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Morphology and viability of adult *Fasciola gigantica* (giant liver flukes) from Philippine carabaos (*Bubalus bubalis*) upon *in vitro* exposure to lead

Aimee Caye G. Chang, Mary Jane C. Flores*

Biology Department, College of Science, De La Salle University, Manila, Philippines

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ABSTRACT

Objective: To evaluate the effects of lead in the morphology and viability of *Fasciola gigantica* (*F. gigantica*) (giant liver fluke) isolated from infected livers of carabaos *in vitro* using the following concentrations of lead: 0, 100, 150 and 200 mg/L.
Methods: *In vitro* viability and motility assay was conducted to evaluate the effects of lead using 1% methylene blue as the vital dye for assessment of the flukes' viability.
Results: Results indicate that *F. gigantica* can tolerate lead exposure as high as 200 mg/L with visible morphological variations. Upon exposure to lead, liver flukes tend to curl and excrete black precipitates as a sign of physiological stress response. Furthermore, the lethal concentration (LC_{50}) of lead against *F. gigantica in vitro* was 160 mg/L.
Conclusions: In conclusion, tolerance of liver flukes to high levels of lead suggests its potential as a possible biomarker of environmental pollution.

1. Introduction

Environmental pollutants such as heavy metals have a tendency to accumulate within living organisms. As these pollutants are unavoidable factor in the environment, this raises a major concern to all. As anthropogenic pollution increases due to man-made activities such as mining and fuel development, animals and humans are exposed to increasing amounts of heavy metals in the atmosphere[1]. Over time, it will bioaccumulate within humans and mammals which could eventually compromise the host. Although tiny amounts may provide beneficial effects, excess amounts may cause heavy metal toxicity that will lead to various health problems[1-3].

One significant rapidly growing field is biomonitoring of heavy metals utilizing living organisms as bioindicators. Biomarkers can either be effect indicators or accumulation indicators. Effect indicators point to changes occurring in the environment by changes in their morphology or physiological responses. On the other hand, accumulation indicators or sentinels are those which are able to accumulate toxins or pollutants at high amounts without compromising themselves or changing their normal responses[4,5]. Therefore, as parasites are used as sentinel organisms in detecting environmental pollutants, it is of great interest to explore the parasites inhabiting the bile ducts where these heavy metals are being deposited into the body. One of which is the liver fluke from the genus *Fasciola* that thrives in the bile ducts in the liver of its host. Furthermore, the hosts of these trematode parasites which are ruminants and plants are remarkably known as heavy metal hyperaccumulators. This makes an interesting connection between metal bioindicator plant species being consumed by ruminants and goes to the liver of a fluke-infected host. According to studies done by Lotfy *et al.* (2013), *Fasciola hepatica* is able to accumulate heavy metals greater than the host tissues, especially compared to the liver. This suggests a way of disposing hyperaccumulator plants by feeding it to fascioliasis-infected ruminants in which heavy metals will be absorbed by the parasite without greatly affecting the host. However, no study has been done yet on the effect of heavy metal exposure in *Fasciola* species[5,6] as most recent studies focus on blood flukes and tegumental efficacy studies[7,8].

Therefore, this study aimed to determine the effect of lead exposure to the viability and motility of *Fasciola gigantica* (*F. gigantica*) isolated from infected livers of locally-slaughtered

*Corresponding author: Mary Jane C. Flores, Biology Department, College of Science, De La Salle University, Manila, Philippines.
Tel: 524 4611 loc 460; +639151122341
E-mail: mary.jane.flores@dlsu.edu.ph

carabaos. The findings from this study could help evaluate the use of *F. gigantica* as effective bioindicators of environmental pollution and contributes preliminary knowledge regarding significant effects of heavy metal accumulation within the tissues of the parasite.

2. Materials and methods

2.1. Collection of F. gigantica

The health and condition of the carabaos was assessed before being slaughtered in a public abattoir in Manila, Philippines. Livers were then examined for the presence of *Fasciola* immediately after slaughter. The criteria for choosing a fluke-infected liver were through signs of rotting, discoloration and enlarged liver size due to calcification and obstruction of bile ducts. The liver, contained in physiological saline solution, was then transported at the Zoology and Parasitology laboratory of De La Salle University, Science and Technology Research Center. Live samples of *F. gigantica* were extracted from the hepatobiliary tracts of naturally-infected carabaos and approximately 50 flukes were collected. Liver flukes used in the study have a body length and width range of 29-49 mm by 6.71-9.70 mm. The flukes were then placed in sterile glass bottles containing physiological saline[9,10] and measured prior to exposure to heavy metal.

2.2. In vitro exposure of Fasciola spp. to heavy metal

Lead nitrate was used as the source of lead for the viability assay. Sterile tissue culture media (Medium 199) was prepared in tissue culture[11-13]. The heavy metal was diluted to the culture media to achieve the desired concentrations: 100, 150 and 200 mg/L. 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 ℃[13,14] and were monitored every three hours until flukes started losing their viability.

2.3. Determination of lethal concentration 50 (LC_{50})

After exposure to heavy metal using 100, 150 and 200 mg/L, LC_{50} was determined by assessing the motility of the flukes using the following criteria[11,12] (Table 1):

Table 1
Criteria for assessing the motility of the flukes exposure to lead.

Motility score	Criteria
3	Movement of the whole body
2	Movement of only some parts of the body
1	Immobile but not dead (Unstained with 1% methylene blue)
0	Immobile and dead (Stained with 1% methylene blue)

The effect of the heavy metals on the viability of the parasite was evaluated by calculating the relative motility (RM) value:

$$\text{RM value} = \frac{\text{MI test}}{\text{MI control}} \times 100$$

$$\text{MI} = \frac{\sum nN_n}{N_n}$$

wherein MI = Motility index; n = score ; Nn = number of flukes with the score n.

A smaller RM value indicates higher mortality rate. Then finally, the lethal concentration that was able to kill half of the population was determined by linear regression analysis.

3. Results

3.1. In vitro exposure of F. gigantica to heavy metal

At 100 mg/L, the relative motility was 0.75 which means 25% of the flukes were dead while at 150 mg/L, the relative motility value was 0.61 which means 39% of the flukes were dead. And finally at 200 mg/L, the relative motility value was 0.30 which means 70% of the flukes were dead (Tables 2 and 3).

Table 2
Motility index and relative motility values of *F. gigantica* after 15 h exposure to lead.

Concentration (mg/L)	Replicates		Average RM
	MI	RM	
0 (control)	3.00	1.00	1.00±0.00
	3.00	1.00	
	3.00	1.00	
100	2.25	0.75	0.75±0.00
	2.25	0.75	
	2.25	0.75	
150	2.50	0.83	0.61±0.25
	1.00	0.33	
	2.00	0.66	
200	0.75	0.25	0.30±0.05
	1.00	0.33	
	1.00	0.33	

Table 3
Percentage of motility of flukes exposure to different concentrations of lead.

Concentration (mg/L)	Motility scores (%)			
	3	2	1	0
0 (control)	100	0	0	0
100	58	17	17	8
150	17	50	33	0
200	0	42	8	50

3.2. Determination of LC_{50}

Using linear regression analysis in determining the LC_{50} (Figure 1), results from the viability and motility assay showed that 160 mg/L was the lethal concentration that was able to kill half of the population of flukes used in the experiment.

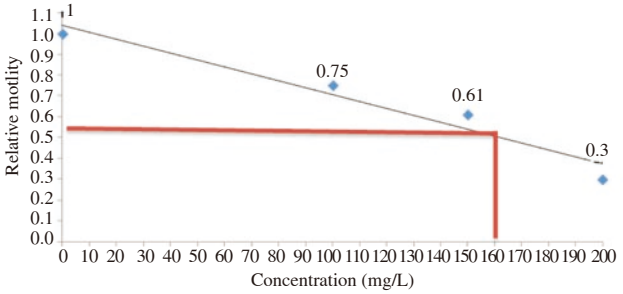

Figure 1. Linear regression analysis for LC_{50} determination using 0, 100, 150 and 200 mg/L of lead concentration.

3.3. Confirmatory run using the LC_{50} value

Another assay was conducted to confirm the tolerance of *F. gigantica* to high levels of lead using the obtained LC_{50} value from the previous assay. Likewise, similar morphological and physiological results were observed until 15 h exposure (Figure 2).

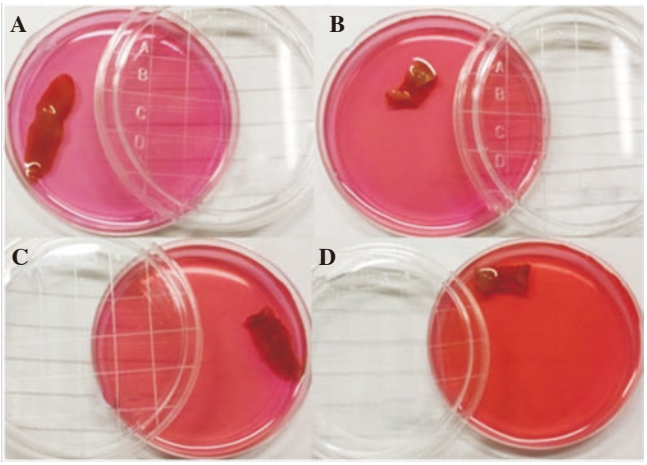

Figure 2. *F. gigantica* in Medium199 after 3 h of exposure to lead showing distinct signs of curling.
A: 0 mg/L; B: 110 mg/L; C: 160 mg/L; D: 210 mg/L.

3.4. Motility state of F. gigantica exposure to heavy metal

No changes in the flukes observed at 0 h of the assay. The flukes were motile both in the control and experimental groups. After 3 h incubation, no significant changes were also observed and all flukes remained motile. After 6 h incubation, the control group remained motile while reduced motility and curling were observed in the flukes from the experimental group. However, despite the slow movements and curling observed from the flukes exposed to lead all flukes were still viable. The presence of black precipitates in the medium of lead-treated flukes which could be waste metabolic products due to stress response was likewise observed. After 9 h incubation, flukes in the control group were still motile. However, in the lead-treated groups, flukes seemed immobile but were still moving when tapped with a forceps. Thus, all flukes were still viable at 9th hour. Curling of flukes and black precipitates were still observed in the experimental group which were not present in the control group. Same observations were noted after 12 h incubation. After 15 h incubation, flukes in the control group remained motile while flukes exposed to different concentrations of lead showed signs of immobility. Therefore, immobile flukes were stained with 1% methylene blue to determine the motility score of each fluke. No significant visual differences observed between flukes with a motility score of 1 and 0.

4. Discussion

Results obtained in this study suggest that liver flukes probably have a capacity to bioaccumulate lead within their bodies to a threshold of 200 mg/L. Noted morphological observation was that flukes tend to curl when exposed to increasing concentrations of lead while excretion of waste metabolic products was observed as possibly the flukes' physiological response to lead exposure. Furthermore, LC_{50} of lead that could kill at least half of *F. gigantica* population *in vitro* was 160 mg/L. In conclusion, tolerance of liver flukes to high levels of lead suggests its potential as a possible biomarker of lead pollution in the environment. However, further studies are necessary to determine the exact amount of heavy metal that the fluke can accumulate within its body. Furthermore, morphological, ultrastructural and physiological studies are recommended to evaluate its potential as bioindicator of heavy metal pollution in the environment.

Conflict of interest statement

We declare that we have no conflict of interest.

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