Chapter 14

Identification and Differential Activity of Glutathione S-Transferase Mu in Strains of *Fasciola hepatica* Susceptible and Resistant to Triclabendazole

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Additional information is available at the end of the chapter

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Abstract

Fasciola hepatica is a helminth parasite that causes fascioliasis in domestic ruminants and humans. Economic losses due to its infection are estimated in US$ 2000–3000 million yearly [1]. The anthelmintics are at present the only weapon against these parasitic helminths [2]. The parasite resistance to different anthelmintics including that of *F. hepatica* to triclabendazole (TCBZ) is growing worldwide. Glutathione S-transferases (GSTs) are enzymes involved in the detoxification of a wide range of substrates through chemical conjugation with glutathione, so that the product becomes more soluble in water, less toxic and easier to excrete. Eight GST isoenzymes are present in *F. hepatica* [3]. Since the different isoenzymes do not necessarily have the same metabolic activity, in the present work, we evaluated the metabolic activity of total cytosolic GST and GST mu and GST pi isoenzymes in adult strains of *F. hepatica* susceptible (*Cullompton*) and resistant (*Sligo* and *Oberon*) to TCBZ of the highest metabolic activity of total GST. The genetic sequence database at the National Center for Biotechnical Information (NCBI) (GenBank ID: KF680281–KF680282) corresponding to the GST mu gene isolated from *Cullompton strain* (TCBZ-susceptible) and (GenBank ID: KF680283–KF680284) corresponding to the GST mu gene isolated from *Sligo* strain (TCBZ-resistant) in *F. hepatica*. Comparative analysis of both strains, *Cullompton* and *Sligo*, showed two nucleotide changes and change of one amino acid in the GST mu isoenzyme of the TCBZ-resistant strain. These results together with the higher enzymatic activity of GST have a potential relevance as it contribute to the understanding the mechanisms that generate resistance to anthelmintics and the activity, metabolism, and disposition of these drugs in the parasite.

Keywords: *Fasciola hepatica*, triclabendazole, isoenzymes, glutathione S-transferases

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 [1]. The anthelmintics are at present the only weapon against these parasitic helminths [2]. The parasite resistance to different anthelmintics including that of *F. hepatica* to triclabendazole (TCBZ) is growing worldwide [3]. In the last few years, a rise in cattle fasciolosis cases has been reported, probably due to weather changes determining a different distribution of the snail *Galba truncatula,* which is a required intermediate host [4]. 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 [5].

In fasciolosis, anthelmintic control is based mainly on the use of TCBZ, a halogenated benzimidazole thiol derivative that shows excellent efficacy against both juvenile (immature) and adult stages. The nematodicidal action of benzimidazoles (BZDs) is based on their binding to beta-Tubulin, which produces subsequent disruption of the tubulin-microtubule dynamic equilibrium [6]. When the *in vivo* effect of TCBZ on the distribution of alpha and beta-Tubulin was evaluated in the testis tubules of *F. hepatica* obtained from bovines exposed to this drug, the results obtained confirmed that TCBZ alters the distribution of the microtubules and reaffirmed that this is one of its main mechanisms of action [7, 8]. TCBZ is metabolized into its anthelmintically active metabolite sulphoxide (TCBZ-SO) by the host liver [9] but also by the parasite's subcellular fractions [10]. It has also been reported that *F. hepatica* has significantly higher sulphoxidation activity than nematode and cestode parasites [10]. The resistance to (BZDs) detected in other helminths such as *Haemonchus contortus* [11]. Although the flukicidal activity of TCBZ remains to be fully understood, there are data to support a microtubule-based action of this compound. However, it has been shown that the TCBZ-resistant *F. hepatica* is not associated with residue changes in the primary amino acid sequence of beta-Tubulin [11]. This suggests that there may be an alternative mechanism of TCBZ resistance in *F. hepatica* [5].

The development of drug resistance can be facilitated by the action of xenobiotic metabolizing enzymes (XMEs) of phase I and phase II of detoxification [12]. In all organisms, XMEs serve as an efficient defense against the potential negative action of xenobiotics. Several phase I enzymes are expressed in mammalians, where they introduce or unmask new functionalities on xenobiotic compounds. Examples of these enzymes include cytochrome P450s (Cyt P450), flavin-containing monooxygenases (FMO), alcohol and aldehyde dehydrogenases, and esterases. To eliminate a large array of chemicals, living organisms have developed, in virtually all tissue enzyme systems, XMEs that transform exogenous and endogenous compounds into more hydrophilic derivatives through reactions collectively known as biotransformation. At present, much less is known about the activity of certain phase II enzymes and relatively less attention has been paid to hydrolytic and conjugative pathways. Many phase II reactions in mammals involve conjugating potentially toxic substances to glutathione. These reactions are mediated by the enzyme glutathione S-transferase (GST) enzymes [13]. Recent research has highlighted the importance of these transferases in the establishment of chronic helminth

infections. 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 [14].

As many as eight GST isoforms have been shown to be present in *F. hepatica* [15]. The genes encoding the mu class of enzymes are organized in a gene cluster on 45 chromosome 1p13.3 and are known to be highly polymorphic. The ability of helminth GSTs to effectively neutralize known cytotoxic products arising from the attack of reactive oxygen species on cell membranes provides evidence that GSTs have the potential to protect the parasite against different xenobiotics. In *F. hepatica*, GSTs are found in the tegument, muscular tissues, parenchymal cells, and the intestine [16]. GSTs account for as much as 4% of the total soluble protein [17] and are major detoxification enzymes in adult helminths, as these organisms appear to lack the important Cyt P450-dependent detoxification reactions [18]. Results obtained in our laboratory confirmed that Cyt P450 are not only involved in detoxification mechanisms but also actively participate in the development of resistance to TCBZ by the trematode [19]. GSTs have been investigated in parasitic worms with respect to their biochemistry and have also been identified as potential vaccine candidates in digenean parasite. This property has been exploited with cGSTs [20–22]. Most studies concerning the metabolic response of *F. hepatica* against the anthelmintic TCBZ have used *in vitro* or *ex vivo* test models [23].

Whereas the background about the interactions with such enzymatic systems may drastically affect the disposition kinetics of different drugs [24], the aim of the present work was to evaluate *in vitro* of total cGST and cGSTmu and cGSTpi isoenzymes in the susceptible (*Cullompton*) and resistant (*Sligo* and *Oberon*) strains of *F. hepatica* and identified and characterized the gene GST mu isolated isolate from *Cullompton strain* (TCBZ-susceptible) and *Sligo* (TCBZ-resistant) in *F. hepatica*.

2. Materials and methods

2.1. Collection of parasite material

Nine (9) parasite-free Corriedale weaned lambs were orally infected each with 200 metacercariae of *F. hepatica*.

2.2. Collection and processing of adult flukes

Adult flukes were collected from bile ducts and liver and processed. The collection of the flukes, their processing to obtain the cytosolic [25].

2.3. Preparation of cytosolic fractions

Parasite specimens (10–15 g) of the TCBZ-susceptible or TCBZ-resistant isolates of *F. hepatica* were rinsed with cold KCl (1.15%) and then transported to the laboratory in flasks filled with phosphate buffer (PB) (0.1 M, pH 7.4) at 4°C. All subsequent operations were performed between 0 and 4°C. Each sample was cut into small pieces and washed several times with PB. Samples were homogenized

(1:1) in PB, pH 7.4 with an Ultra-Turrax homogenizer (IKA Works Inc., Wilmington, USA), centrifuged at 10,000 g for 20 min and the resulting supernatant centrifuged at 100,000 g for 60 min.

The supernatant was collected and stored at −80°C until the analysis. Protein content from the supernatant fractions was determined using bovine serum albumin as a standard [26].

Total cGST activity using 1-chloro, 2,4-dinitrobenzene as substrate (CDNB), GST-pi activity using ethacrynic acid as substrate and GST mu activity using 3,4-dichloronitrobenzene (DCNB) as substrate were monitored by a continuous spectrophotometric method [27].

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

Total RNA was isolated from each strain of adult trematodes ($n = 15$) using Trizol, and reverse transcribed using superscript III RNAase® (Applied Biosystems, Brunn am Gebirge, Austria) following the protocol recommended by the manufacturers [28].

2.5. PCR amplification of cDNA

The PCR product was analyzed by electrophoresis in 1% agarose gel.

3. Results

Total GST activity ($n = 13$) in each strains was different in all strains tested. In the Sligo (1277 ± 32 nmol/min/mg protein) and Oberon (1216 ± 16 nmol/min/mg protein), strains were 59 and 52%, respectively, higher ($P < 0.001$) than in the susceptible *Cullompton strain* (800 ± 60 nmol/min/mg protein) (**Figure 1**). GST mu activity in Oberon (1.37 nmol/min/mg protein)

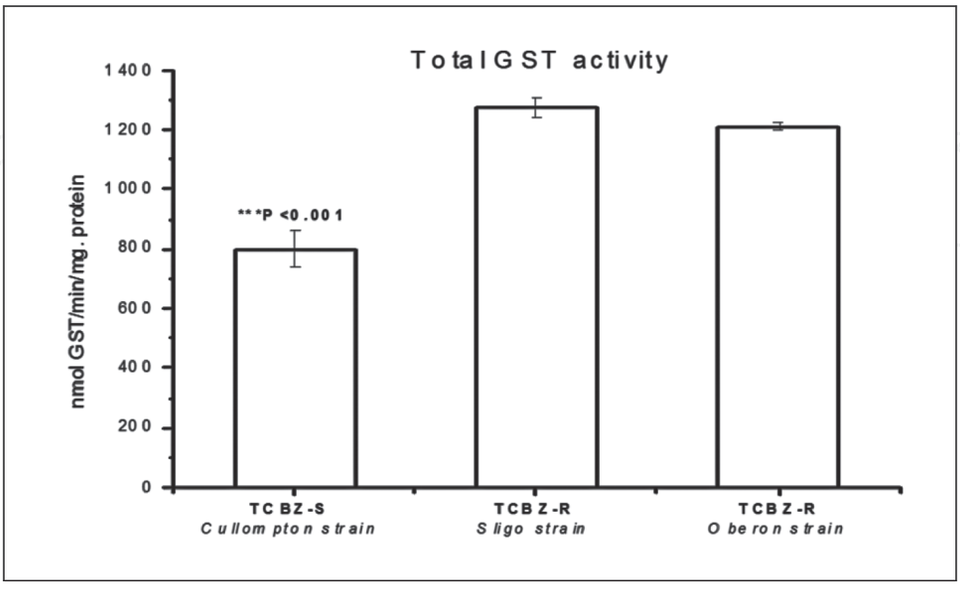

Figure 1. Quantification of total activity of glutathione S-transferase (GST) in *Fasciola hepatica* susceptible (*Cullompton strain*) and resistant (*Sligo* and *Oberon* strains) to triclabendazole.

and Sligo (1.28 nmol/min/mg protein) resistant strains was 17 and 26%, respectively, higher than in the susceptible *Cullompton strain* (0.8 nmol/min/mg protein) (**Figure 2**) while GST-pi activity did not differ between the different strains tested (**Figure 3**). RT-PCR (**Figure 4**). 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) to the GST

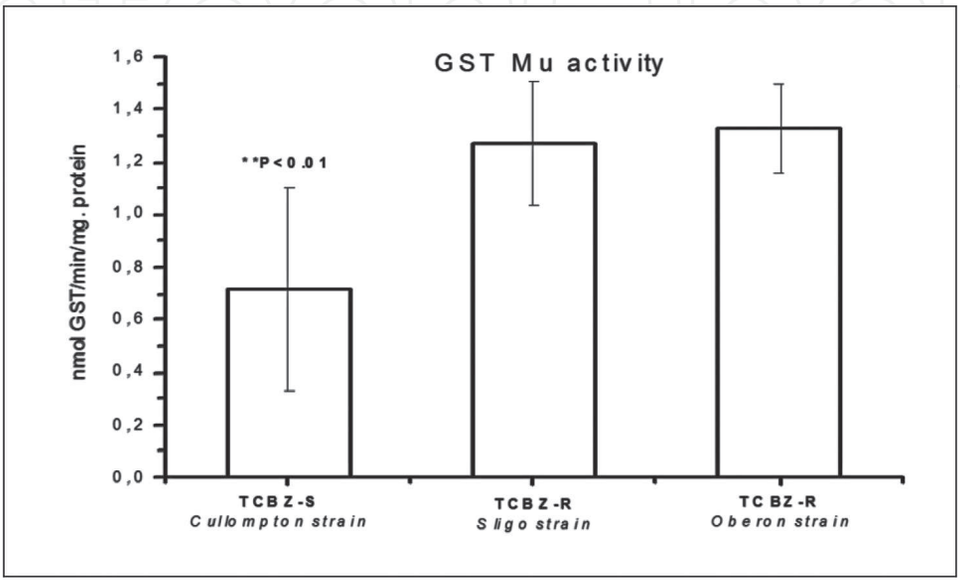

Figure 2. Quantification of activity of mu glutathione S-transferase (GST) in *Fasciola hepatica* susceptible (*Cullompton strain*) and resistant (*Sligo* and *Oberon* strains) to triclabendazole.

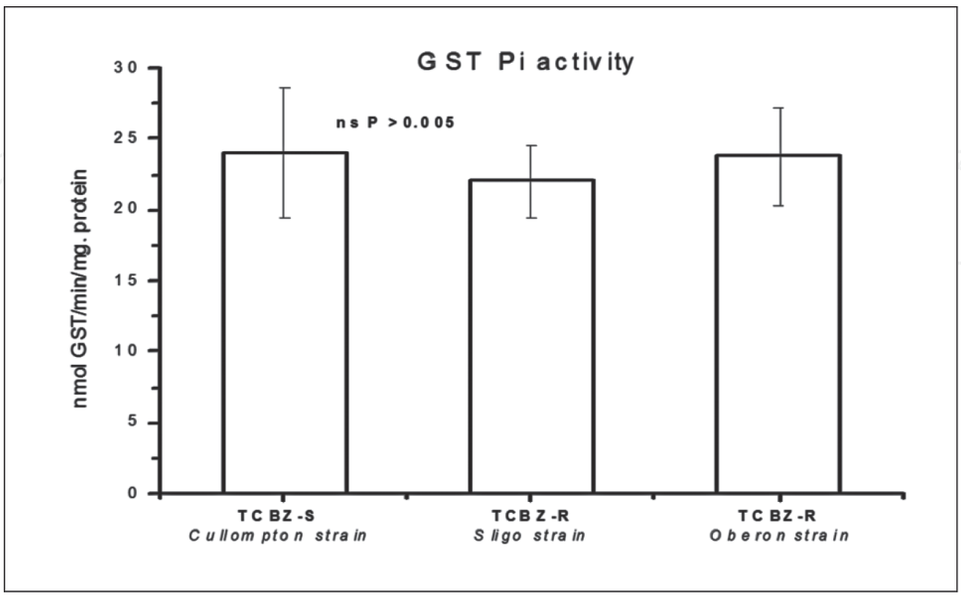

Figure 3. Quantification of activity of pi glutathione S-transferase (GST) in *Fasciola hepatica* susceptible (*Cullompton strain*) and resistant (*Sligo* and *Oberon* strains) to triclabendazole.

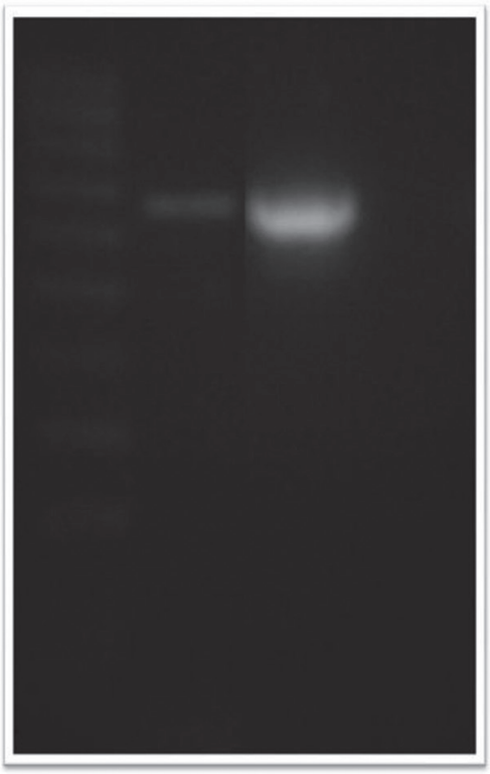

Figure 4. A 657-bp band compatible with the expected size in the 100-bp DNA ladder marker (lane 1), *Cullompton strain* (lane 2), and *Sligo* (lane 3) was obtained.

mu gene isolated from the Sligo strain (TCBZ-resistant) in *F. hepatica* (**Figures 5** and **6**) can be substituted by other polar or small amino acids in particular threonine which differs only in that it has a methyl group in place of a hydrogen group found in serine. Serines are quite common in protein functional centers.

4. Discussion

In the absence of an efficacious vaccine, chemotherapy remains the main tool in treating fasciolosis. Although other alternatives exist, current measures to control fasciolosis are based on the use of drugs such as triclabendazole (TCBZ) [29, 30].

In the anthelmintics, has been adults [9, 29, 31].

> *Parasite defense mechanisms include detoxifying and anti-oxidant enzymes that would suppress its oxidative killing [23]. Therefore, it is necessary to know the mechanisms of detoxification and mechanism of anthelmintic resistance of F. hepatica.*

Other possibilities include enhanced substrate affinity of the enzymes brought about by mutations within their encoding genes. Residue changes may also influence the substrate specificity of the enzymes and could explain why TCBZ-resistant flukes remain susceptible to ABZ [14, 32–35].

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Gst Cullompton strain TATGCCAGCCCAAACTCGGATACTGGAAAATAAGAGGGCTCCAACAACCCGTTCGACTCT 60
Gst Sligo strain      TATGCCAGCCCAAACTCGGATACTGGAAAATAAGAGGGCTCCAACAACCCGTTCGACTCT 60
Gst Cullompton strain ------------------------------AGAGGGCTCCAACAACCCGTTCGACTCT 28
Gst Sligo strain      ----------------------------------GGCTCCAACAACCCGTTCGACTCT 24
                                                         ***********************

Gst Cullompton strain TGCTCGAATACCTGGGTGAAGAGTATGAAGAACATCTGTACGGTCGTGATGATAGGGAGA 120
Gst Sligo strain      TGCTCGAATACCTGGGTGAAGAGTATGAAGAACATCTGTACGGTCGTGATGATAGGGAGA 120
Gst Cullompton strain TGCTCGAATACCTGGGTGAAGAGTATGAAGAACATCTGTACGGTCGTGATGATAGGGAGA 88
Gst Sligo strain      TGCTCGAATACCTGGGTGAAGAGTATGAAGAACATCTGTACGGTCGTGATGATAGGGAGA 84
                      ************************************************************

Gst Cullompton strain AATGGTTTGGCGATAAATTCAACATGGGATTGGATTTGCCAAATTTACCATACTACATTG 180
Gst Sligo strain      AATGGTTTGGCGATAAATTCAACATGGGATTGGATTTGCCAAATTTACCATACTACATTG 180
Gst Cullompton strain AATGGTTTGGCGATAAATTCAACATGGGATTGGATTTGCCAAATTTACCATACTACATTG 148
Gst Sligo strain      AATGGTTTGGCGATAAATTCAACATGGGATTGGATTTGCCAAATTTACCATACTACATTG 144
                      ************************************************************

Gst Cullompton strain ACGATAAGTGCAAACTGACTCAGTCGGTGGCCATAATGCGGTACATTGCGGATAAGCATG 240
Gst Sligo strain      ACGATAAGTGCAAACTGACTCAGTCGGTGGCCATAATGCGGTACATTGCGGATAAGCATG 240
Gst Cullompton strain ACGATAAGTGCAAACTGACTCAGTCGGTGGCCATAATGCGGTACATTGCGGATAAGCATG 208
Gst Sligo strain      ACGATAAGTGCAAACTGACTCAGTCGGTGGCCATAATGCGGTACATTGCGGATAAGCATG 204
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Gst Cullompton strain GAATGCTTGGTTCGACACCCGAGGAACGAGCTCGAATTTCGATGATCGAAGGAGCTGCAA 300
Gst Sligo strain      GAATGCTTGGTTCTACACCCGAGGAACGAGCTCGAATTTCGATGATCGAAGGAGCTGCAA 300
Gst Cullompton strain GAATGCTTGGTTCGACACCCGAGGAACGAGCTCGAATTTCGATGATCGAAGGAGCTGCAA 268
Gst Sligo strain      GAATGCTTGGTTCTACACCCGAGGAACGAGCTCGAATTTCGATGATCGAAGGAGCTGCAA 264
                      ************ ***********************************************

Gst Cullompton strain TGGATCTTCGGATGGGTTTTGTTCGTGTTTGTTACAACCCAAAATTTGAAGAAGTGAAAG 360
Gst Sligo strain      TGGATCTTCGGATGGGTTTTGTTCGTGTTTGTTACAACCCAAAATTTGAAGAAGTGAAAG 360
Gst Cullompton strain TGGATCTTCGGATGGGTTTTGTTCGTGTTTGTTACAACCCAAAATTTGAAGAAGTGAAAG 328
Gst Sligo strain      TGGATCTTCGGATGGGTTTTGTTCGTGTTTGTTACAACCCAAAATTTGAAGAAGTGAAAG 324
                      ************************************************************

Gst Cullompton strain GAGATTATCTGAAAGAACTGCCAACAACGTTGAAGATGTGGTCCGATTTTCTTGGAGATC 420
Gst Sligo strain      GAGATTATCTGAAAGAACTGCCAACAACGTTGAAGATGTGGTCCGATTTTCTTGGAGATC 420
Gst Cullompton strain GAGATTATCTGAAAGAACTGCCAACAACGTTGAAGATGTGGTCCGATTTTCTTGGAGATC 388
Gst Sligo strain      GAGATTATCTGAAAGAACTGCCAACAACGTTGAAGATGTGGTCCGATTTTCTTGGAGATC 384
                      ************************************************************

Gst Cullompton strain GTCACTATTTGACAGGTTCTTCAGTTAGCCATGTGGACTTTATGGTTTACGAAGCATTGG 480
Gst Sligo strain      GTCACTATTTGACAGGTTCTACAGTTAGCCATGTGGACTTTATGGTTTACGAAGCATTGG 480
Gst Cullompton strain GTCACTATTTGACAGGTTCTTCAGTTAGCCATGTGGACTTTATGGTTTACGAAGCATTGG 448
Gst Sligo strain      GTCACTATTTGACAGGTTCTACAGTTAGCCATGTGGACTTTATGGTTTACGAAGCATTGG 444
                      ******************* ************************************

Gst Cullompton strain ACTGTATTCGTTATTTGGCACCACAGTGTCTGGAGGACTTTCCCAAATTGAAGGAATTCA 540
Gst Sligo strain      ACTGTATTCGTTATTTGGCACCACAGTGTCTGGAGGACTTTCCCAAATTGAAGGAATTCA 540
Gst Cullompton strain ACTGTATTCGTTATTTGGCACCACAGTGTCTGGAGGACTTTCCCAAATTGAAGGAATTCA 508
Gst Sligo strain      ACTGTATTCGTTATTTGGCACCACAGTGTCTGGAGGACTTTCCCAAATTGAAGGAATTCA 504
                      ************************************************************

Gst Cullompton strain AGAGTCGTATTGAAGATCTTCCAAAAATCAAGGCATACATGGAATCAGAGAAGTTCATCA 600
Gst Sligo strain      AGAGTCGTATTGAAGATCTTCCAAAAATCAAGGCATACATGGAATCAGAGAAGTTCATCA 600
Gst Cullompton strain AGAGTCGTATTGAAGATCTTCCAAAAATCAAGGCATACATGGAATCAGAGAAGTTCATCA 568
Gst Sligo strain      AGAGTCGTATTGAAGATCTTCCAAAAATCAAGGCATACATGGAATCAGAGAAGTTCATCA 564
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Gst Cullompton strain AGTGGCCTTTGAACTCGTGGATTGCTTC---------------------------------- 628
Gst Sligo strain      AGTGGCCTTTAAACTCGTGGATT------------------------------------- 623
Gst Cullompton strain AGTGGCCTTTGAACTCGTGGATTGCTTCTTTCGGTGGTGGAGACGCTGACGCTGGCCTGC 628
Gst Sligo strain      AGTGGCCTTTGAACTCGTGGATTGCTTCTTTCGGTGGTGGAGACGCTGACGCTGGCCTGC 624
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Gst Cullompton strain ----
Gst Sligo strain      ----
Gst Cullompton strain NAN- 631
Gst Sligo strain      NNNA 628
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Figure 5. CLUSTAL 2.1 multiple sequence alignment.

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> Gst Cullompton strain
LGYWKIRGLQQPVRLLLEYLGEEYEEHLYGRDDREKWFGDKFNMGLDLPNLPYYIDDKCKLTQSVAIMRY
IADKHGMLGSTPEERARISMIEGAAMDLRMGFVRVCYNPKFEEVKGDYLKELPTTLKMWSDFLGDRHYLT
GSTVSHVDFMVYEALDCIRYLAPQCLEDFPKLKEFKSRIEDLPKIKAYMESEKFIKWPLNSWIASFGGGD
A

> Gst Sligo strain
CQPKLGYWKIRGLQQPVRLLLEYLGEEYEEHLYGRDDREKWFGDKFNMGLDLPNLPYYIDDKCKLTQSVA
IMRYIADKHGMLGSTPEERARISMIEGAAMDLRMGFVRVCYNPKFEEVKGDYLKELPTTLKMWSDFLGDR
HYLTGSSVSHVDFMVYEALDCIRYLAPQCLEDFPKLKEFKSRIEDLPKIKAYMESEKFIKWPLNSWIASF
GGGDAD


Gst Cullompton strain ----LGYWKIRGLQQPVRLLLEYLGEEYEEHLYGRDDREKWFGDKFNMGLDLPNLPYYID 56
Gst Sligo strain      CQPKLGYWKIRGLQQPVRLLLEYLGEEYEEHLYGRDDREKWFGDKFNMGLDLPNLPYYID 60
                          ************************************************

Gst Cullompton strain DKCKLTQSVAIMRYIADKHGMLGSTPEERARISMIEGAAMDLRMGFVRVCYNPKFEEVKG 116
Gst Sligo strain      DKCKLTQSVAIMRYIADKHGMLGSTPEERARISMIEGAAMDLRMGFVRVCYNPKFEEVKG 120
                      ************************************************************

Gst Cullompton strain DYLKELPTTLKMWSDFLGDRHYLTGSTVSHVDFMVYEALDCIRYLAPQCLEDFPKLKEFK 176
Gst Sligo strain      DYLKELPTTLKMWSDFLGDRHYLTGSSVSHVDFMVYEALDCIRYLAPQCLEDFPKLKEFK 180
                      ************************* ********************************

Gst Cullompton strain SRIEDLPKIKAYMESEKFIKWPLNSWIASFGGGDA- 211
Gst Sligo strain      SRIEDLPKIKAYMESEKFIKWPLNSWIASFGGGDAD 216
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Figure 6. Amino acid alignments of GST mu isoenzyme of *Fasciola hepatica* susceptible and resistant to TCBZ. Light shading indicates change of one amino acid at position 143 in the TCBZ resistant strain.

GSTs are regulated by a structurally diverse range of xenobiotics, and at least 100 chemicals have been identified to induce GSTs. Many of the compounds that induce GSTs are themselves substrates for these enzymes or are metabolized (by CytP450 or FMO) to compounds that can serve as GST substrates, suggesting that GST induction represents part of an adaptive response mechanism to chemical stress caused by electrophiles [36].

In the present work, cGST was analyzed in three strains of *F. hepatica* (*Cullompton*, a TCBZ-susceptible strain, and *Sligo* and *Oberon*, TCBZ-resistant strains).

The activity of *Sligo* and *Oberon* strains expressed significantly higher metabolic activity than that measured in the cytosolic fractions obtained from the susceptible strain. In this work identified and characterized the GST mu gene isolated from TCBZ-susceptible and TCBZ-resistant *F. hepatica* strains, and comparative analysis of both strains *Cullompton* and *Sligo* showed change two nucleotide and changes GST mu protein: Threonine in the TCBZ-susceptible strain by Serine in the TCBZ-resistant strain can be substituted by other polar or small amino acids in particular threonine which differs only in that it has a methyl group in place of a hydrogen group found in serine [37]. These genetic variations can change an individual's susceptibility to carcinogens and toxins as well as affect the toxicity and efficacy of certain drugs but GST mu protein in active sites not present the classical Asp-His-Ser therefore not would find affected by this change of amino acid mu GST biological activity.

5. Conclusion

GST activity has this great potential importance as it might contribute to generating the phenomenon of resistance to TCBZ. These results contribute to the understanding not only of this

metabolic pathway but also of the mechanism of resistance to TCBZ in *F. hepatica*. The results also add information to the knowledge of the response that the parasites have exposure to different xenobiotics.

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