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Enzymatic and fecundity evaluation of *Fasciola hepatica* exposed to different doses of γ- irradiation in Ethiopian sheep

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Abstract

The upshot of γ-irradiated *Fasciola hepatica* infection on the activity of plasma glutamate dehydrogenase (GLDH) and γ-glutamyl transpeptidase (GGT) was evaluated in 36 sheep infected with a single dose of 30, 60, 120, and 240 grays and those kept as negative and positive control over 17 weeks. During this period, serum and faecal samples, as well as body weight gains, were taken at weekly intervals. Furthermore, the effects of the irradiation dose (500) for oral vaccination and on the recovery of adult flukes were assessed following primary infection. Eggs were first detected in the faeces of infected sheep on week 8 post-infection. The parasite viability was severely affected by doses of γ-irradiation of 120 Gy or 240 Gy. In the aforementioned doses, relatively low numbers of mature flukes of about 60 (17.1%) and 38 (10.8%) were recovered than the control group, respectively. The sensitized lambs also showed less hepatic damage compared with the controls as indicated by lower levels of the serum enzyme glutamate dehydrogenase and γ- glutamyl transferase significant body weight loss was observed between weeks 6 and 8 post-infection followed by a steady increase of the mean weight of infected animals across time. In conclusion, vaccination of sheep with γ irradiated metacercariae of *F. hepatica* appeared to affect the number and development of the fluke population resulting in reduced hepatic damage during migration, reduced fecundity after patency, as measured by worm and egg counts, levels of serum glutamate dehydrogenase and γ- glutamyl transferase.

Keywords: Gamma irradiation; *F. hepatica*; immune response; liver damage; worm and egg recovery; body weight changes.

Introduction

Fasciola hepatica is a trematode parasite with a global distribution, which is responsible for considerable disease and production losses. The commonly known species of *Fasciola* in tropical and subtropical countries is *Fasciola gigantica* whereas *Fasciola hepatica is* found in countries with cold and temperate climates where both species overlapping in subtropical areas (Urquhart *et al*., 1996; Yilma and Malone, 1998; Mas-coma *et al.*, 1999; 2005; Tadesse *et al.*, 2019a). In Ethiopia, *F. hepatica* is mostly associated with disease in sheep, cattle, and goats (Yilma and Malone, 1998; Biffa *et al.*, 2006; Tadesse *et al.*, 2019a, b).

The findings of the abattoir study are inadequate to reveal information regarding the development of the disease in live animals. However, data gathered is useful to disclose the cross-sectional studies (prevalence's, seasonal variations), liver damages, fluke examinations and measurements, assessment of liver fluke burden, and economic importance of the disease (Yilma and Malone, 1998; Biffa *et al.*, 2006; Tadesse *et al.,* 2019a, b).

The presence of eggs in feces is the best way to know if a host is harboring flukes in the live animal. False positives may occur due to the retention of eggs in the gall bladder for at least 2 weeks after successful treatment. Furthermore, it has shown daily variation in egg counts and a maximum rate of egg recovery of only 45-60% (Flanagan *et al.*, 2011; Rojo-Vázquez *et al.*, 2012; Sargison *et al.*, 2016).

Irradiation has been successful in the attenuation of the infective stage of the parasites for use as vaccines against a number of parasites including *Fasciola spp*. Some studies had revealed that the optimal protection was found to be dependent on the dose of irradiation, the number of immunizing cercariae, and the number and time course of immunizations (Minard *et al*., 1978). The evolution of hepatic enzymes during the infection is shown to be important in assessing the level of liver damage by infective flukes. Most work on methods using blood parameters has focused mainly on the determination of enzymes particularly aspartate aminotransferase (AST) and gamma- glutamyltrans-

peptidase (GGT) (Simesen *et al*., 1973; Vyckoff and Bradley, 1985; Urquhart *et al.,* 1996; Taylor *et al*., 2007).

Recovered liver fluke length and width, the total mass of recovered flukes, and liver damage are the major factors often used to indicate the protection against the severity of parasite infection (Valero *et al*., 2002; Valero *et al*., 2006). A detailed study to generate data on all aspects of the host-parasite relationship is still required to better understand the disease dynamics and identify ideal irradiation doses for experimental vaccination trials. Therefore, this study aims to evaluate the enzyme profiles (GLDH and GGT), the enumeration and fecundity assessment with egg counts of recovered flukes as well as assessment of liver and body weight changes in sheep ɣ- irradiated by different doses of *Fasciola hepatica* metacercariae for its vaccination potential.

Materials and methods

Study area

The infective metacercariae of *Fasciola hepatica* used in the experimental sheep have been obtained from artificially infected *Galba truncatula* snails collected from water at Angolela river and villages situated in fasciola endemic areas around Debre Berhan city. The city is located in the Semien Shewa Zone of the Amhara Region, about 120 kilometers northeast of Addis Ababa on the paved highway to Dessie. It has a latitude of 9°41′N and longitude of 39°32′E and an elevation of 2,840 meters. The experiment was conducted at Aklilu Lemma Institute of Pathobiology.

Fasciola hepatica infective metacercariae

F. hepatica metacercariae were produced in laboratory reared snails of *Galba truncatula* from river Angolela. The viability of the metacercariae of *Fasciola hepatica* was checked by stimulation of the metacercariae by artificial digestion before dosing of sheep (Wikerhause, 1960). The experiment consists of 36 six-month-old local breed sheep divided into six groups of animals each. The first four groups were vaccinated with 500 *F. hepatica* metacercariae per os with an increasing level of irradiation dose. The fifth and sixth groups were served as infected control and negative control, respectively. The irradiation doses used were 30, 60, 120, and 240 grays for groups I, II, III, and IV, respectively (see Table 1). The gamma irradiation process uses Cobalt 60 to attenuate the

metacercariae of *F. hepatica*, which has been widely used by SIT (Sterile Insect Technique) project located in Kaliti, Addis Ababa. The 500 metacercariae were separately counted under a dissecting microscope. Administration of the doses was done by giving in a gelatin capsule as described in (Boray, 1983) and (Wikerhause, 1960). At the time of infection, metacercariae were 2 months old and had 82% viability. It was assessed by using the criteria of works done that depict the motility of the worms and clear observation of the excretory granules in some cysts under dissecting microscope.

Table 1. Details of experimental groups exposed to various levels of irradiation doses

Group	Number of sheep	Number of parasites (Metacercarae)	Irradiation dose (Killo radiance or Gray)
I	6	500	3 (30)
II	6	500	6 (60)
III	6	500	12 (120)
IV	6	500	24 (240)
Infected Control (PC)	6	500	-
Non-Vaccinated	6	-	-

Study animals

The animals used in this experiment were the local breed of sheep. They were purchased from the market in the Gojo district in central Ethiopia. Gojo is situated in the West Shewa zone in the Oromiya region of Ethiopia with geographical coordinates of 9° 16' 0" North, 38° 5' 0" East, and with an altitude of 2905 meters above sea level. The sheep were brought to the market from the surrounding areas of Gojo town. The area was supposed to be free or pose a very low risk for fasciola species. The sheep breed was the Arsi Bale type which has a wider distribution in the central and southern parts of the country and is susceptible to fasciola species (Alemu and Merkel, 2009).

Study design and sampling

The study was a randomized controlled experiment where sheep were selected based on approximate similarity in estimated age, body weight, and sex (all male). The sheep were matched and randomly allocated into groups consisting of six animals each to begin the experiment. Upon arrival, all the sheep

have been treated with Triclabendazole (Fasinex, 10-12 mg/kg) to preclude any fluke infection and Oxytetracycline (10mg/kg) to control opportunistic bacterial diseases. All the animals were kept for about a month before the commencement of the actual experiment. Animals were tested for the presence of internal parasites and fasciola species before the beginning of the experiment using simple floatation and sedimentation methods, respectively. These tests have been done throughout the adaptation period. The sheep were maintained with concentrate and well-dried hay free of parasites. Each sheep were provided with 25gms of concentrate (75% wheat bran and 25% Nug cake) and with hay and water provided ad libitum. Sheep were weighed twice before and every other week after the commencement of the experiment. The body and liver weight of each animal was measured using a standard weighing scale at a weekly interval. Each liver was placed in a large basin after evisceration for subsequent weighing. The changes in body weight gain and loss recorded in different groups were compared to determine the effect of vaccinations. Blood samples for biochemical analysis were collected from the external jugular vein into marked vacuum tubes and transported at +4 °C to the laboratories every week starting at week one till the end of the experiment. The serum was separated from blood samples and collected into Eppendorf tubes to be stored at -20°C before analysis. The blood tubes were centrifuged at 3000 rpm for 10 minutes for serum separation.

The faecal sample was examined at the weekly interval after 8 weeks of the experiment. For the estimation of prevalence fresh faeces were randomly collected regularly, directly from the rectum of all animals, and transported to the laboratory in an airtight condition. The specimens were then subjected to qualitative and quantitative coproscopic examination for the presence of characteristic *Fasciola* eggs and egg counts by direct sedimentation technique employing a standard procedure (Urquhart *et al*., 1996). At the end of the experiment, a postmortem examination was carried out on all animals. The bile ducts and liver parenchyma was examined and the number and size of adult flukes inside were observed and counted.

Spectrophotometric analytical methods

Due to the poor stability of enzymes, the serum samples were analyzed within 6 hours after collection. The activity of liver enzymes γ-glutamyl transferase (GGT) and glutamate dehydrogenase (GLDH) were conducted spectrophotometrically to assess liver damage using commercial kits (Chemical analyser)

and adjusted to 37ºC. The levels were expressed as international units per liter (IU/L). The enzymes used are a product of Bio vision Inc.

Statistical analysis

The data collected from each group (number and size of parasites, enzyme parameters) were compared by two-way analysis of variance (ANOVA), followed by Tukey post hoc test for differences between the means of control and experimental samples at each time interval.

Results

Dynamics of hepatic enzymes

The dynamics of hepatic enzymes during the experimental period on sheep γ-irradiated by metacercariae of *F. hepatica* was shown in Figures 1 and 2 below. Animals exposed were responded well to the infection as shown by the evolution of hepatic and gall bladder enzymes. The enzyme level varies with the dose of γ-irradiation and non-sensitized groups of animals. Generally, irrespective of immunization, all the infected animals exhibited increasing trends in the GLDH and GGT levels from week 2 onward post-infection. In all cases, the uninfected control sheep (NC) maintained a low profile of enzymes that were well within the normal ranges of each enzyme (GLDH= 1-12; GGT= 34-100 IU) in sheep.

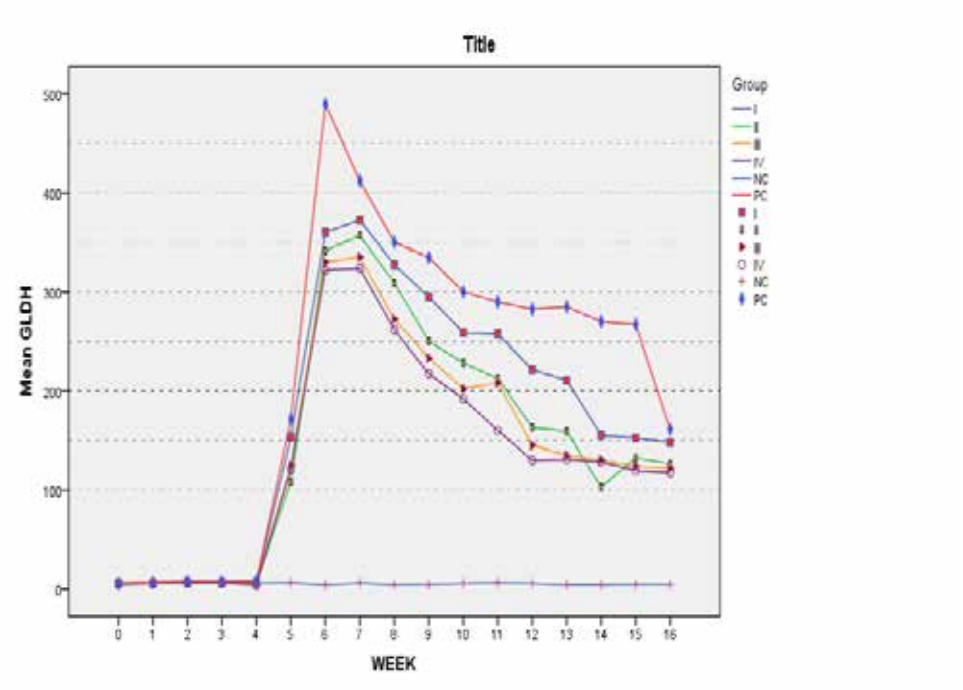

Fig 1. The weekly dynamics of GLDH during the experimental period in sheep

The mean GLDH level of positive control sheep (489.67±16 IU) was significantly (P <0.05) higher compared to the other vaccinated groups at GI (360±9 IU), GII (356.6±8 IU), GIII (335.3±8 IU), and GIV (324.4±12). The comparative progressive decline in the mean levels of serum GLDH of the infected control sheep observed between weeks 6 and 16 was in line with the decline observed in all other treatments.

All vaccinated groups had maintained the serum GLDH level greater than 100 IU at week 16 post-infection. However, sheep that were dosed with 30 Gy (minimum γ-irradiation dose used in the present experiment) had shown significantly higher (P<0.05) mean serum GLDH level between weeks 5 and 16 compared to the relatively lower levels of other treatment groups during the same period.

In the group of sheep dosed with 60, 120, and 240 Gy the mean serum GLDH level lies in a range of 100 and 150 IU after week 6 post infections. However, these values were relatively stable between weeks 12 and 16 post-infection, regardless of a statistically insignificant (P>0.05) difference between the groups. On the other hand, animals vaccinated with a low dose of 30 Gy kept the stable serum GLDH level in the range of 150 - 200 IU between weeks 14 and 16 post-infection. The serum GLDH profile of non-sensitized positive control sheep reached a minimum level of 200 IU only at week 16 following a sharp decline from 267± at week 15 to 200 at week 16 post-infection. The more pronounced changes in levels of the enzyme at positive control and lower dosed groups indicated a relatively higher level of damage to the liver.

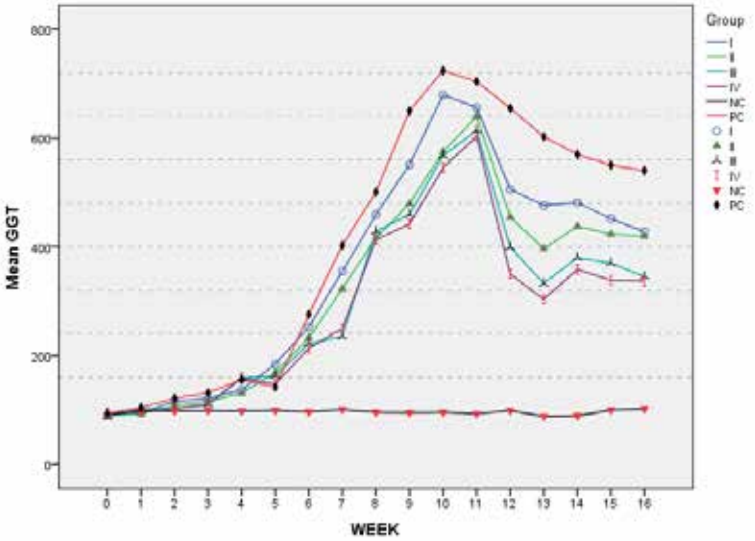

Fig 2. The weekly dynamics of GGT during the experimental period in sheep

The mean serum γ-glutamyl transpeptidase (GGT) concentrations remained altered in all infected sheep except the negative control. In this regard, the mean levels of the enzymes persistently increased from week 5 post-infection onward until the end of the experiment. After week 5 there was no much fluctuation of the enzyme profile among the treatment groups except that levels were raised till week 11. The difference in GGT levels of all infected sheep with respect to the positive control was significant (P>0.05), but these were more pronounced between weeks 10 and 11. In all groups, infected animals had exhibited peak levels of GGT concentrations between weeks 10 and 11 across

time. However, after week 12 to the end of the experiment, all infected sheep had invariably maintained relatively raised constant GGT levels. The difference in the mean peak values of GGT between positive control and other treatment groups as well as between group I and groups II, III, and IV at weeks 10 and 11 were significant ($P< 0.05$).

The discharge of fluke eggs in the faeces and the EPG

All the vaccinated and infected control groups were found to shade eggs except the uninfected negative controls. Eggs were seen to shade starting from the eighth-week post-infection. These were first observed in infected control groups (PC) and those vaccinated with a low dose of the parasite (GI dosed with 30 gray and GII sheep with 60 grays). The animals in high-dose vaccinated groups (GIII received 120 grays and GIV dosed with 240 grays) began to shade eggs only after week 9 post-infection. The uninfected control animals (NC) were never appeared to excrete eggs. The eggs discharged by all infected and vaccinated animals continued to be recovered until the end of the experiment 17 weeks post-vaccination. Higher EPG output was observed in infected control (PC) followed by those animals vaccinated with low doses of the gamma irradiation (GI and GII). The EPG of all groups steadily increased with time (Figure 3).

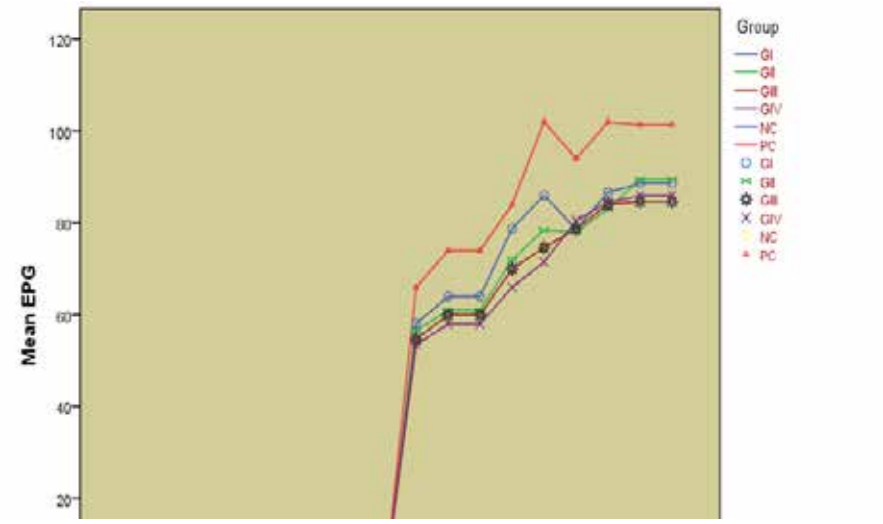

Fig 3. The eggs per gram of faeces in different experimental groups across time

The EPG of all groups steadily increased with time. The pattern of EPG raise was similar in all sheep that had received the irradiated infective metacercariae and those that were infected with the same number of a non-sensitized normal fluke. The clear differences in EPG output were noted between week 12 and 15 post-infection. Infected control sheep had shown significantly (P<0.05) higher EPG level reaching peak 13 post-infection compared to the group of sheep that received different levels of gamma sensitized metacercariae.

The higher EPG of eggs (108) were recorded for positive control animals at the end of the experiment, 17 weeks post-vaccination than the other groups. However, the EPG of higher-dose animals (GIII and IV) appeared lower than those vaccinated with lower doses (GI and II). In all cases, a slight variation of EPG was observed between those higher dosed groups of animals in GIII and GIV. Similarly, the EPG of GI animals was relatively higher than that of GII from the lower dosed animals (Figure 3). Generally, with the exception of infected control animals, a relatively nonsignificant EPG output was induced following vaccination with 500 metacercariae attenuated with 240Gy of γ-irradiation (GIV), or attenuated with 120Gy (GIII) of γ -irradiation compared to metacercariae γ-irradiated at 30Gy and 60Gys. These findings disclosed the effect of the degree of parasite attenuation (dose levels) on starting time of egg shedding and the level of EPG output in sheep irradiated with gamma cells provided, the levels of irradiation-attenuated infective stages were kept constant (500 metacercariae).

Fluke recovery and measurements

Out of the 500 flukes administered per os to each group, the mean (M± SE) number of flukes recovered varies significantly (P<0.05). The present finding indicated that the higher the irradiation dose, the lowest was the recovery of flukes in necropsy examinations (Table 2). That means a strong dose-response was evident in the number of parasites recovered in the groups dosed with incremental doses of γ-irradiation of *F. hepatica* metacercariae. The recovered parasites were morphologically normal, patent, immature, and adult liver flukes. The percentage of the flukes recovered (percentage reduction in fluke burden) unirradiated from the liver of the sheep were highest in infected control groups (22.5%; 112.3±4.3) followed by GI animals dosed with the lowest irradiation dose (18.6%; 93± 3.4). The percentage reduction in the burden of flukes in group II and III sheep was 13.4% (67.8 ± 2.8) and 11.2% (56.5± 7.18) respectively. Similarly, those animals in the highest irradiation dose (GIV)

yield the lowest recovery (7.7%, 38.3± 3.3). Accordingly, the higher the irradiation dose, the lowest was the recovery of flukes in necropsy examinations.

A total of 350 (82%) recovered fluke lengths were measured from sheep in all infected (PC) and vaccinated groups. The overall number of flukes counted from the positive control group was significantly higher (P<0.05) than other vaccinated groups of the experimental animals.

Table 2. The number of metacercariae administered per os and the flukes recovered in different vaccination groups at necropsy

Group	Number of animals	Number of parasites (met)	Irradiation dose (gray)	Fluke recovery				
				Mean ±SE	%	Max	Min	Range
I	6	500	30	93± 3.4	18.6	103	80	23
II	6	500	60	67± 2.8	13.4	75	60	15
III	6	500	120	56.5± 7.18	11.2	75	30	45
IV	6	500	240	38.3± 3.3	7.7	50	30	20
Infected Control (PC)	6	500	-	112.3± 4.3	22.5	123	100	23
Non-Vaccinated	6	-	-	-	-	-	-	-

A relatively large number of flukes were recovered and measured (98; 28%) from sheep in the positive control than the vaccinated groups. The number of flukes measured in groups GI, GII, GIII, and GIV was 77 (22%), 77 (22%), 60 (17.1%), and 38 (10.8%) respectively. The length of flukes recovered ranged from 0.30cm to 2.3cm. The mean length of *Fasciola hepatica* recovered in the positive control group was 2.2cm. The corresponding length in GI, GII, GIII, and GIV was 2.1, 1.8, 1.6, and 1.5cm respectively. However, in all the infected control and vaccinated groups the majority (Mean + SEM) of flukes recovered (69 ± 5.1) had lengths between 1.3cm and 1.5cm. Similarly, a significant number of flukes had a length greater than 1.6cm and less than 1.9cm (66 ± 4.74). In all treatment groups, the mean number of those flukes with lengths less than 0.5cm (43 ± 8.3) and more than 2cm (57) was relatively small. The mean number of flukes measuring less than or equal to 1cm was significantly lower in the group infected with high irradiation dose in GIV (15 ± 1.3) and GIII (27 ± 3.1) respectively in that order. However, the reverse is true for animals that received parasites exposed to lower irradiation dose in GI (37 ± 2.1) and GII

(37 ± 3.4). The overall number of flukes counted from the positive control group was significantly higher (P<0.05) than other vaccinated groups of the experimental animals (Figure 3).

Similar trends were recorded on the width of flukes recovered corresponding to their lengths where the widths of the flukes had seen to rise with the increasing length of liver flukes. The width of flukes recovered ranged from 0.30cm to 1.2cm. However, in all the infected control and vaccinated groups the majority (Mean + SEM) of flukes recovered (68± 5.1) had widths between 0.7cm and 0.9cm. Similarly, a significant number of flukes had a width greater than 0.5cm and less than 0.6cm (66 ± 4.74). In all treatment groups, the mean number of those flukes with lengths less than 0.3cm (43 ± 8.3) and more than 1.2cm (53) was relatively small. The mean number of flukes measuring less than or equal to 0.5cm was significantly lower in the group infected with high irradiation dose in GIV (10 ± 2.3) and GIII (16 ± 2.1) respectively in that order. However, the reverse is true for animals that received parasites exposed to lower irradiation dose in GI (23 ± 3.1) and GII (23 ± 3.4). The overall number of flukes counted from the positive control group (29 ± 5.2) was significantly higher (P<0.05) than other vaccinated groups of the experimental animals (Figure 4). These findings are an indication for strong host immunological responses at higher gamma-irradiated groups of animals (GIII and GIV) than those who received parasites exposed to low irradiation and uninfected controls (PC).

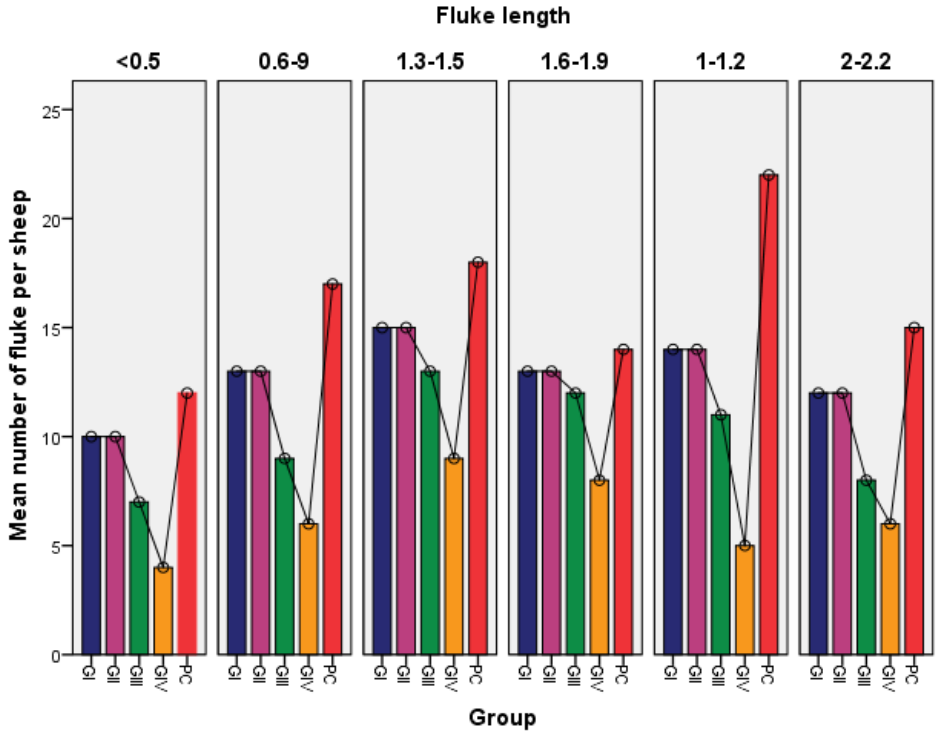

Fig 4. The number of flukes recovered and the measurement of fluke length (cm) in different treatment groups

Similar trends were recorded on the width of flukes recovered corresponding to their lengths where the widths of the flukes had seen to rise with the increasing length of liver flukes. The width of flukes recovered ranged from 0.30cm to 1.2cm (Figure 4).

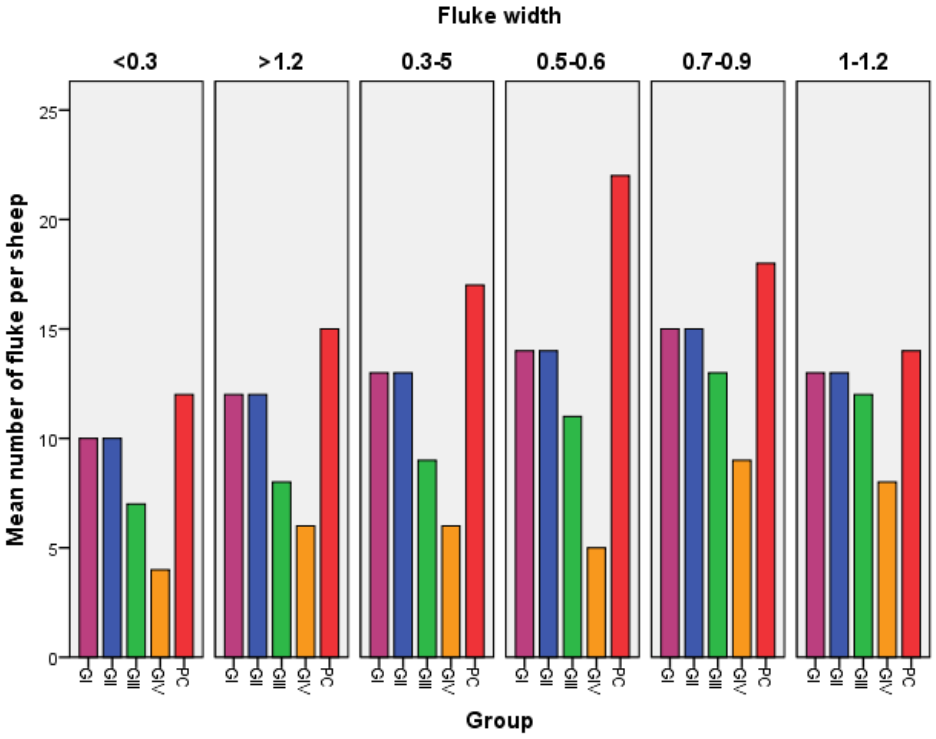

Fig 5. The number of flukes recovered and the measurement of fluke width (cm) in different treatment groups.

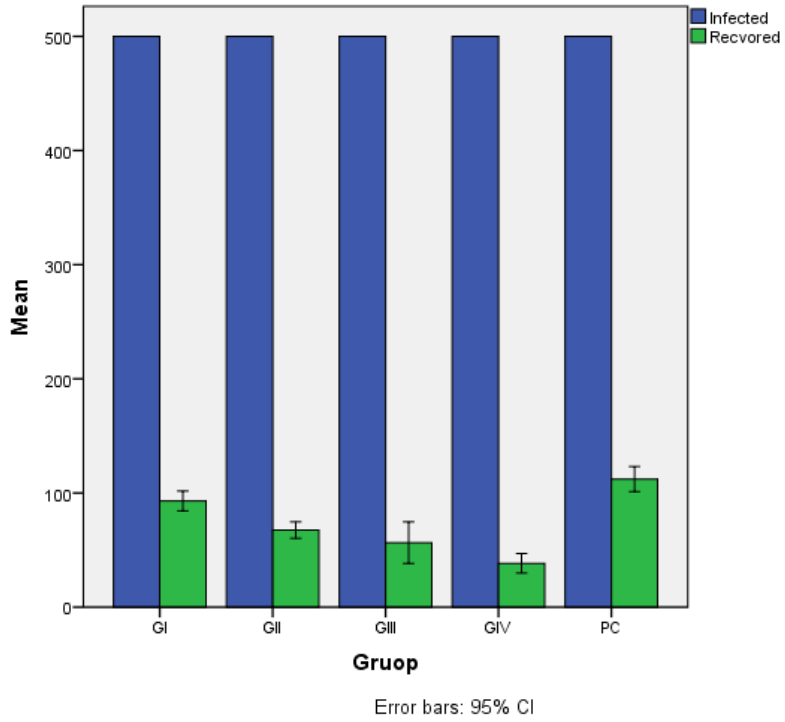

Fig 6. The number of flukes recovered from the different treatment groups at end of the experiment

Table 3. The mean Fluke length, width, and mean liver weight of infected and control groups

Group	Mean number of fluke ± SEM	Mean Length ± SEM (cm)	Mean width ± SEM (cm)	Mean liver weight ± SEM (gm)	Mean differences of liver weight (PC-other groups) ± SEM (gm)
PC	112.3± 4.3	2.3 ± 0.2	1.75 ± 0.1	396	Reference
GI	93± 3.4	2.1 ± 0.3	1.5 ± 0.2	378	18 ± 2.3
GII	67± 2.8	1.8 ± 0.1	1.3 ± 0.1	368	28 ± 3.4
GIII	56.5± 7.18	1.6 ± 0.1	1.2 ± 0.1	356	40 ± 2.3
GIV	38.3± 3.3	1.5 ± 0.15	1.1 ± 0.1	348	48± 3.3
NC	-	-	-	325	71 ± 2.5

The liver weight and the irradiation dose

The results disclosed the difference in the mean change of the liver weight between the infected and control groups at necropsy (Table 3). The highest mean liver weight changes were observed between positive and negative control groups with a difference of 71± 2.5 g and this was significant (P<0.5%). Comparison between highly dosed groups at GIV with that of positive control had shown a mean difference of 48± 3.3 g. This was followed by the mean difference of 40± 2.3 g with that of group III animals. The relative comparative mean live weight changes recorded for GI and GII were 18 ± 2.3 and 28 ± 3.4gm. Generally, the liver weight relatively tends to decline with the progressive increase in irradiation dose and subsequent decrease in the recovery of the fluke and reduction in the size of *F. hepatica* at necropsy examinations.

Bodyweight changes

Comparison of the relative mean weight gain (M± SD) of highly dosed GIV (20.8±3.5kg) sheep and non-infected controls (22.5±4.5 kg) had detected non-significant (4kg) differences (P<0.05). Similarly, relatively lower but statistically insignificant (3.7kg) difference was recorded between uninfected control (22.5±4.5 kg) and group GIII (20.8±3.5kg) animals. The variation detected in mean weight gain between negative controls (22.5±4.5kg) and that of lower dosed group GI (18.8±2.3kg) and GII (18.6± 2.1kg) sheep were significant with a difference of 7.1and 6.9kg respectively. However, the relative difference (8.1kg) was more pronounced between negative control (22.5± 4.5) and infected control (18.4± 0.96) groups. Generally, the mean weight gain of all groups tends to increase with time despite the differences in weight gain of the treatment groups used for the vaccine trials. Despite the nearly linear increase in the mean weight gain of positive controls and the groups received the lower irradiation doses of 30 and 60 gray, a significant body weight loss was observed between weeks 6 and 8 post-infection followed by a steady increase of the mean weight of infected animals across time (Figure 7).

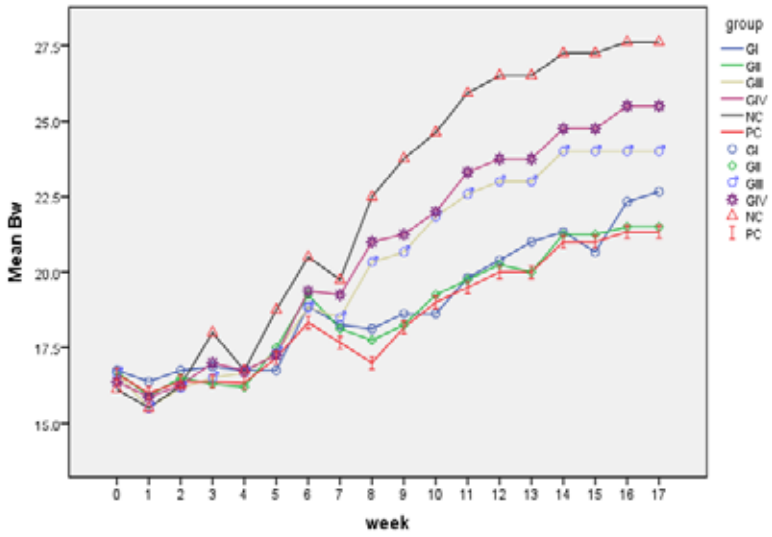

Fig 7. Bodyweight changes of sheep vaccinated with different doses of *Fasciola hepatica* **across time**

Discussion

To deter the detrimental effects of pathogens to the host, huge successes have been made in vaccine development against viruses and bacteria over the past several years. Except for the live attenuated Huskvac vaccine for lungworm, there are no commercially viable vaccines for animal helminth parasitic pathogens (McKeand, 2000). Recent advances in vaccination with recombinant helminth antigens have been successful against cestode infections of livestock and new vaccines are being tested against nematode parasites of animals (Hewitson and Maizels, 2014). More recently, a new vaccine for *H. contortus*, Barbervax, consisting of native gut-derived antigen complex has been launched in Australia www.barbervax.com.au.

Attenuated antiparasite vaccines enable the host to mount a protective immune response against the organism without the development of the pathological symptoms of infection. Despite the practical nature of this strategy, several irradiation-attenuated parasite species have been used experimentally to induce protection in various host species (Smith *et al.*, 1993), including cattle

against infection with the liver fluke, *Fasciola hepatica,* and sheep and cattle against infection with *F. gigantica* (Haroun and Hillyer, 1986).

Several authors previously depicted that plasma activities of liver enzymes are sensitive indicators of liver damage in sheep and cattle (Rowlands and Clampitt, 1979; Bulgin *et al*., 1984; Ferre *et al*., 1994; Fere *et al*., 1995a; Fere *et al*., 1996) and with this regard, our findings were not different. In ruminants (bovine and ovine) fasciolosis young flukes may be found in the hepatic parenchyma for approximately 6-8 weeks, migrating through the tissue and growing. The flukes then enter the biliary system, mature, and lay eggs which can be found in faeces many months after initial infection (Taylor *et al*., 2007).

In the present study, the parasitological findings disclosed that sheep appear to show reduced liver damage against *F. hepatica* infection when dosed with 120 and 240 gray γ-irradiated metacercariae, the size of the inocula being always 500 metacercariae in a single experimental infection. This is evidenced by the significant reduction of worm egg outputs and the recovery of mature flukes in groups γ-irradiated with 120 and 240 grays compared to the positive control and groups kept at 30 and 60 gray of γ-irradiated metacercariae.

In this study, primary vaccination of sheep with 500 γ-irradiated (30 and 60 grays) metacercariae of *Fasciola hepatica* did not generate a significant reduction of liver damage compared with 500 γ-irradiated (120 and 240 grays) metacercariae and the positive control as measured by the reduction of faecal egg output and recovery of flukes from liver and bile ducts. All the vaccinated and infected control groups were found to shade eggs except the uninfected negative controls. Higher EPG output were observed in infected control (PC) followed by those animals vaccinated with low doses of the gamma irradiation (GI and GII). However, a study (Campbell *et al*., 1978) stated that vaccination of sheep with either 100 or 1000 γ-irradiated (25 grays) metacercariae of *Fasciola hepatica*, on two occasions six weeks apart, did not generate significant protection against intraruminal challenge with *F. hepatica* six weeks after the second vaccinating dose as measured by the recovery of flukes from liver and bile ducts, twenty weeks after challenge.

The present results were in agreement with most of the previous studies in that vaccination of sheep with irradiated metacercariae of *F. hepatica* yielded matured and immature parasites with varying levels of reduction across treatment groups. In contrary to some authors (Rickard and Howell, 1982; Haroun

and Hillyer, 1986; Boyce *et al.*, 1987) stated that sheep do not acquire resistance against *Fasciola hepatica* as indicated by the yields of mature parasites from primary and secondary infections, the result of the present study revealed differences in the response of sheep to yield matured parasites in vaccinated and control groups from primary infection. In coincident with this, the mean number of flukes recovered from infected groups varies significantly ($P<0.05$). That means a strong dose-response was evident in the number of parasites recovered in the groups dosed with incremental doses of γ-irradiation with *F. hepatica* metacercariae. Accordingly, the yield of matured parasites from Ethiopian sheep ranged from 7.7% to 22.5%. In the studies with European fleece sheep, yields of *F. hepatica* ranged from 16 to 38% after primary infection and from13 to 31% after secondary infection (Rickard and Howell, 1982). A significant reduction in parasite numbers (from 17% in control animals to 3.4%) was reported in Sudanese desert sheep vaccinated with irradiated metacercariae of *F. gigantica* following a secondary challenge (A'Gadira, *et al.*, 1987).

Presently, the percentage of the flukes recovered (percentage reduction in fluke burden 81.5%) unirradiated from the liver of the sheep were highest in infected control groups (22.5%; 112.3±4.3) followed by GI animals dosed with the lowest irradiation dose (18.6%; 93± 3.4 recovered / 83.4% reduction). The percentage reduction in the burden of flukes in group II and III sheep was 86.7% (13.4%; 67.8 ± 2.8 recovered) and 88.8% (11.2%; 56.5± 7.18 recovered) respectively. Similarly, those animals in the highest irradiation dose (GIV) yield the lowest recovery 92.3% (7.7%, 38.3± 3.3 recovered). Accordingly, the higher the irradiation dose, the lowest was the recovery of flukes in necropsy examinations. There was, however, a significant increase in the proportion of flukes retarded in the parenchyma of vaccinated groups with the significant effect observed in those vaccinated with 120 and 240 grays of γ-irradiated (30 and 60 grays) metacercariae. The percentage of retarded flukes was positively correlated ($r = 0.72$) with the degree of liver damage. According to the works of some authors (Creaney *et al.*, 1995), no significant reduction of fluke burdens was observed in any group, although a non-significant 20% reduction was observed in sheep vaccinated with 2000 metacercariae irradiated with 100 grays.

The present study used parasitological parameters such as recovered liver fluke length and width, the total mass of recovered flukes, and liver damage (enzyme profile) which are major factors often used to indicate the protection against the severity of parasite infection. This method of assessing has been used in evaluating vaccination against fasciolosis by different studies includ-

ing a multivalent vaccine of recombinant stage-specific antigens (Boray, 1983; Valero *et al*., 2002; Valero *et al*., 2006; Jayaraj *et al*., 2009).

Cysteine proteases are common virulence mediators of parasites and are produced by all stages of the fluke lifecycle. They mediate biological functions including excystment, tissue invasion, and immune evasion (Berasain *et al*., 2000). Adult fluke cathepsin L and Newly Excysted Juvenile (NEJ) cathepsin B are the prominent proteolytic enzymes of their respective ES (excrete tory secretory) materials. *F. hepatica* cathepsin L5 (fifth stage larvae) (Creaney et al., 1995; Smooker *et al*., 2000; Irving *et al*., 2003; Law *et al*., 2003; Meemon *et al*., 2004; Beckham *et al*., 2006; Kennedy *et al*., 2006) and *F. gigantica* cathepsin L1 (Grams *et al*., 2001) are promising targets for vaccines against *Fasciola* infection.

Similar to the current finding, other authors (Jayaraj *et al*., 2009) depicted that, in single and multivalent recombinant protein vaccinations of adult stage *F. hepatica* cathepsin L5, metacercarial stage *F. gigantica* cathepsin L1g, and juvenile stage *F. hepatica* cathepsin B against *F. hepatica* challenge infection, the rats vaccinated with recombinant proteins were shown to have significantly fewer and smaller flukes than the control rats. That means maximum protection of 83% was seen in the group vaccinated with a combination of cathepsin B and cathepsin L5 (Jayaraj *et al*., 2009). Although there was a variation in the level of protection, the percentage reduction of the fluke population of gamma-irradiated *Fasciola hepatica* metacercarial infection in Ethiopian sheep ranged from 81.5% (22.5%, 112.3$\pm$4.3 recovered) to 92.3% (7.7%, 38.3$\pm$ 3.3 recovered).

In agreement with the present study, vaccination with the multivalent cathepsin B/L5 leads to less liver damage, lower fluke numbers, and the lowest mean wet weight compared to control rats. This result indicates liver fluke development was retarded in the vaccinated groups. It may be that a fluke vaccine does not need to induce sterile immunity, but reduce the pathology to a level that is tolerated by the animal. A reduction in fluke burdens will also reduce the number of eggs passed by infected animals, reducing pasture contamination. A study demonstrated (Jayaraj *et al*., 2009) that juvenile stage-specific recombinant proteins (B, L1g) can mediate immunity to liver fluke infection, but that protection is greatest when a juvenile stage protease (cathepsin B) is used in concert with an adult stage protease, L5. A work reported (Jayaraj *et al*., 2009), the protective immunity elicited by recombinant protein vaccination appears to evoke effector and memory responses against infection. Although

such vaccination induces strong humoral responses (Jayaraj *et al*., 2009), the precise effecter mechanisms leading to fluke control have not been elucidated. That a cocktail of juvenile and adult stage *Fasciola* recombinant proteins induced better protective immunity than individual protein alone indicates that stage-specific, multivalent recombinant vaccines against parasites may be feasible (Jayaraj *et al*., 2009).

The number and size of fluke's recovered had a significant effect on the observed weight changes of the liver in different groups. The results disclosed the difference in the mean change of the liver weight between the infected and control groups at necropsy. The highest mean live weight changes were observed between positive and negative control groups with a difference of 71± 2.5 g and this was significant (P<0.5%). The presence of flukes in positive control was defiantly responsible for the increased weight of the liver than the uninfected negative control sheep. Comparison between highly dosed groups at GIV with that of positive control had shown a mean difference of 48± 3.3 g. This was followed by the mean difference of 40± 2.3 g with that of group III animals. The relative comparative mean live weight changes recorded for GI and GII were 18 ± 2.3 and 28 ± 3.4gm. Generally, the liver weight relatively tends to decline with the progressive increase in irradiation dose and subsequent decrease in the recovery of the fluke and reduction in the size of *F. hepatica* at necropsy examinations.

The experiment was only resumed after three weeks of acclimatization and all animals began to gain weight after the second week post-infection and this gain was continued till the end of the experimental period. Generally, the mean weight gain of all groups tends to increase with time despite the differences in weight gain of the treatment groups used for the vaccine trials. Despite the nearly linear increase in the mean weight gain of positive controls and the groups received the lower irradiation doses of 30 and 60 gray, a significant body weight loss was observed between weeks 6 and 8 post-infection followed by a steady increase of the mean weight of infected animals across time. Given the young growing age of sheep used in this experiment, the initial live weight gain has been expected. However, the effect of fasciola on body weight loss of treatment groups has been described between weeks 6 and 8. This might be due to damage caused by the migrating flukes to the liver parenchyma. Rates of growth were significantly reduced by 14.7% and 14.1% in steers receiving a superimposed artificial infection rate of 1200 metacercariae and grazed at 3.54 beasts/hectare and 4.39 beasts/hectare respectively. Similarly, group body

weights were depressed 3% and 20% in steers receiving 600 metacercariae and grazed at 3.54 beasts/hectare and 4.39 beasts/hectare respectively (Chick *et al*., 2008).

Along with parasitological tests (faecal examination for fluke eggs and the EPG count), laboratory analysis of the hepatic enzymes has been a useful indicator for damages caused by liver flukes. In this regard, the first is the estimation of plasma levels of enzymes released by damaged liver cells. Two enzymes are usually measured. Glutamate dehydrogenase (GLDH) is released when parenchymal cells are damaged and levels become elevated within the first few weeks of infection. The other, gamma-glutamyl transpeptidase (GGT) indicates damage to the epithelial cells lining the bile ducts; elevation of this enzyme takes place mainly after the flukes reach the bile ducts and raised levels are maintained for a longer period (Taylor *et al*., 2007; Hoffmann and Solter 2008; Washington and Hoosier, 2012).

Serum GGT and GLDH determinations for diagnosis of hepatic disease in domestic animals have received less attention in Ethiopia than in some other countries. The determination of GLDH activity is best done in conjunction with the determination of other hepatic enzymes (GGT) and other indicators of hepatic injury or disease.

Many of the early studies on *F. gigantica* and *F. hepatica* assumed that infection with the former was essentially similar to that of *F. hepatica*. However, based on fluke biomass, average fluke size, degree of liver damage, plasma glutamate dehydrogenase (GLDH) levels, and gamma-glutamyl transpeptidase (GGT) responses during the first 10 weeks of infection, *F. hepatica* develops more rapidly than *F. gigantica*, resulting in increased plasma levels of GLDH, indicating greater damage to the liver parenchyma(Boyd, 1962; Meeusen *et al*., 1995) and an increase in the GGT levels at 10 weeks post-infection, indicating epithelial damage in the bile duct (Chauvin *et al*., 1995; Martínez-Valladares, 2010b).

The results of the present study indicated that a peak in GGT activity was associated with the onset of patency. Tissue damage, which is shown by fluctuations in GLDH and GGT levels after adult flukes have become established in the bile ducts, is considered to be due to the feeding activity of adult flukes and the deposition of immune complexes in the liver parenchyma. In this sense, the serum was successfully assayed for the presence of the enzymes glutamate

dehydrogenase (GLDH) and gamma-glutamyl transferase (GGT), as indicators of liver and bile duct damage respectively.

In the present study, animals that have been exposed to infection of the metacercariae of *F. hepatica* were responded well to sensitization and infection as shown by the evolution of hepatic (GLDH), bile duct, and gall bladder (GGT) enzymes. The degree of visual hepatic damage and burden of *F. hepatica* were significantly positively related to levels of GGT and GLDH. The enzyme level varies with the dose of γ-irradiation and non-sensitized groups of animals. In all cases, the uninfected control sheep (NC) maintained a low profile of enzymes that were well within the normal ranges of each enzyme (GLDH= 1-12; GGT= 34-100 IU).

Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme that catalyzes the removal of hydrogen from L-glutamate to form the corresponding ketimine acid that then undergoes spontaneous hydrolysis to 2-oxoglutarate. The liver has by far the highest concentration of GLDH activity. Lesser amounts are found in the kidney and small intestine, where the GLDH activity is located in the proximal and distal tubular epithelial cells and the mucosal epithelial cells, respectively. The GLDH activity of non-hepatic tissues is relatively small compared to that found in the liver, where GLDH is concentrated in the central areas of the lobule. In all species, increases in serum GLDH activity are considered liver-specific (Boyd, 1962; Keller, 1981).

Serum GLDH activity is used most commonly in food animals. Because of its location within mitochondria, GLDH should be released only with irreversible cell injury. The sensitivity of GLDH activity varies depending on the nature of the disease. For example, in a study of calves with hepatic disease, GLDH activity increased in only 60% of the animals (Pearson *et al*., 1995). Similarly, in cattle, the sensitivity of GLDH activity for the detection of hepatic lipidosis, hepatic abscessation, leptospirosis, and fascioliasis was only 28%, 53%, 71%, and 72%, respectively (West, 1991). The determination of GLDH activity is best done in conjunction with the determination of other hepatic enzymes and other indicators of hepatic injury or disease (Hoffmann and Solter, 2008; Washington and Hoosier, 2012).

The elevation of the enzymes and the damage inflicted on bile ducts have shown that they have positively correlated ($r = 0.68$). Irrespective of the infectious groups, similar trends in the elevation GGT profile (an indicator of epithelial

damage in the bile duct) were observed for all sheep infected with *F. hepatica* metacercariae until the end of the experimental period. All animals kept in the sensitized and infected groups had maintained the steady rise in the level of GGT until week 4 post-infection, then after the GGT profile had shown marked elevation before reaching peak levels between weeks 9 and 11 post-infection where the majority of flukes reach patency (epithelial damage to the bile ducts and gall bladder). The peak in GGT activity could be associated with the onset of patency. Then after the level of enzymes progressively decline before it had shown relatively stable enzyme levels between weeks 14-16 post-infection. However, sheep infected with non irradiated normal *F. hepatica* metacercariae (PC) and Group I animals that received the infective parasite exposed to low levels γ-irradiation of 30Gy had shown significantly higher GGT levels compared to the animals in other infected groups that received 120Gy and 60Gy of irradiated *F. hepatica* metacercariae.

A study in goats immunized with Sm 14 antigen of *F. hepatica* disclosed that plasma levels of GLDH and GGT were within the normal range before challenge (week 10). Three weeks after challenge infection (week 13), GLDH levels were elevated until week 18, GGT increased in week 16. No significant difference was noted between groups during the study (Mendes *et al*., 2010).

Although sheep that received the 30 Gy irradiated metacercariae (500) had a relatively higher GGT profile compared to sheep that were treated with the same number of metacercariae and kept at 60 and 120 Gy, the difference was not statistically significant ($P>0.05$). A statistically significant difference ($P<0.05$) in serum GGT levels was only observed between infected control sheep (PC) and the other sensitized groups (GI, GII, GIII, and GIV) after week 8 post-infection. However, between weeks 9 and 11 a statistically significant ($P<0.05$) rise in serum GGT profile was observed in GI (γ-irradiated with 30Gy) sheep compared to other sheep that had received relatively higher doses of γ-irradiation (60 and 120 Gy). The difference in mean (M±SD) GGT profile of group GII and GIII and also GIII and GIV were not significant ($P >0.05$). Particularly the steady rise of GGT levels of groups GII, GIII, and GIV were indistinguishable until the values had shown clear relative comparative difference after week 12 post-infection.

Conclusions

This trial was the first attempt on experimental vaccination of sheep against γ- irradiated *F. hepatica* metacercariae in Ethiopia. In the present settings, which include faecal examinations for *Fasciola hepatica* eggs and the EPG, recovery of flukes and measurement of the sizes and enzyme analysis (GLDH and GGT) became useful indicators for primary vaccination trial of sheep in determining the irradiation attenuating dose. The determination of GLDH activity was best done in conjunction with the determination of GGT as indicators of hepatic injury. The degree of visual hepatic damage and burden of *F. hepatica* were significantly positively related to levels of GGT and GLDH. The enzyme level varies with the dose of γ-irradiation and non-sensitized groups of animals. The findings disclosed that sheep appear to shows significantly reduced liver damage against *F. hepatica* infection when dosed with 120 and 240 grays of γ- irradiation while the size of the inocula is 500 metacercariae in a single infection. A strong dose-response was evident in the number of parasites recovered in the groups dosed with incremental doses of γ-irradiation with *F. hepatica* metacercariae.

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Conflict of interest

None of the authors have any financial or personal interests that could inappropriately influence or bias the contents of this paper.

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