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Assessment of *Fasciola hepatica* glutathione S-transferase as an antigen for serodiagnosis of human chronic fascioliasis

Vasti Aguayo, Bianca Valdes, Ana M. Espino[#]

Laboratory of Immunology and Molecular Parasitology, Department of Microbiology, University of Puerto Rico, School of Medicine, San Juan, PR 00936-5067.

Abstract

Due to the unsatisfactory performance of parasitological diagnosis of human fascioliasis; the use of immunodiagnosis based on the detection of anti-*Fasciola* antibodies is traditionally used as a diagnostic alternative using total or purified parasite excretory-secretory products (ESPs). Glutathione S-transferase (GST) protein, one of the *F. hepatica* ESP components, possesses well-known roles in the detoxification of xenobiotic and endogenously derived toxins within the host bile environment. GST has shown to be a good target for vaccine or drug development against fascioliasis. The current study aimed to evaluate the potential of GST protein purified from a soluble crude extract of adult flukes as an antigen for serodiagnosis of chronic human fascioliasis by indirect ELISA. The study included a panel of 116 serum samples collected from individuals with confirmed fascioliasis, individuals carrying heterologous parasitic infections and healthy subjects. The parasitological examination was used as gold standard and a previously optimized ESP-ELISA was used to compare the performance of the GST-ELISA method. Results demonstrated that GST-ELISA is 94.3% sensitive, 80.2% specific and exhibits a moderate positive correlation ($r=0.555$) and substantial agreement ($k=0.786$) with the results obtained with the ESP-ELISA method. Moreover, because no sera from patients with early *F. hepatica* infection were available, GST-ELISA was then tested with sera from rabbits experimentally infected with *F. hepatica* metacercariae. The assay was able to detect anti-*Fasciola* antibodies as early as the 3rd week of infection ($p<0.0001$) with peaks at 4th and 10th week post-infection.

INTRODUCTION

The serodiagnosis of fascioliasis has been traditionally recognized as the best alternative to diagnose human fascioliasis. The reason for that is the unsatisfactory performance of parasitological diagnosis based on the detection of eggs in feces or bile drainage, which is the only way to confirm *Fasciola* infection (Chen, 1991; Chen, 1990; Inal et al, 2002; Mas-Coma, 2005). Humans are not a suitable host for *Fasciola*, therefore, the fluke may not attain maturity in humans, which will result in a biliary fascioliasis without egg shedding, or will require long pre-patent periods of 4 months or even longer for maturation (Chen, 1990; Espino et al, 1998; Sarkari & Khabisi, 2017). Moreover, after patency, the parasites often

[#] Address correspondence to Espino, A.M. ana.espino1@upr.edu.

VA and AME equally contributed to this work

Present address: Laboratory of Immunology and Molecular Parasitology, Department of Microbiology, University of Puerto Rico, School of Medicine. PO BOX 365067, San Juan, PR 00936-5067

shed the eggs intermittently, making the coprologic examination very insensitive. Enzyme-linked immunosorbent assay methods (ELISA) developed for determining levels of *Fasciola* antibodies provide an invaluable alternative to replace coprologic examination and have been extensively used, either for diagnosis of individual cases, or for the screening of large human populations (Carnevale et al, 2001b; Gonzales Santana et al, 2013; Valero et al, 2012).

The most popular antigens commonly used in conjunction with ELISA methods for antibody detection are the excretory-secretory products (ESPs). ESPs comprise a myriad of molecules secreted, either by immature, or mature flukes, which are responsible for evoking a powerful and long-lasting antibody response. Using ESPs of *F. hepatica* or *F. gigantica*, anti-*Fasciola* antibody has been detected in 100% or 98% by Carnevale, S. et al. 2001 (Carnevale et al, 2001b) and Mattar MA et al. 2004 (Mattar & El-Toukhy, 2004) studies, respectively. However, because ESPs are a complex mixture of antigens, the cross-reactions with sera from heterologous infections are often frequent. Nowadays, it is a common approach to purify ESP components or produce recombinant antigens and use them in diagnostic testing.

A number of proteomic analyses have revealed that proteins belonging to the cathepsin-like family or the glutathione *S*-transferase (GST) family are the major components of *F. hepatica* ESPs (Jefferies et al, 2001; Perez-Sanchez et al, 2006; Wilson et al, 2011). Cathepsins are the most abundant components comprising ~80% of the total secreted products. There are numerous papers reporting the use of Cathepsin molecules as an antigen to detect anti-*Fasciola* antibodies in humans and animals in a number of serologic formats (Carnevale et al, 2001a; Cordova et al, 1999; Cornelissen et al, 1999; Gottstein et al, 2014; Martinez-Sernandez et al, 2011; Mokhtarian et al, 2016; Nagano et al, 2004; O'Neill et al, 1999; Rokni et al, 2002; Strauss et al, 1999). However, GST proteins, which constitute ~4% of total soluble protein content of *F. hepatica* (LaCourse et al, 2012), have been extensively studied as vaccine candidates (Paykari et al, 2002; Sexton et al, 1990; Sexton et al, 1994) or as drug targets since the GST enzyme family has been associated to the resistance to triclabendazole (Fernandez et al, 2015; Radio et al, 2018) but they have been poorly studied as an antigen for serodiagnosis. In this regards only a paper has been published (Mokhtarian et al, 2016). The present study aimed to purify and characterize native forms of GST by a proteomic approach from a soluble extract of *F. hepatica* adult flukes and assesses the value of the present isoform as an antigen for serodiagnosis on a larger number of sera from patients with confirmed chronic fascioliasis from an endemic area of America.

MATERIALS AND METHODS

Rabbit sera

Five New Zealand White rabbits were orally infected with 60 *F. hepatica* metacercariae each. Adult flukes present in bile ducts at week 12 during necropsy were proof of infection. Animals were bled before infection and then at biweekly intervals during 12 weeks; serum samples were stored at -20°C until use.

Human sera

All serum samples used in this study were kindly donated by collaborators from the University of Cajamarca (Cajamarca, Peru) (approved by the ethic committee of the Regional Hospital of Health from Cajamarca and the General Directions of Zoonosis from the Minister of Health, [MINSA], Lima, Peru), the Department of Sanitary Parasitology, ANLIS, Dr. Carlos G. Malbran (Buenos Aires, Argentina), The Tropical Medicine Institute (IMT) of the Central University of Venezuela (Caracas, Venezuela), (approved by the ethical scientific committee of the IMT) and the Infection Disease Division, Washington University School of Medicine (St Louis MO) (approved by the institutional review boards at Washington University School of Medicine). Serum samples were collected only from adult subjects upon receipt of a written informed consent. Since the serum samples were not collected for this study, the names and identities of donating subjects were not retained. A total of 116 serum samples were analyzed. Of these, 36 serum samples were from individuals with confirmed fascioliasis by coprologic examination (CE) that had lived in Cajamarca, Peru where fascioliasis is highly endemic (Marcos et al, 2007). Thirty-six negative sera from subjects living in Puerto Rico where human fascioliasis is non-endemic were also collected. These subjects were also coprologically negative for other parasitic infections. To evaluate potential cross-reactivity, serum samples were also obtained from confirmed cases of paragonimiasis ($n = 6$), schistosomiasis ($n = 14$), filariasis ($n = 5$), giardiasis ($n = 3$), visceral larva migrans (VLM) ($n = 4$), trichinellosis ($n = 4$), toxoplasmosis ($n = 4$) and hydatidosis ($n = 4$). All samples were frozen at -80°C in glycerol until use and aliquots tested had not been thawed prior to testing in this study.

Fasciola hepatica excretory-secretory products

F. hepatica excretory-secretory products (ESPs) were prepared by *in vitro* maintenance of adult flukes as previously described (Espino et al, 1987) with minor modifications. Briefly, *F. hepatica* adult flukes were collected from infected livers of cattle sacrificed in a local abattoir and transported to the laboratory in RPMI-1640 (Sigma-Aldrich, St Louis MO) medium. Flukes were washed out repeatedly of blood, bile and debris in 0.01M phosphate buffered saline (PBS) pH 7.2 and cultured all together in RPMI-1640 medium for 16h, 37°C at a rate of 1 parasite per each 5-ml of medium. After the incubation, adult flukes were removed and the medium was centrifuged at $6,000 \times g$, 4°C and the supernatant was then concentrated by YM-3 AMICON ultrafiltration membrane.

Total soluble extract of adult flukes

Two grams of adult *F. hepatica* flukes extracted from infected livers, as described above, were homogenized in a tissue grinder with a Teflon pestle in PBS, at a ratio of 5-ml of PBS per gram of dry flukes. PBS was supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA), 8 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM iodoacetamide, and 2 mM leupeptine, as protease inhibitors. The homogenate was centrifuged at $12,000 g$ for 1h at 4°C and the supernatant was collected, filtered by a $0.45\mu\text{m}$ Millipore filter and immediately used for glutathione *S*-transferase (GST) purification.

Enzymatic activity

We measured the enzymatic activity of GST in an assay mediated by CDNB (1-chloro-2, 4-dinitrobenzene), as described in the Affinity Chromatography Handbook Vol. 2 (GE Healthcare Life Science). Briefly, the reagent cocktail was prepared in a 1-ml cuvette (880µl of 0.1M phosphate buffer pH 6.5 + 10µl of 100mM GSH + 10µl CDNB). For each assay (reagent and sample), 5µl PBS or 5µl sample were added to the cocktail, respectively and mixed well. Meanwhile the UV spectrophotometer was set up to 340nm to measure/quantify GST activity. The cuvette was inserted into the barrel of the UV spec and absorbance recorded for 3 minutes.

Native *F. hepatica* glutathione S-transferase (GST) purification, nano-LC MS/MS analysis and homology with heterologous GST molecules

GST was purified by affinity chromatography using a HP 5/5 GStrap™ column (GE Healthcare Life Sciences) previously equilibrated with PBS pH 7.4. Absorbance of eluates was monitored at 280 nm. Approximately 10-ml of the *F. hepatica* soluble extract was loaded onto the column at a flow rate of 1ml min⁻¹. Column was washed with PBS to wash out unbound proteins and then washed with 10mM Tris-HCL pH 8.0 buffer containing 10mM reduced glutathione (GSH) to elute the GST protein. The CDNB (1-chloro-2, 4-dinitrobenzene) enzymatic activity assay was used for the monitoring of the efficiency of the protein binding to the column and recovery during the purification process. Purified GST protein was desalted against PBS using a PD-10 column (sephadex G-25) and then concentrated by AMICON ultrafiltration system using an YM-10 membrane (cut-off >10kDa). Protein concentration was adjusted to 1 mg/ml as determined by BCA method using a Pierce protein assay kit (Pierce, Cambridge, NJ). An aliquot of purified GST was subjected to 15% SDS-PAGE and protein composition was visualized by coomassie blue staining. An aliquot of ~300µg GST was digested with trypsin and analyzed by nano-LC-MS/MS platform (Creative Proteomics, USA) for determining purity and identifying possible GST-isoforms. The sequences of GST proteins identified by nano-LC-MS/MS were aligned with the GST sequences from heterologous parasites available at the GenBank using Clustal Omega (Uniprot) available at the bioinformatics resource portal www.expasy.org

Indirect enzyme-linked immunosorbent assays (ELISA)

GST was used as an antigen in an indirect ELISA following a previously reported basic protocol (Espino et al, 1987) that was optimized by checkerboard titration to maximize the sensitivity of the assay. Briefly, we coated 96-well flat-bottomed polystyrene ELISA plates (Costar, Cambridge, MA) overnight at 4°C with 100µl/well of GST (10 µg/ml) in 0.1 ml of 0.05 M carbonate buffer (pH 9.6). After three washes with PBS containing 0.05% Tween-20 (PBST), the plates were blocked with 5% non-fat, skimmed milk in PBST (300µl/well) at 37°C for 1 h. The sera diluted in PBST were added in duplicate (100µl/well) and incubated at 37°C for 1 h. Human sera were diluted 1:100 and rabbit sera were diluted 1:400. The excess antibody was washed off with PBST. Horseradish peroxidase (HRP) labeled-anti-human IgG (Bio-Rad, Hercules, CA) diluted 1:1,250 or HRP labeled-anti-rabbit IgG (Bio-Rad, Hercules, CA) diluted 1:5,000 was added to the wells (100µl/well) and incubated for 1 h at 37°C. After another washing step, the substrate solution (25 ml of 0.1 M citrate-

phosphate buffer [pH 5.0] containing 20 mg α -phenylenediamine hydrochloride and 30% H_2O_2 was added (100 μ l/well), and the plates were incubated in the dark at room temperature for 30 min. The reaction was stopped by the addition of 50 μ l/well 10% HCl and the absorbance at 490 nm (A_{490}) was measured using an ELISA reader (SpectraMax M3). Indirect ELISA using ESP as antigen was performed exactly as previously described (Figuerola-Santiago et al, 2011) to test the human sera. The optimized reagent concentrations for the ESP-ELISA were: 25 μ g/ml for coating antigen concentration, 1:400 for serum dilution and 1:5,000 for conjugate dilution. Each ELISA determination was performed in duplicate, and the results were expressed as the mean A_{490} value for each determination $\pm$ standard error of mean (SEM).

Data analysis

The optimal cut point values for both ELISA methods were established within a 95% confidence interval (CI), by receiver operating characteristic (ROC) curve analysis using the EpiTools epidemiological calculator (<http://epitools.ausvet.com.au>). Arbitrary guidelines were followed for analyzing the area under the curve (AUC) as follows: non-informative, $AUC = 0.5$; low accurate, $0.5 < AUC < 0.7$; moderately accurate, $0.7 < AUC < 1$; perfect, $AUC = 1$ (Swets, 1988). Intra-plate repeatability was evaluated for GST-ELISA by measuring the coefficient variation (CV) of 56 repeats of 16 sera. Reproducibility was evaluated by performing 3 independent assays for the assay using 6 *F. hepatica* sera of infection, 5 negative controls and 5 sera from individuals carrying other parasites on separate runs. Results obtained with GST-ELISA were compared to those obtained with the ESP-ELISA. Correlation between GST-ELISA and ESP-ELISAs was performed using the Pearson correlation coefficient (95% CI). To evaluate the agreement between GST-ELISA with the coprologic examination (CE), we used the Cohen's Kappa calculator according to Landis and Koch (1977) (Landis & Koch, 1977). The Kappa values (κ) were considered as follows: slight agreement ($\kappa < 0.02$), fair agreement ($\kappa = 0.21-0.4$), moderate agreement ($\kappa = 0.41-0.6$), substantial agreement ($\kappa = 0.61-0.8$); perfect agreement ($\kappa = 0.81-1.0$).

RESULTS

F. hepatica GST purity and isoforms identification

Based on the CDNB enzymatic activity assay we primarily determined that the total enzyme (EA) activity of GST in the ESPs (~2mg/ml) was equivalent to 1.8 Units/min (Specific enzyme activity (EEA) = 0.9 Units/min/mg of protein) whereas in the soluble crude extract (22 mg/ml) the EA was equivalent to 45 Units/min with EEA = 2 Units/min/mg of protein. Based on this result, we proceeded to purify GST from the soluble parasite crude extract using a GSTrap affinity chromatography column. The final product (1.5ml) showed a single protein band of ~25kDa when analyzed by 12.5% SDS-PAGE and a protein concentration of approximately 3.5 mg/ml (Figure-1A). The final purified product retained the 100% of enzyme activity and showed EEA = 12.86 U/min/mg of protein. To confirm the purity of our GST a small sample was subjected to a high-resolution mass spectrometry analysis coupled with nanoflow UPLC. We used the total iBAQ value of three replicates to calculate the purity of GST, which was 96.17%. The analysis also revealed that the purified GST was comprised of five isoforms, four belonging to the mu-class (GST1, GST7, GST47 and

GST51) and one belonging to the sigma-class (Table-1) that shares identity percentages ranging among 73.87 to 98.62%. The isoform GST7 was selected for alignment with GST sequences of heterologous organisms which share sequence identity that ranged from 12.879% to 61.46.0% with heterologous GST proteins of *Homo sapiens*, *Wuchereria bancrofti*, *Paragonimus westermani*, *Echinococcus granulosus* and *Schistosoma mansoni* (Figure-1S).

Immunoreactivity of GST with rabbit sera infection

GST protein was not reactive with negative rabbit sera and was also poorly positive with the sera collected at the 2nd week of infection. However, the immunoreactivity of GST became significantly ($p<0.05$) noticeable with all sera from the 3rd week of infection (mean 0.45 ± 0.045) and increased sequentially with the course of infection. Two main immunoreactivity peaks were observed. The first peak occurred at 4th week of infection (mean absorbance 1.96 ± 0.92) and the second occurred at 10th week of infection (mean absorbance 2.11 ± 0.7) and remained at a plateau until the 12th week of infection (mean absorbance 2.18 ± 0.47), time in which the animals were euthanized and the patent infection was confirmed by the presence of adult flukes into the bile ducts (Figure-1B).

Distribution of absorbance values with human sera and ROC analysis

As expected, the distribution in absorbance values with human sera was much more varied than that observed with rabbit sera between positive and negative samples (Figure-2). We used the CE for *F. hepatica* eggs as a gold standard to build ROC curves on the basis of absorbance values obtained with serum samples from the two reference populations: subjects infected with *F. hepatica* that were all CE positive and serum samples from subjects that were all CE negative to *Fasciola* eggs. In addition, GST's capacity to discriminate true negatives from true positive samples was compared to that of the ESP antigen, which is the commonly used antigen for serodiagnosis of fascioliasis (Carnevale et al, 2001b; Espino et al, 1987; Espino et al, 1992; Figueroa-Santiago et al, 2011). In the GST-ELISA, the absorbance values in the negative control group ranged between 0.08 and 0.59, with a mean $\pm$ SEM A_{490} value of 0.275 ± 0.023 . In the ESP-ELISA, the mean A_{490} values for the negative control group were 0.108 ± 0.08 . Significant differences ($p<0.0001$) were found between the mean A_{490} values obtained for both antigens with the same negative sera population. In the GST-ELISA, the sera from patients with fascioliasis showed absorbance values that ranged among 0.299 to 1.2 with a mean A_{490} value of 0.54 ± 0.038 . This mean value was found to be significantly different from the mean value of the negative control group ($p<0.0001$). With the ESP-ELISA, the fascioliasis sera showed absorbance values that ranged among 0.22 to 2.2 (mean 1.46 ± 0.09). This mean value was also found to be significantly different from the mean value obtained with the GST antigen for the fascioliasis population ($p<0.0001$).

Receiving operator characteristic analysis was used to determine the best cut-points for both assays. The ROC optimized cut-point was 0.344 for GST-ELISA and 0.69 for ESP-ELISA. The selection of these cut-points derived from three different conditions: (a) maximum specificity at which the sensitivity was still 100%, (b) maximum sensitivity at which the specificity of the assay was also maximized, and (c) maximum value for Youden's J index (S

+ Sp-1) and test efficiency (Table-2). The area under curve values (AUC) (accuracy) for the ROC curves of GST and ESP were 0.908 and 0.991, respectively (Figure-3). Based on the established cut-points, there were two seronegatives in the fascioliasis population and 10 seropositives in the negative control population using the GST-ELISA, whereas when using the ESP-ELISA, only one seronegative was detected in the fascioliasis population and no seropositives in the negative control population were detected. When serum samples from parasitic infections other than fascioliasis were tested, 5 seropositive samples were found with the GST-ELISA, which include 1 schistosomiasis serum, 3 paragonimiasis sera and 1 hydatidosis serum (Figure-4A). From these sera, only four cross-reacted with the ESP antigen (3 paragonimiasis and 1 hydatidosis sera). Significant differences were obtained between the mean absorbance values of fascioliasis sera (0.54 ± 0.22) tested with GST-ELISA compared to schistosomiasis (0.17 ± 0.101 , $p < 0.0001$), hydatidosis (0.2 ± 0.13 , $p = 0.0063$) and other parasites sera (0.09 ± 0.08 , $p < 0.0001$). However, no statistical differences were found between mean values of fascioliasis samples compared to paragonimiasis samples (0.41 ± 0.31 , $p = 0.09$). Thus, under these optimized conditions, GST-ELISA reached a sensitivity of 91.4% (Lower 95% CL of 77.6% and upper 95% CL 97%) and specificity of 80.2% (Lower 95% CL 70.3% and upper 95% CL 87.5%) (Youden's J Eff. at $P = 0.3$). The specificity calculated for the ESP-ELISA was 97.1% (Lower 95% CL 85.5% and upper 95% CL 99.5%) and specificity of 98.8% (Lower 95% CL 93.3% and upper 95% CL 99.8%). (Youden's J Eff. at $P = 0.05$). The estimated predictive positive value (PPV) and predictive negative value (PNV) for GST-ELISA were 69.3% and 97%, respectively, whereas that for ESP-ELISA the PPV and PNV was 92.1% and 98.7%, respectively (Table-3). There was a moderate positive correlation ($r = 0.555$) and substantial agreement (Cohen's $\kappa = 0.796$) between the GST- and ESP-ELISA (Figure-4B). We also assessed the reproducibility of the GST-ELISA by calculating the CV of data from 3 different assays and 56 repeats of 16 serum samples. The intra-assay and inter-assay reproducibility values were 9.2% and 12.5%, respectively.

DISCUSSION

Glutathione S-transferases (GSTs; EC 2.5.1.18) have been reported both, in eukaryotes (Driss et al, 2016; LaCourse et al, 2012; Savic-Radojevic et al, 2017) and in prokaryotes (Lisewski, 2014) and constitute a large multifunctional dimeric enzyme family that conjugates reduced glutathione to electrophilic centers in hydrophobic organic compounds. GSTs participate in the detoxification of xenobiotics, which are toxic substances produced and released by the human/animal host during the course of metabolism. The liver in particular is an organ where intense metabolic activity occurs and is precisely the place where juvenile and adult stages of *F. hepatica* localize. Therefore, the parasite may be damaged during normal host metabolism with a xenobiotic attack and thus, GST is essential for the survival of *F. hepatica* in the mammalian host. The current study focused to determine whether native forms of *F. hepatica* GSTs purified from a crude soluble extract of adult fluke could serve as antigen for serodiagnosis of chronic human fascioliasis.

To test the effectiveness of GST as a serodiagnostic antigen, it was necessary to purify the protein at concentrations high enough to optimize and validate the ELISA method. Because GST is recognized as an excretory-secretory antigen (Jefferies et al, 2001; Perez-Sanchez et

al, 2006; Wilson et al, 2011), it would have been much more logical to purify GST from ESPs as it was recently reported (Mokhtarian et al, 2016). However, after demonstrating that the soluble fluke extract had much more enzyme activity than did the ESPs, we considered the crude fluke extract would be a more suitable source. Thus, the whole purification process was performed using a GSTrap HR column that permits the processing of large volumes of starting material. As a result, we obtained a high protein yield (3.5-mg/ml) along with maximized specific enzyme activity yield. In comparison with a previous study, in which GST was purified from 2-mg of ESPs using a small amount of GSH-resin in batch mode recovering 800µg/ml purified protein (Mokhtarian et al, 2016), we consider our strategy much more cost-beneficial because, in addition to the high yield, our approach obviates the requirement of performing the in vitro culture of live parasites to prepare the ESPs, which generally takes 24h (Carnevale et al, 2001b; Espino et al, 1987). However, in terms of purity, both strategies render similar results, as demonstrated in the appearance of a similar single ~25kDa band in SDS-PAGE that is similar to the one reported for GST purified from ESPs (Mokhtarian et al, 2016).

Another important observation is that, although some studies affirm that *F. hepatica* may contain seven GST isoforms (Chemale et al, 2006; Rossjohn et al, 1997), in our study we only identified four isoforms belonging to the mu-class (GST1, 7, 47 and 51) and an isoform belonging to the sigma-class (Table-1). These findings are consistent with those obtained by other authors that have reported these isoforms exist in relative higher abundance in *F. hepatica* (Chemale et al, 2006). All these isoforms have been cloned for different study purposes (Kumar et al, 2012; Mokhtarian et al, 2018; Paykari et al, 2002; Sexton et al, 1994). The finding that our GST protein was highly reactive with sera from rabbits with early *F. hepatica* infection is also consistent with the fact that these isoforms localize toward parenchymal cytoplasm in the sub-tegumental area, either of the adult, or the juvenile stages (Creaney et al, 1995). The peak of antibody observed at the 4th week of infection corresponds with the time in which juvenile flukes initiate their migration through liver parenchyma and the peak observed at 10–12 weeks of infection corresponds with the time in which the parasites had already become adult flukes in the bile ducts and began egg shedding. Such a dynamic antibody response is consistent with the notion that GST isoforms are expressed throughout the parasite development in the mammalian host, and evidences that these proteins are continuously exposed to the immune system participating in the elicitation of humoral responses as has been reported to occur for other *F. hepatica* defined antigens (Caban-Hernandez et al, 2014; Cornelissen et al, 2001; Raina et al, 2006). Moreover, the finding that GST detects anti-*Fasciola* antibodies at early stages of the infection may also suggest that GST could be a good antigen for detecting human fascioliasis at an acute phase. Unfortunately, the acute phase in humans is often non-symptomatic or presents no-pathognomonic symptoms that are very difficult to identify and consequently, the possibility of obtaining human sera of early infections for us to test is remote. In contrast, most clinical manifestations of fascioliasis in humans occur at a chronic phase and this is why all sera tested in the current study were from chronic fascioliasis patients.

To determine the suitability of our GST as antigen for serodiagnosis, we used a panel of 36 serum samples from patients with confirmed fascioliasis that lived in Cajamarca, Peru, one

of the most important endemic focus areas of human fascioliasis in the planet. The diagnostic capabilities of the GST-ELISA method was evaluated by determining parameters such as sensitivity, specificity, accuracy and positive and negative predictive values (Xu et al, 1997), which are highly dependent of the cutoff point established. Therefore, to minimize the stochastic uncertainty in cutoff point selection (<http://epitolls.ausvet.com.au>), we allotted the sera into two categories: positive to *F. hepatica* eggs (true positive population) and negative to *F. hepatica* eggs (true negative population) and this later population included either, the sera from healthy subjects that were not infected with any parasite (n=36), or sera from persons carrying parasitic infections other than fascioliasis (n=44). By using ROC curve analysis we were able to select the cutoff value that gave the best balance between sensitivity and specificity for GST-ELISA (Zweig & Campbell, 1993).

The high sensitivity shown by GST-ELISA (94.3%) demonstrates that during the active infection, humans develop antibodies against GST proteins, which is also consistent with our finding that GST was highly reactive with sera from rabbits with 3 to 12 weeks of *F. hepatica* infection (Fig. 1B). Only two fascioliasis sera were recorded as false negatives in the GST-ELISA. Since all sera tested in our study were properly preserved at -80°C and had not been repeatedly thawed, it is possible to speculate that these false negatives could be due to a low intensity of infection since human subjects living in endemic areas often might ingest low doses of metacercariae, and such low infection dose could affect the development of detectable levels of antibodies to a particular antigen (Cornelissen et al, 1999).

When we analyzed the reactivity of GST with the sera from individuals carrying other parasitic infections, we found that only 5 out of 44 sera reacted with GST, specifically 2 sera of paragonimiasis, 1 of hydatidosis and 1 of schistosomiasis. The possibility that these sera belonged to persons with subclinical infection with *Fasciola* cannot be ruled out because the history of watercress consumption for these patients is unknown to us. Additionally, we were unaware as to whether these persons had lived in endemic regions of fascioliasis prior to sample collection or were cases of cured fascioliasis that still had anti-*Fasciola* antibodies which could remain at detectable levels for many months or years after effective treatment (Espino et al, 1992) (we also have no information about previous history of previous triclabendazole treatment in these patients). However, it is also highly possible that these seropositive sera could be true cross-reactions due to the existence of common epitopes between GST *F. hepatica* and proteins of other parasites. The cross-reactivity between helminth antigens is common because there are a large number of proteins that are conserved during evolution and share common epitopes (Hillyer & Serrano, 1983; Yamano et al, 2009). However, what was really surprising was that GST reacted with 10 of 36 (27%) sera from the negative control population, in some cases with absorbance values as high as those observed for the true positive population, in contrast with ESP, which did not show reactions with any of these sera. The possibility that these 10 seropositive sera came from persons that had had a previous exposition to *F. hepatica* is remote because these subjects lived in Puerto Rico where the human fascioliasis is not endemic. In addition, Puerto Rico is neither, an endemic area for paragonimiasis, or for hydatidosis and has not been an endemic area for schistosomiasis for more than 20-years. Therefore, the probability that these false-positives can be due to cross-reactions with antigens of other parasites is remote. In addition, the possibility that these persons were suffering of any sub-clinical parasitic infection

acquired in recent travels in endemic areas (the history of travel within the sample pool was unknown to us) should also be ruled out due to the fact that all these sera resulted negative with the ESP antigen, which has showed a high capacity to discriminate true positive from true negative populations with high level of sensitivity and specificity (Carnevale et al, 2001b; Figueroa-Santiago et al, 2011). Thus, when we represented the distribution of absorbance obtained for *F. hepatica* positive and *F. hepatica* negative sera population in the form of histograms, we observed that the one obtained for ESP (Fig. 2 C–D) exhibits a clear bell-shaped distribution with only a short overlapping region, whereas the histograms obtained for GST (Fig. 2A–B) showed a larger overlapping region that made the discrimination between positive and negative sera populations much more problematic.

We have speculated that the reactivity of GST with some negative sera “in absence of parasitic infection” could be due to cross-reactions with antibodies elicited by GST derived from allergenic organisms such as mites, cockroaches, or pollen, which have been implicated as responsible agents for asthma in the US and other countries (Deifl et al, 2014; Mueller et al, 2015; Sookrung et al, 2018). Unfortunately, since allergic conditions were not excluded when the serum samples were collected and because the medical history of allergic diseases from our negative control population was unknown to us, it is only possible to make this a speculative, and not a final conclusion. However, based on previous studies, the frequency of allergies in this negative population could very well be high (CDC, 2004; Perez-Perdomo et al, 2003), especially since GST is one of the many organisms that have been commonly reported as human allergens (Acevedo et al, 2013; Sookrung et al, 2018). The cross-reactivity of GST between different mite species (Huang et al, 2006; Liao et al, 2013) and fungal sources (Huang et al, 2006; Liao et al, 2013), as well as the cross-reactivity of GST between mites and cockroaches (Huang et al, 2006; Liao et al, 2013) and recently even between cockroaches and helminthes has been demonstrated (Santiago et al, 2012). The prevalence of helminthiasis is high in undeveloped countries, specifically in tropical, rural areas (Bethony et al, 2006), whereas the prevalence of allergic diseases is high in industrialized and developed countries (Devenny et al, 2004; Nicolaou et al, 2005). Such an inverse gradient of distribution of both groups of diseases could explain the notable difference in reactivity of *F. hepatica* GST with the sera of other parasitic infections, which all came from rural areas in undeveloped countries, compared to the reactivity with sera from healthy subjects that were obtained from persons that live in urban areas in a developed country. Based on this premise, if GST were to be tested with a bigger sample number of both positive and negative sera from endemic areas of fascioliasis, the discrimination between positive and negative sera could be significantly improved.

Despite GST-ELISA being problematic for discriminating true positive from true negative, the assay showed a good correlation with the ESP-ELISA method, which indicates that most of sera were correctly discriminated by GST. However, the fact that ESP was as good antigen was not surprising. Although ESP is comprised by myriad of molecules (Jefferies et al, 2001) these antigens have showed a high capacity to discriminate true positive from true negative populations with high level of sensitivity and specificity (Carnevale et al, 2001b; Figueroa-Santiago et al, 2011). However, due to the logical variations in the ESP preparation among different laboratories and the potential presence of cross-reactions, other purified and well defined *F. hepatica* antigens such as cathepsin-L1 (Carnevale et al, 2001a; Mokhtarian

et al, 2016; Tantrawatpan et al, 2007), fatty acid binding protein (FABP) (Mokhtarian et al, 2016) or saposin-like-2 (Figuerola-Santiago et al, 2011; Shin et al, 2016) have been used. These antigens show significantly better diagnostic performance than ESPs for diagnosis of human or animal fascioliasis and much better diagnostic performance than GST, either in native or recombinant form (Mokhtarian et al, 2016).

In conclusion, in this study we developed an indirect ELISA method for detecting antibodies in sera from humans with chronic fascioliasis using GST as a native antigen comprising five active protein isoforms. Despite GST-ELISA demonstrated high sensitivity (94.3%), and reproducibility, it was a poor discriminator between true positive and true-negative sera (specificity 80.3%). Notably, the poor performance of our GST as an antigen is consistent with those previously reported for other recombinant or native variants of GST (Mokhtarian et al, 2016), which indicate that irrespective of the genotypic diversity of the *F. hepatica* isolate used for GST purification, as well as the origin and precedence of sera used in the validation, GST could not be a good antigen for serodiagnosis of fascioliasis. However, to confirm this assumption it could remain necessary to design a larger validation study with more positive serum samples during both acute and chronic phases of fascioliasis and expand the number of sera from individuals carrying other food-borne acquired helminthiasis, specifically schistosomiasis, paragonimiasis and clonorchiasis, which are major causes of cross-reactivity (Ikeda et al, 1996). Finally, it is also important to determine the possible implications that *F. hepatica* GST could be having in the cross-reactions with GSTs from orthologous species causative of allergic diseases in human populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure-1. Electrophoretic evaluation of purified and immunoreactivity with rabbit infection sera. Two different batches of GST were purified from a crude extract of *Fasciola hepatica* adult flukes using a GSTrap affinity chromatography column. **(A)** Purified proteins were analyzed by 15% SDS-PAGE stained with coomassie blue. Lanes-1 & 2: represent different batches of soluble crude extract. Lanes-3 & 4: represent purified GST from crude extracts 1 & 2, respectively, which when were analyzed in triplicate by nano-LC MS/MS showed a 96.17%. **(B)** Purified GST was used as coated antigen in an indirect ELISA to study the kinetic of anti-*Fasciola* antibody in sera from rabbits infected with 60 *F. hepatica* metacercariae.

Figure-2. Distribution of absorbance values obtained with the GST- and ESP-ELISAs. Indirect ELISA methods were optimized using as antigen GST and *F. hepatica* excretory-secretory products, respectively. Absorbance values were distributed in form of frequency histograms to clearly visualize the separation between true positives and true negative populations. (A & B) Represents the frequency of distribution for *F. hepatica* infection sera and negative sera population tested with GST-ELISA and (C & D) represents the frequency of distribution for *F. hepatica* infection sera and negative sera population tested with ESP-ELISA.

Figure-3. Receiver-operator characteristic (ROC) curves.

The ROC curves were built for 36 sera from healthy subjects, 44 sera from individuals carrying other parasitic infections and 36 sera from patients with confirmed diagnosis of fascioliasis by finding *F. hepatica* eggs in fecal samples. The area under the ROC curve (accuracy) was 0.908 for the GST-ELISA and 0.991 for the ESP-ELISA.

Figure-4. Analysis of serum samples obtained from subjects with other parasites and correlation between GST-ELISA and ESP-ELISA.

(A & B) Serum samples from individuals carrying parasitic infections other than *F. hepatica* were studied by GST-ELISA and ESP-ELISA, respectively. The study included 14 serum samples from patients with schistosomiasis mansoni (Sm), 6 serum samples of paragonimiasis westermani / kellicotti (Pw/k), 4 serum samples from patients with hydatidosis (Hyd) caused by *Echinococcus granulosus* and 20 serum samples from patients with other parasites (OP) including filariasis bancrofti (n=5), giardiasis (n=3), visceral larva migrans (n=4), trichinelosis (n=4) and toxoplasmosis (n=4). Dashed lines represent the cutoff point that gives the best balance between sensitivity and specificity according to the ROC curves. (C) Correlation between the results of GST-ELISA and ESP-ELISA was determined. Results show that both assays exhibit a moderate positive correlation ($r=0.555$) and substantial agreement (Cohen's $\kappa=0.796$).

Table-1.Protein Isoforms identified in the purified *Fasciola hepatica* GST

Protein ID	Peptide Counts	Fasta headers	MW	Score
P31670	13	GST27_FASHE Glutathione S-transferase class-nu 26kDa isoenzyme 47 OS= <i>Fasciola hepatica</i> PE =1 SV=3	25.412	323.1
P31671	15	GST28_FASHE Glutathione S-transferase class-mu 26kDa isoenzyme 7 OS= <i>Fasciola hepatica</i> PE =2 SV=3	25.327	323.1
P56598	14	GST29_FASHE Glutathione S-transferase class-mu 26kDa isoenzyme 1 OS= <i>Fasciola hepatica</i> PE =1 SV=2	25.729	323.1
V5W6S2	21	V5W6S2_FASHE Glutathione S-transferase isoenzyme 51 OS= <i>Fasciola hepatica</i> PE=2 SV=1	25.364	323.1
Q06A71	10	FASHE Glutathione transferase sigma class OS= <i>Fasciola hepatica</i> PE=1 SV=1	24.53	323.3

Table-2.

Specificity and sensitivity of GST and ESP proteins based on cut-point results obtained from *Fasciola hepatica* infected compared to un-infected subjects.

Antigen	Cut-point	Sensitivity	Specificity	AUC (95% CI)
GST	>0.288	100.0%	74.1%	0.908 (0.857–0.96)
	>0.299	97.1%	74.1%	
	>0.34	94.3%	80.2%	
	>0.376	80.0%	84.0%	
ESP	>0.22	100.0%	86.2%	0.994 (0.984–1)
	>0.69	97.1%	98.8%	
	>0.75	91.4%	98.8%	
	>1.03	80.0%	98.8%	

Table-3.

Results for serum samples from patients with fascioliasis confirmed by coprologic examination (CE), serum samples from healthy subjects, and from persons with other parasitic infections.

ELISA results	CE-positive		CE-negative		Total No.
	Finding	No.	Finding	No.	
GST ^a					
Positive	True-positive	34	False-positive	15	49
Negative	False-negative	2	True-negative	65	67
Total		36		80	116
ESP ^b					
Positive	True-positive	35	False-positive	3	38
Negative	False-negative	1	True-negative	77	78
Total		36		80	116

^aSensitivity = 94.3% calculated as true-positive number/ (true-positive number + false-negative number) × 100; specificity = 80.2%, calculated as true-negative number/(true-negative number + false-positive number) × 100. True-positive samples were positive in CE, true-negative samples were negative in CE, false-negative samples were positive in CE but negative in serological assays, and false-positive samples were negative in CE but positive in serological assays. CE was used as the gold standard.

^bSensitivity = 97.1%, calculated as true-positive number/ (true-positive number + false-negative number) × 100; specificity = 98.8%, calculated as true-negative number/(true-negative number + false-negative number) × 100.