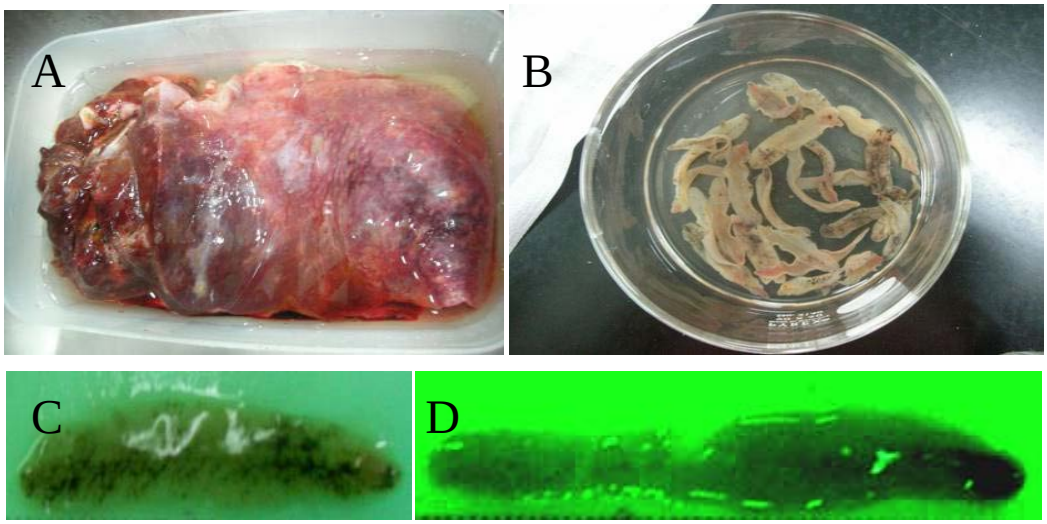

Figure 1. Cattle fasciolids. A: Infected liver tissue. B: Collected flukes. C: *F. hepatica*. D: *F. gigantica*

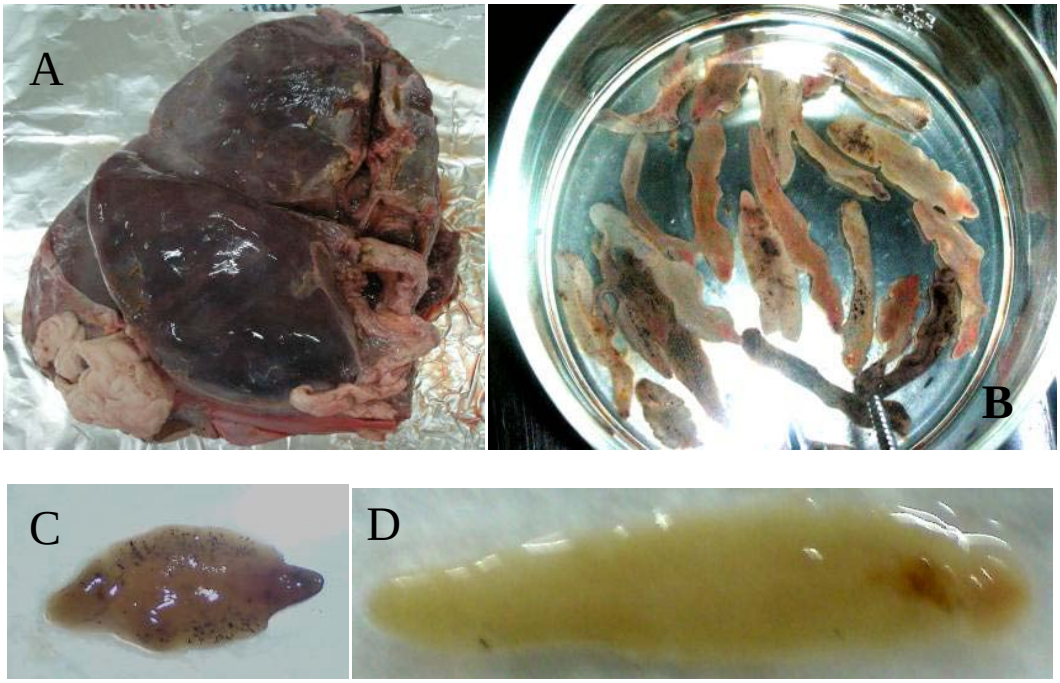

Figure 2. Water buffalo fasciolids. A: Infected liver. B: Isolated flukes. C: *F. hepatica*. D: *F. gigantica*

Extracts varied in total protein concentrations with fluke size (Fig. 3). While an increase in crude protein concentration is logical to assume with increasing age and size, the protein harvest may have been influenced by presence of both endogenous and host–derived proteins, the existence of intermediate form(s) difficult to ascertain based only on morphometric data, variation in worm burden that can influence growth rate in the liver tissue, and possible loss or degradation of proteins during homogenization and protein extraction.

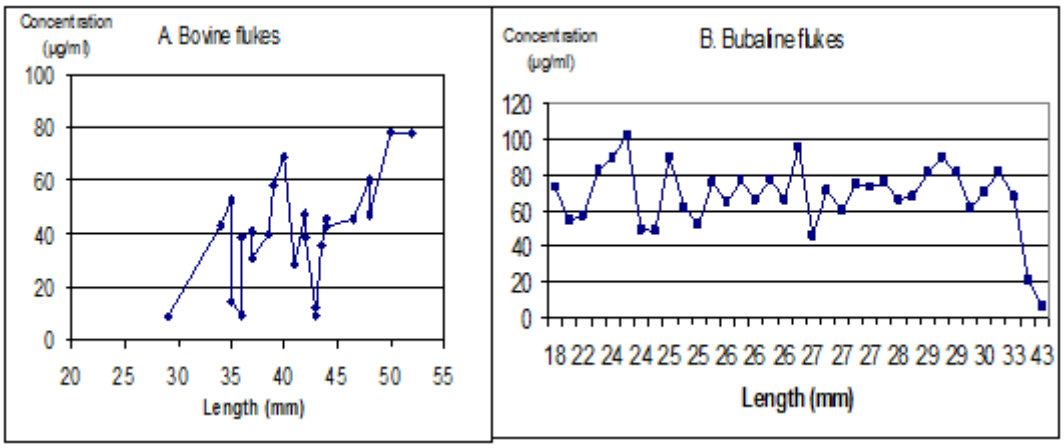

Figure 3. Concentrations of total proteins extracted from individual flukes of varying body length. A: Cattle. B: Bubaline.

Co-existing cattle *F. hepatica* and *F. gigantica* revealed several bands of which five appeared as major bands (Fig. 4A). Between co-existing bubaline *F. hepatica* and *F. gigantica*, there were 12 common bands, several of which were heavy and intensely-stained (Fig.4B), suggestive of the higher level of protein expression. Comparison between cattle and bubaline *F. hepatica* revealed seven common dominant bands and six bubaline-specific bands (220 kDa, 150 kDa, 115 kDa, 67 kDa, 34-37 kDa, 30 kDa) (Fig. 5A). Moreover, between cattle and bubaline *F. gigantica*, there were eight common major and four bubaline-specific bands (212 kDa, 150 kDa, 70 kDa, 30 kDa) (Fig. 5B). Three distinct bands (150 kDa, 67-70 kDa, 30 kDa) were shared by bubaline *F. hepatica* and *F. gigantica*.

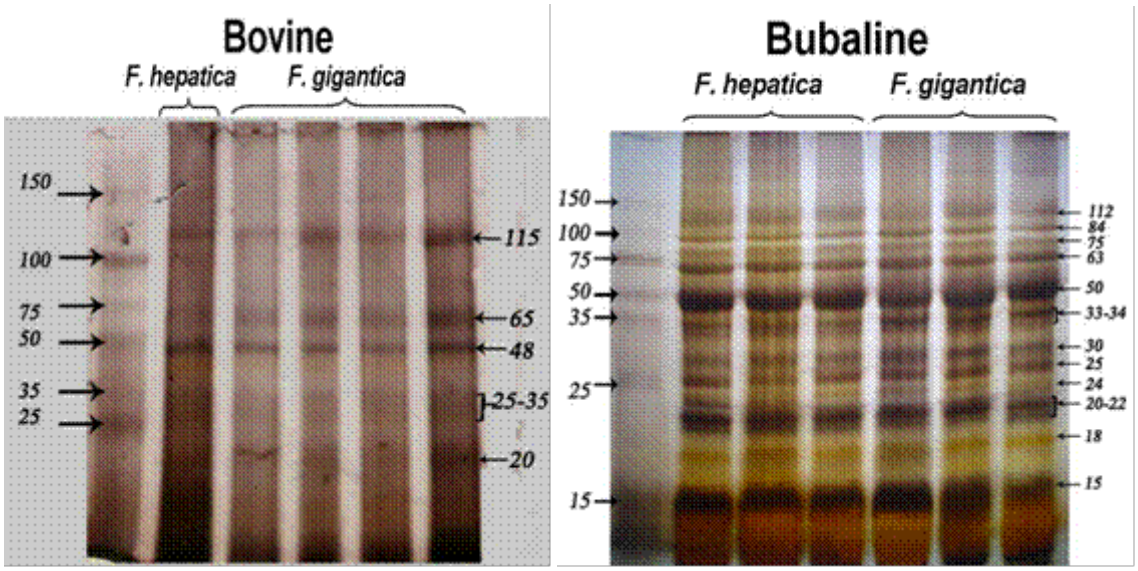

Figure 4. A. Bovine: Protein profile of extracts of co-existing *F. hepatica* (lane 2: 29 mm) and *F. gigantica* (lanes 3-6: 34-39 mm, 40-43 mm, 44-48 mm & 50-52 mm. B. Bubaline: Protein profile of co-existing *F. hepatica* (lanes 2-4: 18-21 mm, 22-25 mm & 26-29 mm) and *F. gigantica* (lanes 5-7: 5: 31-32 mm; 33-35 mm & 43 mm). Lane 1: molecular marker. CB: common bands

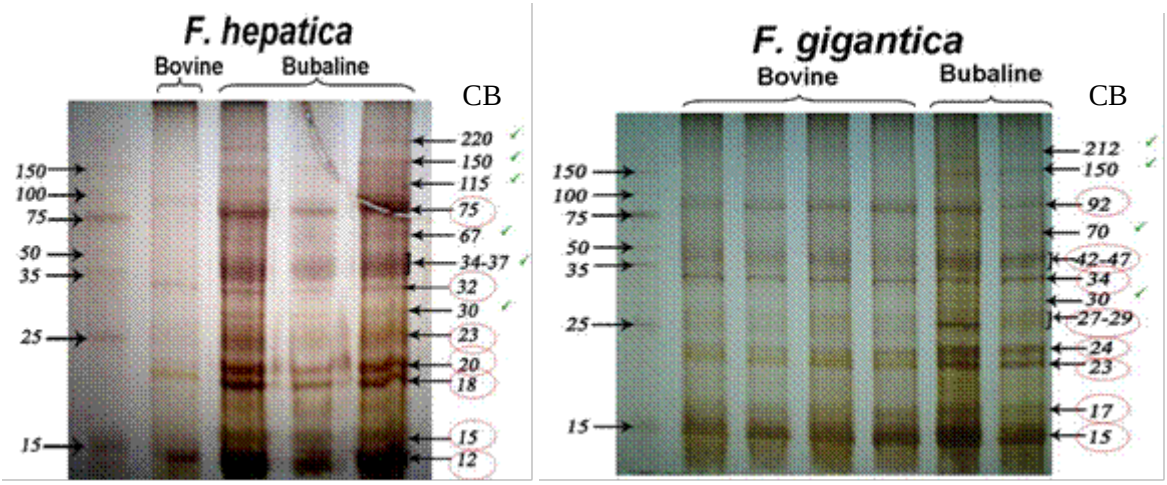

Figure 5. A. *Fasciola hepatica*: Protein profile of extracts from bovine (lane 2: 29 mm) and bubaline (lanes 3-5: 18-21 mm, 22-25 mm & 26-29 mm) flukes. B. *Fasciola gigantica*: Protein profile of extracts from bovine (lanes 2-5: 34-39 mm, 40-43 mm, 44-48 mm & 50-52 mm) and bubaline (lane 6-7: 33-35 mm & 43 mm). Lane 1: Molecular marker. CB: Common bands (encircled) detected in bovine and bubaline *F. hepatica* and *F. gigantica*. Bubaline fluke specific and shared protein bands (checked).

DISCUSSION

Present findings are consistent with earlier observations of the preponderance of *F. gigantica* in cattle and of *F. hepatica* in carabaos in the Philippines (De Leon and Juplo, 1966), Iran (Ashrafi *et al*., 2004) and India (Sharma *et al*., 1989; Jithendran and Bhat, 1999; Jithendran, 2000). However, species identification and preponderance should be verified in future studies using additional morphometric data, considering that the flukes were isolated only from liver slices of only one cattle and one water buffalo,

The similarity in banding pattern between *F. hepatica* and *F. gigantica* that co-existed in cattle and bubaline hosts is indicative of their close genetic relationship. Protein bands specific to co-existing *F. hepatica* and *F. gigantica* in cattle and bubaline hosts suggest host-related influence. Using isoelectric focusing, Lee and Zimmerman (1993) detected similar dominant peak points at pH range 4.6-9.3 between *F. hepatica* and *F. gigantica* and interpreted the difference as host-species influenced. Between cattle and bubaline *F. hepatic*, six bands were bubaline-fluke specific; between cattle and bubaline *F. gigantica*, four were bubaline-specific; and of these specific bands, at least three were shared by bubaline *F. hepatica* and *F. gigantica*. Intermediate or hybrid forms of *F. hepatica* and *F. gigantica* based on morphometric data (Periago *et al*., 2006; Ashrafi *et al*., 2006), and molecular data (Lin *et al*., 2007; Huang *et al*., 2004; Agatsuma *et al*., 2000; Itagaki *et al*., 1998; Hashimoto *et al*., 1997; Alasaad *et al*., 2007) have been inferred. While we observed similarities in protein profile between co-existing *F. hepatica* and *F. gigantica*, the possibility of the presence of both endogenous and host–derived proteins attributable to conditions like thawing of the liver tissue during fluke isolation and during homogenization and protein extraction cannot be discounted. Also, the pooled/mixed extracts of flukes grouped according to proximity in body length may have included potential hybrid within the purportedly *F. hepatica* or *F. gigantica* samples.

Current preliminary findings represent the first in the country. In view of the prevalence of fascioliasis and the dearth of baseline information, we highly recommend parasite surveillance in different susceptible hosts in farms, jointly with analysis of morphological and morphometric data of co-existing and inter-host fasciolid species. Future related studies should take into account wider sampling areas of animal hosts and the profiling of proteins of individual flukes to avoid muddling of protein composition. To

circumscribe host-derived proteins from endogenous components, protein profiling should also include extracts of infected and uninfected liver tissue samples.

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