Vaccine 37 (2019) 3234–3240

Contents lists available at ScienceDirect

Vaccine

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

Moderate protection is induced by a chimeric protein composed of leucine aminopeptidase and cathepsin L1 against *Fasciola hepatica* challenge in sheep

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ARTICLE INFO

Article history:
Received 17 December 2018
Received in revised form 16 April 2019
Accepted 18 April 2019
Available online 26 April 2019

Keywords:
Fasciola hepatica
Chimeric protein
Immunization
Sheep

ABSTRACT

Leucine aminopeptidase (FhLAP) and cathepsin L1 (FhCL1) of *Fasciola hepatica* play a critical role in parasite feeding, migration through host tissue, and immune evasion. These antigens have been tested for immune protection as single components with variable degrees of success. The chimeric-protein approach could improve protection levels against fasciolosis. Previously, we reported the design and construction of a chimeric protein composed of antigenic sequences of FhLAP and FhCL1 of *F. hepatica*. The goal of the present study was to express and evaluate the immune-protective capacity of this chimeric protein (rFhLAP-CL1) in sheep. Animals were randomly allocated into five groups with five animals in each group. Groups 1, 2 and 3 were immunized twice with 100 μg, 200 μg and 400 μg of rFhLAP-CL1 emulsified with Quil A adjuvant, whereas groups 4 and 5 were the adjuvant control and infection control groups, respectively. The animals were then challenged with 200 metacercariae two weeks after the rFhLAP-CL1 booster. The fluke burden was reduced by 25.5%, 30.7% ($p < 0.05$) and 46.5% ($p < 0.01$) in sheep immunized with 100 μg, 200 μg and 400 μg of chimeric protein, respectively, in comparison to the infection control group. There was a reduction of 22.7% ($p < 0.05$) and 24.4% ($p < 0.01$) in fecal egg count in groups 2 and 3, respectively, compared to the infection control group. Sheep immunized with chimeric protein produced *F. hepatica* excretion-secretion product-specific total IgG antibody, which were increased after challenge. Moreover, the levels of rFhLAP-CL1-specific IgG1 and IgG2 isotypes in immunized sheep increased rapidly two weeks after the first immunization and were significantly more elevated than those of the control groups, indicating a mixed Th1/Th2 response. This is a preliminary evaluation of the chimeric protein rFhLAP-CL1 as a possible immunogen against *F. hepatica* infection in sheep.

1. Introduction

Fasciolosis is caused by the digenetic trematode *Fasciola hepatica*, a major parasitic disease of livestock, particularly sheep, goats and cattle. The economic losses to livestock production by fasciolosis are estimated to be over US$3 billion per annum, with over 600 million animals infected [1]. Furthermore, fasciolosis is recognized as an emerging human disease with an estimated 2.4 million people infected and 180 million at risk of infection [1,2]. Currently, the control of *F. hepatica* is based on anthelmintic treatment, and triclabendazole is the drug of choice, which is active against both juvenile and mature flukes [3]. However, due to the overuse of anthelmintics, resistance to triclabendazole and other fasciolicides has been reported [4,5]. In addition, the fasciolicides do not prevent reinfections, and continuous treatment leads to resistance and increased treatment expenses. This, along with consumer pressure for chemical free foods, increases interest in new control strategies, and the development of a vaccine would offer an alternative method of control for this disease [1,6].

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https://doi.org/10.1016/j.vaccine.2019.04.067
0264-410X/

There are several antigens of *F. hepatica* that have been characterized and proposed as possible vaccine candidates in the form of native or recombinant proteins, including glutathione S-transferase [7], saposin-like protein-2 [8], cathepsin L1 [9] and leucine aminopeptidase [10], with variable protection levels when administered as single components.

Cathepsin L1 (FhCL1) and leucine aminopeptidase (FhLAP) of *F. hepatica* are involved in crucial activities such as migration, feeding and immune evasion, and both proteases have been identified in FhES products, used in immunoassays [11,12] and proposed as promising candidates for vaccination [13,14]. Previous vaccine trials with rFhCL1 and rFhLAP have shown reductions in fluke burden ranging from 37.6% to above >80% in cattle and sheep, respectively [9,10], depending on the strategy used to obtain these immunogens. Chimeric proteins have the ability to present multiple antigenic epitopes from different proteins at the same time and thus can induce better levels of protection against pathogens [15]. To date, few studies have focused on the use of chimeric proteins to induce better levels of immunoprotection against parasites, including *Schistosoma mansoni* [16], *Babesia bovis* [17] and *Plasmodium vivax* [18]. The immunization of mice with two chimeric proteins composed of tegument proteins of *S. mansoni*, SmTSP-2 fused to the N- or C-terminus of Sm29, induced a reduction in worm burden and liver pathology compared to control groups [19]. To our knowledge, there are no reports of the use of chimeric proteins based on sequences of LAP and CL1 as possible immunogens against *F. hepatica* in ruminants. Our research group previously designed and constructed a chimeric protein composed of four and six antigenic determinants of LAP (GenBank; AAV59016.1; 192–281 aa) and CL1 (GenBank CAC12806.1; 173–309 aa), respectively. The selection of these sequences was based on bioinformatics analyses that predicted these antigenic regions (BCEPred, ABCPred and Kolaskar & Togaonkar) [20]. The CL1 region of the chimeric protein comprised B-cell epitopes; two epitopes within the chimeric sequence (181–191 aa and 283–289 aa) were previously evaluated as immunogens and showed protection of 47.61% and 30.08% against *F. hepatica* in sheep and goats, respectively [21,22]. Another region (245–264 aa) of CL1 induced high levels of IgG, IFN-γ, IL-4 and IL-17 in mice immunized with peptide B7/B8 [23]. In addition, the sequence of CL1 (288–296 aa) has been recognized by sera of cattle vaccinated with FhCL1/FhCL3 [9].

The aim of this study was to express and evaluate the level of protection and the immune response in sheep immunized with an rFhLAP-CL1 chimeric protein formulated with Quil A adjuvant and challenged with *F. hepatica* metacercariae. The worm burden, fecal egg count and serum levels of antibodies were analyzed.

2. Materials and methods

2.1. Expression and purification of the rFhLAP-CL1 recombinant chimeric protein

The 681 bp chimeric gene contains 270 bp of LAP and 411 bp of CL1 and was previously subcloned into the expression vector pET15b [20]. The pET15b-LAP/CL1 recombinant plasmid was transformed into Rosetta (DE3) *E. coli*, spread onto agar plates containing 100 μg/ml carbenicillin (Carb) and incubated at 37 °C overnight. After that, a clone was selected and grown in 3 ml of Luria-Bertani medium supplemented with 100 μg/ml of Carb (LB/Carb) and incubated at 37 °C with shaking at 220 rpm overnight. A culture of the recombinant strain was used to subculture fresh medium (1:100 dilution) containing antibiotic selection. When the cultures reached an OD_{600} = 0.5–0.6, expression was induced using 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich, México). After four hours, bacterial cells were harvested by centrifugation, washed twice with 1× PBS pH 7.4 and resuspended in 50 mM Tris-HCl pH 8.0, 1 mM PMSF, 100 mM NaCl, and 20 mM imidazole, incubated at 4 °C for 45 min and sonicated (10 burst cycle of 30 s with 30 s pauses). The lysate was centrifuged at 20,196 × g for 40 min at 4 °C, and the pellet was washed with PBS-1% Triton X-100. Then, the pellet was solubilized in binding buffer (50 mM $NaH_2PO_4{\cdot}H_2O$, 50 mM NaCl, 8 M urea, and 10 mM Imidazole, pH 8.0) and incubated at 4 °C with gentle stirring. Following incubation, cell debris was removed by centrifugation, and the supernatant was filtered through a 0.45 μm Millipore filter.

The purification of His-tagged chimeric protein was carried out using an AKTA-FPLC purification system (GE Healthcare) by HisTrap™ HP columns (GE Healthcare Life Sciences, USA) under denaturing conditions. The filtered supernatant was applied to a HisTrap™HP column previously equilibrated with binding buffer, the column was washed with buffer containing 50 mM $NaH_2PO_4{\cdot}H_2O$, 50 mM NaCl, 8 M urea, and 20 mM Imidazole at pH 8.0, and bound proteins were eluted through a stepwise gradient with elution buffer (50 mM $NaH_2PO_4{\cdot}H_2O$, 50 mM NaCl, 8 M urea, and 500 mM Imidazole, pH 8.0). The protein-containing fractions were pooled and dialyzed against phosphate buffer (50 mM $NaH_2PO_4{\cdot}H_2O$ and 50 mM NaCl, pH 8.0) with a step gradient of 6 M to zero M urea. The protein concentration was determined using a bicinchoninic acid assay (ThermoFisher Scientific, USA). Sample purity was evaluated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels stained with Coomassie Blue. The chimeric protein was confirmed by Western blot using anti-$(His)_6$ antibody (1:3000) (H1029, Sigma-Aldrich) and anti-rFhLAP-CL1 polyclonal antibody generated in rabbits (1:8000). The chimeric protein was kept at −20 °C until use.

2.2. Vaccine formulation

The Quil A adjuvant (Accurate Chemical & Scientific Corp, USA) was dissolved at a concentration of 1 mg/ml (w:v) in sterile PBS (pH 7.0) and filtered through a 0.20 μm filter [24]. Subsequently, an adjuvant volume was emulsified with an equal volume of the chimeric protein and stirred overnight at 4 °C.

2.3. Experimental animals

Twenty-five 6- to 8-month-old Katahdin × East Friesian male sheep were obtained and maintained at the Teaching and Research Centre for Animal Health and Production (CEPIPSA) of the National Autonomous University of México, Topilejo, México. This center is a liver fluke-free area; sheep were reared and housed in covered pens with cement floors and shown to be free of infection by faecal egg count and detection of anti-*F. hepatica* excretory-secretory products antibody by ELISA. The animals were weighed and provided hay and pellets and water *ad libitum* throughout the course of the test. The experiment was approved by the Institutional Subcommittee for the Care and Use of Experimental Animals (SICUAE, UNAM), protocol number DC-2017/1-20.

2.4. Metacercariae of F. hepatica

The metacercariae of *F. hepatica* used to challenge the sheep were collected from laboratory colonies of *Lymnaea cubensis* experimentally infected with miracidia of a fluke of bovine origin. The metacercariae were stored at 4 °C until use, and the viability was confirmed by microscopy prior to the infection. Gelatin capsules containing 200 metacercariae were administered *per os* to each animal with esophageal tongs. Only metacercariae less than two months old were used in this experiment.

2.5. Vaccination and experimental infection

All sheep were randomly allocated into five groups with five animals in each group. Groups 1, 2 and 3 were subcutaneously immunized with 100 μg, 200 μg and 400 μg of rFhLAP-CL1 chimeric protein emulsified with Quil A adjuvant, respectively. Groups 4 (Quil A adjuvant and infected) and 5 (unimmunized) were control groups. Immunization was carried out at weeks 0 and 2 in the neck. On week 4 all animals were orally challenged with 200 metacercariae. The experimental period was 24 weeks.

2.6. Protection assessment by vaccination

All sheep were humanely euthanized 24 weeks after the first immunization, and the liver and gallbladder were collected for dissection. The gall bladder was opened, and the flukes were recovered. Then, the major bile ducts were carefully opened with blunt-blunt scissors, and the worms were removed with blunt forceps and counted. The liver of each sheep was cut into small pieces (1 cm^2), soaked in water at 37 °C for 45 min, squeezed, and forced through a 300 μm-mesh sieve; the retained material was analyzed for the presence of damaged flukes [25]. Percentage protection for immunized animals was calculated [10] with the formula: $[(U - V)/U] \times 100$, where U is mean value of the fluke burden of the unvaccinated-infected-control group and V is the mean value of the fluke burden of the vaccinated groups.

2.7. Fecal egg count (FEC)

To assess egg output, fecal samples were collected biweekly from each animal directly from the rectum. The fecal samples were placed in plastic bags, labeled and transported with refrigerant to the laboratory for analysis. Three grams of each sample were processed individually using the sedimentation technique as described by Sexton et al. (1990), and the egg counts were expressed as eggs per gram of feces (EPG) ± standard deviation [26].

2.8. Humoral responses induced by vaccination

Blood samples were collected from all sheep prior to primary immunization (0 week) and every two weeks until the end of the experiment at week 24. The samples were centrifuged, and the serum obtained was stored at −20 °C until use. The humoral response elicited by immunization with chimeric protein and infection was analyzed by enzyme-linked immunosorbent assay (ELISA). Excretion-secretion products of *F. hepatica* (FhES) were obtained and used to determine total IgG antibody titers in the serum samples [21]. The production of rFhLAP-CL1-specific IgG1 and IgG2 isotypes was measured. Flat bottomed micro ELISA plates (Nunc-Immuno MaxiSorp, *USA*) were coated with 100 μl containing 2 μg/ml of FhES or 1 μg/ml of rFhLAP-CL1 chimeric protein in carbonate buffer, pH 9.6 and incubated overnight at 4 °C with gentle shaking. After four washes with PBST (0.05% Tween 20 in PBS pH 7.4), the remaining binding sites were blocked with 200 μl of 3% bovine serum albumin (BSA) in PBST for 1 h at 37 °C and then washed 4 more times. The serum samples were diluted 1:400 in dilution buffer (PBST with 1% BSA) and incubated at 37 °C for 1 h. The plates were washed as described above and incubated with HRP-conjugated rabbit anti-sheep IgG (Ab6747, Abcam), HRP-conjugated mouse anti-goat/sheep IgG1 (A9452, Sigma), and mouse anti-human IgG2 biotinylated isotype (B3773, Sigma; this antibody reacts with sheep IgG2) for 1 h at room temperature in dilution buffer. After incubation and washing under the same conditions as previously described, the reaction was developed with O-phenylenediamine (OPD) in citrate buffer and incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 2 M H_2SO_4 (50 μl/well), and the absorbance was measured at 492 nm using an ELISA reader (*Multiskan* 60, *Thermo Scientific*). The results expressed as the mean of the optical density were obtained from triplicate samples.

2.9. Statistical methods

The mean ± standard deviation (SD) was calculated for fluke burden and fecal egg count. The percent of protection was calculated as previously described [10]. Data were checked for normality (Shapiro-Wilk's test) and variance homogeneity (Levene's test). Differences between groups in the number of worms recovered, egg count and absorbance values were tested by a nonparametric Kruskall-Wallis test. Correlations were calculated with Spearman's nonparametric correlation test to assess the relationship among total IgG, IgG1 and IgG2 in serum and fluke burden. All tests were performed with the software IBM SPSS Statistics 21 for Windows. A $p < 0.05$ was considered significant.

3. Results

3.1. Expression and purification of the rFhLAP-CL1 chimeric protein

Chimeric protein was expressed as inclusion bodies and purified under denaturing conditions (8 M urea) by nickel-affinity chromatography, which eluted at 10%, 30% and 50% of imidazole (0.5 M). The procedure for protein refolding resulted in a substantial loss of protein via precipitation but yielded sufficient soluble protein to be used for immunization experiments. The purified protein was resolved as a single band with an apparent molecular mass of 31 kDa on 12% SDS-PAGE (Fig. S1, lane 1). This value is in agreement with the value estimated from the deduced amino acid sequence of rFhLAP-CL1 plus the hexahistidine tag.

Western blot analysis revealed that the chimeric protein was recognized by mAb anti-histidine as a single band (Fig. S1, lane 2). In addition, rabbit antibodies generated against the chimeric protein reacted with rFhLAP-CL1 at the expected size (~31 kDa) (Fig. S1, lane 3).

3.2. Parasite burden in sheep vaccinated with rFhLAP-CL1 chimeric protein

At the end of the experiment, all sheep were humanely euthanized at 24 weeks after immunization. The parasites in the bile ducts and liver tissue were recovered and counted, and the results were expressed as the mean liver fluke count ± SD (Table 1).

The number of worms recovered from the infection control group exhibited the highest parasitic burden compared to that of the rFhLAP-CL1 vaccinated groups. The percentage of protection was calculated in relation to the infection control group. The three groups vaccinated with rFhLAP-CL1 chimeric protein showed a reduction in fluke burden with the level of protection ranging from 25.5% to 46.5%. The highest degree of protection was observed in sheep vaccinated with 400 μg of the rFhLAP-CL1 chimeric protein, with a mean reduction in fluke burden of 46.5% ($p < 0.01$). Animals vaccinated with 100 μg and 200 μg of rFhLAP-CL1 showed a mean reduction in fluke burden of 25.5% and 30.7% ($p < 0.05$), respectively. In animals treated with Quil A adjuvant only, the mean reduction in fluke burden was 9.8%. The overall protection level achieved in sheep vaccinated with rFhLAP-CL1 was 34.2% ($p > 0.05$). Fluke burdens were compared between the vaccinated groups and adjuvant control group. The levels of protection in sheep vaccinated with 100 μg, 200 μg and 400 μg of rFhLAP-CL1

Table 1
Fluke burden (mean ± S.D) and percentages of recovery from vaccinated and control groups after challenge with *F. hepatica* metacercariae.

Group (antigen/adjuvant)	Number of flukes recovered		(%) Protection	(%) Recovery
	Individual date	Mean ± S.D		
Group 1 (100 μg rFhLAP-CL1 + QS)	50, 69, 26, 75, 68	57.6 ± 19.9	25.5	28.8
Group 2 (200 μg rFhLAP-CL1 + QS)	52, 45, 70, 44, 57	53.6 ± 10.5	30.7	26.8
Group 3 (400 μg rFhLAP-CL1 + QS)	46, 52, 30, 47, 32	41.4 ± 9.7	46.5**	20.7
Quil A adjuvant	68, 84, 64, 71, 62	69.8 ± 8.6	9.8	34.9
Infection control	54, 86, 105, 62, 80	77.4 ± 20.1		38.7

S.D standard deviation.

were 17.4% ($p > 0.05$), 23.2% ($p > 0.05$) and 40.6% ($p < 0.01$), respectively.

In the infection control group, the mean worm burden from the original inoculum of 200 metacercariae was 38.7%, and the mean worm burden in the adjuvant control group was 34.9%. The fluke implantation rate was 28.8% for group 1, 26.8% for group 2 and 20.7% for group 3. The weight of sheep was significantly correlated with the parasite burden (r = -0.4031; p = 0.045, Fig. S2).

3.3. Fecal egg count (FEC)

Eggs of *F. hepatica* first appeared at week 10 postinfection and gradually increased until the end of the experiment (week 24). The average and standard deviation of the fecal egg count of the immunized and control groups are show in Fig. 1.

The mean total FEC of rFhLAP-CL1-vaccinated animals was lower than the adjuvant control group or infection control group. The adjuvant and infection control groups showed 304.7 ± 43.5 and 315.4 ± 56 FECs, respectively. The groups immunized with 100 μg, 200 μg and 400 μg of rFhLAP-CL1 showed 296.8 ± 27.3, 243.8 ± 45.2 and 238.4 ± 22.9 FECs, respectively. There was a reduction in the egg output of 22.7% ($p < 0.05$) and 24.4% ($p < 0.01$) in animals vaccinated with 200 μg and 400 μg of rFhLAP-CL1, respectively, compared to the infection control group. There was no significant difference in the FECs ($p > 0.05$) between sheep immunized with 100 μg of rFhLAP-CL1 (group 1) and control groups, probably due the high variability found.

It was observed that at week 20, the number of eggs released from animals vaccinated with 200 μg ($p < 0.01$) and 400 μg ($p < 0.01$) of rFhLAP-CL1 was significantly lower than that released from the infection control group. In addition, group 3 ($p < 0.05$) showed a statistically significant reduction in FEC compared with the adjuvant control group.

3.4. Humoral responses induced by vaccination and detected by indirect ELISA

Serum samples collected from sheep were tested for anti-FhES products IgG, rFhLAP-CL1-specific IgG1 and IgG2 isotypes, by indirect ELISA. Fig. 2 shows the reactivity of sera from all sheep towards the FhES products. A slight but significant antibody response was elicited in the groups vaccinated with 200 μg (group 2) and 400 μg (group 3) of rFhLAP-CL1 protein two weeks after the first immunization. In addition, an increase in the absorbance of the groups immunized with rFhLAP-CL1 protein was observed within 2 weeks after challenge (week 4), with a peak 10 weeks after the first immunization that remained high until 16 weeks, after which, the level decreased gradually until the end of the experiment (week 24). FhES-specific total IgG levels were higher in the serum of sheep immunized with 200 μg and 400 μg of rFhLAP-CL1 protein than in sheep immunized with 100 μg of rFhLAP-CL1 protein or in control groups. The absorbance of IgG in serum against adult fluke FhES products was found at background levels before challenge to *F. hepatica* infection in the adjuvant control group or infection control group. After that, an increase in antibody titers was observed two weeks postchallenge with a peak at weeks 8–16 that decreased gradually thereafter. The absorbance of IgG in serum against adult fluke FhES products was

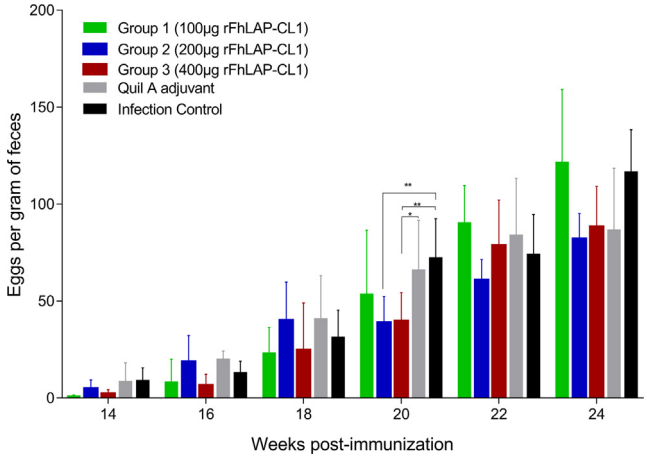

Fig. 1. Egg count dynamics throughout the experiment of sheep immunized with 100 μg (group 1), 200 μg (group 2) and 400 μg (group 3) of rFhLAP-CL1 chimeric protein, as well as adjuvant control group and infection control group. Data are presented as the means ± standard deviation of five animals per group. Groups 2 and 3 showed significant differences compared with group 4 denoted by ** ($p < 0.01$). Only group 3 ($p < 0.05$) showed a statistically significant difference in FEC compared with the adjuvant control group.

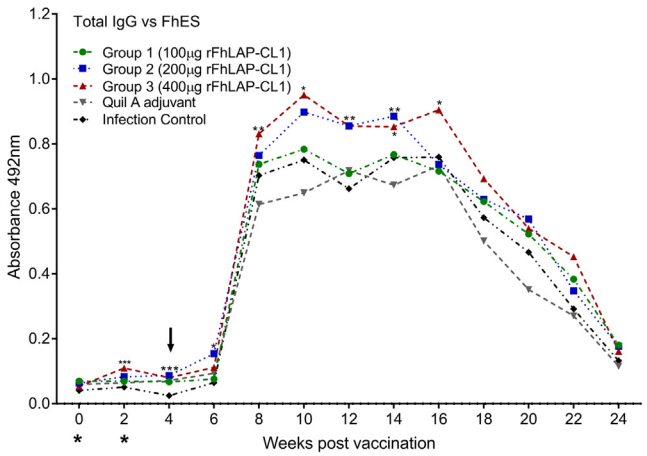

Fig. 2. Total IgG levels against ES products of *F. hepatica* in the serum of immunized and control animals. Sheep were given two immunizations at 0 and 2 weeks with 100 μg (group 1), 200 μg (group 2) and 400 μg (group 3) of rFhLAP-CL1 chimeric protein in Quil A adjuvant and challenged with 200 metacercariae orally (black arrow). Antibody titers are expressed as OD means of 1:400 diluted sera in triplicate wells. * = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$.

lower in the adjuvant control group or the infection control group than in the immunized sheep.

Fig. 3 shows the levels of IgG1 and IgG2 reacting to the rFhLAP-CL1 chimeric protein. The rFhLAP-CL1-specific IgG1 levels were produced in all vaccinated sheep after the first immunization with an increase in antibodies with the 2nd booster (Fig. 3a). The maximum levels of IgG1 in immunized sheep were observed at 2 weeks postchallenge (except group 3, where a peak was observed at week 10) and then decreased gradually until the end of the experiment (week 24). Of the vaccinated groups, group 3 showed the highest IgG1 levels compared to groups 1 and 2. IgG1 levels were significantly higher in the immunized groups compared with the adjuvant control group or the infection control group throughout the experiment. In animals from both control groups, a slight increase in IgG1 levels after challenge was observed, with no significant increase throughout the experimental phase.

In immunized sheep, rFhLAP-CL1-specific IgG2 levels increased with the 1st and 2nd booster of recombinant protein (Fig. 3b). Group 3 (immunized with 400 μg rFhLAP-CL1), showed the highest IgG2 titers at the time of the challenge (week 4). After infection, the immunized groups showed higher rFhLAP-CL1-specific IgG2 titers than the adjuvant control group or the infection control group at weeks 6, 8 and 10. A slight increase in the rFhLAP-CL1-specific IgG2 levels in the sera of animals in the adjuvant control group or infection control group was detected at two weeks postinfection (week 6), without a significant increase throughout the experiment.

Using a nonparametrical Spearman correlation's test, a statistically significant correlation was observed between IgG1 ($r^2 = -0.523$, $p = 0.007$) and IgG2 ($r^2 = -0.688$, $p = 0.001$) titers and parasite burden when analysis was performed including all animals, but not when analysis was performed by each group. In addition, no statistically significant correlation was obtained between total IgG ($r^2 = -0.354$, $p = 0.083$) titers and parasite burden.

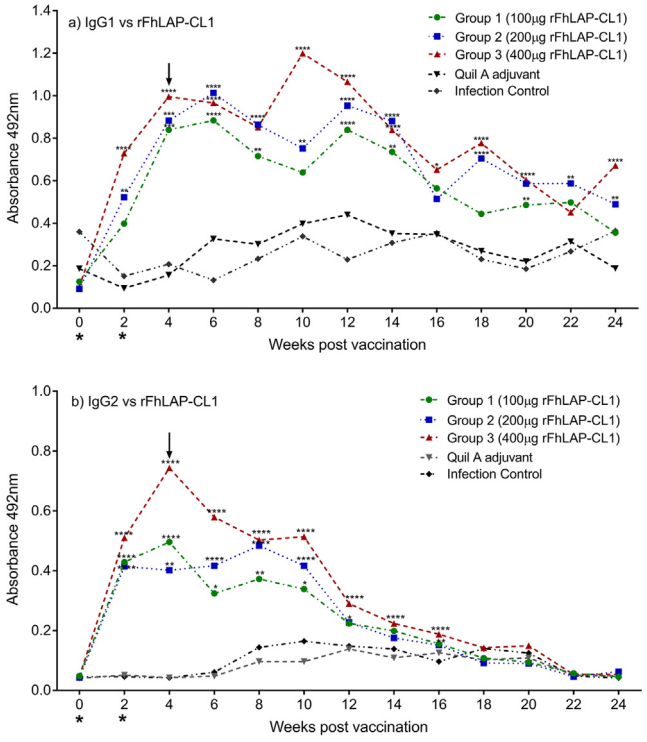

Fig. 3. Analysis of IgG1 (a) and IgG2 (b) antibody responses specific to rFhLAP-CL1 in the serum of vaccinated and control sheep during the experiment. Sheep were given two immunizations at 0 and 2 weeks with 100 μg (group 1), 200 μg (group 2) and 400 μg (group 3) of rFhLAP-CL1 chimeric protein in Quil A adjuvant and challenged with 200 metacercariae orally (black arrow). Antibody titers are expressed as OD means of 1:400 diluted sera in triplicate wells. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; and **** = $p < 0.0001$.

4. Discussion

Recombinant chimeric proteins used as immunogens have been minimally explored in the field of parasitology. Chimeric proteins have the advantage of containing several antigenic and immunogenic epitopes, at least two or more proteins from the different stages of the life cycle of parasite, that induce protective effects against infection [27,28].

In trematodes, especially in *F. hepatica*, the development and use of chimeric proteins as immunogens has not been explored. For the development of a vaccine against *F. hepatica*, many researchers have focused on proteins that play important roles in the biology of the parasite, such as cathepsin L [14] and leucine aminopeptidase [13], and are therefore targets for vaccination. Immunization of cattle with the recombinant protein CL1 followed by a challenge infection with *F. hepatica* resulted in a reduction of 48.2% of worm burden [29]. However, other immunization trials have not shown a similar reduction in fluke burden [30,31]. Moreover, the vaccination of sheep with recombinant LAP formulated with different adjuvants reduced worm burden up to 86.7%, which is the highest level of protection achieved thus far in large animals [10], although more trials are necessary.

To improve protection with CL1 and LAP in vaccination trials in ruminants, our research group designed and constructed a chimeric protein called rFhLAP-CL1, which contains antigenic sequences of both proteins. This chimeric protein was highly immunogenic in immunized rabbits, inducing strong and specific antibody responses towards rFhLAP-CL1. In addition, their antigenicity was evaluated using serum samples from bovines naturally infected with *F. hepatica* through Western blot analyses [20].

We evaluated the immune-protective capacity of rFhLAP-CL1 against *F. hepatica* in sheep by a preliminary study conducted with a small number of animals using different concentrations of immunogen (100 μg, 200 μg and 400 μg). We demonstrated that it is possible to induce moderate protection along with a high humoral immune response in sheep vaccinated with rFhLAP-CL1 formulated with Quil A adjuvant. This adjuvant is a complex mixture of triterpenoid saponins extracted from the bark of *Quillaja saponaria* tree [32] and has been used in vaccine trials against *Taenia multiceps* [33] and *Haemonchus contortus* [34]. Upon assessment of the effect of the adjuvant Quil A against *F. hepatica* in sheep, a significant reduction in FEC and higher *F. hepatica*-specific serum antibody levels (IgG1, IgG2, IgA and IgE) were observed, suggesting that Quil A adjuvant boosted a pro-inflammatory immune response (Th1) [24].

Reduction in fluke burden is a key parameter for evaluating vaccine efficacy after challenge with *F. hepatica*. Immunization with recombinant chimeric protein rFhLAP-CL1 can reduce liver fluke burden in vaccinated and infected animals. The highest reduction in liver fluke burden was observed in sheep immunized with 400 μg of rFhLAP-CL1, which induced a significant level of protection of 46.5% compared with the infected control group. In the group immunized with 200 μg of rFhLAP-CL1, there was a reduction of 30.7% ($p < 0.05$) in fluke burden, while in animals immunized with 100 μg of protein, there was a reduction of 25.5% ($p > 0.05$); this result was not statistically significant, probably due to the high variability found. Similar results have been obtained with other recombinant proteins evaluated as immunogens against *F. hepatica*. Goats immunized with a synthetic peptide of Sm14 antigen induced 45.9% protection against *F. hepatica* [35].

Immunization of goats with peroxiredoxin and FhCL1 emulsified with Quil A adjuvant induced reductions in fluke burden of 33.2% and 38.7%, respectively [30]. Golden et al., 2010 reported that cattle vaccinated with an inactive mutant of rmFhCL1, expressed in *Pichia pastoris*, showed a significant reduction in fluke burden of 48.2%, and Garza-Cuartero et al., 2018 found that cattle vaccinated with rmFhCL1 showed a fluke burden reduction of 37.6%. However, other trials have not shown a similar reduction in worm burden with different proteins, such as glutathione S-transferase [36], leucine aminopeptidase [37] or thioredoxin glutathione reductase [38].

In this trial, the mean fluke implantation rate (38.7% in unimmunized-infected control group) was similar to that in other reported trials, e.g., 34% in Merino sheep [39] and 33% in Corriedale sheep [10], except for a trial in goats that was slightly higher with 46%[30]. Sheep immunized with 100 μg, 200 μg and 400 μg of rFhLAP-CL1 had implantation rates of 28.8%, 26.8% and 20.7%, respectively, slightly higher than those reported in previous vaccine trials [10,36]. The mechanism of protection induced by the rFhLAP-CL1 protein is unknown but may be related to antibody production after immunization, neutralizing the activity of both enzymes; as a consequence, the mechanism of parasite immune evasion and nutrient acquisition is inhibited. However, further studies are necessary to explain the mechanism of immune protection in sheep.

Another important criterion to assess the efficacy of an immunogen against *F. hepatica* is the reduction in egg production because it can potentially affect pasture contamination. The fecundity of *F. hepatica* in sheep vaccinated with 100 μg of rFhLAP-CL1 does not appear to have been affected after establishment in the bile ducts, as evidenced by the reduction in FEC. However, in sheep immunized with 200 μg and 400 μg of rFhLAP-CL1, the protein showed significant reductions of 22.7% ($p < 0.05$) and 24.4% ($p < 0.01$), respectively. Immunization assays with recombinant antigens of *F. hepatica* did not significantly reduce fecal egg output in immunized animals [30,40].

Immunization with rFhLAP-CL1 chimeric protein elicited a strong humoral immune response as measured by single dilution, and all vaccinated sheep had significantly increased antibodies compared to the control groups. This result confirms the immunogenicity of the rFhLAP-CL1 protein observed in rabbits by high production of IgG after immunization with this antigen [20].

The rFhLAP-CL1 chimeric protein used in this trial elicited an IgG response of low but significant magnitude against the FhES products two weeks after the first dose, especially in sheep immunized with 200 μg and 400 μg of immunogen. After challenge, FhES-specific total IgG levels increased in all the experimental animals, with a peak at weeks 8 to 16 after the first immunization. Of the immunized sheep, the group immunized with 200 μg and 400 μg of rFhLAP-CL1 showed higher antibody titers than the group immunized with 100 μg of rFhLAP-CL1 or control groups, suggesting that the antibody levels were from native antigen-boosted memory B cells and that sequences of epitopes from rFhLAP-CL1 chimeric protein exist in the native molecule. This antibody response elicited by rFhLAP-CL1, coincided with high levels of IgG elicited by CL1 mimotopes evaluated as immunogens in sheep (181–191 aa; YPYTAVEGQCRY) and goats (283–289 aa; RGYIRMA) along with a reduction in fluke burden [21,22].

The analysis of IgG subclasses by indirect ELISA, showed a significant increase in rFhLAP-CL1-specific IgG1 and IgG2 isotypes in all immunized sheep two weeks after the first immunization compared to the adjuvant and infection control groups, suggesting a mixed Th1/Th2 response. The highest IgG1 and IgG2 levels were observed in sheep immunized with the highest concentration of rFhLAP-CL1 chimeric protein, which was related to the highest reduction in worm burdens. Previous studies showed that predominant IgG1 production has been associated with nonprotective Th2 responses in naturally infected sheep and cattle [41]. In contrast, a strong IgG2 response was associated with a low fluke burden in cattle immunized with cathepsin L2 and hemoglobin (Hb) [41]. The mixture Th1/Th2 response induced by chimeric protein could be associated with epitopes contained in CL1. The region 245–264 of CL1 (QTCSPLSVNHAVLAVGYGTQ) overlaps with peptides B7 and B8 that induced a mixed Th1/Th2/Th17 response in mice immunized with both peptides [23]. In this trial, the adjuvant-infected control group elicited a nonprotective response, as indicated by the reduction in fluke burden. Moreover, low levels of rFhLAP-CL1-specific IgG1 and IgG2 were observed in sheep of both control groups, suggesting a Th2 nonprotective response.

Correlation between fluke burden and antibody titers (IgG1 and IgG2) at the time of challenge (4 weeks postpriming) was found when analysis was performed with immunized sheep and control animals, but when analysis was performed by each group or at the time of necropsy, no significant correlations were found. A similar result has been reported by Maggioli (2011), where a significant correlation between antibody titers (IgG and isotypes) and fluke burden was obtained at the time challenge when all animals were included [10]. In contrast, no significant correlation between titers of IgG, IgG1 or IgG2 and fluke burden at time necropsy was observed in cattle immunized with thioredoxin glutathione [38]. The mixed IgG1/IgG2 antibody isotypes in sheep immunized with chimeric protein rFhLAP-CL1 in this trial were associated with protection, and although the role of antibodies in the protection against *F. hepatica* is not clear, this mixed antibody response has been reported in other immunization trials [8,22].

5. Conclusion

In conclusion, we have demonstrated that immunization of sheep with recombinant chimeric protein rFhLAP-CL1 combined with Quil A adjuvant induced moderate protection against *F. hepatica* infection in terms of reduction in fluke burden and reduction in FEC. In addition, a marked humoral immune response was elicited, with production of both IgG1-and IgG2 specific antibodies towards recombinant chimeric protein, suggesting a mixed Th1/Th2 immune response associated with protection. Although at this point, the magnitude of the humoral immune response in immunized animals is unknown, for which serial dilutions are necessary. This moderate protection achieved by this chimeric protein against liver flukes in large animals is a preliminary protection test, and thus more trials with a larger number of animals emulsified with different adjuvants are necessary to determine the potential capacity of the rFhLAP-CL1 protein as a vaccine candidate.

Financial support

This study was supported by the project PAPIIT- IN 214,915 DGAPA-UNAM as well the scholarship 401,769 granted by the National Council of Science and Technology (CONACYT), México.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.04.067.

References

[1] Spithill TW, Smooker PM, Sexton JL, Bozas E, Morrison CA, Creaney J. Development of vaccines against *Fasciola hepatica*. First edit. London UK: CABI Publishing; 1999. p. 377–409.

[2] Mas-Coma S. Epidemiology of fascioliasis in human endemic areas. J Helminthol 2005;79:207–16.

[3] Smeal MG, Hall CA. The activity of triclabendazole against immature and adult *Fasciola hepatica* infections in sheep. Aust Vet J 1983;60:329–31. https://doi.org/10.1111/j.1751-0813.1983.tb02833.x.

[4] Ortiz P, Scarcella S, Cerna C, Rosales C, Cabrera M, Guzmán M, et al. Resistance of *Fasciola hepatica* against Triclabendazole in cattle in Cajamarca (Peru): a clinical trial and an in vivo efficacy test in sheep. Vet Parasitol 2013;195:118–21. https://doi.org/10.1016/j.vetpar.2013.01.001.

[5] Novobilský A, Höglund J. First report of closantel treatment failure against *Fasciola hepatica* in cattle. Int J Parasitol Drugs Drug Resist 2015;5:172–7. https://doi.org/10.1016/j.ijpddr.2015.07.003.

[6] Molina-Hernández V, Mulcahy G, Pérez J, Martínez-Moreno Á, Donnelly S, O'Neill SM, et al. *Fasciola hepatica* vaccine: we may not be there yet but we're on the right road. Vet Parasitol 2015;208:101–11. https://doi.org/10.1016/j.vetpar.2015.01.004.

[7] Morrison CA, Colin T, Sexton JL, Bowen F, Wicker J, Friedel T, et al. Protection of cattle against *Fasciola hepatica* infection by vaccination with glutathione S-transferase. Vaccine 1996;14:1603–12. https://doi.org/10.1016/s0264-410x(96)00147-8.

[8] Rivera F, Espino AM. Adjuvant-enhanced antibody and cellular responses to inclusion bodies expressing FhSAP2 correlates with protection of mice to *Fasciola hepatica*. Exp Parasitol 2016;160:31–8. https://doi.org/10.1016/j.exppara.2015.11.002.

[9] Garza-Cuartero L, Geurden T, Mahan SM, Hardham JM, Dalton JP, Mulcahy G. Antibody recognition of cathepsin L1-derived peptides in *Fasciola hepatica* - infected and/or vaccinated cattle and identification of protective linear B-cell epitopes. Vaccine 2018;36:958–68. https://doi.org/10.1016/j.vaccine.2018.01.020.

[10] Maggioli G, Acosta D, Silveira F, Rossi S, Giacaman S, Basika T, et al. The recombinant gut-associated M17 leucine aminopeptidase in combination with different adjuvants confers a high level of protection against *Fasciola hepatica* infection in sheep. Vaccine 2011;29:9057–63. https://doi.org/10.1016/j.vaccine.2011.09.020.

[11] O'neill SM, Parkinson M, Strauss W, Angles R, Dalton JP. Immunodiagnosis of *Fasciola hepatica* infection (fascioliasis) in a human population in the Bolivian altiplano using purified cathepsin L cysteine proteinase. Am J Trop Med Hyg 1998;58:417–23.

[12] Marcilla A, De la Rubia JE, Sotillo J, Bernal D, Carmona C, Villavicencio Z, et al. Leucine aminopeptidase is an immunodominant antigen of *Fasciola hepatica* excretory and secretory products in human infections. Clin Vaccine Immunol 2008;15:95–100. https://doi.org/10.1128/cvi.00338-07.

[13] Acosta D, Cancela M, Piacenza L, Roche L, Carmona C, Tort JF. *Fasciola hepatica* leucine aminopeptidase, a promising candidate for vaccination against ruminant fasciolosis. Mol Biochem Parasitol 2008;158:52–64. https://doi.org/10.1016/j.molbiopara.2007.11.011.

[14] Collins PR, Stack CM, O'Neill SM, Doyle S, Ryan T, Brennan GP, et al. Cathepsin L1, the major protease involved in liver fluke (*Fasciola hepatica*) virulence: propetide cleavage sites and autoactivation of the zymogen secreted from gastrodermal cells. J Biol Chem 2004;279:17038–46. https://doi.org/10.1074/jbc.M308831200.

[15] Dias DS, Ribeiro PAF, Martins VT, Lage DP, Costa LE, Chávez-Fumagalli MA, et al. Vaccination with a CD4+ and CD8+ T-cell epitopes-based recombinant chimeric protein derived from *Leishmania infantum* proteins confers protective immunity against visceral leishmaniasis. Transl Res 2018;200:18–34. https://doi.org/10.1016/j.trsl.2018.05.001.

[16] Yang W, Jackson DC, Zeng Q, McManus DP. Multi-epitope schistosome vaccine candidates tested for protective immunogenicity in mice. Vaccine 2000;19:103–13. https://doi.org/10.1016/s0264-410x(00)00165-1.

[17] Jaramillo Ortiz JM, Molinari MP, Gravisaco MJ, Paoletta MS, Montenegro VN, Wilkowsky SE. Evaluation of different heterologous prime–boost immunization strategies against *Babesia bovis* using viral vectored and protein-adjuvant vaccines based on a chimeric multi-antigen. Vaccine 2016;34:3913–9. https://doi.org/10.1016/j.vaccine.2016.05.053.

[18] Shabani SH, Zakeri S, Salmanian AH, Amani J, Mehrizi AA, Snounou G, et al. Biological, immunological and functional properties of two novel multi-variant chimeric recombinant proteins of CSP antigens for vaccine development against *Plasmodium vivax* infection. Mol Immunol 2017;90:158–71. https://doi.org/10.1016/j.molimm.2017.06.033.

[19] Pinheiro CS, Ribeiro APD, Cardoso FC, Martins VP, Figueiredo BCP, Assis NRG, et al. A multivalent chimeric vaccine composed of *Schistosoma mansoni* SmTSP-2 and Sm29 was able to induce protection against infection in mice. Parasite Immunol 2014;36:303–12. https://doi.org/10.1111/pim.12118.

[20] Hernández-Guzmán K, Sahagún-Ruiz A, Vallecillo AJ, Cruz-Mendoza I, Quiroz-Romero H. Construction and evaluation of a chimeric protein made from *Fasciola hepatica* leucine aminopeptidase and cathepsin L1. J Helminthol 2016;90:7–13. https://doi.org/10.1017/s0022149x14000686.

[21] Villa-Mancera A, Quiroz-Romero H, Correa D, Ibarra F, Reyes-Pérez M, Reyes-Vivas H, et al. Induction of immunity in sheep to *Fasciola hepatica* with mimotopes of cathepsin L selected from a phage display library. Parasitology 2008;135:1437. https://doi.org/10.1017/s003118200800471x.

[22] Villa-Mancera A, Reynoso-Palomar A, Utrera-Quintana F, Carreón-Luna L. Cathepsin L1 mimotopes with adjuvant Quil A induces a Th1/Th2 immune response and confers significant protection against *Fasciola hepatica* infection in goats. Parasitol Res 2014;113:243–50. https://doi.org/10.1007/s00436-013-3650-6.

[23] Rojas-Caraballo J, López-Abán J, Pérez del Villar L, Vizcaíno C, Vicente B, Fernández-Soto P, et al. vitro and in vivo studies for assessing the immune response and protection-inducing ability conferred by *Fasciola hepatica*-derived synthetic peptides containing B- and T-cell epitopes. PLoS ONE 2014;9:e105323. https://doi.org/10.1371/journal.pone.0105323.

[24] Haçarız O, Sayers G, McCullough M, Garrett M, O'Donovan J, Mulcahy G. The effect of Quil A adjuvant on the course of experimental *Fasciola hepatica* infection in sheep. Vaccine 2009;27:45–50. https://doi.org/10.1016/j.vaccine.2008.10.035.

[25] Piacenza L, Acosta D, Basmadjian I, Dalton JP, Carmona C. Vaccination with cathepsin L proteinases and with leucine aminopeptidase induces high levels of protection against fascioliasis in sheep. Infect Immun 1999;67:1954–61.

[26] Sexton JL, Milner AR, Panaccio M, Waddington J, Wijffels G, Chandler D, et al. Glutathione S-transferase. Novel vaccine against *Fasciola hepatica* infection in sheep. J Immunol 1990;145:3905–10.

[27] Anugraha G, Madhumathi J, Prince PR, Jeya Prita PJ, Khatri VK, Amdare NP, et al. Chimeric epitope vaccine from multistage antigens for lymphatic filariasis. Scand J Immunol 2015;82:380–9. https://doi.org/10.1111/sji.12340.

[28] Kaba SA, Price A, Zhou Z, Sundaram V, Schnake P, Goldman IF, et al. Immune responses of mice with different genetic backgrounds to improved multiepitope, multitarget malaria vaccine candidate antigen FALVAC-1A. Clin Vaccine Immunol 2008;15:1674–83. https://doi.org/10.1128/cvi.00164-08.

[29] Golden O, Flynn RJ, Read C, Sekiya M, Donnelly SM, Stack C, et al. Protection of cattle against a natural infection of *Fasciola hepatica* by vaccination with recombinant cathepsin L1 (rFhCL1). Vaccine 2010;28:5551–7. https://doi.org/10.1016/j.vaccine.2010.06.039.

[30] Buffoni L, Martínez-Moreno FJ, Zafra R, Mendes RE, Pérez-Écija A, Sekiya M, et al. Humoral immune response in goats immunised with cathepsin L1, peroxiredoxin and Sm14 antigen and experimentally challenged with *Fasciola hepatica*. Vet Parasitol 2012;185:315–21. https://doi.org/10.1016/j.vetpar.2011.09.027.

[31] Pacheco IL, Abril N, Morales-Prieto N, Bautista MJ, Zafra R, Escamilla A, et al. Th1/Th2 balance in the liver and hepatic lymph nodes of vaccinated and unvaccinated sheep during acute stages of infection with *Fasciola hepatica*. Vet Parasitol 2017;238:61–5. https://doi.org/10.1016/j.vetpar.2017.03.022.

[32] Sun H-X, Xie Y, Ye Y-P. Advances in saponin-based adjuvants. Vaccine 2009;27:1787–96. https://doi.org/10.1016/j.vaccine.2009.01.091.

[33] Gauci C, Vural G, Öncel T, Varcasia A, Damian V, Kyngdon CT, et al. Vaccination with recombinant oncosphere antigens reduces the susceptibility of sheep to infection with *Taenia multiceps*. Int J Parasitol 2008;38:1041–50. https://doi.org/10.1016/j.ijpara.2007.11.006.

[34] González-Sánchez ME, Cuquerella M. Alunda JM. Vaccination of lambs against *Haemonchus contortus* with the recombinant rHc23. Effect of adjuvant and antigen dose. PLoS ONE 2018;13:e0193118. https://doi.org/10.1371/journal.pone.0193118.

[35] Zafra R, Buffoni L, Martínez-Moreno A, Pérez-Écija A, Martinez-Moreno FJ, Pérez J. A study of the liver of goats immunized with a synthetic peptide of the Sm14 antigen and challenged with *Fasciola hepatica*. J Comp Pathol 2008;139:169–76. https://doi.org/10.1016/j.jcpa.2008.06.004.

[36] Buffoni L, Zafra R, Pérez-Écija A, Martínez-Moreno FJ, Martínez-Galisteo E, Moreno T, et al. Immune response of goats immunised with glutathione S-transferase and experimentally challenged with *Fasciola hepatica*. Parasitol Int 2010;59:147–53. https://doi.org/10.1016/j.parint.2009.12.005.

[37] Raina OK, Nagar G, Varghese A, Prajitha G, Alex A, Maharana BR, et al. Lack of protective efficacy in buffaloes vaccinated with *Fasciola gigantica* leucine aminopeptidase and peroxiredoxin recombinant proteins. Acta Trop 2011;118:217–22. https://doi.org/10.1016/j.actatropica.2011.02.008.

[38] Maggioli G, Bottini G, Basika T, Alonzo P, Salinas G, Carmona C. Immunization with *Fasciola hepatica* thioredoxin glutathione reductase failed to confer protection against fasciolosis in cattle. Vet Parasitol 2016;224:13–9. https://doi.org/10.1016/j.vetpar.2016.05.007.

[39] Pérez-Caballero R, Siles-Lucas M, González-Miguel J, Martínez-Moreno FJ, Escamilla A, Pérez J, et al. Pathological, immunological and parasitological study of sheep vaccinated with the recombinant protein 14–3-3z and experimentally infected with *Fasciola hepatica*. Vet Immunol Immunopathol 2018;202:115–21. https://doi.org/10.1016/j.vetimm.2018.07.006.

[40] De Bont J, Claerebout E, Riveau G, Schacht AM, Smets K, Conder G, et al. Failure of a recombinant Schistosoma bovis-derived glutathione S-transferase to protect cattle against experimental *Fasciola hepatica* infection. Vet Parasitol 2003;113:135–44.

[41] Mulcahy G, O'Connor F, McGonigle S, Dowd A, Clery DG, Andrews SJ, et al. Correlation of specific antibody titre and avidity with protection in cattle immunized against *Fasciola hepatica*. Vaccine 1998;16:932–9. https://doi.org/10.1016/S0264-410X(97)00289-2.