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Comparison of recombinant cathepsins L1, L2, and L5 as ELISA targets for serodiagnosis of bovine and ovine fascioliasis

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Abstract

Infections caused by *Fasciola hepatica* are of great importance in the veterinary field, as they cause important economic losses to livestock producers. Serodiagnostic methods, typically ELISA (with either native or recombinant antigens), are often used for early diagnosis. The use of native antigens, as in the MM3-SERO ELISA (commercialized as BIO K 211, BIO-X Diagnostics), continues to be beneficial in terms of sensitivity and specificity; however, there is interest in developing ELISA tests based on recombinant antigens to avoid the need to culture parasites. Of the antigens secreted by adult flukes, recombinant procathepsin L1 (rFhpCL1) is the most commonly tested in ELISA to date. However, although adult flukes produce three different clades of CLs (FhCL1, FhCL2, and FhCL5), to our knowledge, the diagnostic value of recombinant FhCL2 and FhCL5 has not yet been investigated. In the present study, we developed and tested three indirect ELISAs using rFhpCL1, rFhpCL2, and rFhpCL5 and evaluated their recognition by sera from sheep and cattle naturally infected with *F. hepatica*. Although the overall antibody response to these three rFhpCLs was similar, some animals displayed preferential recognition for particular rFhpCLs. Moreover, for cattle sera, the highest sensitivity was obtained using rFhpCL2 (97%), being equal for both rFhpCL1 and rFhpCL5 (87.9%), after adjusting cut-offs for maximum specificity. By contrast, for sheep sera, the sensitivity was 100% for the three rFhpCLs. Finally, the presence of truncated and/or partially unfolded molecules in antigen preparations is postulated as a possible source of cross-reactivity.

Keywords Cathepsin · *Fasciola hepatica* · Fascioliasis · ELISA · Cross-reactivity · Serodiagnosis

Introduction

Fascioliasis (or fasciolosis) is recognized as one of the most important food-borne trematodiasis in the veterinary field because of the economic impact it has on livestock (mainly sheep and cattle) producers (Schweizer et al. 2005; Charlier

et al. 2007; Charlier et al. 2009; Mezo et al. 2011). The genus *Fasciola* includes two species, *F. hepatica* and *F. gigantica*, the former of which is distributed worldwide and the second of which is restricted to several regions of Africa and Asia (Mas-Coma et al. 2009). The broad distribution of *F. hepatica* seems to be associated with its ability to adapt to new definitive hosts (Robinson and Dalton 2009) and with its capacity to infect different snail species (intermediate hosts) living in diverse habitats and under different environmental conditions (Correa et al. 2010).

When humans or animals are infected by ingestion of the metacercariae (present in vegetables or contaminated water), the parasites excyst in the intestine, transverse the intestinal wall and the peritoneal cavity, and then migrate to the liver where they feed and grow for 8–12 weeks (Andrews 1999). Finally, the parasites move into biliary ducts where they mature and begin producing eggs. Nevertheless, sometimes a small proportion of parasites reaches ectopic locations (Mas-

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Coma et al. 2013, 2014), thus preventing elimination of the eggs through the biliary ducts. Considering the characteristics of the biological cycle of *Fasciola*, diagnosis of infections by either *F. hepatica* or the more pathogenic *F. gigantica* (Valero et al. 2016) has traditionally been carried out by microscopic observation of parasite eggs in fecal samples from infected hosts (Mas-Coma et al. 2014). However, these methods are tedious, have poor sensitivity, and depend to a greater or lesser degree on the experience of the examiner. Moreover, fecal examination cannot detect acute infections when the parasites feed on liver parenchyma, before egg production has started (Charlier et al. 2014; Mas-Coma et al. 2014).

In the past two decades, considerable effort has been made to develop rational ELISA methods for the diagnosis of human and animal infections caused by *F. hepatica* and *F. gigantica* in order to prevent the limitations inherent in microscopic examination of fecal samples (Espino and Dumenigo 2003). Such methods include the use of whole or purified natural antigens from *Fasciola* as well as recombinant antigens, to detect anti-*Fasciola* circulating antibodies (Cornelissen et al. 1999; Carnevale et al. 2001b; Espinoza et al. 2007; Mezo et al. 2007; Gonzales Santana et al. 2013; Gottstein et al. 2014). They also include ELISA methods capable of detecting secreted antigens present in serum or fecal samples from infected humans and animals (Espino and Finlay 1994; Abdel-Rahman et al. 1998; Mezo et al. 2004; Ubeira et al., 2009; George et al., 2017). Because antibody titers may remain elevated for long periods of time after treatment of the infection, ELISA methods for the detection of specific antibodies cannot distinguish between current or past infections. Nevertheless, these ELISAs offer the advantage of being able to diagnose early prepatent (acute) infections (Salimi-Bejestani et al. 2007; Mezo et al. 2010a,b; Mas-Coma et al. 2014), and, for this reason, they are frequently used to screen herds of domestic animals in serum and mostly in milk samples (Mezo et al. 2010b; Duscher et al. 2011; Mezo et al. 2011). Most of these methods are indirect or capture ELISAs that include *Fasciola* cathepsins L (CLs) as antibody targets, as these antigens probably induce the highest proportion of anti-*Fasciola* antibodies during natural infections. However, except for ELISAs based on the use of single recombinant CLs (rCLs) or recombinant procathepsins L (rpCLs), typically L1 clade members, the suitability of each of the several cathepsins secreted by *Fasciola* for detecting anti-*Fasciola* antibodies by ELISA has not previously been investigated. This aspect is relevant, as it has been reported that the pattern of cathepsins that are secreted by *F. hepatica* varies during its biological cycle, so that immature parasites begin producing FhCL3, FhCL4, and cathepsins B, but that expression is gradually replaced by FhCL1, FhCL2, and FhCL5 in adults (Robinson et al. 2008; Smooker et al. 2010; Cwiklinski et al. 2015). Moreover, production of these FhCLs by adult flukes has been reported to be imbalanced, with proportions of 69, 22 and 9% for FhCL1, FhCL2, and FhCL5,

respectively (Robinson et al. 2009). It is therefore important to determine which of these molecules are most relevant from the point of view of their antigenicity and suitability as target antigens for serodiagnosis of human and animal infections by *Fasciola*. In the present study, we developed and tested indirect ELISAs based on recombinant procathepsin L1 (rFhpCL1), rFhpCL2, or rFhpCL5, in order to investigate which of these targets are best recognized by sera from sheep and cattle naturally infected with *F. hepatica*. We also evaluated if any of these rFhpCLs is advantageous over the use of native CLs using as reference method of this study the MM3-SERO ELISA, in which the monoclonal antibody (mAb) MM3 captures native *Fasciola* CLs (Mezo et al. 2007).

Material and methods

Collection of biological samples

Cattle

A slaughterhouse that processes cattle from the whole region was visited fortnightly during a year. At each visit, 15 adult cows (over 2 years old; Friesian breed, females) were selected at random, and their livers as well as stool and blood samples were collected and transported to the laboratory promptly. In total, samples from 100 naturally *F. hepatica*-infected and 200 *F. hepatica*-free adult cattle, as determined, respectively, by the presence or the absence of flukes in livers (gold standard), were collected. For recovering and counting all flukes, each liver was thoroughly examined. In the first step, we proceeded to the opening of the bile ducts and the gallbladder to obtain the most of intact adult flukes and then the livers were cut into slices (approximately 1 cm thick) which were manually squeezed to obtain the immature flukes inhabiting the parenchyma. This procedure prevented any possible problems of low sensitivity when liver inspection is not performed thoroughly, as has been described in previous studies (Rapsch et al. 2006; Charlier et al. 2008).

In addition to fluke counts, samples of feces from all animals were subjected to sedimentation (Anderson et al. 1999) and flotation (MAFF 1971) procedures to concentrate eggs from *Fasciola* or other parasites, and then they were microscopically examined. The remaining fecal material was frozen at −25 °C for subsequent further quantitation of *Fasciola* antigens by the MM3-COPRO ELISA (Mezo et al. 2004; Martínez-Sernández et al. 2016).

As we prioritized testing simultaneously the sera with the four *F. hepatica* antigens in a single run, the sample size was limited to 33 *F. hepatica*-infected and 22 *F. hepatica*-free cows, which was optimal for the conditions of our laboratory. For the first group, the infected animals ($n = 100$) were stratified in three categories according to their parasite burden

(low: 1–10 flukes, middle: 11–25 flukes, and high: ≥ 26 flukes) and animals from each group were selected by random sampling using EPIDAT 3.1 software (Consellería de Sanidade, Xunta de Galicia, Spain). To assure a better comparison of the performance of the different ELISAs evaluated in the present study, animals with low parasite burdens represented the 60% ($n = 20$) of the sample, while animals from the remaining categories constituted both the $\approx 20\%$ ($n = 6$ and 7) of the sample. Of the 200 *F. hepatica*-free cattle, 22 were selected by simple random sampling.

Sheep

Serum and fecal samples used in the present study were obtained from the herd of a commercial farm suffering from fascioliasis (infected sheep) and from the fluke-free herd maintained at INGACAL (non-infected sheep). The sheep in both herds were an autochthonous Galician breed ("raza gallega"). The sample size was limited by the availability of animals to conduct the study. In the first herd, the 15 sheep suffering chronic fascioliasis (using both data from coprology and MM3-COPRO ELISA as gold standard) were sampled. In the second herd, samples were taken from another 15 sheep chosen completely at random. As for cattle samples, the feces from all sheep were examined for the presence of eggs from *Fasciola* and other parasites. Fecal aliquots were also frozen at -25 °C for further quantitation of *Fasciola* antigen by the MM3-COPRO ELISA (Mezo et al. 2004; Martínez-Sernández et al. 2016).

Parasites and antigens

The *F. hepatica* excretory-secretory antigens (ESAs) used in the MM3-SERO ELISA (see below) were obtained as previously reported (Mezo et al. 2003). Briefly, live adult flukes were collected from the bile ducts of naturally infected cows and washed, first in sterile saline solution containing antibiotics (penicillin/streptomycin) and glucose (2 g/L), at 38 °C, and then in RPMI 1640 cell culture medium supplemented with 20 mM HEPES, 0.3 g/L L-glutamine, 2 g/L sodium bicarbonate and antibiotics, at 38 °C under 5% CO_2 in air. The flukes were then transferred to 75-cm^2 tissue culture flasks and maintained in culture medium (3 mL/fluke) at 38 °C under 5% CO_2 in air. After 24 h incubation, the medium containing the secreted antigens was removed and centrifuged at 10,000g for 20 min at 4 °C in the presence of protease inhibitors (SigmaFast Protease Inhibitor Tablets, Sigma-Aldrich, Madrid, Spain). The supernatant was then passed through a 0.45-μm pore filter disk, concentrated in an Amicon 8050 ultrafiltration cell (Amicon, Inc., Beverly, MA) equipped with a YM10 membrane (10 kDa molecular weight cut-off), dialyzed against PBS, sterilized by filtration and, finally, stored at -80 °C until required. The protein concentration was measured using the Micro BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Barcelona, Spain).

The recombinant Ani s 1 allergen (rAni s 1) (Anadón et al. 2010; Cuéllar et al. 2012) included in the Trisakis 170 kit (Lin et al. 2014), was produced in *E. coli* and purified and refolded as previously described (Anadón et al. 2010).

Production and purification of mAb MM3

Hybridoma cells secreting mAb MM3 were obtained as previously described (Mezo et al. 2004; Martínez-Sernández et al. 2014). The secreting hybridoma cells were grown intraperitoneally in pristan-primed BALB/c mice, and the anti-*F. hepatica* IgG1/κ antibodies were isolated from the ascitic fluid by affinity chromatography on a protein G column (HiTrap Protein G, GE Healthcare, Madrid, Spain) according to the manufacturer's protocol.

Cloning of the genes encoding FhpCL1, FhpCL2, and FhpCL5 and expression of recombinant proteins

Nucleic acid isolation

Messenger RNA (mRNA) was obtained from adult specimens of *F. hepatica* as previously reported (Muiño et al. 2011). Briefly, whole *F. hepatica* mRNAs were obtained using the Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA), according to the manufacturer's instructions, and the mRNA concentrations were determined by spectrophotometry (NanoDrop; Thermo Fisher Scientific). A collection of cDNA was prepared with one microgram of mRNA using the Marathon cDNA Amplification kit (Clontech, Palo Alto, CA), according to the manufacturers' instructions. The double-strand cDNAs were subsequently ligated to the Marathon adaptors (AP1).

Cloning of FhpCL1

The procedures used to clone and subclone FhpCL1 are reported elsewhere (Muiño et al. 2011). The deduced amino acid sequence was annotated under GenBank accession number CCA61803.1. For expression of the corresponding protein, the FhpCL1 gene was directionally cloned into the pQE-30 expression vector (Qiagen; Qiagen Iberia SL, Madrid, Spain) and further transformed into the *E. coli* strain M15 [pREP4].

Cloning of FhpCL2

Primers corresponding to sequences described by Kofta et al. (2000) were synthesized to amplify the full cathepsin molecules: F-kofta (5′ ATGTGGTTCTTCGTATTAGC 3′) and R-kofta (5′ CCAAGTATTTTTAACAATCCAATA 3′). PCR

reactions were carried out under conditions of low stringency, in order to amplify genes corresponding to different types of cathepsins. The cDNA described above was used as template. The PCR products were cloned into the pGEM-T vector (Promega Biotech Ibérica SL, Madrid, Spain), and DNA from recombinant plasmids was automatically sequenced using fluorescence-base labeling with the ABI PRISM system (Perkin Elmer, Langen, Germany) and the universal primers D and SP6. A clone similar to the FhCL2 (gb|CCA61803.1) was obtained. The FhpCL2 without the signal peptide was directionally cloned (Sac I – Sma I) into the pQE-30 expression vector (Qiagen) using primers F-pCL2 (5′ TCGAATGA CGATTTGTG 3′) and R-pCL2 (5′ TTCACGGAAATC 3′), and was then further transformed into the *E. coli* M15 [pREP4] cells. The selected FhpCL2 sequence was annotated in GenBank under accession number KY464953.

Cloning of FhpCL5

Generic primers, F-pCL5 (5′ TCAAATGACGATTT GTGGCATCAATGGAAG 3′) and R-pCL5 (5′ TCACGGAAATTGTGCCACCATCGGGAC 3′), were designed to directly clone the FhpCL5 gene using the cDNA collection as template. Considering that the FhpCL1, FhpCL2, and FhpCL5 sequences were almost identical at the amino terminal region and quite similar at the carboxy terminal region (both used to design the different sets of primers), and as FhpCL5 is the least abundant member of the FhCLs expressed by adult worms (Robinson et al. 2009), numerous clones had to be sequenced to obtain a sequence compatible with the characteristics previously reported by Norbury et al. (2012) for the CL5 clade. The selected FhpCL5 sequence was annotated in GenBank under accession number KY392883. For protein expression, competent *E. coli* M15 [pREP4] cells were transformed with the FhpCL5 gene directionally cloned (Bam H I – Sac I) into the pQE-30 expression vector (Qiagen) with the primers F2-pCL5 (5′ TCAAATGACGATTTGTGG 3′) and R2-pCL5 (5′ TCACGGAAATTGTGC 3′).

Expression, purification, and refolding of rFhpCL1, rFhpCL2, and rFhpCL5

Recombinant FhpCLs expression was induced by adding 1 mM IPTG to the transformed *E. coli* cultures (Muiño et al. 2011). Once the expression was induced, cultures were maintained 4 h at 37 °C. Then, *E. coli* cells were harvested by centrifugation, and the insoluble recombinant proteins were purified with B-PER reagent (Thermo Fisher Scientific), solubilized and purified by affinity chromatography with HIS-Select Nickel Affinity Gel (Sigma-Aldrich) under denaturing conditions (8 M urea), as indicated by the supplier. The different rFhpCLs were refolded as previously described (Muiño

et al. 2011) with a few modifications. Briefly, each elute from the affinity column was pretreated with 10 mM DTT for 1 h at room temperature (RT), and subsequently diluted at a ratio of 1/10 in TBS (50 mM Tris, 150 mM NaCl, pH 8) plus 10 mM cysteine, 1 mM cystine, and 1 mM EDTA. Once dialyzed against TBS (pH 8), each rFhpCL was dialyzed against PBS and concentrated by membrane-filtration in an Amicon-stirred ultrafiltration cell equipped with a Filtron Omega Series membrane (10 kDa nominal molecular weight limit; Pall Corporation, Port Washington, NY). Finally, the protein concentration was determined with the Micro BCA Protein Assay kit, and the refolded recombinant proteins were stored at − 80 °C until use.

Adjustment of rFhpCL1, rFhpCL2, and rFhpCL5 concentration in ELISA

To investigate the optimal concentration of each rFhpCL for use as target in indirect ELISA, we tested several concentrations of each antigen in the range of 1–10 μg/mL in PBS. Polystyrene microtiter plates (Greiner Bio-One; Soria-Melguizo, Madrid, Spain) were coated with 100 μL/well of each rFhpCL dilution and incubated for 2 h at 37 °C. The plates were then washed three times with PBS and blocked with 200 μL/well of 1.5% sodium caseinate in PBS for 1 h at RT. Aliquots of 100 μL of an appropriate dilution of mAb MM3 (1/2000) in PBS containing 0.05% Tween 20 and 1% skimmed dry milk (PBS-T-SM) were then added to each well, and the plates were incubated for 30 min at RT with shaking at 750 rpm. The plates were then washed five times with PBS-T, and bound MM3 antibodies were detected after incubation with HRP-labeled goat anti-mouse IgG secondary antibodies (Bio-Rad, Madrid, Spain) diluted 1/3000 in PBS-T-SM for 30 min at RT with orbital shaking at 750 rpm. The plates were then washed, as above, and incubated for 20 min at RT with 100 μL/well of the enzyme substrate OPD (SigmaFast OPD, Sigma-Aldrich). Finally, the optical density (OD) was measured at 492 nm. Recombinant procathepsin dilutions containing the same equivalent concentration (i.e., yielding the same OD signal with mAb MM3) were selected for use in an indirect ELISA, as indicated below.

Indirect ELISAs with rFhpCL1, rFhpCL2, rFhpCL5, and rAni s 1

The wells of ELISA plates were coated with 100 μL of each rFhpCL (rFhpCL1, rFhpCL2, or rFhpCL5) or with the *Anisakis simplex* allergen rAni s 1, all at a concentration of 2.5 μg/mL in PBS, and incubated for 2 h at 37 °C. The plates were then washed three times with PBS and blocked with 200 μL/well of 1.5% sodium caseinate in PBS for 1 h at RT. Aliquots of 100 μL of sheep or cattle sera (from *F. hepatica*-infected and *F. hepatica*-free animals) diluted 1/100 in PBS-T-

SM were added to each well of the plates in duplicate, and incubated for 30 min at RT with orbital shaking at 750 rpm. The plates were then washed five times with PBS-T, and bound IgG antibodies were detected with either HRP-conjugated mouse anti-sheep/goat IgG monoclonal antibodies (1/30,000 in PBS-T-SM; Sigma-Aldrich), or HRP-conjugated sheep anti-bovine IgG1 polyclonal antibodies (1/6000 in PBS-T-SM; Bio-Rad), and OPD, as indicated above.

MM3-SERO ELISA

The MM3-SERO ELISA was performed as previously described (Mezo et al. 2007) but with some modifications. Briefly, ELISA plates were coated with purified mAb MM3 (100 μL/well at 5 μg/mL), incubated ON at 4 °C, washed three times with PBS, and blocked with 200 μL/well of 1.5% sodium caseinate in PBS for 1 h at RT. Aliquots of 100 μL of *F. hepatica* ESAs at 1 μg/mL in PBS or PBS only were added to each well in odd (Ag+) and even (Ag−) plate rows, respectively. The plates were incubated for 2 h at RT and then washed three times with PBS, before 100 μL of each serum sample (from sheep or cattle) diluted 1/100 in PBS-T-SM was added to each Ag+ and Ag− well in duplicate. The plates were then incubated for 30 min at RT with shaking at 750 rpm, washed five times with PBS-T, and specific sheep or bovine IgG was detected as described above. The OD value for each sample was calculated as OD1-OD2, where OD1 is the value for the Ag+ well, and OD2 is the value for the Ag− well.

Statistical analysis

The significance of the differences in coproantigen values for fecal samples obtained from parasitized cattle classified according to their parasite burden (low, middle, high; see above) was determined by the Kruskal-Wallis test (nonparametric ANOVA) and Dunn's multiple comparison test. The analysis was conducted using the GraphPad Instat statistical package (GraphPad Software Inc., San Diego, CA). Differences were considered significant at $p < 0.05$. Pearson's correlation coefficients (r) were calculated to compare the data obtained analyzing samples from infected animals with the different indirect and capture ELISAs using OriginPro 7.5 (OriginLab Corporation, Northamptom, CA).

The cut-off values for each ELISA and species tested (sheep or cattle) were calculated from the OD values obtained testing the sera from *F. hepatica*-free animals. Two methods were used to calculate the cut-off values for the different indirect ELISAs. In method A, designed to guarantee maximum specificity (i.e., 100%; see Martínez-Sernández et al. 2016), the cut-off values were calculated as the sum of the highest OD value obtained on testing the negative sera plus 1SD. These cut-offs were used to obtain the sensitivities of each test, which were calculated using EPIDAT 3.1 software as the number of true positives (correctly

identified by each test), divided by the total number of infected animals. In method B, the cut-off values were obtained by receiver operating characteristic (ROC) analysis. The ROC curves were generated using the MedCalc software (MedCalc Software, Ostend, Belgium) following the methodology proposed by DeLong et al. (1988). The cut-off values defined by the Youden index (i.e., that maximize the sum of sensitivity and specificity) were used to estimate the sensitivity and specificity of each test. Finally, the cut-offs and the corresponding sensitivities of the MM3-SERO ELISA were determined considering method A only.

Results

Comparison of the FhpCL1, FhpCL2, and FhpCL5 amino acid sequences

The sequence alignments of the three FhpCLs used in this study are shown in Fig. 1. The displayed sequences corresponded to the full-length propeptide (residues S^1-R/L^{91}) lacking the deduced signal peptide (residues 1–15). The alignments revealed a high percentage of sequence identity among the three FhpCLs, although the percentage of identity was higher between FhpCL1 and FhpCL5 (87%), in comparison with the 84% calculated for FhpCL2 and FhpCL5, and the 78% for FhpCL1 and FhpCL2. Assignation of the sequence gb|CCA61803.1 to FhCL1 was previously reported (Muiño et al. 2011). Regarding the sequence gb|KY464953, we observed that it differs by three nucleotides, but only by one residue ($L^{52}S$), from sequence gb|ABQ95351.1 (Baspinar et al. unpublished results) and by seven nucleotides, which translate into five residue changes ($L^{52}S$, $F^{86}Y$, $N^{110}D$, $P^{149}T$, $L^{152}F$), from the sequence gb|AAC47721.1 (Dowd et al. 1997), both of which were classified as FhCL2. Interestingly, these three sequences totally coincide with the key residues Y^{69}, M^{70}, A^{135}, L^{160}, T^{161}, A^{163}, and L^{209} (mature protein numbering) reported by Norbury et al. (2012) as being typical of the FhCL2 clade. The sequence gb|KY392883 was classified as FhCL5 because of its similarity to the sequence gb|AAF76330 reported by Smooker et al. (2000) and classified as FhCL5 by Norbury et al. (2012). Our sequence has four nucleotide changes, two of which translate into two amino acid substitutions ($I^{263}T$ and $M^{295}T$), relative to gb|AAF76330. However, both sequences share the characteristic residues L^{69}, M^{70}, A^{135}, L^{160}, N^{161}, G^{163}, and L^{209} reported by Norbury et al. (2012) in the mature FhCL5.

Characteristics of the cattle and sheep included in the study

As indicated in the previous section, fasciolosis in cattle was determined by liver necropsy and further confirmation by

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FhpCL1    1  SNDDLWHQWKRMYNKEYNGDDDQHRRNIWEKNVKHIQEHNLRHDLGLVTYTLGLNQFTDMTFEEFKAKYL   70
FhpCL2    1  SNDDLWHQWKRIYNKEYNGADDEHRRNIWGKNVKHIQEHNLRHDLGLVTYKSGLNQFTDLTFEEFKAKYL   70
FhpCL5    1  SNDDLWHQWKRIYNKEYNGADDYHRRNIWEQNVKHIQEHNLRHDLGLVTYKLGLNQFTDMTFEEFKAKYL   70

FhpCL1   71  TEMSRASDILSHGVPYEANNRAVPDKIDWRESGYVTEVKDQGNCGSCWAFSTTGTMEGQYMKNERTSISF  140
FhpCL2   71  IEIPRSSELLSRGIPYANKLAVPESIDWRDYYVTEVKDQGQCGSCWAFSTTGAVEGQFRKNERASASF    140
FhpCL5   71  TEMPRASELLSHGIPYANKRAVPDRIDWRESGYVTEVKDQGGCGSCWAFSTTGAMEGQYMKNQRTSISF  140

FhpCL1  141  SEQQLVDCSGPWGNNGCSGGLMENAYQYLKQFGLETESSYPYTAVEGQCRYNKQLGVAKVTGYYTVHSGS  210
FhpCL2  141  SEQQLVDCTRDFGNYGCGGGYMENAYEYLKHNGLETESYYPYQAVEGPCQYDGRLAYAKVTGYYTVHSGD  210
FhpCL5  141  SEQQLVDCSRDFGNYGCNGGLMENAYEYLKRFGLETESSYPYRAVEGQCRYNEQLGVAKVTGYYTVHSGD  210

FhpCL1  211  EVELKNLVGAEGPAAVAVDVESDFMMYSGGIYQSQTCSPLGLNHAVLAVGYGTQGGTDYWIVKNSWGSYW  280
FhpCL2  211  EIELKNLVGTEGPAAVALDADSDFMMYQSGIYQSQTCLPDRLTHAVLAVGYGSQDGTDYWIVKNSWGTWW  280
FhpCL5  211  EVELQNLVGAEGPAAVALDVESDFMMYRSGIYQSQTCSPDRLNHGVLAVGYGTQDGTDYWIVKNSWGTWW  280

FhpCL1  281  GERGYIRMARNRGNMCGIASLASLPMVARFP  311
FhpCL2  281  GEDGYIRFARNRGNMCGIASLASVPMVARFP  311
FhpCL5  281  GEDGYIRMVRKRGNTCGIASLASVPMVAQFP  311
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Fig. 1 Alignments of the amino acid sequences corresponding to FhpCL1 (gb|CCA61803.1), FhpCL2 (gb|KY464953), and FhpCL5 (gb|KY392883). The portion of the whole sequences corresponding to the mature enzymes is marked in bold type, while the region corresponding to the propeptides (residues 1–91 in the three sequences) is shown in normal type. Residues representative of the each clade (residues at positions 69, 70, 135, 160, 161, 163, and 209 in the mature sequence) according to Norbury et al. (2012) are underlined. Amino acid substitutions in clades L2 and L5 with respect to clade L1 are marked in cyan or yellow

fecal egg counting and coproantigen detection (MM3-COPRO ELISA). The non-infected group tested negative in fluke, egg, and coproantigen determinations. Coprology also revealed that most cows in both groups had intestinal nematodes from one or more genera of Trichostrongylidae, Trichuridae (*Trichuris* spp.), and Strongylidae. Additionally, the farm records for individual cows revealed that most were routinely treated with albendazole during the dry period and vaccinated against infectious bovine rhinotracheitis and bovine respiratory disease (bovine respiratory syncytial virus, parainfluenza virus type 3, and *Mannheimia haemolytica*). Some were also vaccinated at the end of the gestation period against coronavirus, rotavirus, and *E. coli*.

Regarding sheep, *F. hepatica* eggs (ranging between 0.3 and 9 eggs per gram of feces) and coproantigens (fecal antigen concentration ranging from 7.1 to 200.5 ng/mL) were detected in all sheep from the infected herd but were absent in the fluke-free herd. As for cattle, infections by gastrointestinal nematodes were detected in most animals from both infected and non-infected herds. Specifically, nematodes in the families Trichostrongylidae, Molineidae (*Nematodirus* spp.), Ancylostomatidae, Strongylidae, and Trichuridae (*Trichuris* spp.) were identified. The fecal antigen concentrations, parasite load and other characteristics from the infected animals included in the study are shown in Table 1.

Recognition of rFhpCL1, rFhpCL2, and rFhpCL5 by sera from infected and non-infected cattle and sheep

The antigens rFhpCL1, rFhpCL2, and rFhpCL5 were evaluated in indirect ELISA testing sera from cows and sheep naturally infected with *F. hepatica* and from non-infected animals. As indicated in the previous section, the optimal concentration of the target antigens was determined in ELISA plates by their recognition by mAb MM3, which binds to an epitope that is present in the three FhCL clades. Moreover, as the MM3 epitope is conformational in nature (Muiño et al. 2011), their recognition by this mAb in ELISA strongly suggests correct folding of these recombinant molecules. The results presented in Fig. 2a (*Fasciola*-infected) and Fig. 2b (*Fasciola*-free) show the individual IgG antibody response of the 33 and 22 cows, respectively, to the three recombinant cathepsins in indirect ELISA, and to native cathepsins in capture ELISA (MM3-SERO). Most of the sera from *Fasciola*-infected cows produced OD signals higher than 0.5 to the three rFhpCLs although, considering the samples individually, the antibodies in some sera displayed a preference for certain rFhpCLs. For example, this was the case with serum #5, which reacted preferentially with rFhpCL5 and rFhpCL1, and serum #15 which produced the highest OD value with rFhpCL2. Finally, only two serum samples (#14 and #29) produced low responses with ≤0.25 OD (Fig. 2a). With respect to sera from *Fasciola*-free cattle (Fig. 2b), unlike for the ELISA MM3-SERO, several serum samples produced high backgrounds in indirect ELISA, with OD values ≥0.1. The highest background values were obtained with rFhpCL1 (sera #10, #17, and #19) and with rFhpCL5 (sera #10, #15, and #21), although one sample (serum #22) also produced a considerable background (OD > 0.2) with rFhpCL2.

The antibody responses of sera from *Fasciola*-infected and *Fasciola*-free sheep are shown in Fig. 3a, b. Regarding infected sheep, the response to the three recombinant antigens follows the same pattern as indicated above for cattle, with good ELISA signals and only one sample yielding an OD signal

Table 1 Basic characteristics of *Fasciola*-infected cattle and sheep from which the serum samples were obtained and analyzed

	Cows			Sheep[c]
Fluke number	1–10	11–25	≥ 26	NS
Parasitized animals	20	6	7	15
Coproantigen ng/ml (range)	6.91 (0.6–38.8)	64.23 (5.4–233.3)[a]	137.47 (36.5–229.4)[b]	46.03 (7.1–200.5)
Age (range)	8.8 (4–12)	8.5 (9–12)	7.1 (4–10)	4.7 (3–8)

[a] Statistic differences ($p < 0.05$) with group harboring 1–10 flukes

[b] Statistic differences with group harboring 1–10 flukes ($p < 0.01$) and with group harboring 11–25 flukes ($p < 0.05$)

[c] Animals in production, not sacrified at the moment of sample collection

below 0.5. However, unlike for cattle, the sera from *Fasciola*-free sheep (Fig. 3b) yielded very low backgrounds, and none of the values were higher than OD = 0.075.

Diagnostic value of the indirect ELISA with rFhpCL1, rFhpCL2, or rFhpCL5 versus the MM3-SERO capture ELISA

For a better comparison of the signals obtained in indirect ELISA with rFhpCL1, rFhpCL2, and rFhpCL5 with those of the classical MM3-SERO ELISA, we elaborated a graph grouping the response given by each individual serum in each of the four ELISAs tested (Fig. 4a, b). In these figures, the corresponding cut-offs, calculated as the maximum OD value from negative sera plus one SD (method A) or obtained by ROC analysis (method B) are represented, respectively, by horizontal dashed red and blue lines. The sera from infected cattle produced similar mean OD signals ($p > 0.05$ for all comparisons), ranging from OD = 0.177 to OD = 2.398 for rFhpCL1-ELISA, from OD = 0.192 to OD = 2.268 for rFhpCL2-ELISA, from OD = 0.186 to OD = 2.673 for rFhpCL5-ELISA, and from OD = 0.254 to OD = 2.238 for MM3-SERO ELISA (Fig. 4a). However, due to the big differences observed between the cut-off of the reference test MM3-SERO ELISA (OD = 0.043) and the calculated cut-

offs for the indirect ELISAs (OD = 0.632, 0.310, and 0.597, respectively, with method A, and 0.265, 0.265, and 0.285, respectively, with method B) several positive sera were incorrectly classified by these latter. Specifically, $n = 4$, $n = 1$, and $n = 4$ sera from infected cattle were misclassified by rFhpCL1, rFhpCL2, and rFhpCL5 ELISAs, respectively, considering the cut-off obtained by method A, while $n = 2$, $n = 1$, and $n = 2$ of these sera were incorrectly classified using the cut-off obtained by method B (ROC analysis). These results yielded sensitivity values of 87.9, 97.0, and 87.9%, respectively, considering the former cut-offs (method A), and sensitivities of 93.9, 97.0, and 93.9%, respectively, considering the latter cut-offs (method B). Although the sensitivity values of the rFhpCL1 and rFhpCL5 ELISAs obtained using cut-off values calculated with ROC curves (method B) are apparently better than those obtained with method A, it is relevant to consider that this increase in sensitivity was done at expenses of lowering the specificity of the assays by about 5% (see Table 2).

With respect to infected sheep, the distribution of OD signals obtained in indirect ELISA with the three rFhpCLs was similar to that obtained for infected cattle (the lowest OD signal obtained with rFhpCL5 (OD = 0.185) and the highest with rFhpCL2 (OD = 2.260)). Interestingly, as the cut-off values calculated with method A (rFhpCL1, OD = 0.140; rFhpCL2, OD = 0.114; rFhpCL5, OD = 0.095; MM3-SERO,

Fig. 2 ELISA OD values obtained testing sera from *Fasciola*-infected (**a**) and *Fasciola*-free (**b**) cattle. The symbols on each vertical line represent the mean ELISA OD values obtained with each recombinant *F. hepatica* procathepsin or with the MM3-SERO ELISA for each individual serum sample tested in duplicate. Abbreviations are as follows: rpCL1, rFhpCL1; rpCL2, rFhpCL2; rpCL5, rFhpCL5; MM3, MM3-SERO ELISA
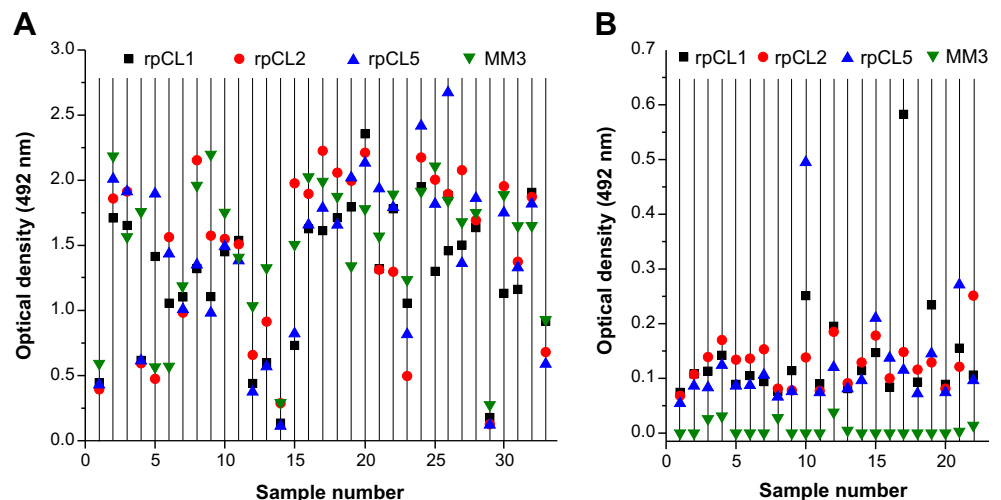
Fig. 3 ELISA OD values obtained testing sera from *Fasciola*-infected (**a**) and *Fasciola*-free (**b**) sheep. The symbols on each vertical line represent the mean ELISA OD values obtained with each recombinant *F. hepatica* procathepsin or with the MM3-SERO ELISA for each individual serum sample tested in duplicate. Abbreviations are as follows: rpCL1, rFhpCL1; rpCL2, rFhpCL2; rpCL5, rFhpCL5; MM3, MM3-SERO ELISA
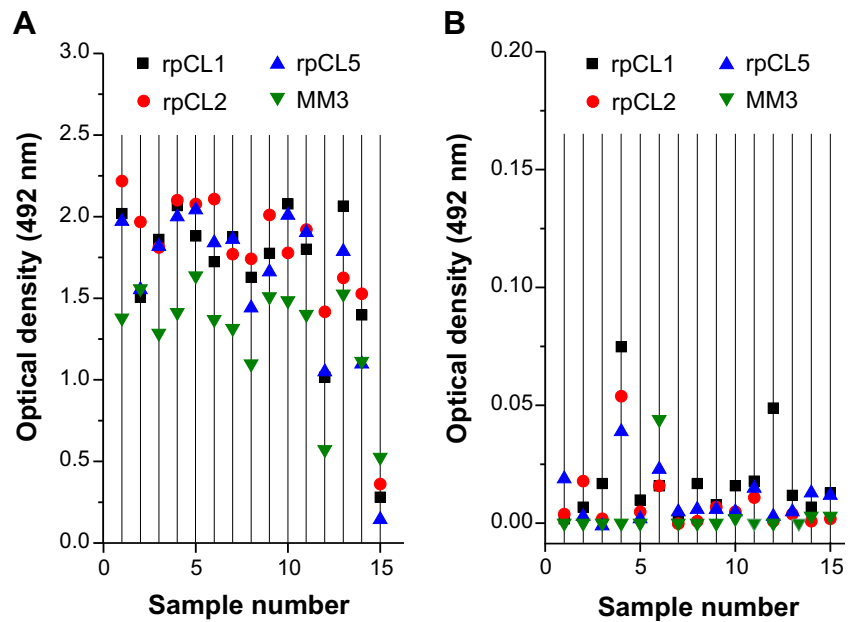
OD = 0.055) or the method B (rFhpCL1, OD = 0.121; rFhpCL2, OD = 0.100; rFhpCL5, OD = 0.085) were much lower than for cattle, the three ELISA variants and the MM3-SERO ELISA were capable of correctly classifying all sera from infected and non-infected sheep, i.e., with a sensitivity and specificity of 100% (Table 2). Nevertheless, the comparison of the OD signals obtained in any of the indirect ELISAs with those yielded by the MM3-SERO capture ELISA (either for cattle or sheep) revealed that the signal-to-noise ratio was much more favorable in the latter.

Finally, the *r* values obtained on comparing the four ELISA methods for *Fasciola*-infected cattle and sheep sera are shown in Table 3. The correlation was strongest between the data obtained with rFhpCL5 and rFhpCL1 ELISAs for both cow and sheep sera, while the lowest *r* value corresponded to the comparison between the OD values of the tested cow sera in the MM3-SERO and rFhpCL5 ELISAs.

Assessment of cross-reactive antibodies to other recombinant and non-recombinant antigens in sera from *Fasciola*-infected and *Fasciola*-free cattle and sheep

The relatively large proportion of sera from *Fasciola*-free cattle with cross-reactive antibodies to one or more of the recombinant rFhpCLs used in this study (see Fig. 2b) led us to
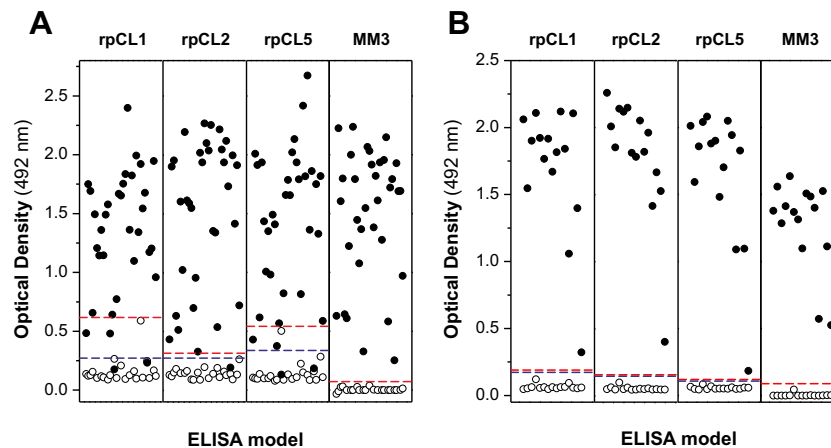

Fig. 4 ELISA OD values obtained testing sera from cattle (**a**) and sheep (**b**) grouped by each ELISA model. Each point in the figure represents the mean OD obtained for an individual serum sample tested in duplicate (*Fasciola*-infected animals: filled circles; *Fasciola*-free animals: open circles). The horizontal lines represent the cut-off values for each ELISA model, which were obtained by two different methods (method A, red line; method B, blue line; see "Material and Methods" section) considering data from negative sera. Abbreviations are as follows: rpCL1, rFhpCL1; rpCL2, rFhpCL2; rpCL5, rFhpCL5; MM3, MM3-SERO ELISA

Table 2　Analysis of the performance of rFhpCL1, rFhpCL2, and rFhpCL5 for serodiagnosis of fascioliasis

		Method A		Method B (ROC analysis)		
		Sensitivity[a] (95%CI)	Specificity[a] (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	Youden index J[b]
Sheep	rpFhCL1	100 (96.7–100)	100 (96.7–100)	100 (78.2–100)	100 (78.2–100)	1.00
	rpFhCL2	100 (96.7–100)	100 (96.7–100)	100 (78.2–100)	100 (78.2–100)	1.00
	rpFhCL5	100 (96.7–100)	100 (96.7–100)	100 (78.2–100)	100 (78.2–100)	1.00
	MM3-SERO	100 (96.7–100)	100 (96.7–100)	–	–	–
Cattle	rpFhCL1	87.9 (75.2–100)	100 (97.7–100)	93.9 (79.8–99.3)	95.4 (77.2–99.9)	0.89
	rpFhCL2	97.0 (89.6–100)	100 (97.7–100)	97.0 (84.2–99.9)	100 (84.6–100)	0.97
	rpFhCL5	87.9 (75.2–100)	100 (97.7–100)	93.9 (79.8–99.3)	95.4 (77.2–99.9)	0.89
	MM3-SERO	100 (96.7–100)	100 (96.7–100)	–	–	–

[a] Sensitivity and specificity values obtained considering the cut-offs calculated with method A, which maximizes specificity (see "Material and Methods" section)

[b] Youden Index [J = max (sensitivity + specificity − 1)]: graphically, it is the maximum vertical distance between the ROC curve and the diagonal line

investigate whether these cows also had cross-reactive antibodies to other recombinant or non-recombinant antigens. For this purpose, we evaluated the presence of IgG antibodies reactive with a recombinant *A. simplex* allergen (rAni s 1) expressed in *E. coli* and purified using the same procedure as for rFhpCLs, or reactive with mouse IgG1 (purified mAb MM3 without *F. hepatica* antigens, which is used as control for each individual serum in the MM3-SERO ELISA). A relevant proportion of sera from cattle infected with *F. hepatica* had IgG antibodies reactive with rAni s 1, three of which produced OD signals above 1.0 (see Fig. 5a). Although the reactivity of sera from the *Fasciola*-free population of cattle was much lower (Fig. 5b), these samples still showed a notable reactivity to rAni s 1 (Fig. 5b). Moreover, anti-mouse IgG1 cross-reactivity was also observed for a significant number of sera within the samples from *Fasciola*-infected and *Fasciola*-free cows (Fig. 5e, f). On the other hand, regarding serum samples from sheep, the population infected with *F. hepatica* did not recognize the rAni s 1 (Fig. 5c) allergen, and only two serum samples from non-infected sheep produced OD values > 0.2 (Fig. 5d). Also, no

reaction to mouse IgG1 antibodies was observed when testing sheep sera from both populations (Fig. 5g, h).

Discussion

This is the first study that comparatively evaluates the performance of three rFhpCLs (rFhpCL1, rFhpCL2, and rFhpCL5) as targets antigens in ELISA for the serodiagnosis of fasciolosis in sheep and cattle. These molecules were selected since (i) they are produced by adult flukes and thus continue stimulating the immune system during the chronic phase of the illness, and (ii) *Fasciola* cathepsins L1, L2, and L5 contain a common epitope recognized by mAb MM3, which is the capture antibody in the MM3-SERO ELISA (Mezo et al. 2007). Although rFhpCLs from the L1 clade have already been successfully used to develop a very sensitive and specific lateral flow test for immunodiagnosis of human fascioliasis (Martínez-Sernández et al. 2011), as well as to design ELISA tests for human (O'Neill et al. 1999; Carnevale et al. 2001a, b; Gonzales Santana et al. 2013; Gottstein et al. 2014) and animal use (Cornelissen et al. 2001; Kuerpick et al. 2013; Selemetas et al. 2014; Bloemhoff et al. 2015), the performance of rFhpCLs from L2 and L5 clades as ELISA targets has not previously been investigated. From a functional point of view, a single amino acid substitution may be sufficient to affect substrate specificity in cysteine proteases from *F. hepatica* (Smooker et al. 2000). The comparison of the new FhpCL2 (gb|KY464953) and FhpCL5 (gb|KY392883) sequences described in the present study with other reported cathepsins L2 (gb|ABQ95351.1; gb|AAC47721.1) and L5 (gb|AAF76330) revealed some nucleotide and amino acid changes. However, as none of the amino acid changes were in the positions referred by Norbury et al. (2012) as being typical of the

Table 3　Correlation coefficients (*r*) of *Fasciola*-infected sheep and cattle sera results obtained in the four ELISA models tested

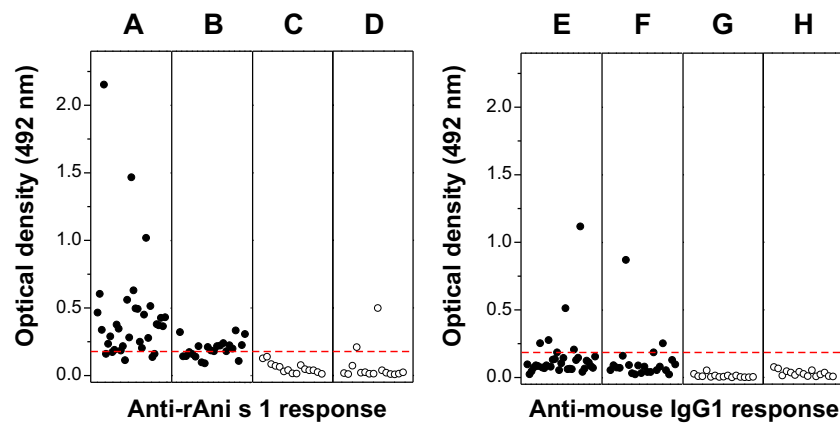|		rpCL2	rpCL5	MM3
Sheep	rpCL1	0.865	0.965	0.859
	rpCL2	–	0.912	0.815
	rpCL5	–	–	0.859
Cows	rpCL1	0.761	0.917	0.632
	rpCL2	–	0.743	0.765
	rpCL5	–	–	0.599

Fig. 5 ELISA OD values obtained testing sera from *Fasciola*-infected and *Fasciola*-free cattle (closed circles) and sheep (open circles) to the Ani s 1 recombinant *Anisakis* allergen (letters A–D) or to mAb MM3 (letters E–H). Each point in the figure represents the mean OD obtained for one individual serum sample tested in duplicate. Columns A and E: sera from *Fasciola*-infected cattle; columns B and F: sera from *Fasciola*-free cattle; columns C and G: sera from *Fasciola*-infected sheep; columns D and H: sera from *Fasciola*-free sheep. An indicative cut-off of 0.2 is drawn (red dashed line) for comparison purposes

Fasciola L2 and L5 clades, the assignation of our recombinant molecules as FhpCL2 and FhpCL5 was probably correct.

The alignment of the sequences of the three FhpCLs used in this study showed a mean of amino acid changes of 17%, which, besides affecting substrate specificity, it may be enough to induce variations in the level of circulating antibodies produced in infected animals. The results showing preferences in procathepsin recognition in the population of sera from *Fasciola*-infected animals, mainly cattle (see Fig. 2a), are consistent with this hypothesis. More importantly, our results indicated that indirect ELISAs based on rFhpCL1, rFhpCL2, and rFhpCL5 show differences in sensitivity, despite the fact that the target antigens were expressed and purified in the same way. Specifically, considering the OD values obtained with the different immunoassays, the cut-off values calculated to maximize specificity (method A), and the number of false negatives obtained for each rFhpCL, the rFhpCL2 might be a better target antigen than the other rFhpCLs. However, the MM3-SERO ELISA had a better performance, as this method classified correctly all samples from sheep and cattle. There are at least two explanations for the superiority of the MM3-SERO ELISA over the ELISAs based on the use of individual rFhpCLs: (i) mAb MM3 is able to virtually capture all native cathepsin isoforms of the CL1, CL2, and CL5 clades from *Fasciola* (Muiño et al. 2011; and this study), including those from the more pathogenic *F. gigantica* species (Valero et al. 2009; Valero et al. 2016). This may explain the fact that OD values obtained in MM3-SERO ELISA tended to be higher than in indirect ELISAs among infected animals with low anti-*Fasciola* antibody levels (see Fig. 2a), and (ii) the MM3-SERO ELISA includes an individual control for each serum, which ensures that particular animals with anti-species cross-reactive antibodies or antibodies against other irrelevant proteins present in the ELISA plate (see Fig. 5e, f) can be easily detected, thus allowing this signal to be subtracted from

that of the corresponding positive well. Consequently, good OD signals and low backgrounds (which translates into lower cut-offs) are normally achieved, yielding an excellent signal-to-noise ratio, for both cattle and sheep sera. These results suggest that the use of a single recombinant cathepsin/procathepsin as target antigen in ELISAs for serodiagnosis of fascioliasis may limit the sensitivity of the assay when testing sera from some species, particularly cattle.

Considering the above characteristics of the MM3-SERO ELISA, its highest sensitivity (see Table 2) over the indirect ELISAs based on the use of a single *F. hepatica* cathepsin/procathepsin L1, L2, or L5 is not surprising. However, considering that representatives of all mature *Fasciola* CLs are captured in MM3-SERO, the high backgrounds obtained with the rFhpCL1, rFhpCL2, and rFhpCL5 antigens for *Fasciola*-free cattle (see Fig. 2b) may appear unexpected at first sight. Moreover, this phenomenon was not limited to the samples investigated in this study, nor to the expression of rpFhCLs in *E. coli*. In agreement with this, a similar finding was recently reported in a study where the performance of an indirect ELISA based on a rFhpCL1 expressed in the yeast *Pichia pastoris* was compared with that of a commercial indirect ELISA test containing a purified fraction from *Fasciola* ESAs (f2 antigen; Institute Pourquier, Montpellier, France) (Kuerpick et al. 2013). Cross-reactivity was also observed by Cornelissen et al. (2001) who tested sera from sheep and cattle harboring other parasites, mainly nematodes, suggesting that the cross-reactivity may be due to common epitopes between recombinant *Fasciola* cathepsins and antigens present in other parasites. However, as such cross-reactivity did not occur with the three tested rFhpCLs (see Fig. 2b), this explanation seems unlikely.

Another possible source of cross-reactivity is that rFhpCLs produced in *E. coli* were contaminated by bacterial proteins retained in the IMAC column during the affinity purification

process. Coelution of native *E. coli* proteins (e.g., proteins containing clusters of histidine residues) with recombinant proteins is a recognized feature, mainly when recombinant proteins are expressed at low level (Robichon et al. 2011). However, this seems also unlikely for several reasons: (i) rFhpCLs are produced as inclusion bodies, and contaminating proteins are removed during the washing procedures before denaturation and subsequent IMAC purification of rFhpCLs; (ii) as indicated above, the reactivity of a particular serum does not occur with the three rFhpCLs, despite the fact that all of them were produced and purified following the same procedure; and (iii) preincubation of the cross-reactive sera with a bacterial extract prior to the incubation in ELISA plates, or the use of a rFhpCL1 expressed with a second tag in order to eliminate possible remaining contaminants in a capture ELISA did not eliminate the observed backgrounds (data not shown). Conversely, we hypothesize that the phenomenon may be related to a greater opportunity of cross-reactive antibodies to bind non-specific linear epitopes present in rpFhCL preparations than in native CLs. While in native antigens, the availability of non-specific linear epitopes may be low due to the correct folding of the protein, such epitopes may be exposed for antibody-binding when truncated, partially folded, or unfolded molecules are present, as occurs frequently when recombinant proteins are overexpressed in bacterial or yeast cell systems (Lilie et al. 1998; Cornelissen et al. 2001). In addition, an unexpectedly high number of samples from *Fasciola*-infected and non-infected cattle reacted with a recombinant form of the *A. simplex* Ani s 1 allergen (Fig. 5a, b), an antigen that is present in a fish parasite never naturally in contact with cattle, or with other herbivorous animals (Anadón et al. 2010). Such allergen is structurally related with Kunitz-type serine protease inhibitors (Audicana and Kennedy 2008). Thus, with respect to *Fasciola*-infected cows, it could be hypothesized that cross-reactivity with rAni s 1 is due to the presence of common epitopes between this allergen and the Kunitz-type molecules secreted by *Fasciola* (Bozas et al. 1995; Di Maggio et al. 2016; Smith et al. 2016). Nevertheless, the fact that the response was irregular among these cows, and that it also occurred, to a lesser extent, among *Fasciola*-free cows, suggests again that cross-reactive antibodies may be induced against antigens not related with *Fasciola* but which share some linear epitopes.

Considering the above argumentations and the sensitivity obtained in the indirect ELISAs tested, the usual purification conditions of recombinant proteins expressed in bacterial or yeast systems may render these antigens not suitable for use as target in ELISAs for serodiagnosis of fascioliasis in certain animal species. Besides, there is also experimental data suggesting that cross-reactivity may not occur in the same way in different host species. In this sense, we observed that the OD responses to rFhpCLs were more homogeneous in infected sheep than in cattle (see Figs. 3a and 2a, respectively), which

was also reflected by the higher *r* values obtained for sheep sera relative to cattle sera, when comparing the OD values obtained on testing the different rFhpCLs (see Table 3). Moreover, interspecific differences in the generation of cross-reactive antibodies to *Fasciola* procathepsins can also be deduced from a previous study in which no cross-reactivity was observed for human sera in a lateral flow test based on the same rFhpCL1 (Martínez-Sernández et al. 2011) used in the present study. This reflects that some species may be more prone to induce cross-reactive antibodies than others or that the antigens to which humans are exposed have fewer common epitopes with *Fasciola* CLs than those to which ruminants are exposed.

Limitations of the study

The limited number of positive and negative sera tested in this study may not be sufficient to obtain precise values of sensitivity and specificity for the three indirect ELISAs based on recombinant rFhpCLs and to obtain cut-off values valid for use under field conditions. However, this did not prevent drawing conclusions about their performance with respect to the MM3-SERO ELISA, as they were tested simultaneously with the same sera. Moreover, it did not condition the fact that recombinant CLs purified by IMAC may be more prone to be recognized by cross-reactive antibodies than the corresponding natural antigens. Finally, although liver necropsy and coproantigen detection may not be perfect gold standards, since some animals may only have migrating or ectopic flukes, considering the age of cattle in our study (4–12 years old), the probability of having a single contact within the previous weeks before slaughtering is minimal.

Conclusion

In summary, our results show that rFhpCL2 may be more suitable than rFhpCL1 and rFhpCL5 as target antigen in indirect ELISA for the serodiagnosis of fascioliasis in ruminants, particularly in cattle, as these molecules, unless purified to homogeneity and correctly folded, may be more frequently recognized by cross-reactive antibodies than the corresponding natural antigens. These results reflect that further research into methods of producing and purifying recombinant *Fasciola* cathepsins is required to overcome the need to collect live adult parasites in abattoirs, or from experimental infections, to obtain natural antigens.

Compliance with ethical standards

Blood and fecal samples were collected from naturally infected sheep and cattle by veterinarians from the "Centro de Investigaciones Agrarias de Mabegondo" (INGACAL, A Coruña, Spain). The samples were collected either during routine control and treatment of herds (sheep) or in the slaughterhouse (cattle). All procedures were carried out in strict accordance with Spanish and EU legislation (Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU).

Conflict of interests   The authors declare that they have no competing interests.

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