Original article

Humoral response (IgG) of goats experimentally infected with *Fasciola hepatica* against cysteine proteinases of adult fluke

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Abstract – The use of cysteine proteinases from *Fasciola hepatica* adult flukes for the serodiagnosis of caprine fasciolosis by means of an indirect ELISA test was studied. Two proteolytic fractions from adult fluke homogenates, with apparent molecular weights of 28 and 34 kDa (P28 and P34 respectively), were characterised as cysteine proteinases using azocasein assays and gelatin gel analysis. Both P28 and P34 fractions were electroluted and used as antigens in two different indirect ELISA tests. Serum IgG levels against P28 and P34 in goats given an experimental primary infection with 200 metacercariae or in goats given two experimental infections with 200 metacercariae were determined and compared with those observed in an uninfected control group. ELISA tests using both cysteine proteases showed a rapid and consistent detection of specific IgG in all experimentally infected goats. The IgG response to P28 was the first to be detected as early as 2–3 weeks post-infection and remained elevated throughout the experiment. The response to P34 was detected later (4–6 wpi) and disappeared in some animals at 18 wpi, while flukes were still present in the bile ducts. No significant differences were observed between the anti-P28 and anti-P34 IgG responses between animals receiving a primary or a challenge infection. The results of our study, although preliminary, are promising since the P28 ELISA described here may be a reliable method for the immunodiagnosis of *F. hepatica* infection in goats.

Fasciola hepatica / cysteine proteinases / IgG / ELISA / goat

1. INTRODUCTION

Although fasciolosis in goats is considered to be less frequent than in other ruminant species, in some geographical areas its prevalence reaches high values [14]. Similarly, goats are described as a species very sensitive to *Fasciola hepatica* in which the disease can appear in its acute form [17].

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The detection of infections in ruminants relies on the microscopic observation of *F. hepatica* eggs in the faeces of infected animals. However, early diagnosis by coprological examination is not possible because eggs are not found in the faeces until 10–12 weeks after infection, when flukes reach maturity, and when hepatic injury has been produced [33]. To prevent this hepatic damage, several immunological methods have been developed for the detection of early and specific antibodies to *Fasciola hepatica*, especially in cattle and sheep [31] but few of them have been focused on goats [18].

Serodiagnosis of sheep and cattle fasciolosis was initially developed for the detection of specific antibodies against antigens derived from adult fluke extracts or their excretory/secretory products (ESP) [34, 35]. Antibodies to these antigens can be detected during the first 2–4 weeks after infection, providing a rapid and sensitive procedure for the diagnosis of the disease [31]. However, cross-reactivity with other parasites has been reported, decreasing the specificity of the techniques [4, 28].

Another alternative based on the detection of specific antigens in the serum [29] or in the faeces of infected animals [11] has been developed. Although these assays can give additional information about the presence of the parasite in the host and an estimation of fluke burden, their value is also limited. Coproantigens cannot be detected until 6 weeks post-infection [1] and antigenemia disappeared at about 8–10 weeks, probably due to the formation of immune complexes [5]. This kind of assay has never been evaluated in goats.

Recently, partially purified or well-defined *F. hepatica* antigens have been evaluated in order to enhance the specificity of the diagnostic assay. Cysteine proteinases, from the adult fluke or from ESP, are one of the more widely used. They have been used as antigens in the serodiagnosis of human and animal fasciolosis (in cattle, sheep and some camelids such as alpacas) [6, 8, 9, 23], with an excellent potential for the sensitive and specific detection of the disease. In fact, serum from patients infected with *Schistosoma mansoni*, cysticercosis, hydatidosis and Chagas disease does not cross-react with *Fasciola hepatica* proteinases [24].

The aim of this work was to study the potential use of cysteine proteinases of *F. hepatica* adult fluke as diagnostic markers of fasciolosis in experimentally infected goats. The humoral response (IgG) against electroluted cysteine proteinases was analysed and compared with the faecal egg output in goats receiving a primary and a challenger infection. In the same way, a preliminary study on the cross-reactivity of the fluke proteinases was evaluated in goats experimentally infected with *Teladorsagia circumcincta* and *Toxoplasma gondii,* two common parasites of this species.

2. MATERIALS AND METHODS

2.1. Animals

Fifteen eight-month-old male Serrana goats were used for experimental infections. They were randomly allocated into three groups of five animals each (groups A to C). All animals were housed in covered pens and fed with hay and a commercial pelleted ration for goats. Water and mineralised salt were available "ad libitum".

2.2. Experimental infection

All animals were left for fifteen days to acclimatise to pen conditions. Group A was used as the uninfected control. Goats of groups B and C were orally infected with viable *F. hepatica* metacercariae (kept for five months at +4 °C) of bovine origin provided by the Central Veterinary Laboratory, Weybridge (UK). Group B received four doses of 50 metacercariae on weeks 0, 1, 2 and 3. Group C also received four doses of 50 metacercariae at the same

time and were challenged with 200 meta-cercariae on week 8. From week 0 until week 19, blood samples were taken weekly by jugular venipuncture. Serum samples were stored at –20 °C until serological analysis. Infected and control goats were killed by intravenous injection of a lethal dose of thiobarbital at the end of the experiment. This experiment was carried out in accordance with the Code of Practice for the Housing and Care of Animals used in Scientific Procedures.

2.3. Parasitological studies

Faecal samples were collected from each animal every week and the faecal examinations were performed using a sedimentation method. At necropsy, the liver and the gallbladder were dissected and all flukes were recovered, counted, fixed by standard methods and measured.

2.4. Preparation of parasite homogenates

Adult parasites were harvested from naturally infected cattle after opening the bile ducts. Parasites were washed extensively in sterile 0.1 M PBS, pH 7.4, blotted dry and then homogenised on ice using a glass/glass homogeniser. Homogenates were centrifuged at $10\,000 \times g$ for 30 min at 4 °C. The supernatant was collected and kept at –80 °C until use for enzyme determinations.

2.5. Evaluation of the proteolytic activity of parasite products

The proteolytic activity and the effect of pH on that activity in the parasite homogenate was measured spectrophotometrically using the chromogenic non-specific substrate azocasein (Sigma Chemical. St. Louis, USA) [15]. Ten microliters of homogenate (1.5 mg/mL) (or H_2O for blanks) were incubated with 100 µL of substrate (1 mg/mL in buffer at different pH from 3.0 to 11.0) for 16 h, then the undigested proteins were precipitated by addition of an equal volume of 1.0 M perchloric

acid. The supernatant was harvested by centrifugation for spectrophotometrical determinations at 405 nm.

The sensitivity of azocasein degradation to a variety of proteinase inhibitors was also examined at pH 5 and 7. Homogenate or H_2O for blanks were pre-incubated with different inhibiting buffers at the following final concentrations: PmsF: phenylmethyl-suphonylfluoride, 1.0 mM; EDTA: ethylenediaminotetra-acetic acid, 10.0 mM; E64: L-trans-epoxysuccinyl-L-leucylamide-(4-guanidino)-butane, 0.1 mM; pepstain: 0.1 mM.

The molecular weights of the proteins with proteolytic activity were determined using gelatin-substrate gel analysis. The homogenates were run under non-reducing conditions into a discontinuous SDS-polyacrylamide gel containing 0.1% (w/v) gelatin. Fractions of the gels were washed several times with 3% Triton X-100 and then incubated for 16 h with buffer only or buffer containing proteinase inhibitors. The Coomassie blue stain was used to detect the proteolytic activity in the gels [16].

2.6. Electroelution of proteins from SDS-page gels

F. hepatica adult worm homogenate was subjected to SDS-PAGE and the proteins of Mr 28 (P28) and 34 (P34) kDa with proteolytic activity were localised in gels using a coloured marker protein. The electroelutions were performed using a GE 200 SixPac Gel Eluter (Hoefer Scientific Instruments, San Francisco, USA) following the manufacturer's instructions. Briefly, P28 and P34 bands were cut into approximately 2–3 mm squares and placed into the elution tube. Elution was run at 50 V for 2 h and a reverse polarity at the end of the elution for 5 s in 0.025 M Tris pH 8.3, 0192 M glycine and 0.1% (w/v) SDS. SDS-PAGE under non-reducing conditions of both proteolytic fractions and *F. hepatica* adult fluke homogenates are
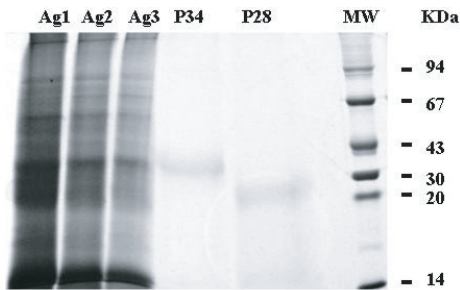
Figure 1. SDS-PAGE under non-reducing conditions of *F. hepatic*a adult flukes homogenate at different concentrations (Ag1, Ag2, Ag3) and electroluted fractions with cysteine proteinase activity (P34, P28). MW, molecular weight markers in kDa.

shown in Figure 1. Collected fractions were used independently as the antigen in two indirect ELISA (ELISA-28 and ELISA-34).

2.7. Indirect enzyme-linked immunosorbent assay (ELISA)

ELISA conditions for IgG anti-P34 and anti-P28 were carried out using the checkerboard technique. A positive serum pool, from naturally infected goats with high titers against *F. hepatica* ESP [18], and negative serum pool (control group) were used in the checkerboard protocol. Both of them were diluted at 1:100 and used to establish optimal concentration of antigen and conjugate (anti-goat IgG-peroxidase, Sigma) according to the results obtained by successive two-fold dilutions. Finally, an antigen concentration of 5 µg/mL and a 1:4000 dilution of conjugate were used. OPD (Sigma) at a final concentration of 0.4 mg/mL in 0.012% (v/v) H_2O_2 citrate buffer was used as the substrate. For each plate, test samples and both positive and negative reference pools were included and diluted at 1:100. The OD at a wavelength of 492 nm of each serum sample was expressed as a percentage (% relative) of the OD value observed in a positive pool.

To study the cross-reactivity of P34 and P28, the sera of goats experimentally infected with common parasites in goats such as *Toxoplasma gondii* and *Teladorsagia circumcincta* were analysed under the same conditions [7, 22].

2.8. Statistical methods

The data were analysed either by the Student *t* test or by analysis of variance (F test). *p* values of 0.05 or lower were considered statistically significant.

3. RESULTS

3.1. Development of the infection

F. hepatica eggs were observed in the faeces of the infected animals between 9 and 13 weeks pi and were recovered until the end of the experiment. Peak egg sheddings (870 and 910 eggs per gram respectively) were recorded at the end of the experiment in groups B and C respectively. The uninfected group A showed negative counts throughout the experiment.

The mean number of flukes recovered from infected goats at necropsy was 48.4 for group B (24.3% of the infective dose) and 83 for group C (20.1% of the infective dose). Recovered flukes from each group were measured and no significant differences in the mean fluke lengths were found between both groups.

3.2. Proteolytic activity of adult *F. hepatica* homogenates

Gelatin gels revealed two major fractions with proteolytic activity in the homogenates of *F. hepatica* with apparent molecular weights of approximately 28 and 34 kDa (P28 and P34 respectively) (Fig. 1). The two proteins were characterised as cysteine proteases, according to the inhibition of their proteolytic activity by
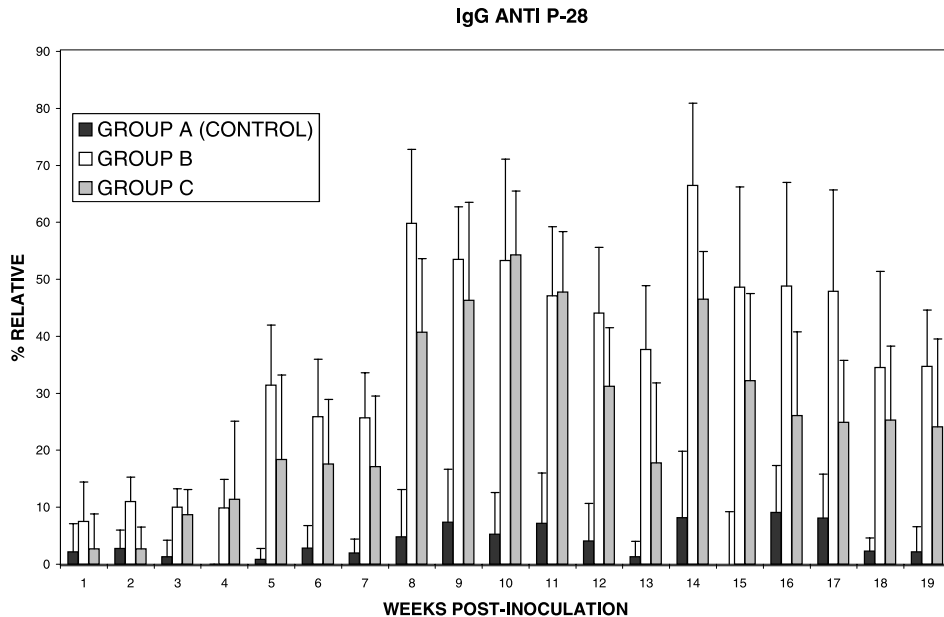
Figure 2. IgG response (mean value ± standard deviation) anti P-28 in control (group A) and experimentally infected (groups B and C) goats. OD results have been expressed as a percentage (relative %) of the OD observed with a positive pool.

the E64 inhibitor using both azocasein and gelatin as substrates.

3.3. IgG response against P28

Figure 2 shows the IgG levels obtained using the ELISA test with P28 as the antigen. Infected groups (B and C) showed significant higher levels of IgG anti-P28 compared to those of the control group ($p < 0.001$). A significant increase in specific antibodies against P28 was first detected at 2 weeks post-infection (wpi) in group B and 3 wpi in group C and was maintained throughout the rest of the experiment. Serum levels of specific IgG reached peak values at 8 wpi in group B (59.8%) and at 10 wpi in group C (54.3%) and then declined until the end of the study. There were no significant differences between the specific IgG levels of groups B and C during the experiment.

3.4. IgG response against P34

Specific anti-P34 IgG levels are shown in Figure 3. Antibody responses against P34 in infected goats (groups B and C) were significantly higher than those observed in the control group ($p < 0.001$). Specific serum IgG levels increase significantly from 4 wpi and 6 wpi onwards in groups B and C respectively. Peak values were observed at 8 wpi in group B (89.6%) and 9 wpi in group C (64.6%) and then decreased gradually. IgG anti P34 levels were not significantly increased at 18 wpi. No statistical difference was observed in the evolution of specific antibody response between groups B and C.

3.5. Cross-reactivity with other parasites

The levels of IgG anti-P28 and P34 were lower than 5% in serum samples of
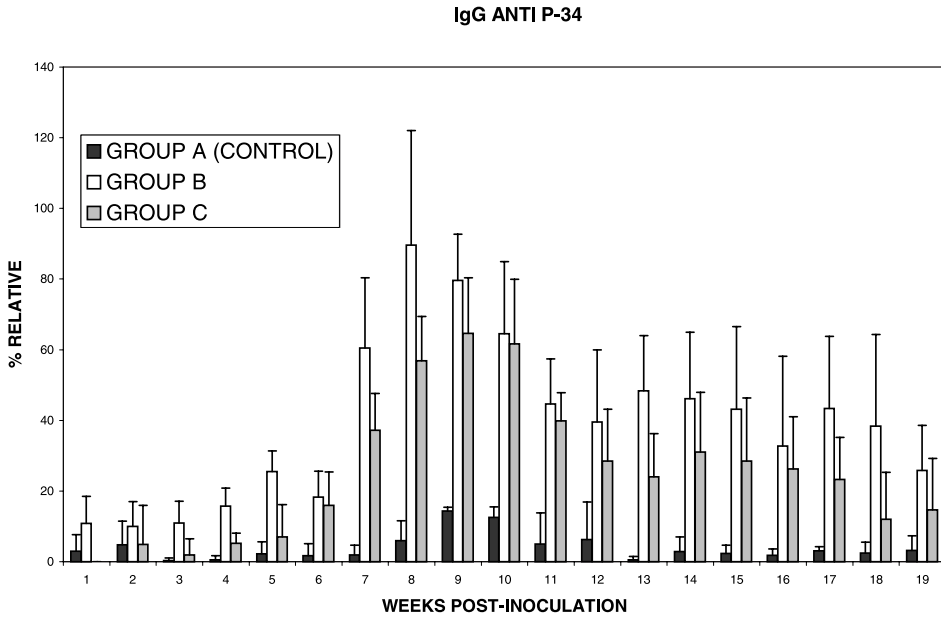
IgG ANTI P-34

Figure 3. IgG response (mean value ± standard deviation) anti P-34 in control (group A) and experimentally infected (groups B and C) goats. OD results have been expressed as a percentage (relative %) of the OD observed with a positive pool.

goats experimentally infected with either *T. gondii* or *T. circumcincta.*

4. DISCUSSION

Cysteine proteinases with molecular weights ranging between 10 and 150 kDa have been isolated from excretory-secretory products [10] and parasite homogenates [27] of both immature and mature *F. hepatica*. Several functions have been suggested for these proteinases, including tissue invasion, feeding and immune evasion [3, 32]. In the present work, two cysteine proteinases of 28 and 34 kDa were purified, by means of preparative electrophoresis and electroelution in polyacrylamide gels. This method has been previously used in the isolation of antigens of diagnostic utility in fasciolosis [30] and other parasitic diseases [12].

These molecules have been considered as valuable antigens for the immunodiagnosis of the disease and some of them have been evaluated. Fagbemi and Guobadia [13] showed that a 28 kDa cysteine protease obtained from *F. gigantica* adult worms ensured high sensitivity and specificity of the ELISA test for ruminant fasciolosis due to this parasite. A protein band of 28 kDa present in *F. hepatica* adult preparations was immunodominant in cattle naturally exposed to the parasite [25]. Purified cysteine proteases of *F. hepatica* have been used in highly sensitive and specific ELISA for detecting human fasciolosis [6, 24].

In this study, ELISA using both cysteine proteases P28 and P34 allowed a rapid and consistent detection of specific IgG in all experimentally infected goats. The profiles of the IgG anti-P28 and anti-P34 were slightly different. The IgG response to P28

occurred earlier than the response to P34. Besides, increased values of IgG anti-P28 were sustained throughout the whole experiment, but disappeared at 18 wpi in the P34 ELISA test, while flukes were still present in the bile ducts and faecal egg outputs were high. Accordingly, P28 seems to be a better antigen than P34 to detect and evaluate *F. hepatica* infections in goats.

The antibody response to P28 (and, to a lesser extent, to P34) followed a pattern similar to the response to adult *F. hepatica* ESP described previously [20]. Both P28 and ESP specific antibodies were produced early, reached a peak at 8–12 wpi and decreased thereafter. There was no significant correlation between anti-P28 or anti-ESP IgG levels and fluke burdens and no differences could be detected between primarily and secondarily infected goats. This similarity may be explained by the fact that the ESP of adult *F. hepatica* included cysteine proteinases [32], secreted or excreted from the tegument or the gut of the flukes [2].

Any ELISA used for the immunodiagnosis of *F. hepatica* infections must have high sensitivity and specificity, and be able to detect infection early, before liver damage has occurred. In our experience, with experimentally infected and challenger goats, the P28 ELISA exhibited a high sensitivity and revealed no cross-reactions with sera from goats infected with *T. circumcincta* and *T. gondii*. Cross-reactivity with other goat parasites has not yet been evaluated because of the lack of monospecific sera. Early detection was also achieved, especially with P28, which was recognised at 2–3 wpi, before significant liver damage occurred as has been reported in previous studies [19]. The recognition of P28, obtained from adult flukes, at 2–3 wpi, when adult flukes have not yet developed, suggests that juvenile and adult parasites produce the same cysteine proteinases [21] or the existence of some common epitopes shared by juvenile and mature proteinases [4]. It is also noteworthy that early detec-

tion (at 2 wpi) was performed with relatively low doses of infecting parasites: group C was infected with only 100 metacercariae, an amount smaller than what is usual in natural infections [26].

The results of the present study, although preliminary, are promising: the P28 ELISA described here may be a reliable method for the immunodiagnosis of *F. hepatica* infection in goats. Further studies will be carried out to determine the cross-reactivity with *Dicrocelium dendriticum*, *Moniezia* spp. and some other parasites and to apply the test in field studies.

ACKNOWLEDGEMENTS

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