

Original Research Paper

Differential Activities of Glutathione S-Transferase Isoenzymes in Strains of *Fasciola Hepatica* Susceptible and Resistant to Triclabendazole

¹V. Fernández, ²P. Ortiz, ^{1,3}M.V. Solana and ^{1,4}H. Solana

¹Laboratorio de Biología Celular y Molecular. Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET, FCV-UNCPBA, Tandil, Argentina

²Facultad de Ciencias Veterinarias, Universidad Nacional de Cajamarca, Cajamarca, Peru

³ANPCyT, Argentina

⁴CICBA, Argentina

Article history

Received: 10-08-2014

Revised: 31-10-2014

Accepted: 27-11-2014

Corresponding Author:

V. Fernández

Laboratorio de Biología Celular y Molecular. Centro de Investigación Veterinaria de Tandil, Argentina

Email: vanesaf@vet.unicen.edu.ar

Abstract: Fasciolosis, a parasitic zoonosis of intrahepatic location, is caused by the trematode *Fasciola hepatica*. Its control is mainly based on the use of the anthelmintic Triclabendazole (TCBZ). The indiscriminate use of this drug has favored the development of anthelmintic resistance. The Glutathione S-Transferases (GSTs) are multifunctional enzymes involved in the detoxification of xenobiotics and endogenous compounds using conjugation with endogenous glutathione. Recently, it has been shown an active participation of this family of enzymes in the detoxification of TCBZ related to the phenomenon of resistance. In *F. hepatica*, eight isoenzymes of the GST are present. Since it is well known that different isoenzymes do not necessarily have the same metabolic activity, this study evaluated the cytosolic activity of mu and pi GST isoenzymes in TCBZ resistant (*Sligo* and *Oberon* strains) and TCBZ susceptible (*Cullompton* strains) of *F. hepatica*. The results obtained in this study confirm that, although both isoenzymes are involved in different processes of detoxification in *F. hepatica*, only the GSTmu isoenzyme is involved in the manifestation of resistance to TCBZ.

Keywords: *Fasciola Hepatica*, Triclabendazole Resistance, Glutathione S-Transferases Isoenzymes

Introduction

Fasciolosis is a parasitic zoonotic disease caused by the trematode *Fasciola hepatica*, a parasite of ruminants reared in temperate regions of the world. This parasitic disease is of great economic importance because annual losses due to this infection are estimated at more than \$ 3 billion annually (Boray, 1994). In addition, fasciolosis has become an emerging zoonosis in many countries (Mas-Coma *et al.*, 2009a) estimating that 17 million people are currently infected and 180 million are at risk of infection (WHO, 2007). In Europe, in recent years an increase in cases of cattle fasciolosis have been reported due to meteorological changes that determine a different distribution of the snail *Galba truncatula*, that it is the intermediate host required (Mas-Coma *et al.*, 2009b).

The control of fasciolosis is mainly based on the use of the anthelmintic Triclabendazole (TCBZ), a halogenated benzimidazole which shows excellent

efficacy against the juvenile and adult stages. On the other hand, parasites activate the induction of certain metabolic enzymes that deactivate Xenobiotics (XME) like anthelmintics, thus facilitating the survival of the exposed helminth (Brennan *et al.*, 2007). This metabolic response to anthelmintics can initiate the phenomenon of resistance including resistance of *F. hepatica* to TCBZ. This ability to deactivate anthelmintics through biotransformation processes represents an advantageous defense strategy of parasites (Robinson *et al.*, 2004). In parasitic helminths the Glutathione S-Transferases (GSTs) are the main phase II detoxification system. In *F. hepatica*, GST enzyme represents 4% of the total soluble protein distributed in various tissues with important physiological functions (Chemale *et al.*, 2006).

The increase in the activity of enzymes such as Flavin monooxygenase (XME phase I) (Alvarez *et al.*, 2005) and GST (XME phase II) (Scarcella *et al.*, 2012)

in TCBZ resistant trematodes (*Sligo* strain) provides an interesting contribution to the understanding of the phenomenon of resistance that these worms possess. This overexpression confirms that more than one metabolic pathway may be involved in the phenomenon of resistance against TCBZ (Scarcella *et al.*, 2012). In *F. hepatica*, eight GST isoforms have been reported (Wijffels *et al.*, 1992), some associated to cell membranes (mGST) that structurally and functionally is distinct from the cytosolic homotrimeric GST (cGST) (Mannervik and Widersten, 1995). Therefore the mGST differs from the cGSTs (Morgenstern *et al.*, 1982) in the fact that can activate by-sulphydic reagents, such as N-ethylmaleimide. This property is important to establish that it is not contaminated with cGST (Andersson *et al.*, 1994). Most of the "in vitro" or "ex vivo" studies on the metabolic response of *F. hepatica* against TCBZ have shown a significant decrease in the activity of superoxide dismutase and an increase in the level of GST in both young and adult flukes after incubation with TCBZ sulphoxide (Shehab *et al.*, 2009). Therefore, the interactions with these enzymatic systems can drastically affect the kinetic disposition of these drugs.

The present work evaluated the activity "in vitro" of total cGST and cGSTmu and cGSTpi isoenzymes in the susceptible (*Cullompton*) and resistant (*Sligo* and *Oberon*) strains of *F. hepatica*.

Material and Methods

Fifteen (15) weaned Corriedale lambs free of parasites were infected orally with 200 metacercariae of *F. hepatica* contained in a gelatin capsule. Five animals were infected with a *F. hepatica* susceptible to TCBZ (*Cullompton* strain) and five with the resistant strains respectively (*Sligo* and *Oberon* strains). For more details on the history of the three isolates see corresponding work (Robinson *et al.*, 2004). Intrahepatic infection was confirmed 16 weeks later by the presence of eggs in feces and the indirect estimation of liver damage was determined by measuring the levels of the serum enzymes GLDH and GT (Fig. 3).

The animals were sacrificed by procedures and management protocols approved by the Ethics Committee in accordance with the policy of Animal Welfare (law 087/02) of the Faculty of Veterinary Medicine of the National University of the Center of the Province of Buenos Aires (UNCPBA), Tandil, Argentina (<http://www.vet.unicen.edu.ar>) (AVMA, 2001). The adult flukes were collected of the bile ducts and liver and subsequently processed to obtain the Cytosolic fractions (Cyt). The test conditions were as described elsewhere (Solana *et al.*, 2009).

Fasciola hepatica (10 g) were washed with cold KCl (1.15%) and then transported to the laboratory covered with Phosphate Buffer Saline (PBS) (0.1 M, pH 7.4) at 4°C. All subsequent operations were between 0 and 4°C.

Each specimen was cut into small pieces and washed several times with PBS. The samples were homogenized (1:1) in PBS with a Homogenizer Ultra-Turrax (IKA Works Inc., Wilmington, USA), centrifuged at 10,000 g for 20 m and the resulting supernatant centrifuged at 100,000 g for 60 min. The supernatants of ultracentrifugation (Cyt) were collected and then stored at -80°C until subsequent analysis. The protein content of the Cyt fractions was determined using bovine serum albumin as standard (Lowry *et al.*, 1951). The total Cytosolic GST (cGST) activity was determined using 1-chloro, 2, 4-Dinitrobenzene (CDNB) as substrate. Ethacrynic acid was used as substrate to measure the GST-Pi activity and 1,4-Dichlorobenzene (DCNB) to measure GST-Mu activity. All these procedures were supervised by the continuous Spectrophotometric method (Habig *et al.*, 1974).

Data on the activity of total cGST, GST-Mu and GST-Pi in the Cytosolic protein fraction (n = 13) of the trematodes were compared statistically using an Analysis of Variance (ANOVA). Statistical comparisons were performed using a two way ANOVA using the Bonferroni analysis by the software GraphPad INSTANT 3.00 (Graph Pad Software, Inc.). Differences with $p \leq 0,05$ were considered significant.

Results and Discussion

The cytosolic fractions of *F. hepatica* resistant to TCBZ (*Sligo* and *Oberon* strains) expressed greater metabolic activity of cGST with respect to the susceptible strain (*Cullompton*) (Table 1). The total activity of GST (n = 13) in *Sligo* and *Oberon* resistant strains was 59 and 52% respectively higher ($p < 0.001$) than in the susceptible *Cullompton* strain.

When were analyzed the different isozymes are found different enzymatic activities. While the GST-Pi activity (n=13) did not differ between the different *F. hepatica* strains tested (Fig. 1), the GST-Mu activity had significant differences (Fig. 2).

The GST-Mu activity (n = 13) in the resistant *Oberon* strain was 1.37 nmol/min/mg of protein and in the *Sligo* strain was 1.28 nmol/min/mg of protein compared to the susceptible *Cullompton* strain (0.7 nmol/min/mg of protein) (Fig. 2).

This activity was 71 and 86% higher in the resistant strains compared to the susceptible strain (Table 2). Confirming that this isoenzyme is actively involved in the mechanisms of TCBZ detoxification.

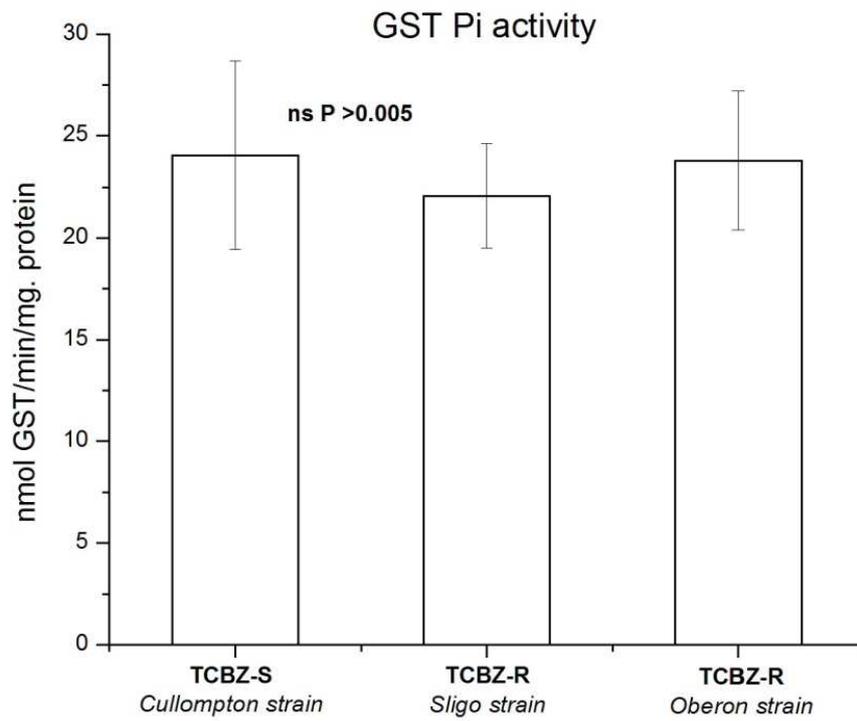


Fig. 1. Activity of the isoenzyme *pi* of Glutathion-S-Transferase (GST-Pi) in *Fasciola hepatica* Susceptible (TCBZ-S) and resistant (TCBZ-R) to triclabendazole

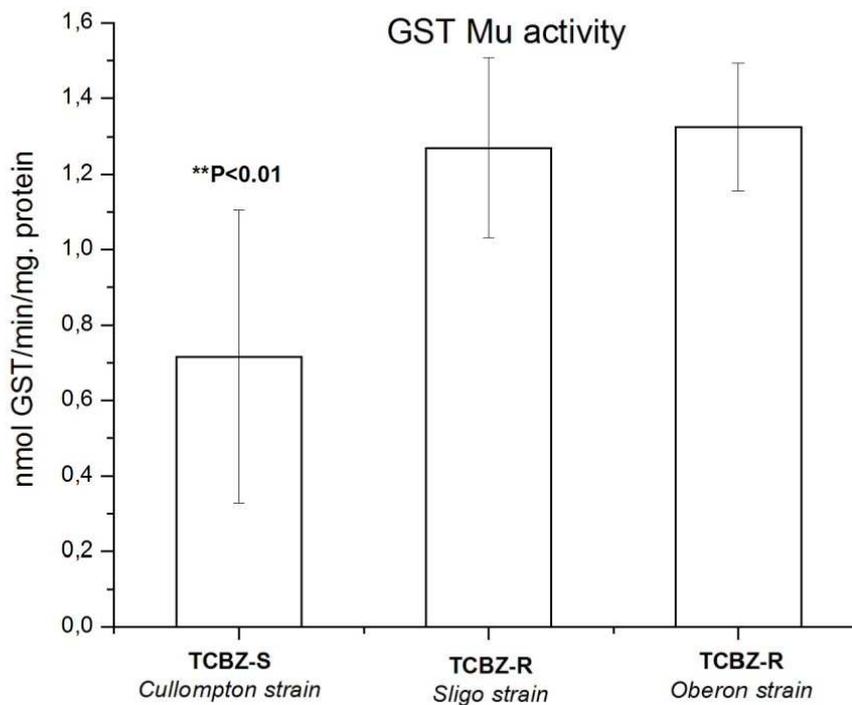


Fig. 2. Activity of the isoenzyme *mu* of Glutathion S-Transferase (GST-Mu) in *Fasciola hepatica* Susceptible (TCBZ-S) and resistant (TCBZ-R) to triclabendazole

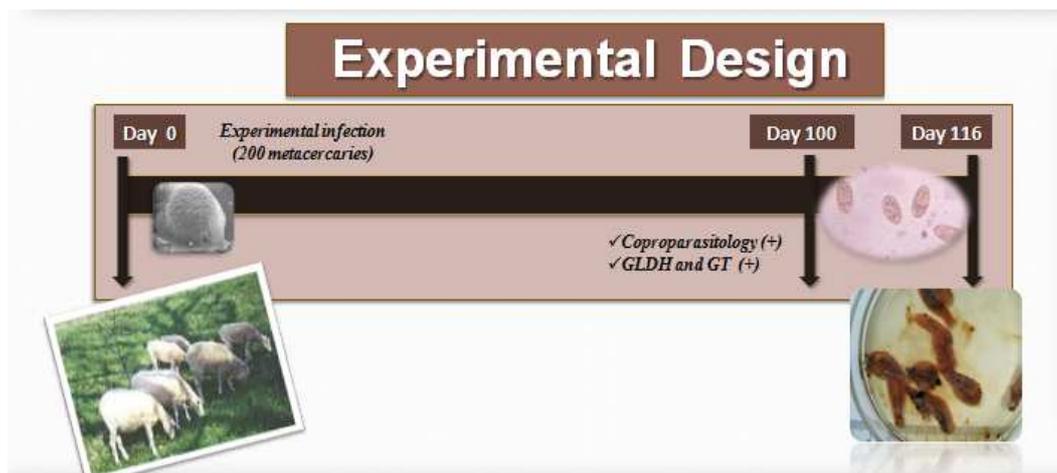


Fig. 3. Experimental Design: At day 0, each lambs free of parasites were infected orally with 200 metacercariae of *F. hepatica* contained in a gelatin capsule. The infection was confirmed 14 weeks later (Day 100 pt.) by the presence of eggs in feces and the indirect estimation of liver damage was determined by measuring the levels of the serum enzymes GLDH and GT. The adult flukes were collected of the bile ducts and liver (Day 116 pt.)

Table 1. Activity of total cytosolic Glutathione S-Transferase (cGST) in strains of *Fasciola hepatica* susceptible (TCBZ-S) and resistant (TCBZ-R) to triclabendazole

	<i>Cullompton</i>	<i>Sligo</i>	<i>Oberon</i>
cGST Activity (nmol /min/mg protein)	800±60	1277±32	1216±16
% Activity	100	159	152

Table 2. Differential activity of Glutathione S-Transferase isoenzymes in strains of *Fasciola hepatica* susceptible and resistant to triclabendazole

Strain	Activity	%	Activity	%
<i>Cullompton</i>	24,2±0,4	100	0,7±0,3	100
<i>Sligo</i>	22,8±0,5	92	1,28±0,2	171
<i>Oberon</i>	23,6±0,3	96	1,37±0,1	186

Conclusion

The increased activity of the total cGST (XEM Phase 2) in both strains TCBZ-R (*Sligo* and *Oberon*) observed in this study provides an understanding of the phenomenon of resistance and adds information to the knowledge of the response that the parasites have exposure to different xenobiotic. While the GST-Pi activity (substrate: Ethacrynic acid) did not differ between the different strains tested confirms its non-participation in the phenomenon of resistance to TCBZ, for another hand the isoenzyme GST-Mu (substrate: DCNB) has respectively 71 and 86% higher activity in the TCBZ-R strains (*Sligo* and *Oberon*) confirming that this isoenzyme is actively involved in the expression of increased metabolic activity. These results contribute to the understanding of this pathway and too add information at the knowing over the parasites and their answer to different xenobiotics.

Acknowledgement

The metacercariae of *F. hepatica* were kindly provided by Professor I. Fairweather, School of Biology and Biochemistry, The Queens University of Belfast, United Kingdom, Northern Ireland.

Funding Information

H. Solana is Adjunct Professional from the Comisión de Investigaciones Científicas (CIC-BA) de la Provincia de Buenos Aires, Argentina.

Research at the Laboratorio de Biología Celular y Molecular is supported by SECAT-UNCPBA and CIVETAN CONICET, both of Universidad Nacional del Centro de la Prov. de Bs. As. and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT PICT 2012/ N° 865-Préstamo BID) (all Argentina) and Contrato N°:017-2012-CONCYTEC-OAJ (CONCYTEC –Perú).

Author's Contributions

V. Fernandez and M.V. Solana: Carried out all different experiments.

P. Ortiz: Participated in the design of different experiments.

H. Solana: Participated in experimental design, overall coordination and drafting of the final work.

Ethics

The animals were sacrificed by procedures and management protocols approved by the Ethics Committee in accordance with the policy of Animal Welfare (law 087/02) of the Faculty of Veterinary

Medicine of the National University of the Center of the Province of Buenos Aires (UNCPBA), Tandil, Argentina (<http://www.vet.unicen.edu.ar>) (AVMA, 2001).

References

- Alvarez, L.I., H.D. Solana, M.L. Mottier, G.L. Virkel and I. Fairweather *et al.*, 2005. Altered drug influx/efflux and enhanced metabolic activity in triclabendazole-resistant liver flukes. *Parasitology*, 131: 501-10. PMID: 16174415
- Andersson, C., E. Mosialou, R. Weinander and R. Morgenstern, 1994. Enzymology of microsomal glutathione s-transferase. *ADV Pharmacol.*, 27: 19-35. DOI: 10.1016/S1054-3589(08)61028-5
- AVMA, 2001. Report of the AVMA panel on euthanasia. *J. AVMA*. 218: 669-696. DOI: 10.2460/javma.2001.218.669
- Boray, J.C., 1994. Disease of domestic animals caused by flukes. Rome, Food and Agricultural Organization of the United Nations.
- Brennan, G.P., I. Fairweather, A. Trudgett, E. Hoey and M.M. McConville *et al.*, 2007. Understanding triclabendazole resistance. *Experimental Molecular Pathology*, 82: 104-9. DOI: 10.1016/j.yexmp.2007.01.009
- Chemale, G., R. Morphew, J.V. Moxon, A.L. Morassuti and E.J. Lacourse *et al.*, 2006. Proteomic analysis of glutathione transferases from the liver fluke parasite, *Fasciola hepatica*. *Proteomics*, 6: 6263-6273. PMID: 17078019
- Habig, W.H., M.J. Pabst and W.B. Jakoby, 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.*, 249: 7130-7139. PMID: 4436300
- Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265-75.
- Mannervik, B. and M. Widersten, 1995. Human Glutathione Transferases: Classification, Distribution, Structure, Functional Properties and Tissue. In: *Advances in Drug Metabolism in Man*, Pacifici, G.M. and G.N. Fracchia (Eds.), Commission of the European Communities, Luxembourg.
- Mas-Coma, S., M.A. Valero and M.D. Bargues, 2009a. Chapter 2 fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Adv. Parasitol.*, 69: 41-146. DOI: 10.1016/S0065-308X(09)69002-3
- Mas-Coma, S., M.A. Valero and M.D. Bargues, 2009b. Climate change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiasis. *Vet. Parasitol.*, 163: 264-280. DOI: 10.1016/j.vetpar.2009.03.024
- Morgenstern, R., C. Guthenberg and J.W. Depierre, 1982. Microsomal glutathione S-transferase: Purification, initial characterization and demonstration that it is not identical to the cytosolic glutathione S-transferases A, B and C. *Eur. J. Biochem.*, 128: 243-248. DOI: 10.1111/j.1432-1033.1982.tb06958.x
- Robinson, M.W., J. Lawson, A. Trudgett, E.M. Hoey and I. Fairweather, 2004. The comparative metabolism of triclabendazole sulphoxide by triclabendazole-susceptible and triclabendazole-resistant *Fasciola hepatica*. *Parasitol Res.*, 92: 205-210. DOI: 10.1007/s00436-003-1003-6
- Scarcella, S., P. Lamenza, G. Virkel and H. Solana, 2012. Expression differential of microsomal and cytosolic glutathione-S-transferases in *Fasciola hepatica* resistant at triclabendazole. *Mol. Biochem. Parasitology*, 181: 37-39. DOI: 10.1016/j.molbiopara.2011.09.011
- Shehab, A., S.M. Ebeid, M.Y. El-Samak and M.N. Hussein, 2009. Detoxifying and anti-oxidant enzymes of *Fasciola gigantica* worms under Triclabendazole Sulphoxide (TCBZ-SX): An *in vitro* study. *J. Egypt Soc. Parasitol.*, 39: 73-83. PMID: 19530611
- Solana, H., S. Scarcella, G. Virkel, C. Ceriani and J. Rodríguez *et al.*, 2009. Albendazole enantiomeric metabolism and binding to cytosolic proteins in the liver fluke *Fasciola hepatica*. *Vet. Res. Commun.*, 33: 163-173. DOI: 10.1007/s11259-008-9166-3
- WHO, 2007. Report of the WHO Informal Meeting on Use of Triclabendazole in Fascioliasis Control. WHO Headquarters, Geneva, Switzerland.
- Wijffels, G.L., J.L. Sexton, L. Salvatore, J.M. Pettitt and D.C. Humphris *et al.*, 1992. Primary sequence heterogeneity and tissue expression of glutathione S-transferases of *Fasciola hepatica*. *Exp. Parasitol.*, 74: 87-99. DOI: 10.1016/0014-4894(92)90142-W