P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article

Gross and Molecular Comparison of *Fasciola hepatica* and *Fasciola gigantica* from the Field in the Philippines

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Article History: Received: November 04, 2017 Revised: November 27, 2017 Accepted: December 09, 2017

ABSTRACT

The study established the morphologic and molecular differentiation of *Fasciola hepatica* and *Fasciola gigantica* in buffaloes. Specifically, the study described the gross structure and morphometry of *F. hepatica* and *F. gigantica* and validated the identification of *Fasciola* spp. based on gross morphology and PCR results. Sixty (60) samples were evaluated grossly and morphometrically using body length, body width, cone width, and cone length as parameters to differentiate the two species. Ten representative samples from each species identified based on the parameters were subjected to single step-duplex polymerase chain reaction (PCR) for molecular identification of the species identity. Results showed significant differences in body length, cone width and cone length between *F. gigantica*-like and *F. hepatica*-like samples. *F. hepatica*-like samples have significantly shorter (P=0.001) body length (29.20±3.04mm) as compared to *F. gigantica*-like samples (46.23±4.54mm). Mean cone width was also found to be significantly (*P*=0.001) longer in *F. gigantica*-like (3.67±0.55mm) compared to *F. hepatica*-like (3.33±0.48mm Cone length was significantly higher (P=0.001) in *F. gigantica* (3.67±0.55mm) than *F. hepatica* (2.90±0.33mm). Body width (BW) showed no significant difference (P=0.186) between the two species. *F. gigantica*-like samples had mean BW measurements of 10.63±0.99mm which had a very slight difference with *F. hepatica*-like samples at 10.33±0.71mm. PCR products obtained from the single-step duplex PCR targeting either the cytochrome c oxidase subunit I or the large subunit ribosomal RNA revealed that all 20 samples that were morphometrically differentiated as *F. gigantica* or *F. hepatica* were indicative of *F. gigantica* identity. The present findings suggested that conventional gross and morphometric analysis of *Fasciola* spp. alone was not completely reliable in differentiating the two species thus a use of molecular identification assay would yield a more dependable result.

Key words: *Fasciola hepatica, Fasciola gigantica,* Morphometry, Single-step duplex PCR

INTRODUCTION

Fasciola gigantica and *Fasciola hepatica* are liver flukes that cause fasciolosis, a disease affecting both livestock animals and humans (Rokni *et al.*, 2010). Infected animals suffer high morbidity rates and reduced production of milk and meat as well as liver condemnation that results to important economic losses (Mas-Coma *et al.*, 2005).

F. hepatica is widely distributed worldwide while *F. gigantica* is more widespread in the tropical and subtropical regions (Esteban *et al.*, 2003; Ashrafi *et al.*, 2004; Moghaddam *et al.*, 2004; Mas-Coma *et al.*, 2005). However, the distribution of both *F. hepatica* and *F. gigantica* may overlap in subtropical areas (Mas-Coma *et al.*, 1999; 2005). Furthermore, hybridization/introgression phenomena might take place where both species coexist (Periago *et al.*, 2008). Fasciola intermediate forms between *F. hepatica* and *F. gigantica* have been reported from Asian countries including Korea (Agatsuma *et al.*, 2000; Choe*et al.*, 2011), Japan (Itagaki *et al.*, 2005), Iran (Ashrafi *et al.*, 2006; Amor *et al.*, 2011), China (Peng *et al.*, 2009; Ai *et al.*, 2011) and Vietnam (Le *et al.*, 2008; Itagaki *et al.*, 2009), as well as African countries including Egypt (Periago *et al.*, 2008; Amer *et al.*, 2011).

F. hepatica and *F. gigantica* can generally be distinguished on the basis of their morphology (Ashrafi *et al.*, 2006). Fasciolids are identified primarily on difference

Cite This Article as: Valino LSI, Venturina VM and Mingala CN, 2017. Gross and molecular comparison of *Fasciola hepatica* and *Fasciola gigantica* from the field in the Philippines. Inter J Vet Sci, 6(4): 216-221. www.ijvets.com

in body shape and size of adults, with the smaller *F. hepatica* exhibiting wide and defined shoulders compared to the slender *F. gigantica* having less defined shoulders and shorter cephalic cones (Kimura *et al.*, 1984; Kahn, 2004).

The use of molecular methods and markers are useful to distinguish intermediate forms and between species (Marcilla *et al.*, 2002). Molecular techniques have become widely accepted throughout the world. They provide a more specific method than methods conventionally employed in epidemiological studies (Coote, 1990; Erlich et al., 1991; Barker, 1994; Rognlie *et al.*, 1994; Heckeroth *et al.*, 1999). The first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA) which occurs between the 18S, 5.8S, and 28S coding regions, have been used for diagnostic purposes at the level of species (Morgan and Blair, 1995; Leon-Regagnon *et al.*, 1999; Tkach *et al.*, 2000; Kostadinova *et al.*, 2003; Scholz *et al.*, 2004; Tandon *et al.*, 2007; Prasad *et al.*, 2008).

Despite the importance of differentiating between the infection by either fasciolid species, due to their distinct epidemiological, pathological and control characteristics, there is, unfortunately, neither a direct coprological nor an indirect immunological test available for their diagnosis. The specific differentiation can only be made by either a morphological study of adult flukes (Tkach *et al.*, 2000) or by molecular tools (Le *et al.*, 2012). Hence, subjects diagnosed are currently referred to as infected by *Fasciola* spp. (Mas-Coma *et al.*, 2005).

The study aimed to compare morphometrical and molecular parameters between the two species of *Fasciola* collected from buffaloes in slaughter houses of San Jose City and Cabanatuan City, Nueva Ecija, Philippines.

MATERIALS AND METHODS

Specimen collection

Samples were obtained from the province of Nueva Ecija, Philippines, specifically from San Jose City and Cabanatuan City slaughter houses. Adult liver flukes were collected from carefully incised biliary tracts and gall bladders of infected buffaloes, these were where adult liver flukes could be found. Worms were removed with the use of forceps, and placed in bottles with lukewarm water for ~30 minutes to relax their bodies and prevent them from curling up or forming wrinkles on the sides, before preserving them in 70% ethanol. A total of 519 samples were collected from three infected livers, from the buffaloes of the two slaughter houses. Sixty (60) specimen samples were randomly selected for morphological examination. On the basis of the gross appearance particularly the general shape and body length as described in previous studies (Narva *et al.*, 2008; Ghavami *et al.*, 2009; De Vera *et al.*, 2009), thirty representatives each of *F. hepatica* and *F. gigantica* samples were subjected to morphological analysis and was grouped as *F. hepatica*-like and *F. gigantica*-like.

Morphologic examination

Gross morphologic examination involved the measurements of 60 adult flukes using a caliper as follows (Fig. 1): BL (body length), maximum BW (body width at shoulder level), CW (cone width) and CL (cone length).

Molecular analysis

Twenty (20) out of the 60 samples which were classified as *F. gigantica* and *F. hepatica* based on morphometric evaluation were used for molecular identification using PCR. Samples for PCR analysis were initially washed with physiologic buffered saline thrice to remove the host blood, bile and contaminating microorganisms. The DNA of each worm samples was extracted using the Wizard Genomic DNA Purification protocol (Promega, Wisconsin, USA).

Amplification was done with a total volume of 10 µl. The master mix was composed of 2.55 µl double distilled water (DDW), 3 µl PCR buffer, 0.75 µl $MgCl_2$, 0.5µl dinucleotide Triphosphate (dNTPs), 0.5 µl each of the three-primers set (10 pmol FHF 5'-GTTTTTTAGTTGTTTGGGGTTTG-3'/ 10 pmol FGF5'-TGTTATGATTCATTGTTTGTAG-3'/ 20 pmol FHGR 5'-ATAAGAACCGACCTGGCTCAC-3'), targeting the cytochrome c oxidase subunit I, large subunit ribosomal RNA and a reverse universal primer, respectively, 0.5 µl of Taq polymerase and 2.0 µl of DNA template. A negative control containing DDW instead of DNA was used and a positive control of *F. gigantica* extract was also included in the run.

The samples were loaded in a thermo-cycler under the following conditions: 95°C for 3 min (initial denaturation) followed by 30 cycles of 95°C, 30s (denaturation), 52°C, 30s (annealing), 72°C, 2 min (extension), and a final extension of 72°C for 7 min.

Gel electrophoresis and UV illumination

An aliquot (3 µl) of each amplified PCR products was examined on 1.5% agarose gels stained with gel red. A 7 µl 1-kb plus ladder was placed in the first well of the gel while 3 µl of negative control was placed on the wall next to the last sample. The gel was run in the electrophoresis machine for 30 min. Visualization of the gel was done under short UV wave illumination. Amplicons were also sent for sequencing to further confirm the identity of the samples.

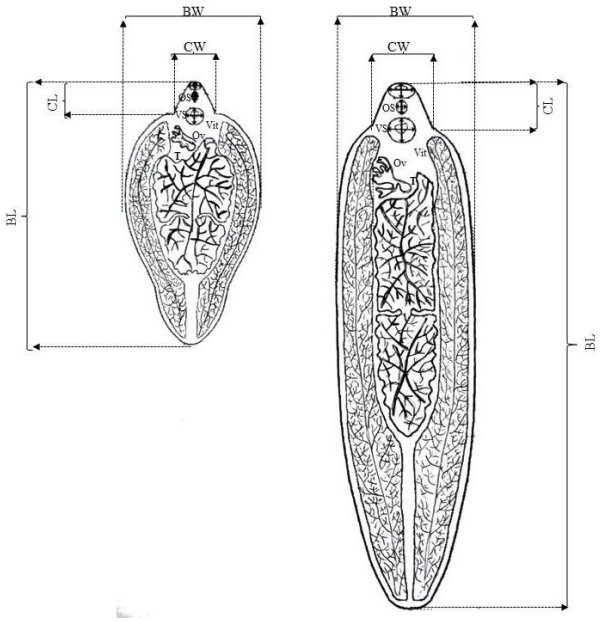

Fig. 1: Morphological measurements (Periago*et al.*, 2006).

Data analysis

T-test was performed to determine the significant difference between the measurements made between *F. hepatica*-like and *F. gigantica*-like samples set at P<0.05 with the use of Statistica v.10 program.

RESULTS AND DISCUSSION

Gross morphologic identification of *Fasciola* spp.

Out of 519 samples collected, only 57 (10%) showed *F. hepatica*-like characteristics such as broad and prominent shoulders (Fig. 2) and shorter body length as compared to the rest of the samples (462) which displayed *F. gigantica*-like characteristics. The gross visual differentiation was based on the studies conducted by Ghavami *et al.* (2009). Thirty (30) of the *F. hepatica*-like were used to represent *F. hepatica* and 30 of *F. gigantica*-like specimens were subjected to morphological measurements and analysis.

Statistical analysis revealed significant difference in body length, cone width and cone length between *F. gigantica*-like and *F. hepatica*-like (Table 1) samples. *F. hepatica*-like samples have significantly smaller (P=0.001) body length (29.20±3.04mm) as compared to *F. gigantica*-like samples (46.23±4.54mm). Mean cone width was also found to be significantly (P=0.001) longer in *F. gigantica*-like (3.67±0.55mm) compared to *F. hepatica*-like (3.33±0.48mmConsistently, cone length measurement was found to be significantly higher (P=0.001) in *F. gigantica* (3.67±0.55mm) than *F. hepatica* (2.90±0.33mm). The measured body width (BW) on the other hand showed no significant difference (P=0.186) between the two types of samples. *F. gigantica*-like samples had a mean BW measurement of 10.63±0.99mm which had a very slight difference with *F. hepatica*-like samples at 10.33±0.71mm. The present findings coincide with other literatures that dealt with similar work using the same criteria adapted in the study (Narva *et al.*, 2008; Ghavami *et al.*, 2009; De Vera *et al.*, 2009).

It can be generalized that visual gross inspection and morphometric evaluation can be distinguished between *F. hepatica* and *F. gigantica*. However, it must be noted that the range of measurements done on the representative samples have overlapping values. The minimum range of BL for *F. gigantica* was 36.0mm which was very close to the maximum range for *F. hepatica* at 35.0mm. Similar observation was noted for CW with range of 3.00-5.00mm and 3.00-4.00mm for *F. gigantica* and *F. hepatica* respectively. Furthermore, the minimum value for the CL of *F. gigantica* (3.0mm) was similar with *F. hepatica*. Similar results were noted in previous work involving morphometric differentiation of the two *Fasciola* species. Indeed, overlapping morphological measurements between adult liver flukes identified as *F. gigantica* and *F. hepatica* collected from the province of Sultan Kudarat in Mindanao were recorded (Narva *et al.*, 2008). Furthermore, El-Rahimy *et al.* (2012) supported the current finding as they concluded that conventional morphological and metric assessments were not useful for differentiation between *F. gigantica* and *F. hepatica* due to extensive overlap in the relative ranges. Similar conclusion was reached concerning protein band characterization where the patterns of protein banding were mostly similar. For specific identification, the group recommended genotyping using RFLP-PCR which gave consistent results and clear differentiation between the two species (Rokni *et al.*, 2010).

The foregoing discussion may be explained by the claims of Ghavami *et al.*(2009) that external measurements of the differences in body length, width, cone length and width of fasciolids can be influenced by intensity of infection, host species, age and immune reactions due to a possible previous exposure to the infection.

Table 1: Gross morphometry of adult liver flukes (*Fasciola* spp.) from buffaloes (*Bubalus bubalis*) in San Jose City and Cabanatuan City, Nueva Ecija, Philippines using T-Test.

	Range (mm)	Mean (mm)	SD	Df	t-value	p
BL						
FG	36.00-55.00	46.23	4.54	58	17.11	0.001*
FH	24.00-35.00	29.20	3.04			
BW						
FG	09.00-13.00	10.63	0.99	58	01.34	0.186
FH	09.00-12.00	10.33	0.71			
CW						
FG	03.00-05.00	03.67	0.55	58	02.51	0.001*
FH	03.00-04.00	03.33	0.48			
CL						
FG	03.00-05.00	03.67	0.55	58	06.70	0.001*
FH	02.00-03.00	02.90	0.30			

BL-Body length, BW- Body width, CW-Cone width, CL-Cone length, FG- *F. gigantica,* and FH-*F. hepatica.*; *P≦0.05 significant.

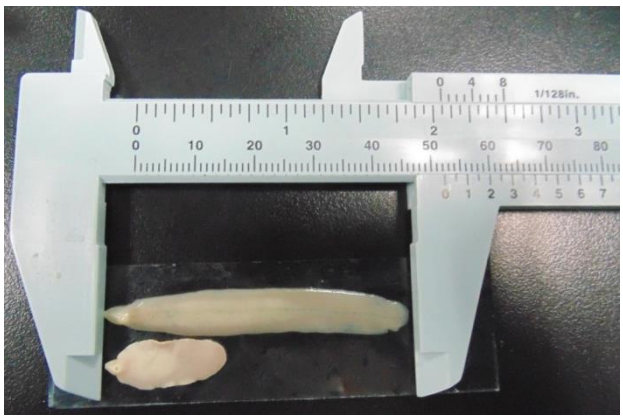

Fig. 2: *Fasciola* spp. samples collected from water buffaloes. The worms were measured with the use of a caliper.

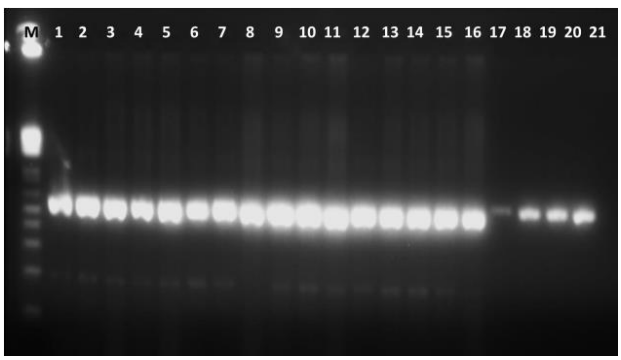

Fig. 3: Gel electrophoresis of duplex PCR using mtDNA template from 10 morphologically-identified *F. hepatica* and 10 *F. gigantica*; M, 1-kb plus ladder marker; Lanes: 1 to 10, morphologically-identified *F. hepatica*; 11-20, represents *F. gigantica*; lane 21, (-) negative control (no DNA).

The age of buffaloes where liver fluke specimens were sourced ranged from three to five years. Also, the age of the *Fasciola* isolates were not known. Both factors may affect the morphometrical values obtained from the study because the possibility of juvenile isolates that were subjected to the analyses may contribute to the overlapping of the analyzed morphometrical data.

Moreover, Ghavami *et al.* (2009) observed that *Fasciola* isolates from high altitude showed different morphological and physiological characteristics from those inhabiting at low altitude. Thus, establishing a fix consistent range on the morphometrical value on Fasciolids was possibly unattainable.

The possibility that intermediate forms of *Fasciola*spp. exist was not discounted in the present work. The morphometric measurements of fasciolid eggs from cattle in Pakistan were found between *F. hepatica* and *F. gigantica* standard populations (Afshan *et al.*, 2013). Hence, the overlaps in the measurements could possibly be due to the presence of intermediate forms.

Molecular analysis

Mitochondrial DNA extracted from samples of 10 morphological representative of *F. hepatica* and 10 *F. gigantica* were used as templates in the single-test duplex PCR run. Duplex PCR targeting mitochondrial DNA (using 3 primers: 2 forward and 1 reverse) was used to simultaneously identify and differentiate the DNA templates. The PCR protocol used in the study was based on an optimized assay developed in the Philippine Carabao Center (PCC) Animal Health laboratory by Belotindos (personal communication).

PCR products amplified from DNA templates of all 20 samples used in the study had similar sizes of 615 bp suggestive of *F. gigantica* identity and further confirmed by sequencing (Fig. 3). Similar amplicon was generated using the same methodology in a study by Le *et al.* (2008) in the molecular identification of *F. gigantica.*

None of the ten (10) representative samples of *F. hepatica* were identified positively based on the PCR product size of 1031 bp. Difference in the amplicon size could be easily distinguished and categorized from one another using the duplex primer, hence, making the differentiation of the two species easy.

Analysis of the data suggests that morphometric criteria alone were not useful in differentiating *F. gigantica* and *F. hepatica.* Samples that were initially identified as *F. hepatica* based on BL, CL, and CW turned out to be *F. gigantica* based on the results of PCR runs. This finding coincided with the study by Ghavami *et al.* (2009) wherein positive differentiation between *F. gigantica* and *F. hepatica* based on morphological analysis was contradicted by the results of the PCR as both samples were confirmed to be *F. hepatica*. However, the *Fasciola* spp. samples were collected from cattle and sheep in a slaughterhouse in Iran and the primers were derived from the nuclear ITS2 sequences of *Fasciola* spp. The present study utilized samples from buffaloes, while primers were derived from the mitochondrial DNA of the fluke. Confirmation of the identity of amplified PCR product could be done; however, this would not be covered in the study because of the limited time available. The collaborators from PCC would however continue sequencing the work of the samples to further strengthen the evidence shown in the study.

It is concluded that morphometric analysis alone is not sufficient and reliable in the species-specific identification and differentiation of *Fasciola* isolates. To make it more conclusive, molecular identification assay using PCR and further confirmation by sequencing are highly recommended.

Acknowledgments

We thank Dr. Arnel N. Del Barrio, Executive Director of PCC, for his valuable support. We also thank all the staff of the Biosafety and Environment Section of PCC for providing support and assistance to conduct the study.

Authors contribution

All authors contributed to sample collection, data analysis and manuscript preparation; LSIV preformed sample collection and laboratory analysis, VMV and CNM sample collection, CNM edited and prepared the final version of the manuscript for publication.

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