Experimental Parasitology 159 (2015) 274–279

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research brief

A single amino acid substitution in isozyme GST mu in Triclabendazole resistant *Fasciola hepatica* (*Sligo* strain) can substantially influence the manifestation of anthelmintic resistance

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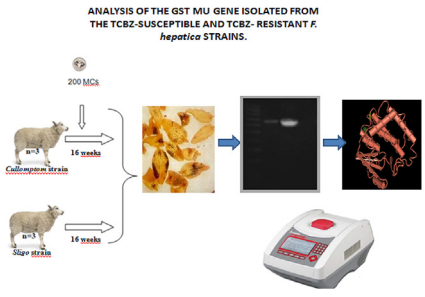
HIGHLIGHTS

- Caracterization the GST mu gene isolated from the TCBZ susceptible and TCBZ resistant *Fasciola hepatica* strains.
- The comparative analysis of the genes of both, TCBZ-susceptible and TCBZ-resistant strain showed three nucleotide changes.
- In the GST protein: the amino acid Threonine in the TCBZ-susceptible strain is replaced by Serine in the TCBZ-resistant.
- The mutation is in the domain C terminal alpha helical GST protein, and in the area of the active.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 May 2015
Received in revised form
25 August 2015
Accepted 29 October 2015
Available online 2 November 2015

Keywords:
Fasciola hepatica
Triclabendazole
Isozyme
Glutathione S-Transferases mu

ABSTRACT

The helminth parasite *Fasciola hepatica* causes fascioliasis in human and domestic ruminants. Economic losses due to this infection are estimated in U$S 2000–3000 million yearly. The most common method of control is the use of anthelmintic drugs. However, there is an increased concern about the growing appearance of *F. hepatica* resistance to Triclabendazole (TCBZ), an anthelmintic with activity over adult and young flukes.

F. hepatica has eight Glutathione S-Transferase (GST) isozymes, which are enzymes involved in the detoxification of a wide range of substrates through chemical conjugation with glutathione. In the present work we identified and characterized the GST mu gene isolated from the TCBZ-susceptible and TCBZ-resistant *F. hepatica* strains. Total RNA was transcribed into cDNA by reverse transcription and a 657 bp amplicon corresponding to the GST mu gene was obtained. The comparative genetic analysis of the GST mu gene of the TCBZ susceptible strain (*Cullompton*) and TCBZ resistant strain (*Sligo*) showed three nucleotide changes and one amino acid change at position 143 in the GST mu isozyme of the TCBZ-resistant strain.

Abbreviations: TCBZ, Triclabendazole; GST, Glutathione S-Transferase; BZDs, benzimidazoles anthelmintics; XMEs, xenobiotic metabolizing enzymes.

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http://dx.doi.org/10.1016/j.exppara.2015.10.007

These results have potential relevance as they contribute better understand the mechanisms that generate resistance to anthelmintics.

1. Introduction

Fasciola hepatica is a helminth parasite that causes fascioliasis in domestic ruminants and humans. Economic losses due to its infection are estimated in U$S 2000–3000 million yearly (Boray, 1994). Anthelmintics are currently the most used method of control of parasitic helminths (Mas-Coma et al., 2009a). In fascioliasis, anthelmintic control is based mainly on the use of Triclabendazole (TCBZ), a halogenated benzimidazole thiol derivative that shows excellent efficacy against both juvenile (immature) and adult stages of *F. hepatica* (Boray et al., 1983).

Helminth parasites possess different biochemical mechanisms for detoxification. Overall, parasites may evade drug antiparasitic effects by: i) mutation of target receptors, ii) overexpression of efflux transport pumps and/or iii) overexpression of metabolic enzymatic systems (Alvarez et al., 2005). Parasite resistance to different anthelmintics, including that of *F. hepatica* to TCBZ, is growing worldwide (Mas-Coma et al., 2009a). In the last few years, an increase in cattle fascioliasis has been reported, probably due to weather changes and there are other factors, like man-made modifications of the environment as the use of artificial irrigation to improve the quality and quantity of fodder to animals and floods that also have contributed to increase the prevalence and determining a different distribution of the snail *Galba truncatula*, which is one of the intermediate hosts (Bargues et al., 2012; Mas-Coma et al., 2009b).

The action of the benzimidazoles anthelmintics (BZDs) is based on the binding to beta-Tubulin of the parasite, which produces subsequent disruption of the tubulin-microtubule dynamic equilibrium and the consequent death of helminth (Lacey, 1988). Evaluation of the effect of TCBZ on the distribution of alpha and beta-Tubulin "*in vivo*" in the testis tubules of *F. hepatica* obtained from bovines exposed to this drug confirmed that TCBZ alters the distribution of the microtubules and that this is one of its main mechanisms of action (Solana et al., 2009; Scarcella et al., 2011).

TCBZ is metabolized into its anthelmintically active metabolite sulphoxide by the host liver (Scarcella et al., 2011) but also by the parasite's subcellular fractions (Virkel et al., 2006). It has also been reported that *F. hepatica* has significantly higher sulphoxidation activity than nematode and cestode parasites (Mottier et al., 2004). The resistance to BZDs detected in other helminths such as *Haemonchus contortus* has been associated with the loss of high-affinity binding and alteration of the beta-Tubulin isoform pattern, correlated with several mutations, the most prominent of which is a conserved mutation at amino acid 200 (phenylalanine to tyrosine) in beta-Tubulin isotype 1 (Robinson et al., 2002). Although the activity of TCBZ remains to be fully understood, data support an action of this compound over the microtubule. However, it has been shown that TCBZ resistance in *F. hepatica* is not associated with residue changes in the primary amino acid sequence of beta Tubulin (Robinson et al., 2002). This suggests that there may be an alternative mechanism of TCBZ resistance in *F. hepatica* (Alvarez et al., 2005). The development of drug resistance can be facilitated by the action of xenobiotic metabolizing enzymes (XMEs) of phase I and phase II of detoxification (Cvilink et al., 2009). In all organisms, XMEs serve as an efficient defense against the potential negative action. The increased *"in vitro"* activity of Flavin monooxygenase (a phase I XME) (Alvarez et al., 2005) and Glutathione S-Transferase (GST) (a phase II XME) (Scarcella et al., 2012) in TCBZ-resistant flukes (*Sligo* strain) provides an understanding of the phenomenon of resistance. This overexpression of different enzymes is a multienzymatic response involving more than one metabolic pathway (Scarcella et al., 2012). The actions of GSTs in mammals involve conjugating potentially toxic substances to glutathione (Farahnak and Barrett, 2001). These proteins appear to be the main phase II detoxification system present in parasitic worms. General biological roles of helminth GSTs include xenobiotic detoxification and ligand binding/transport functions (Wijffels et al., 1992). In *F. hepatica*, GSTs are found in the tegument, muscular tissues, parenchymal cells, and the intestine (Panaccio et al., 1992). GSTs account for as much as 4% of the total soluble protein (Brophy et al., 1990) and are major detoxification enzymes in adult helminths (Brophy et al., 1990). This property has been exploited with cytosolic GSTs (cGST) (Mannervik and Widersten, 1995; Morgenstern et al., 1982; Andersson et al., 1994). Eight classes of GST isozymes have been shown to be present in *F. hepatica* (Creaney et al., 1995). Results in our laboratory have shown that total cGST activity is different in all strains tested. In the TCBZ-resistant (*Sligo strain)* (1277 ± 32 nmol/min/mg protein) and TCBZ-resistant (*Oberon strain)* (1216 ± 16 nmol/min/mg protein), total cGST activity are 59% and 52% respectively higher ($P < 0.001$) than that in the TCBZ-susceptible (*Cullompton* strain) (800 ± 60 nmol/min/mg protein). Regarding the isozymes, cGST mu activity in the *Oberon* (1.37 nmol/min/mg protein) and *Sligo* (1.28 nmol/min/mg protein) strains are 17% and 26% higher than *Cullompton* strain, respectively (0.8 nmol/min/mg protein). In contrast, cGST pi activity does not differ between the different strains tested (Fernández et al., 2014). By observing the degree of involvement of the isoenzyme, in the present work, we identified and characterized the GST mu gene isolated from the TCBZ-susceptible and TCBZ-resistant *F. hepatica* strains.

2. Materials and methods

2.1. Collection of parasite material

Six parasite-free Corriedale weaned lambs were orally infected each with 200 metacercariae of *F. hepatica* contained in a gelatin capsule and kindly provided by Professor I. Fairweather, School of Biology and Biochemistry, Queen's University Belfast, Northern Ireland, UK. Three animals were infected with the TCBZ-susceptible strain *Cullompton*, and three with the TCBZ-resistant strain *Sligo*. For details of the history of the two strains, see Scarcella et al. (2012). Sixteen weeks later, the infection was confirmed by the presence of eggs in feces and liver damage was indirectly estimated by the determination of serum glutamatedehydrogenase (GLDH) and gamma glutamyl transferase (γGT) activities.

Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar), and to internationally accepted animal welfare guidelines (AVMA, 2001).

2.2. Collection and processing of adult flukes

Adult flukes were collected from bile ducts and liver, and were stored in RNAlater®, (Applied Biosystems, Brunn am Gebirge, Austria) until extraction.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from each strain of adult trematodes (n = 50) using Trizol®, one *F. hepatica* in 1 ml of Trizol®, (Applied Biosystems, Brunn am Gebirge, Austria) following the protocol recommended by the manufacturers. The reverse transcription was performed using superscript III RNAase® (Applied Biosystems, Brunn am Gebirge, Austria). The synthesized cDNA was preserved at −20 °C until further use.

2.4. PCR amplification of cDNA

The cDNA from each strain of adult flukes was used as a template for PCR amplification, using the FgGST-F (5′-ATG CCA GCC AAA CTC GGA TAC-3′) and FgGST-R (5′-TCA AGC CGG TGC AGC GTC TC-3′) primers (Jedeppa et al., 2010). Gene amplification was performed with 0.2 mM dNTPs, 1.5 mM MgCl2. Taq DNA polymerase buffer (1 ml), 2.5 units of Taq DNA polymerase (Promega Corporation, Madison, Wisconsin, USA) and 2 ml of template cDNA, with initial denaturation for 5 min at 94 °C, followed by annealing for 45 s at 52 °C and elongation for 1 min at 72 °C for 35 cycles. The oligonucleotides used to conserve regions of the GST mu gene, which amplifies a 657 bp fragment. The oligonucleotides were thermodynamically analyzed with the help of the Oligo Tech DNAstar software. Total Amplicons (n = 50) were cloned using the TOPO® TA Cloning kit for sequencing (Invitrogen, Vienna, Austria) and recombinant DNA from single clones was prepared (PeqGold Plasmid Miniprep Kit II; PeqLab, Erlangen, Germany) and sequenced (Macrogen, Korea).

2.5. Data analysis

Sequence analyses were either performed using the Basic Local Alignment Search Tool (BLAST) or Clustal 2.1. The information obtained from the peptide sequencing was carried out with the Expansy-Translate tool and compared with sequences in GenBank, using BLASTp. From the amino acid sequence was analyzed domain search, families and functional sites through the tool Prosite. GST mu sequence was used to search 3D structure of the protein NCBI (National Center for Biotechnology Information) is determined by the database with the programme Cn3D.

3. Results

RT-PCR resulted in an amplification product of 657 bp corresponding to the GST mu isozyme (Fig. 1). The genetic sequence database at the National Center for Biotechnical Information (NCBI) (GenBank ID: KF680281-KF680282) corresponding to the GST mu gene isolated from the *Cullompton* strain (TCBZ-susceptible) and (GenBank ID: KF680283-KF680284). Using BLASTn, the comparative analysis of the GST mu gene isolated from the *Sligo* strain (TCBZ-resistant) in *F. hepatica,* yielded values of 91–100% identity with other *F. hepatica* sequences previously identified by GST mu. Using Clustal 2.1, the comparative analysis of the genes of both, *Cullompton* (TCBZ-susceptible) and *Sligo* (TCBZ-resistant) strain showed three nucleotide changes (Fig. 2). The Expansy-Translate tool allowed us to detect a change of one amino acid at position 143 in the GST protein: the amino acid Threonine in the TCBZ-susceptible strain is replaced by Serine in the TCBZ-resistant strain (Fig. 3). Using Prosite allowed us to detect that the mutation is in the C terminal alpha helical domain GST protein, and in the area of the active site (Fig. 4). The structure 3D of the protein demonstrated that the mutation its located in the outside of the protein, can bind ligands and thus modify its activity (Fig. 5).

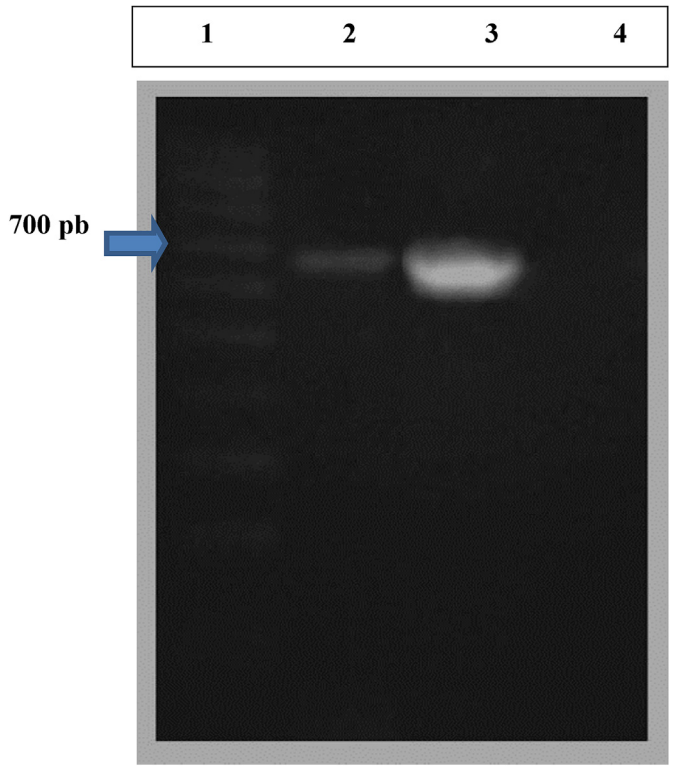

Fig. 1. RT-PCR amplification of GST mu gene of *F. hepatica* susceptible (*Cullompton*) and resistant (*Sligo*) to TCBZ. DNA ladder marker (lane 1), TCBZ-S, *Cullompton* strain (lane 2), TCBZ-R, *Sligo* strain (lane 3) and negative control (lane 4).

4. Discussion

Due to the lack of an efficacious vaccine, chemotherapy remains the main tool in the control of fascioliasis. Although other alternatives exist, current measures to control fascioliasis are based on the use of drugs such as Triclabendazole (TCBZ) (Fairweather, 2009; Brennan et al., 2007). Metabolic deactivation has been reported in the larvae and adults of helminth parasites (Fairweather, 2009; Virkel et al., 2006). It is thought that the repeated contact with an anthelmintic may make parasites defend against the chemical stress by the induction of biotransformation enzymes or transporters.

Fasciolicides interact with a number of different systems within the fluke and exert a variety of effects. So, it is difficult to determine whether flukicidal action is due to a single effect or a combination of effects (Gusson et al., 2006). Parasite defense mechanisms include detoxifying and anti-oxidant enzymes that would suppress its oxidative killing (Betts and Russell., 2003). In *F. hepatica,* there is a lack of knowledge about the mechanisms of detoxification and anthelmintic resistance.

The XMEs of helminth parasites may protect these organisms from the toxic effects of anthelmintics. It is well recognized that species-related differences in XME expression may affect not only the persistence of drugs and poisons in the body but also their susceptibility (Gusson et al., 2006). Other possibilities include enhanced substrate affinity of the enzymes through mutations

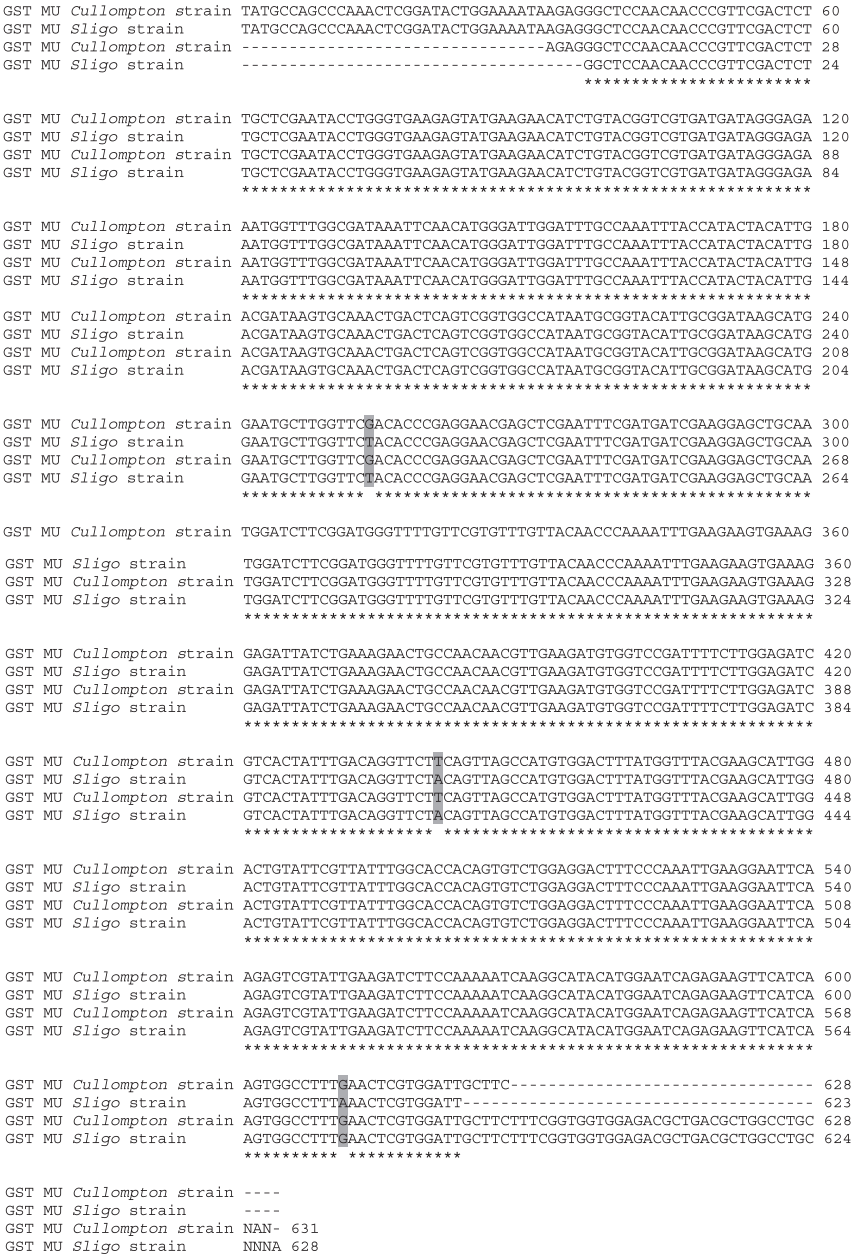

Fig. 2. Alignment of GST mu gene of *F. hepatica* susceptible (*Cullompton*) and resistant (*Sligo*) to TCBZ. Light shading indicates replacement of three nucleotides in the TCBZ-resistant strain. For GenBank Accession numbers of cDNA sequences see text.

within their specific genes.

In this work, we identified and characterized the GST mu gene isolated from TCBZ-susceptible and TCBZ-resistant *F. hepatica* strains. The comparative analysis of the *Cullompton* and *Sligo* strains (TCBZ-susceptible and TCBZ-resistant respectively) showed a change of three nucleotides changes and one amino acid change at position 143 in the GST mu isozyme of the TCBZ-resistant strain. It was found a replacement: Threonine in the TCBZ-susceptible strain by Serine in the TCBZ-resistant strain, that the mutation is in the C terminal alpha helical domain.

Serines are quite common in protein functional centers. The hydroxyl group is fairly reactive, being able to form hydrogen bonds with a variety of polar substrates. Perhaps the best known role for serine in protein active sites is exemplified by the classical Asp-His-Ser catalytic triad found in many hydrolases (Betts and Russell., 2003). These genetic variations can change an individual's susceptibility to carcinogens and toxins as well as affect the toxicity and efficacy of certain drugs. GST mu protein showed a change of one amino acid, this change of Threonine in the TCBZ-susceptible strain by Serine in TCBZ-resistant strain may affect the biological activity of GST mu. This property has been exploited with cytosolic GSTs (cGST) (Mannervik and Widersten, 1995; Morgenstern et al., 1982; Andersson et al., 1994). Glutathione S-transferase (GST) family, the mutation is in the C terminal alpha helical domain. C-terminal alpha helical domain; a large, diverse group of cytosolic dimeric proteins involved in cellular detoxification by catalyzing the conjugation of glutathione (GSH) with a wide range of endogenous and xenobiotic alkylating agents, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress. In addition, GSTs also show GSH peroxidase activity and are

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> GST MU Cullompton strain
LGYWKIRGLQQPVRLLLEYLGEEYEEHLYGRDDREKWFGDKFNMGLDLPNLPYYIDDKCKLTQSVAIMRYIADKHGMLGSTPEERARISMIEGAAMDLRMG
FVRVCYNPKFEEVKGDYLKELPTTLKMWSDFLGDRHYLTGSTVSHVDFMVYEALDCIRYLAPQCLEDFPKLKEFKSRIEDLPKIKAYMESEKFIKWPLNSW
IASFGGGDA

> GST MU Sligo strain
CQPKLGYWKIRGLQQPVRLLLEYLGEEYEEHLYGRDDREKWFGDKFNMGLDLPNLPYYIDDKCKLTQSVAIMRYIADKHGMLGSTPEERARISMIEGAAMD
LRMGFVRVCYNPKFEEVKGDYLKELPTTLKMWSDFLGDRHYLTGSSVSHVDFMVYEALDCIRYLAPQCLEDFPKLKEFKSRIEDLPKIKAYMESEKFIKWP
LNSWIASFGGGDAD


GST MU Cullompton strain ----LGYWKIRGLQQPVRLLLEYLGEEYEEHLYGRDDREKWFGDKFNMGLDLPNLPYYID 56
GST MU Sligo strain      CQPKLGYWKIRGLQQPVRLLLEYLGEEYEEHLYGRDDREKWFGDKFNMGLDLPNLPYYID 60
                            ************************************************************

GST MU Cullompton strain DKCKLTQSVAIMRYIADKHGMLGSTPEERARISMIEGAAMDLRMGFVRVCYNPKFEEVKG 116
GST MU Sligo strain      DKCKLTQSVAIMRYIADKHGMLGSTPEERARISMIEGAAMDLRMGFVRVCYNPKFEEVKG 120
                         ************************************************************

GST MU Cullompton strain DYLKELPTTLKMWSDFLGDRHYLTGSTVSHVDFMVYEALDCIRYLAPQCLEDFPKLKEFK 176
GST MU Sligo strain      DYLKELPTTLKMWSDFLGDRHYLTGSSVSHVDFMVYEALDCIRYLAPQCLEDFPKLKEFK 180
                         ************************* ********************************

GST MU Cullompton strain SRIEDLPKIKAYMESEKFIKWPLNSWIASFGGGDA- 211
GST MU Sligo strain      SRIEDLPKIKAYMESEKFIKWPLNSWIASFGGGDAD 216
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Fig. 3. Amino acid alignments of GST mu isoenzyme of *F. hepatica* susceptible (*Cullompton*) and resistant (*Sligo*) to TCBZ. Light shading indicates change of one amino acid at position 143 in the TCBZ-resistant strain. For GenBank Accession numbers of cDNA sequences see text.

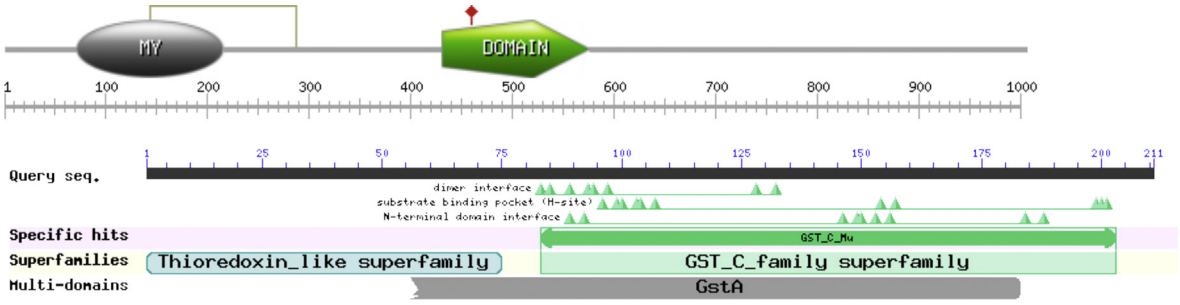

Fig. 4. Domain of GST mu isoenzyme of *F. hepatica* resistant (*Sligo*) to TCBZ. Indicates with an arrow change of one amino acid at position 143 in the region is preserved for in the in the C terminal alpha helical domain GST protein, corresponding to the GST superfamily.

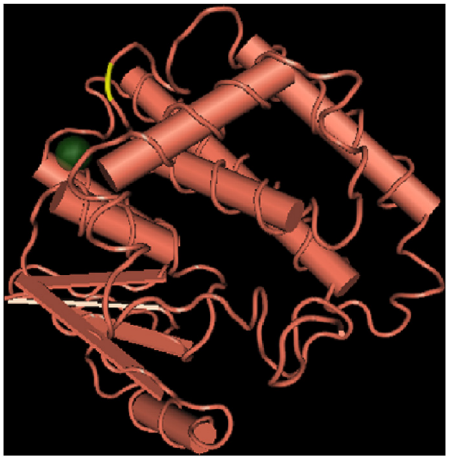

Fig. 5. Structure 3D of GST mu isoenzyme of *F. hepatica* resistant (*Sligo*) to TCBZ. Light shading indicates change of one amino acid at position 143.

involved in the synthesis of prostaglandins and leukotrienes. The GST fold contains an N-terminal thioredoxin-fold domain and a C-terminal alpha helical domain, with an active site located in a cleft between the two domains. Based on sequence similarity, different classes of GSTs have been identified, which display varying tissue distribution, substrate specificities and additional specific activities. In humans, GSTs display polymorphisms which may influence individual susceptibility to diseases such as cancer, arthritis, allergy and sclerosis.

The mutation and consequent modification of an amino acid in the GST mu in TCBZ-resistant strain observed in this study match with the overexpression of metabolic enzymatic system detected in previous studies provides an understanding of the phenomenon of resistance and adds information to the knowledge of the response that the parasites have exposure to different xenobiotics. These results contribute to better understand not only this metabolic pathway, but also the mechanism of resistance to TCBZ in *F. hepatica.*

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