

Efficacy of Purified Glutathione -S- Transfere in Providing Protection against *Haemonchus Contortus* Infection in Sheep

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Abstract

The efficacy of purified glutathione-s-transferase (GST) to protect sheep against haemonchosis was assessed. Fifteen lambs, 3.5-5month-old, were bisected into five groups (n=3). Two groups were vaccinated with 250µg of each prepared antigen (crude adult antigen (CAA), purified GST) in combination with mineral oil adjuvant at zero, 14th days of the experiment. The other three groups were kept as control groups; non-immunized infected, non-immunized non-infected and adjuvant control. One week after the last booster dose, the lambs in all groups except control negative were challenged with 400 L3/kg live weight orally. Protein characterization of each antigen was done by SDS-PAGE and immunoblotting. The vaccine efficacy was estimated by the fecal egg count, where the vaccinated groups showed 58.90% and 79.38% reduction in eggs in CAA and GST respectively. Moreover, 31.45% and 92.10% reduction in abomasal worm count was reported in CAA and GST vaccinated lambs, respectively. The protection was found corresponsive to the sera antibody levels in the immunized groups. It was concluded that the GST had protective efficacy against *H. contortus* infection in lambs.

Keywords: *Haemonchus contortus*; Sheep; GST; Fecal egg count; Worm burden; ELISA

Introduction

Haemonchus contortus (*H. contortus*) is a bloodsucking nematode inhabits the abomasum of sheep and goats. It has been categorized as the most important parasite of small ruminant worldwide [1]. This nematode causes huge financial losses in sheep industry besides altering the well-being of their host [2]. The main way for controlling of such nematode is accomplished by the use of anthelmintic medications that provide >99% efficacy [3]. Nevertheless, among the drawbacks of using this approach include environmental contamination and the drug residues in the marketable product [4,5]. In addition, anthelmintic resistance developed against new successive class of the drug [6]. Consequently, noticeable efforts have exerted towards the development of state-of-the art vaccines [7]. The accessibility of successful antiparasitic vaccine would insert a major novel protective weapon added to the other methods used for the management and control of infection in sheep [8]. Immunization with crude extract of *H. contortus* and proteins purified from it provides a prospective source of protective immunity [9]. The glutathione S-transferases (GSTs) are a multipurpose protein included cellular detoxification and/or by binding to a wide range of toxic molecules [10]. Reviewed parasitic GST for its role in detoxification through involved in removing of endogenous toxic compounds or reactive oxygen species of the host immune-initiated [11]. It also, has a role in transportation or metabolism of the essential materials for parasites life. It plays a critical role in detoxify cytotoxic immune-initiated products of lipid peroxidation [12]. Consequently, its protective effects against some parasites considered it a potential target for pharmaceutical and vaccine purposes [13]. The GST was predominantly abundant in *H. contortus* and has a partial protective effect against its infection [14,15]. Using of GST in different forms has variable protective efficacy against helminths; lymphatic filariasis *Schistosoma japonicum* and *Trichenella spiralis* [16-19]. In addition, other studies showed that GSTs from schistosomes and cestodes are antigenic and immunogenic and provide variable degrees of protection to animals following experimental infection [20,21]. Moreover, used GST antigen for diagnosis of Haemonchosis in sheep [22]. The present study aimed to investigate the potential protective effect of purified *H. contortus* GST compared to the crude adult antigen against challenge with *H. contortus* in lambs.

Material and Methods

Ethical approval

The procedures in all experimental native breed animals were performed in agreement with the recommendations and guidelines stated by the ethical Committee of the National Research Centre under certificate number (16050).

H. contortus isolate

Adult worms of *H. contortus* were collected from all abomasal content of infected sheep at El-monib slaughter house, Giza, Egypt according to [23]. Intact washed worms were incubated at 37 °C for 2-3hr to obtain eggs. Eggs were then used in fecal culture to recover third larval stage.

Crude adult and GST antigens preparation

The crude adult antigen was prepared from the collected worm as stated by [24]. The purification of *H. contortus* adult GST was done as reported by [25]. Briefly, the sepharose 4B column (GE Healthcare., UK) was applied for GST purification. Equilibration of glutathione sepharose was conducted through 4B packed column using Tris buffer (Tris-HCl pH 8). The crude extract of adult worms was dispensed onto the column. Then it was washed to remove unbound fractions by equilibration buffer. The elution buffer (50 mM Tris-HCl, 10 mM Glutathione reduced pH 9.6) was used on the column to elute the glutathione bound fractions in 1 ml fractions. The latter was pooled and subjected to further dialysis concentration using polyethylene glycol (MW 8000). The specific activity of purified GST was determined according to using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate [26]. The CDNB molar extinction is 0.0096 μ M⁻¹/cm. The purified GST was stored at -20 °C until use. The protein amount in the CA and GST antigens was detected according to [27].

Preparation of hyper- immune sera

Nine healthy white New Zealand male rabbits were classified into three groups, each group contained three rabbits; first group was inoculated with CA antigen and second group was injected with GST according to and the third one used as control negative rabbit [28]. Briefly, 200 μ g of each prepared antigens emulsified with an equal amount of mineral oil was injected subcutaneously in each rabbit. After two weeks, two booster dosages were injected subcutaneously with a week interval. After one week from the last booster dose, sera samples were collected and stored at -20 °C.

Western blot hybridization

The different antigens were analyzed under reducing condition on 10% SDS-PAGE each lane was loaded with 70 μ g protein and subsequently stained with Coomassieblue according to the method described by [29]. The protein bands were electro-blotted on nitrocellulose paper as described by using mini trans-blot electrophoretic transfer cell (Biorad, USA) to react with the hyper immune sera against each antigen [30].

Experimental immunization of lambs

Fifteen male native lambs, 3.5–5 months of age (20-25Kg weight), kept in our amenities under strict hygienic measures, were segregated in to five groups. The first and second groups (each of n=3) were vaccinated with 250 μ g of each prepared antigen emulsified in 1ml of mineral oil adjuvant. Booster dose was done with the same dose at 14 day first immunization. One week after the last dose, the lambs were challenged with 400 L3/kg live weight (*H. contortus* third larvae) for each lamb orally with buccesophgeal catheter according to [15]. The third group (n=3) received the mineral oil adjuvant only then subjected to the challenge infection at the main time. The fourth group (n=3) received the challenge infection only as positive control. The fifth group (n=3) was kept uninfected unimmunized as control negative. Sera were collected weekly from each lamb from 0 day until the end of the trial to estimate antibodies titer during the experiment.

Vaccination evaluation

The protection level was evaluated by measuring sera anti- CAA, GST antibody levels, fecal egg output reduction %, worm burden in abomasum and the hematocrit value as described by [31].

Parasitological parameters (Fecal egg count, worm burden and establishment rate): Samples of feces were collected on the expected days for egg appearance (16, 17, 18, 20, 21, 22 post challenge) then, weekly till the end of experiment. Egg count for each animal in the group was carried out weekly, from the onset of egg shedding until the 10th week post challenge. The fecal samples were collected rectally and were examined by using the salt flotation technique then egg count per gram feces (epg) was done through McMaster technique according to [23]. At the end of the experiment, all animals were sacrificed at the local slaughter house. The abomasum were taken in ice tank to the laboratory to estimate the adult parasite burden. The abomasum was washed carefully with warm normal saline solution to eliminate adhering worms and the worm count was done [32]. The establishment rate was calculated by dividing the abomasal worm count upon the infective dose and multiplied by 100.

Immunoglobulins measurement by ELISA: ELISA was performed according to [33]. Antigen concentration and sera dilutions were determined checkerboard titration. Sera samples from immunized lambs were collected at weekly intervals from 0 to 14 weeks of the experiment. ELISA plates were coated with a 100 μ l of each antigen (CAA or GST), which was diluted in carbonate bicarbonate buffer (pH 9.6) as 2 μ g/well and incubated at 4 °C overnight. Tested sera was diluted at 1:100 with dilution buffer (Phosphate buffer saline, 0.05% Tween 20), consequently incubated with a 1:1000 dilution of donkey anti-sheep peroxidase labelled IgG (whole molecule) (Sigma-Aldrich, USA) in (Phosphate buffer saline, 0.05% Tween 20). After washing in PBS-Tween20, 50 μ l of O-Phenylenediamidine (OPD) (Sigma chemicals) was added and incubated in dark place till color appearance. The reaction was hindered by adding 50 μ l / well of 1 N Na OH. The optical densities (OD) were read with a micro-ELISA reader system at 450 nm.

Statistical analysis

All the records (Worm burden, EPG and ELISA titers) were statistically analyzed by using SPSS 16.0 and were expressed as the mean \pm SEM (Standard Error of the Mean). Statistical analyses were carried out by the One Way ANOVA. Differences were considered significant at $p < 0.05$.

Results

Electrophoretic pattern of prepared antigens

Gel electrophoresis of the two antigens indicated various protein bands at different molecular weight. The electrophoretic profile of CAA showed 23 protein bands (175 to 15.5 KDa). While GST antigen characterization gave 2 epitopes with molecular weight 23.2 and 24 KDa. Analysis of the resultant band demonstrated that there was common band between CAA and GST, 24 KDa. The immune-blot reaction against rabbit sera showed that antigenic bands recognized positive sera using CAA contains 13 immunogenic reactive bands (175 to 18.6 KDa) while GST antigen has 2 bands (24.6 and 26 KDa). The common band in immunoblotting between CAA and GST was 24.6 KDa (Figure 1).

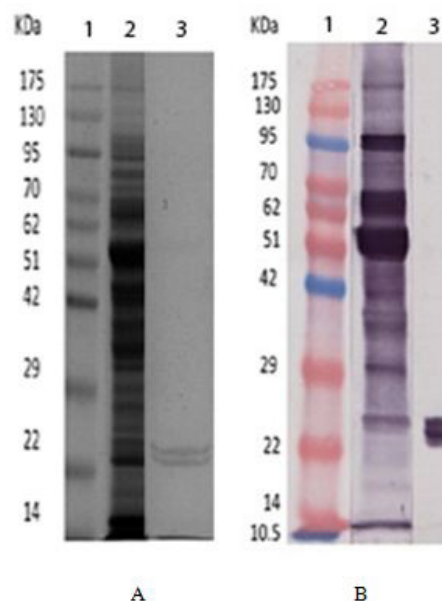


Figure 1: (A) The protein profile of CAA and GST antigens demonstrated by 10% SDS-PAGE. Lane M, M.W pre-stained protein marker in KDa Lane 1, CAA antigen, lane2, GST antigen; (B) Western blot analysis of CAA and GST antigens (Lane M, M.W pre-stained protein marker in KDa, Lane1, CAA, Lane 2, GST antigen)

Vaccination evaluation

Parasitological parameters: The egg shedding in feces appeared at the 18th dpi in adjuvant and control infected unimmunized groups. While in CAA and GST immunized groups, the eggs appeared at the 28th and 22nd dpi, respectively. The mean count of eggs per gram feces (epg) in control groups reached the peak at the 6th week post challenge. While the peak in egg count in immunized groups reached at 7th week post infection. From the 5th week post infection, there was a significant difference in egg count until the end of the experiment between the vaccinated groups ($p < 0.001$) (Figure 2). The reduction in epg was 59.90% and 79.38% in CAA and GST immunized groups, respectively (Table 1). Regarding, the cumulative FEC, vaccinated lambs had lower values than control groups (adjuvant and infected) lambs (Table 1). GST vaccinated animals had the lowest elimination in compared to the CAA vaccinated lambs. Furthermore, the GST immunized group showed 92.10% reduction in worm burden and it was significantly higher than CAA immunized group (31%) (Table 2). Moreover, the establishment rate was significantly low in GST group (2.13%) in comparison with other groups.

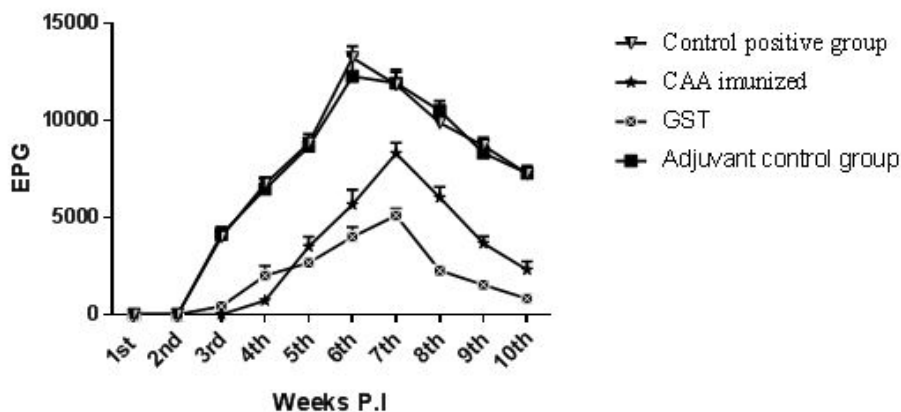


Figure 2: Egg count in all groups along the experiment, egg appeared from the 18th day post infection in control groups (infected non-immunized and adjuvant). It appeared at the 28th day PI in the CA immunized group and at 22nd day PI in GST immunized lambs

Item	CAA immunized group	GST immunized group	Adjuvant control group	Control infected non-immunized group
Cumulative egg count	31079+3400	15601 +2350	68632 +7570	75603+7860
Reduction % in egg count	59.90	79.38	9.22	0.00

Table 1: Cumulative fecal egg count along the experiment and reduction percentage in the egg count in different groups

Group	Female worms	Male worms	Total worm burden	Establishment rate (worm burden/infective dose) %	Worm burden reduction %
CAA immunized group	1060.6±6.2 ^a	630.3±7.3 ^a	1700.0±12.5 ^a	21.25	31.45
GST immunized group	110.3±.3 ^b	60.3±.3 ^b	170.6±0.3 ^b	2.13	92.1
Adjuvant control group	1250.0±14.4 ^c	940.6±5.2 ^c	2190.6±19.2 ^c	27.40	11.45
Control infected non-immunized group	1400.0±11.6 ^c	1080.0±9.9 ^c	2480.0±21.4 ^c	31.00	0
P	<0.001	<0.001	<0.001	<0.001	

Small letters represent differences between groups in the same column

P= probability value between groups

CAA= Crude adult antigen

GST= glutathione -S- transferase antigen

Table 2: Mean of worm burden reduction percentage after post-mortem examination of experimentally infected sheep ± stander error

The immunoprotective efficacy detection against used antigens by ELISA: Immunization with GST and CAA elicited a strong IgG antibody (Ab) specific response against GST & CAA in the vaccinated groups (Figure 3). The antibody titer increased by time

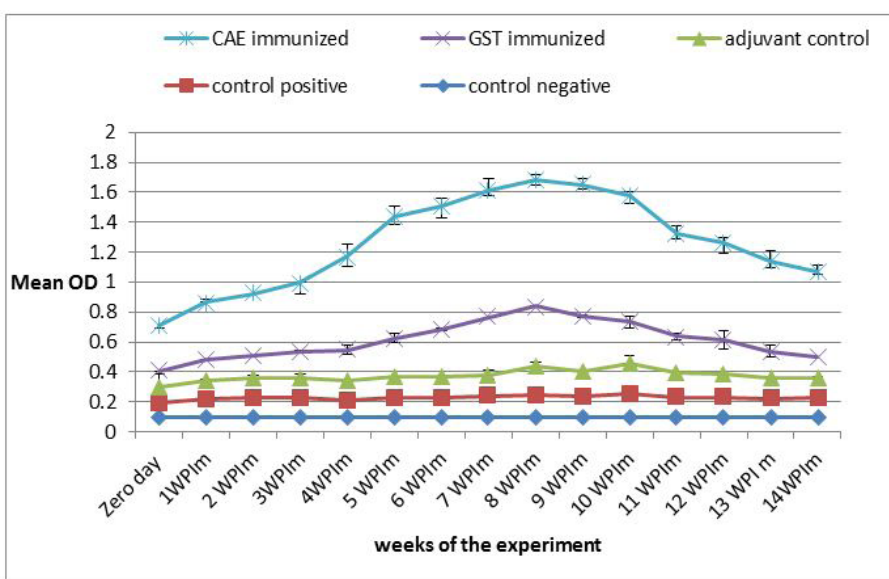


Figure 3: ELISA mean optical density of CA immunized, GST immunized, control positive group and adjuvant control groups for detection of antibody levels at zero day, after 3 week post immunization (WPI m) and a weekly intervals until the 10th weeks post infection

until reach the peak at the 4th and 5th weeks post infection in GST and CAA respectively then declined at the end of the experiment. The immunized lambs had significantly Ab levels higher than infected unimmunized one ($p<0.05$). Moreover, CAA vaccinated lambs were significantly different to non-vaccinated group through the whole period of the experimental. While, GST immunized lambs showed a less sustained Ab response in compare to CAA. In addition, the infected unimmunized group did not elicit any significant Ab titer without any difference with uninfected control group. In addition, the OD highest average value was found in the lambs receiving CAA.

Discussion

In present study, glutathione affinity chromatography successfully isolated GST protein from *H. contortus*. In addition, the immunoblot reaction showed common band between CAA and GST (24.6 KDa). This result was similar to GST of other helminthes; it was as a single band for *Ancylostoma. caninum* (24KDa) and was 24 KDa, 26 KDa and 24 KDa for *Hymenolepis diminuta*, *Fasciola hepatica* and *H. contortus* L3, respectively [12,34]. The GST antigen was used in early diagnosis of *H. contortus* infection and they added that GST has an immunogenic protein with detection of Abs as early as two weeks post-infection [22]. However, the SDS-PAGE and western blot of GST showed a band around 24 KDa. This band was considered an immunogenic band as it stimulated IgG production [22,35,36].

The previous studies on GSTs from helminthes were documented its induction of immune protection, *Fasciola hepatica*, *Seteria cervi* and *Echinococcus granulosus* [37-39]. Therefore, here in, a trial for lamb vaccination by purified GST of *H. contortus* was conducted. The results showed reduction of worm burden percentage and egg count for CAA immunized group (58.9% and 79.38% respectively). In the same time, GST immunized group caused major reduction in worm and epg (92.10% and 79.38% respectively). Moreover, the pre-patent period extended to 28 and 22 dpi in CAA and GST immunized groups respectively in comparison to infected unimmunized lambs and adjuvant control groups. Our results found that the immunized sheep were partially protected against haemonchosis. These results were in accordance with results of previous studies in lamb's immunization by adult somatic, larval and excretory secretory antigens [8,22,40,41]. The GST of other helminthes recorded remarkable results of protection; for *Setaria cervi* for *Schistosoma*, *Wuchereria bancrofti*, *Fasciola gigantica* and *N. americanus* [16-18,42,43]. Recently, clinical trials were conducted using the Na-GST-1 from *Necator americanus* in Brazilian and American volunteers [44,45]. On contrast, no protection was reported when used crude adult extract [46]. Moreover, ineffectiveness of GST was recorded in vaccination trial against *H. contortus* [47]. Additionally, *H. contortus* GST concerning binding and/or transporting heme-related compounds and hadn't protective effect in parasite infection [35]. They added that the lack of protection attributed to production of low titer of anti GST antibodies. We think that the latter finding of controversially may be refer to the method of GST preparation in which herein it is purified GST.

Referring to the abomasal worm burden is among the important parameters that categorized to evaluate any vaccine trial against *H. contortus*. Herein, it is high (92.10%), this result was higher than recorded by who recorded it was 71% by using rHc23 vaccination [31]. It is constant that GST is an essential protein for parasite development and survival. Where found that *H. contortus* L3s resist desiccation due to Hc-gst protect it from oxidative damage via enzymatic antioxidant defense mechanism. In addition, GST is belonging to a well-known family of cytosolic enzymes involved in detoxification [48,49]. Furthermore, the reduction in adult abomasal worm count may be referring to the anti-GST antibodies could be neutralized GST enzyme activity leading to a significant disturbance in its function. The detoxification of heme is corrupted. The oxidative iron in heme damages the parasite which leads to reduction in worm burden in the immunized animals [34,50]. Moreover, the schistosome GST is accessible to host immune system due to it is an excretory secretory protein [51]. Additionally, the low epg and reduced worm burden attributed that the vaccine acts on the immature stages rather than adult worms. This augmented by who said that the vaccination did not perfectly get rid of adult nematodes from vaccinated animals, but it caused decreasing pasture contaminations by infective larvae and subsequently decreasing the level of reinfection [52]. Thus, neutralizing the activity of GST may be a strategy to induce protection against some parasitic infections. Interestingly, there are some studies on GST of other helminthes of fantastic results. For example, used Na-GST-1 from *N. americanus* as a vaccine which currently in phase 1 clinical trial in healthy adult volunteers in the United States and Brazil. In contrast, obtained 35.71% reduction in adult worms in mice immunized with rTsGST of *Trichinella spiralis* [45,53]. This low protection rate referred to the TsGST is a cytoplasmic protein, not an ES protein, and of low antigenicity which reflected in sufficient antibodies production by vaccination. The current results showed that immunized lambs by CAA and GST elicited high levels of circulating anti-GST *Haemonchus* IgG. As reported previously, antibodies might have a function in protecting immunity against *H. contortus* [54-56]. The purified antigen induced the highest effect in minimizing number of worm burden and egg count among the tested antigens. It is worthy to mention that an absence of detectable specific anti-*Haemonchus* Ab after challenge in non-immunized lambs. This matched with previous observations of [42,31]. Since no significant difference between unimmunized infected and adjuvant groups in egg count and worm burden. This denotes that the protection is due to the vaccination by GST or CAA, which was dependent on the used antigens [31].

Conclusion

In conclusion, depending on the efficacy criteria of *H. contortus* vaccine that pronounced by which must have reduction in the cumulative fecal egg count and adult abomasal worms post challenge in addition to serum anti-GST antibody levels [31]. These

criteria were found in the lambs vaccinated by purified GST. Therefore, the present results confirmed the significant effect of GST vaccine on protection of lambs against haemonchosis. Finally, a commercial vaccine is now available to *H. contortus* in sheep, Barbervax vaccine, was launched for commercial use in October 2014 (barbervax.com.au). This action gives us hopefulness for further development of another vaccine for Haemonchosis from GST.

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