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Tegumental Studies of Adult *Fasciola gigantica* (Giant liver fluke) from Philippine Carabaos (*Bubalus bubalis*) Using Scanning Electron Microscopy for Lead Bio-Indicator Analysis

Aimee Caye G. Chang and Mary Jane C. Flores*
Biology Department, De La Salle University, 2401 Taft Avenue, Manila 0922, Philippines

*Corresponding Author: mary.jane.flores@dlsu.edu.ph

ABSTRACT

Recent studies show that parasites such as liver flukes have a capacity to bioaccumulate heavy metals significantly than the liver and muscle tissues of the host without compromising effects on them. One such heavy metal is lead (Pb) which is an abundant pollutant in the environment. Therefore, this study aimed to evaluate the effects of lead in the tegument of *Fasciola gigantica* (giant liver fluke) isolated from infected livers of carabaos *in vitro* using the following concentrations of lead: 0ppm, 110ppm, 160ppm, and 210ppm. Results indicate that upon exposure to lead, liver flukes tend to curl and exhibit wrinkled appearance after 15 hours. Furthermore, scanning electron microscopy studies of the tegument showed that chemical exposure of the flukes to lead caused similar alterations observed when flukes are exposed to anthelmintic drugs which are sloughing, blebbing, furrowing, and folding. Moreover, two novel types of alterations were observed in this study which are wrinkling and crumpling of the tegumental surface. In conclusion, distinct morphological and tegumental characteristics observed in liver flukes due to lead tolerance suggests its potential as bio-indicator of environmental pollution and thus promotes its use as a good candidate of an effect indicator.

Keywords: *Fasciola gigantica*, Giant liver flukes, Philippine carabaos, Tegument, Scanning electron microscopy, Blebbing, Crumpling

INTRODUCTION

Heavy metals such as lead have a tendency to build up within living organisms. As these pollutants are always present in the environment, this poses a health threat to individuals. As these pollutants continuously increase due to man-made activities such as mining and fuel development, animals and humans are exposed to increasing amounts of heavy metals over time (Sures, 2004). Consequently, it will bioaccumulate within the bodies which could eventually cause deleterious effects to the host. Small amounts may provide beneficial effects; however, huge amounts may cause heavy metal toxicity that will lead to several health problems such as cancer, organ damage, abnormality of the nervous system, infertility, growth retardation, and sometimes death (Lotfy et al., 2013).

One morphological structure of *Fasciola* spp. reported to be prone to damages is the tegument. The tegument is between the surface of the parasite and host which is an integrating layer that facilitates maintenance of the parasite's homeostasis. Some of its functions include absorption and means of nutrition and waste exchange, regulation of ion transfer between the parasite and the surrounding fluid, and also evasion of immunological mechanisms exhibited by the host. Therefore, targeting the integrity and usual functions of the tegument could compromise the viability of the parasite and could hasten the antagonistic mechanisms of the hosts' immune responses such as production of immune effector cells (Sobhon et al., 1998).

Therefore, the tegument can be used as a tool for assessing bioindicator potentials of parasites. It was established that liver flukes have a capacity to bioaccumulate heavy metals in their flesh significantly than liver and muscle tissues of the host (Lotfy et al., 2013). Thus, analysis for bioindicators using parasites of terrestrial hosts is a rapid growing area of research to indicate environmental pollution within the area. Bioindicators can either be effect indicators, accumulation indicators, or both. Effect indicators show changes occurring in the surroundings by changes in their morphology or physiological responses. On the other hand, accumulation indicators or sentinels are those which are capable of accumulating toxins or pollutants at elevated amounts without compromising themselves or changing their common responses (Lotfy et al., 2013; Sures, 2004; Sures et al., 1999). As *Fasciola* spp. is already known as accumulation indicator, this paper aims to evaluate the potential of *Fasciola gigantica* as effect indicator of lead using the tegument as the basis for distinct alterations.

METHODOLOGY

Collection of *Fasciola gigantica*

The health and condition of the carabaos were assessed prior to slaughter in a local abattoir in Manila. Afterwards, livers were examined for *Fasciola* infection. The criteria for choosing a fluke-infected liver are through signs of rotting, discoloration, and enlarged liver size due to calcification and obstruction of bile ducts. The liver, contained in a clean cooler, was then transported at the Zoology and Parasitology laboratory of De La Salle University – Science and Technology Research Center (STRC). Live samples of *Fasciola* spp. were then extracted from the hepatobiliary tracts of naturally-infected livers of carabaos. The flukes were then placed in sterile glass bottles containing physiological saline (Kawano et al., 1989) prior to *in vitro* exposure to lead. Measurements of the specimens were compared to other published standard size ranges for species identification of adult *Fasciola gigantica*.

In Vitro Exposure of *Fasciola gigantica* to Lead

Sterile tissue culture media (Medium 199) was prepared in tissue culture plates (Diab et al., 2010; Keiser & Morson, 2008). Lead nitrate as the source of lead was diluted to the culture media to achieve the desired concentrations: 110ppm, 160ppm, and 210ppm. For the negative control, flukes were exposed in M199 medium only. Four flukes were placed into each plate in triplicates. Both the experimental and the control groups were then incubated in a modified incubator with 5% CO_2 at 37°C for 7 and 15 hours (Keiser & Morson, 2008).

Scanning Electron Microscopy (SEM)

Preparation for SEM. Treated flukes after heavy metal exposure were obtained in each concentration and incubation time. The flukes were rinsed with 0.85% NaCl (w/v) for 24 hours at room temperature (Keiser & Morson, 2008). Afterwards, the flukes were washed repeatedly (up to three brief washes) in cold double-distilled water and dehydrated through a graded series of ethanol. The flukes were then freeze-dried using liquid carbon dioxide. ***SEM observation and description.*** The specimens were processed in the Surface Physics Laboratory, Physics Department at Science and Technology Research Center (STRC) of De La Salle University Manila. The general morphology of the flukes was assessed. Specifically, the following parts were observed under SEM: apical cone region, ventral and oral sucker, surface of the tegument, ventral and dorsal tail regions, and lateral margins (Shalaby et al., 2009; Keiser & Morson, 2008). Table 1 describes the morphological abnormalities that can be observed in all areas of the tegumental surface.

Table 1. Definition of Tegumental Surface Alterations (Keiser & Morson, 2008)

Types of damages	Description
Sloughing	shedding of the surface
Blebbing	small bubble-like formations
Swelling	enlargement of a certain part or organ
Empty spine sockets	observable holes in the center of each spine
Furrowing	groove pattern on the surface
Folding	bending of a part
Eruption	breakage or disruption of an organ

RESULTS AND DISCUSSION

In Vitro Exposure of *Fasciola gigantica* to Lead

All flukes prior to *in vitro* exposure to lead were measured and confirmed as *Fasciola gigantica* species and possess body lengths and widths falling within the range of 26-40mm by 7.46-14.30mm, cone lengths and widths within 1.62-6.92mm by 2.50-4.54mm, oral suckers with diameters within 0.33-0.86mm, and ventral suckers with diameters within 0.38-0.86mm. One apparent observation after 15 hours of exposure to lead was that the flukes appeared wrinkled though still viable. The flukes in the control group were monitored until 17 hours and viability of flukes was still evident.

Morphological observations through scanning electron microscopy

Minor tegumental deformity and loss of spines were observed in all experimental groups due to prolonged exposure to physiological saline solution during morphometrics of live flukes prior to lead exposure. Figures 1 and 2 show the tegument of the control fluke wherein signs of disintegration of the tegument can be seen. However, despite this untoward occurrence, alterations between the control and the lead-exposed flukes can still be distinguished. Table 2 summarizes alterations observed in each concentration.

Table 2. Summary Table of Tegumental Surface Alterations Observed[1] in Each Concentration After Exposure to Lead

Conc.(ppm)	0		110		160		210	
Hours / Alterations	7	15	7	15	7	15	7	15
Empty spine sockets	*	*	*	*	*	*	*	*
Sloughing			*	*	*	*	*	*
Blebbing			*	*	*	*	*	*
Furrowing				*				
Folding					*	*	*	*
Wrinkling					*		*	
Crumpling						*		*

[1] Asterisk (*) indicates that an alteration was observed.

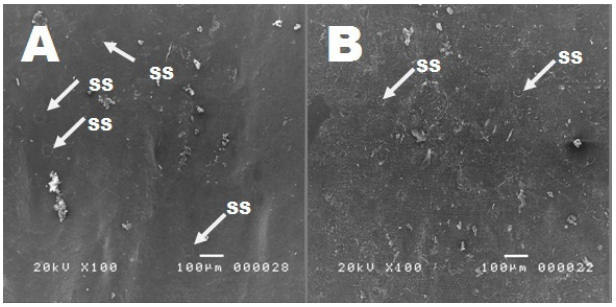

Figure 1. *F. gigantica* tegument of 0 ppm (control) at 0 hour. (A) Spine Sockets (SS were observed at the dorsal midbody portion of the fluke and (B) resence of SS and network of fibrous projections appear at the ventral midbody.

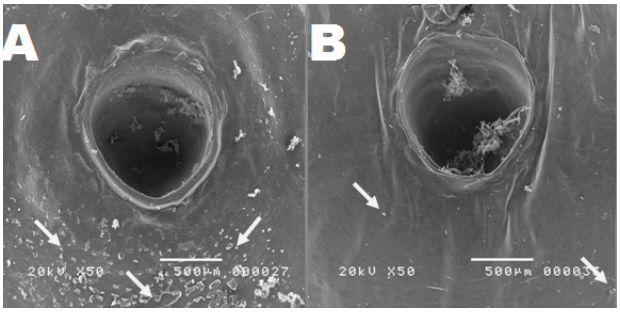

Figure 2. *F. gigantica* tegument of anterior ventral region of 0ppm (control) at (A) 7 hours incubation showing remnants of spines (arrows) left and (B) 15 hours incubation where spines are no longer visible.

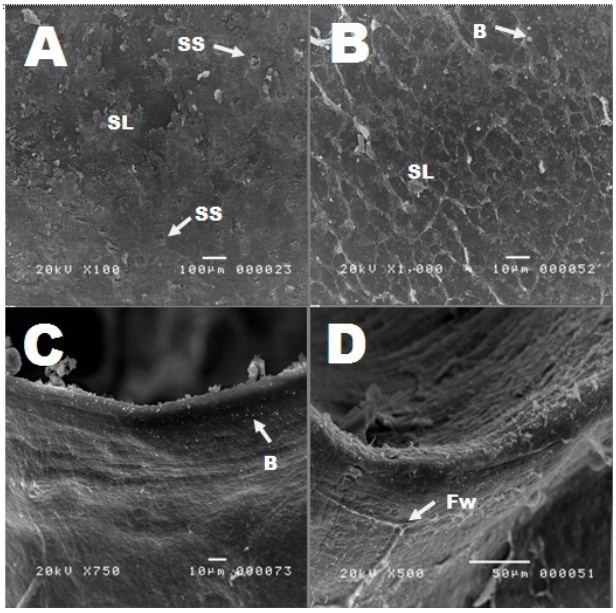

Figure 3. *F.gigantica* tegument of 110ppm lead exposure at (A) 7 hours incubation showing empty spine sockets (SS) and sloughing (SL) at dorsal posterior region; (B) 15 hours incubation showing blebs (B) and sloughing (SL) of surface at dorsal tail region; (C) 7 hours incubation showing blebbing (B) near margin of ventral sucker and (D) 15 hours incubation showing furrowing (Fw) near margin of ventral sucker.

At 110ppm concentration, traces of spine sockets were evident after seven hours exposure but due to the loss of spines observed in the tegument of the control fluke, comparison cannot be construed that the empty spine sockets were due to the lead exposure. However, sloughing or shedding of the tegumental surface was visible at the dorsal tegumental region wherein intensity of sloughing was more severe after 15 hours exposure to lead (Figure 3A-B). Appearance of scattered blebs in the tegument was also noticeable near the ventral sucker margin after seven hours incubation of the flukes and particularly at the dorsal tail region after 15 hours lead exposure (Figure 3C-D). According to Bennett, Hughes, and Harness (1980), blebbing occurs as a result of the parasites' persistent exertion to shed its surface membrane and replace the damaged portions with a new one. It is therefore a tegumental coping mechanism of the parasites when chemical or drug agents are being introduced (Tansatit et al., 2012; Halferty et al., 2009). Slight furrowing near the ventral sucker was also observed after 15 hours (Figure 3D). The intensity of the alterations observed was more prominent as incubation time increases while no significant differences are observed in the alterations found in the dorsal and ventral side of the flukes.

At 160ppm concentration, a wrinkled appearance of the tegument was commonly observed both dorsally and ventrally. This supports the observation during the viability assay that the flukes appeared wrinkled after retrieval of flukes from the culture plates at 15th hour of the assay. Presence of blebs was observed at the dorsal anterior region (Figure 4A) and an inward fold was evident at 15 hours-exposed flukes (Figure 4B). The ventral side of the flukes shows wrinkling near the ventral sucker after seven hours incubation (Figure 4C) while a more severe wrinkled manifestation was apparent after 15 hours exposure that resulted to a crumpled appearance (Figure 4D). Consistent with the findings at 110ppm concentration, no significant differences are observed in the alterations found in the dorsal and ventral side of flukes and severity was apparent at higher incubation time. At this point, intensity of alterations became more prominent compared to the alterations observed at lower concentration of lead.

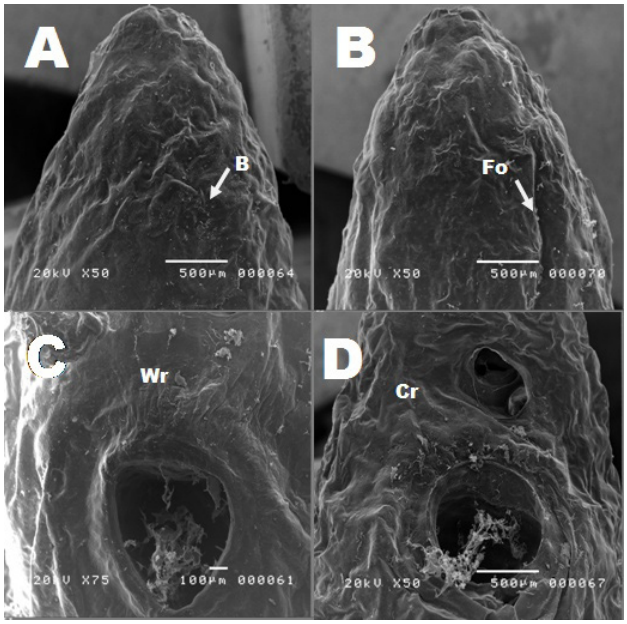

Figure 4. *F.gigantica* tegument of 160ppm lead exposure at (A) 7 hours incubation showing blebs (B) at dorsal anterior region; (B) 15 hours incubation showing folding (Fo) at dorsal anterior region; (C) 7 hours incubation showing wrinkling (Wr) at ventral anterior region and (D) 15 hours incubation showing crumpling (Cr) at ventral anterior region.

At 210 ppm concentration, crumpling of the tegumental surface became more prominent. A burnt exterior was evident particularly after 15 hours exposure of the flukes. At seven hours exposure, wrinkled appearance started to form at the dorsal anterior region while prolonged exposure to lead resulted to crumpled tegument (Figure 5A-B). At the ventral midbody region, blebs were present after seven hours incubation and an outward

fold was also observed (Figure 5C). After 15 hours, the basal lamina was seen exposed which might be due to excessive sloughing of the surface (Figure 5D). The observations at 210ppm were consistent with the previous results wherein tegumental alterations were more intense as concentration and time increased. No significant differences were also found in the alterations between the dorsal and ventral side as well as the anterior, midbody, and posterior regions. According to Tansatit et al. (2012), the ventral portion of the fluke is usually thicker than the dorsal side of the tegument. Thus, the insignificant differences in the alterations on both regions noted in this study suggest a homogenous uptake of lead throughout the entire body of the fluke (Tansatit et al., 2012).

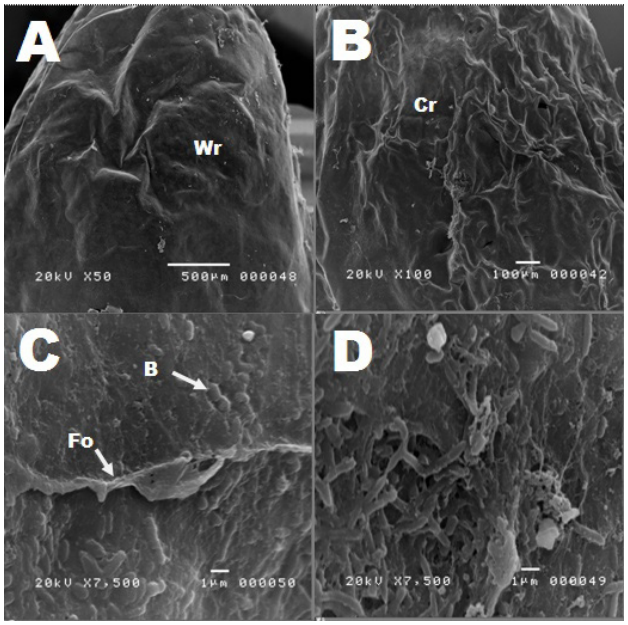

Figure 5. *F.gigantica* tegument of 210ppm lead exposure at (A) 7 hours incubation showing wrinkling (Wr) at anterior dorsal region; (B) 15 hours incubation showing crumpling (Cr) at dorsal anterior region; (C) 7 hours incubation showing blebbing (B) and folding at ventral midbody region and (D) 15 hours incubation showing exposed basal lamina due to intensive sloughing.

CONCLUSION AND RECOMMENDTIONS

Scanning electron microscopy studies of the tegument showed that exposure of the flukes to lead caused similar alterations observed when flukes are exposed to anthelmintic drugs. These are sloughing, blebbing, furrowing, and folding. However, two types of tegumental alterations that were not previously reported in anthelmintic tegumental studies were observed in this study which are wrinkling and crumpling of the surface.

In conclusion, due to their apparent morphological changes after heavy metal exposure, this study promotes the potential of *Fasciola gigantica* as a good candidate of an effect indicator. However, further studies are necessary to determine the exact amount of heavy metal that the fluke can accumulate within its body. Furthermore, molecular, ultrastructural, and physiological studies are recommended to evaluate its potential as a bioindicator of heavy metal pollution in the environment.

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