Induction of immunity in sheep to *Fasciola hepatica* with mimotopes of cathepsin L selected from a phage display library

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(*Received 25 February 2008; revised 11 April 2008; accepted 8 May 2008; first published online 24 September 2008*)

SUMMARY

An M13 phage random 12-mers peptide library was used to screen cathepsin L mimotopes of *Fasciola hepatica* and to evaluate their immunogenicity in sheep. Seven clones showed positive reactivity to a rabbit anti-cathepsin L1/L2 antiserum in ELISA, and their amino acid sequences deduced by DNA sequencing were tentatively mapped on the protein. Twenty sheep were randomly allocated into 4 groups of 5 animals each, for immunization with 1×10^{14} phage particles of clones 1, 20, a mixture of 7 clones and PBS, without adjuvant at the beginning, and 4 weeks later. All groups were challenged with 300 metacercariae at week 6 and slaughtered 16 weeks later. The mean worm burdens after challenge were reduced by 47·61% and 33·91% in sheep vaccinated with clones 1 and 20, respectively; no effect was observed in animals inoculated with the clone mixture. Also, a significant reduction in worm size and burden was observed for those sheep immunized with clone 1. Animals receiving clone 20, showed a significant reduction in egg output. Immunization induced a reduction of egg viability ranging from 58·92 to 82·11%. Furthermore, vaccinated animals produced clone-specific antibodies which were boosted after challenge with metacercariae of *F. hepatica*.

Key words: *Fasciola hepatica*, cathepsin L, phage display, mimotopes, vaccine.

INTRODUCTION

Fasciolosis or liver fluke disease is caused by *Fasciola hepatica* in a wide range of mammals. The human infection is estimated in 2·4 million people and it seems to be increasing (Mas-Coma *et al*. 1999; Mas-Coma, 2005). Fasciolosis also causes important economic losses in the livestock industry (Torgerson and Claxton, 1999). Triclabendazole is the only effective drug against early stages of the parasite; however, resistance to it has been extensively reported (Overend and Bowen, 1995; Mitchell *et al*. 1998; Moll *et al*. 2000; Gaasenbeek *et al*. 2001). Other control measures such as vaccination should be developed for sustainable control of this disease. There are numerous studies on the use of purified native or recombinant molecules as candidate vaccines (Hillyer, 2005; McManus and Dalton, 2006). These include cathepsins L, a family of proteases involved in activities such as immune evasion (Carmona *et al*. 1993; Smith *et al*. 1993), invasion of tissues (Dalton and Heffernan, 1989; Berasaín *et al*. 1997), nutrition (Yamasaki *et al*. 1989; Smith *et al*. 1993) and egg output (Wijffels *et al*. 1994; Dalton *et al*. 1996). These activities may interfere with their protective effect; thus, it would be desirable to isolate the immunizing motifs for vaccination purposes. Moreover, while native cathepsins L could be purified in sufficient quantities for experimental trials, large-scale production requires a cheaper system, i.e. competitive with current drug treatments (Dalton *et al*. 2003).

Phage display is a powerful tool to select peptides or proteins with high affinity and specificity to almost any molecular target of interest. Its utility is based on collections of bacteriophage particles displaying random peptide sequences on their surfaces, as a fusion product with one of the phage coat proteins (Smith and Pretenko, 1997). Libraries of random peptides have been successfully used to identify peptides that mimic vaccine epitopes in the case of *Plasmodium vivax* or *Plasmodium yoelii* malaria, and *Schistosoma japonicum* schistosomiasis (Demangel

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doi:10.1017/S003118200800471X Printed in the United Kingdom

et al. 1996; Tang *et al*. 2004; Wang *et al*. 2005, Narum *et al*. 2006; Wu *et al*. 2006). The mimotope vaccine strategy has not been reported for *Fasciola hepatica* cathepsins L in sheep; thus, this was the objective of the present study.

MATERIALS AND METHODS

Excretion/secretion products (*E/S*)

Mature flukes were removed from the bile ducts of infected cattle livers from the local abattoir of the City of Toluca, washed 6 times in sterile 0·01 M phosphate-buffered saline (PBS), pH 7·2, and incubated for 16 h at 37 °C in RPMI-1640, pH 7·3, with penicillin (100 IU/ml) and streptomycin (100 μg/ml). The medium was removed and centrifuged at 14 000 ***g*** for 30 min at 4 °C. The supernatant was collected and concentrated using Amicon Ultra-15 centrifugal filter tubes with a 10 000 molecular weight cut-off membrane (Millipore, USA). After determination of the protein concentration as described by Bradford (1976) using the Bio-Rad commercial kit, the supernatant was aliquoted and stored at −20 °C until use.

Purification of cathepsin L proteinases

Fasciola hepatica cathepsins L1/L2 were purified from the E/S products by HPLC ion-exchange chromatography on a Q-Sepharose column (Amersham Biosciences, USA), equilibrated in 20 mM Tris-HCl, pH 8, followed by gel-filtration chromatography on a Superdex 75 column (Amersham Biosciences, USA), equilibrated with 20 mM Tris-HCl, pH 7.

Mass spectrometric analysis

The purified proteinases were separated by 12% reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with 0·1% Coomassie brilliant blue; the observed double band was cut and digested in gel with trypsin (Promega, USA), using the technique described previously (Shevchenko *et al*. 1996). Samples containing peptides resulting from proteolytic cleavage were analysed by matrix-associated laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in a Voyager-DE PRO MALDITOF system (Applied Biosystems, USA).

Preparation of polyclonal antibodies against cathepsin L, and Western blot analysis

One New Zealand rabbit was subcutaneously immunized with 75 μg of the purified enzymes; the initial injection was prepared in Freund's Complete Adjuvant and the 4 subsequent injections, given at 2-week intervals, were in Freund's Incomplete Adjuvant (Sigma, USA). The immunoglobulin G (IgG) fraction of the serum was purified by affinity chromatography with protein A-Sepharose (Amersham Biosciences). For Western blotting, the purified fraction was separated by 12% reducing SDS-PAGE, transferred to polyvinylidene difluoride (PVDF, Millipore) membrane and probed with the polyclonal specific anti-cathepsin L antibody. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma, USA) was used as a secondary antibody at a dilution 1 : 2500. The blots were visualized with 3,3′-diaminobenzidine (DAB, Sigma, USA).

Panning with the polyclonal anti-cathepsin L antibody

The Ph.D.-12 Phage Display Peptide Library (New England Biolabs, USA) is composed of a combinatorial library of random peptide 12-mers fused to a minor coat protein (pIII) of the M13 phage and with a complexity of $2{\cdot}7 \times 10^9$ electroporated sequences. The phage library was subjected to affinity selection as described by the Ph.D.-12 kit. Briefly, the wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Nunc-Immuno, MaxiSorp) were coated with 150 μg/ml of purified antibodies against cathepsins L1/L2 in 100 μl of 0·01 M phosphate-buffered 0·15 M saline (PBS, pH 7·3) overnight at 4 °C in a humidified container. The wells were blocked for 1 h at 4 °C with 300 μl of blocking solution (1% bovine serum albumin in PBS). The library phages ($1{\cdot}0 \times 10^{11}$ particles in 100 μl of PBS-T, 0·1% Tween 20) were added to the wells and left for 1 h at room temperature with gentle stirring, followed by 10 washes with PBS-Tween 20. Bound phages were eluted with 100 μl of 0·2 M Glycine-HCl, pH 2·2, for 10 min at room temperature and neutralized by adding 15 μl of 1 M Tris-HCl, pH 9·0.

The titre of the eluted phage was calculated by infecting a log-phase culture of ER 2738 *E. coli* (New England Biolabs, USA); the colonies were counted and expressed as plaque-forming units per millilitre (pfu/ml). Subsequently, the phage eluates were amplified and concentrated using polyethylene glycol precipitation by the standard procedure. The second and third panning round were similar to the first one, except that the purified antibodies against cathepsins L1/L2 were used at 75 μg/ml and 37·5 μg/ml, respectively.

Phage ELISA and DNA sequencing

ELISA plates were coated with 10 μg/ml of purified anti-cathepsins L1/L2 antibodies in 100 μl of PBS, and incubated overnight at 4 °C. The wells were washed 5 times with PBS-T, and blocked for 1 h at 37 °C with blocking solution. Specific phage clones (1×10^{10} pfu) in PBS containing 0·2% BSA-0·2% Tween 20, were incubated for 2 h at room

https://doi.org/10.1017/S003118200800471X Published online by Cambridge University Press

temperature with gentle shaking. After 5 washes with PBS-T, the bound phage particles were detected utilizing an anti-M13-HRP antibody (Amersham Biosciences, USA) at a dilution of 1 : 5000 in blocking solution for 1 h at room temperature. The reaction was developed with 2, 2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma, USA) in citrate buffer. The absorbance values were measured with a Beckman Coulter DTX 880 ELISA reader.

The selected clones were amplified and the purified single-strand phage DNA fragments sequences were determined with the 96gIII sequencing primer 5′-HOCCCTCATAGTTAGCGTAACG-3′ (New England Biolabs, USA) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). Alignments of the selected clones with the published sequences of cathepsins L1/L2 were performed using the software Clone Manager Professional Suite (Sci Ed Central) software. The aligned amino acid sequences of cathepsin L mimotopes and the consensus sequences were performed using a web-based tool called MIMOX (Huang *et al*. 2006).

Phage Western blot

The phage ($1{\cdot}0 \times 10^{12}$ particles) from each selected clone were separated by 10% SDS-PAGE, and transferred to PVDF membrane. The membrane was blocked in 5% skimmed milk for 2 h at room temperature and the polyclonal rabbit anti-cathepsin L serum was added and incubated for 1·5 h. The membrane was washed thrice in PBS-T. HRP-conjugated goat anti-rabbit IgG (Sigma, USA) was used as secondary antibody at a dilution 1 : 2500, incubated for 1 h; afterwards, the washings were repeated. The blots were visualized with DAB as indicated above.

Vaccination and challenge

Twenty cross-bred 1-year-old sheep were obtained and kept at the Teaching and Research Centre for Animal Health and Production (CEPIPSA), from the National Autonomous University of Mexico. All animals were checked for absence of *F. hepatica* eggs in the feces and antibodies in serum by ELISA, and were randomly allocated into 4 groups of 5 animals each. Each group was subcutaneously immunized with clone 1, clone 20 and a mixture of 7 clones (1, 2, 3, 5, 17, 19, 20); the dose was 1×10^{14} phage particles without adjuvant per animal, at the beginning and then at week 4. Control group animals were injected with PBS.

Metacercariae of *F. hepatica* used for challenging sheep were collected from laboratory colonies of *Lymnea cubensis* snails experimentally infected with miracidia. Two weeks after the second immunization (week 6), each group member was orally challenged with 300 metacercariae given in a gelatin capsule administered with oesophageal tongs.

Protection assessment

All sheep were humanely slaughtered 22 weeks after the first immunization, and the livers and gall bladders were removed. The number of adults present in each animal were recovered and counted, and their length measured. The feces were removed from the rectum after slaughter and fecal examinations were performed using the sedimentation technique as described by Sexton *et al*. (1990). The number of *F. hepatica* eggs in the sediment was expressed as number of eggs per gram (EPG) of feces. Eggs were collected from the gall bladder, washed with tap water and incubated in darkness at 22 °C for 14 days (Dalton *et al*. 1996). They were examined microscopically for evidence of miracidium development and hatching. Eggs which hatched upon exposure to light or those containing a clearly-developed, mobile miracidium were scored as viable.

Analysis of antibody responses by ELISA

Blood samples were collected from all sheep prior to the first immunization and every 2 weeks until the time of slaughter (week 22). The serum was obtained and then stored at −80 °C. The antibody responses were analysed by ELISA, using 96-well microtitre plates coated with 2 μg of E/S products incubated in PBS overnight at 37 °C. The excess binding sites were blocked for 1 h at 37 °C with 200 μl of blocking solution. The serum samples were diluted 1 : 800 and incubated at 37 °C for 1 h. Bound antibodies were detected with donkey anti-sheep IgG-HRP (Sigma, USA) and ABTS as substrate. The absorbance values were measured with an ELISA reader (Beckman Coulter DTX 880). The protocol used for the ELISA using the phages as antigen was similar to that described above, except that the samples were diluted 1 : 400.

Statistical analysis

The non-parametric Kruskall-Wallis test was used to compare the results of the number of worms recovered, the egg count and viability, as well as the size of *F. hepatica*. Correlations were calculated with Spearman's non-parametric correlation test. Differences in frequency of infection were tested by Chi-square, or when specified, by the Fischer exact test. All tests were performed using the software *SPSS 15 for Windows*. A $P \leqslant 0{\cdot}05$ was considered significant.

RESULTS

Western blot analysis using purified anti-cathepsin L antibodies

Cathepsin L identification was done by mass spectrometry; in which 5 masses assigned to expected peptides comprising 65 amino acids out of the 326

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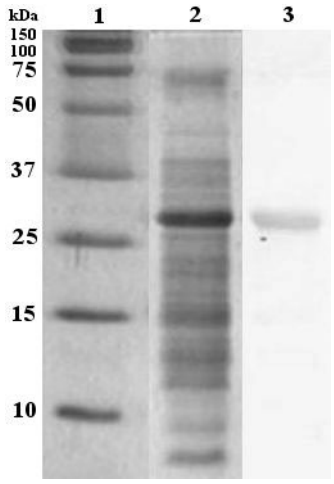

Fig. 1. Western blot analysis of anti-cathepsin L IgG response against E/S products of *Fasciola hepatica*. Molecular weight marker (lane 1), E/S products from mature flukes, separated by SDS-PAGE (12% gel) under reducing conditions (lane 2), and then transferred to PVDF membrane, recognized by the anti-cathepsin L antiserum (lane 3).

were demonstrated. Likewise, cathepsins L1/L2 migrated as a double 27–29·5 kDa band and were recognized by the anti-cathepsins L antiserum by Western blot analysis (Fig. 1).

Panning results and amino acid sequences of phage clones selected

To identify peptides that can mimic structural features of cathepsins L, we used the purified polyclonal anti-cathepsin L1/L2 antibodies to pan a 12-mer phage display library. In order to determine the effect of enrichment after each round of panning, input and output phages were titrated. The eluted phage of each round was increased from 2×10^4 pfu in the first round to $5{\cdot}2 \times 10^6$ pfu in the third round (Table 1). After 3 rounds of panning 36 recombinant phage clones were randomly selected. The reactivity of the phage clones with anti-cathepsin L antibodies was measured by ELISA (Fig. 2). We selected 7 positive clones on the basis of the absorbance values above the negative controls and these were analysed by DNA sequencing. The deduced amino acid sequences of the selected peptides were aligned to several complete *F. hepatica* cathepsin L1/L2 sequences available from the GenBank database. The 7 phage clones selected and aligned with cathepsin L1/L2 were located at the middle of the sequence and/or the C-terminal end (Fig. 3A, B). The consensus amino acid residues of the mimotopes from the phage clones selected from of the 12-mer peptide display library are presented in Fig. 3C. To examine whether the purified anti-cathepsin L1/L2 antibodies could recognize the peptides displayed by the bacteriophages, these were separated by Western blot with the purified IgG; 4 of 7 phage clones were clearly recognized (Fig. 3D).

Table 1. Enrichment of specific phages during panning

Round	Input phage (pfu)*	Eluted phages (pfu)	Phage recovery
1	$1{\cdot}0 \times 10^{11}$	$2{\cdot}0 \times 10^4$	$2{\cdot}0 \times 10^7$
2	$1{\cdot}0 \times 10^{11}$	$4{\cdot}6 \times 10^5$	$4{\cdot}6 \times 10^6$
3	$1{\cdot}0 \times 10^{11}$	$5{\cdot}2 \times 10^6$	$5{\cdot}2 \times 10^5$

* pfu: plaque-forming units.

Parasite burden and size in sheep vaccinated with phage clones

Animals vaccinated with clones 1 and 20 showed a mean reduction in fluke burden of 47·61% ($P<0{\cdot}05$) and 33·91% ($P>0{\cdot}05$), respectively (Table 2). No reduction in the fluke counts were found in those sheep inoculated with the mixture of clones. Also, the size of the adult parasites of the group vaccinated with clone 1 was smaller those of the other groups.

FEC and fluke egg viability

The fecal egg counts for each group are shown in Table 3, where a statistically significant reduction can be seen in the group inoculated with clone 20 ($P<0{\cdot}05$). Immunization with clone 1 or the mixture had no effect, although significant differences in the viability of eggs were seen between control and all vaccinated animals (Table 3).

Humoral response induced by the selected phage clones

In vaccinated sheep a low but detectable humoral response against the homologous phages was observed, with a peak 2 weeks after the first immunization, and a slight decrease until the end of the experiment (Fig. 4). The response was stronger against clone 1, followed by that to clone 20. The group vaccinated with the mixture of clones showed the lowest response, but a second increase in antibody levels after challenge was observed.

Figure 5 shows the reactivity of all groups towards the E/S products. As it can be seen, a slight antibody response was produced in the groups vaccinated with clones 1 and 20 two weeks after the first immunization. In addition, an increase in the absorbance of the 4 groups was observed within 2 weeks following challenge (at week 6) and remained high throughout the infection. The absorbance against adult fluke E/S products was higher in the serum of vaccinated animals than in controls. A significant negative correlation between the absorbance of the group vaccinated with clone 1 and the parasite burden was observed (Spearman's correlation coefficient $r = -0{\cdot}958$; $P<0{\cdot}05$).

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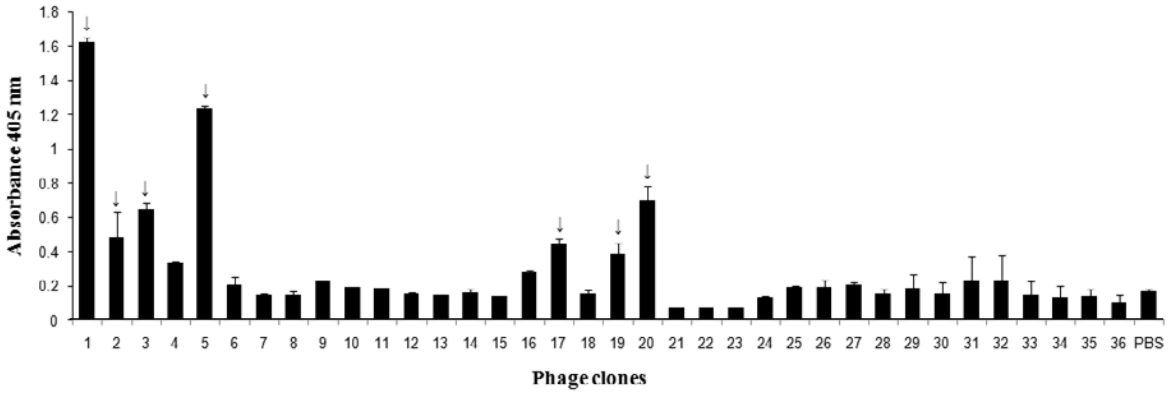

Fig. 2. Sandwich ELISA to select phage clones specifically bound to anti-cathepsin L antibodies. Arrows indicated phage clones selected. Data represent the mean of 3 determinations and bars represent standard deviations.

A

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  1 snddlwhqwkrmynkeyngaddehrrniweenvkhiqehnlrhdlglvtytlglnqftdmtfeef

 66 kakyltemprasdilshgipyeannravpdkidwresgyvtgvkdqgncgscwafsttGTMEGQY
                                                              03 NTLSGQY

131 mknektsisfseqqlvdcsgpwgnngcsgglmenayeylkrfgletessYPYRAVEGQCryneql
                                                       01 YVYRWVEAEC

196 gvakvtgyytvhsgsevelknlvgsegpaaiaveaesdfmmyRSGIYQSQTCLPfalnhavlavg
                                              02 REPIYHKLHRLT

                                         20 FSPAYLRDAALK
261 ygtqdgtdyWIVKNSWglswgergyirmarnrgnmcgiASLASLPMVARFP
                                              01 VA
          05 WHVPRTW                      05 WVLPP
                                              03 FWRMP
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B

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  1 mrffvlavltvgvfasnddlwhqwkriynkeyngaddehrrniwgknvkhiqehnlrhglglvty

 66 klglnqftdltfeefkakylieiprssellsrgipykanklavpesidwrdyyYVYEvkdqgqcg
                                                       19 YVYE

131 scwafsttgavegqfrknerasasfseqqlvdctrdfgnygcgggymenayeylkhngletesyy

196 pyqavegpcqydgrlayakvtgyYTVHSGDEIELKnlvgtedlpavaldadsdfmmyqsgiyqsq
                          17 YIDHIVLTIYLE

261 tclpdrlthavlavgygsqdgtdywivknswgtwwgedGYIRFARNrgnmcgiaslasvpmvarfp
                                         19 GRSRVRRP
```

C

Clone	Amino acid sequence										
01	Y	V	Y	R	W	V	E	A	E	C	V	A
19	Y	V	Y	E	G	R	S	R	V	R	R	P
05	W	H	V	P	R	T	W	W	V	L	P	P
03	N	T	L	S	G	Q	Y	F	W	R	M	P
02	R	E	P	I	Y	H	K	L	H	R	L	T
20	F	S	P	A	Y	L	R	D	A	A	L	K
17	Y	I	D	H	I	V	L	T	I	Y	L	E
Consensus	Y	V	+	-	+	V	-	-	V	R	L	P

D

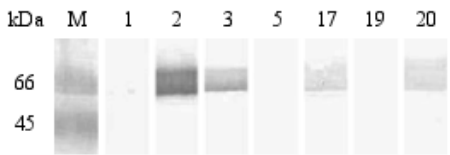

Fig. 3. Amino acid analysis of clones selected using purified rabbit anti-cathepsin IgG. Analysis of the 7 putative mimotopes and amino acid sequences of cathepsin L1 (A) and L2 (B) of *Fasciola hepatica* (GenBank database Accession numbers: L1, U62288; L2, U62289). (C) Sequence of peptides displayed by the phage clones and consensus amino acid residues. (D) Western blot analysis of phage clones with anti-cathepsin L antibodies. M: Molecular weight marker.

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Table 2. Reduction in worm number and size due to vaccination of sheep with phage clones

Immunization with:	Number of flukes recovered: Individual data	Mean ± S.D.	Reduction rate (%)	Mean size (mm) ± S.D.
Clone 1	30, 48, 60, 74, 29	48·20 ± 19·40*	47·61*	22·39 ± 3·20*
Clone 20	55, 26, 73, 54, 96	60·80 ± 25·88	33·91	24·47 ± 3·46
Mixture of clones	110, 95, 27, 68, 176	95·20 ± 55·09	–	25·39 ± 3·55
PBS (control)	90, 126, 118, 57, 69	92·00 ± 29·96	–	26·35 ± 3·33

* Significant differences versus control group ($P < 0·05$).
S.D.: standard deviation.

Table 3. Reduction of FEC and viability of *Fasciola hepatica* eggs recovered from gall bladders of vaccinated sheep

Immunization with:	Fecal egg counts (mean ± S.D.)	Reduction in FEC (%)	Egg viability (%)
Clone 1	147·00 ± 38·82	25·85	58·92*
Clone 20	108·00 ± 30·75	45·55*	73·76*
Mixture of clones	141·20 ± 49·96	28·78	82·11*
PBS (control)	198·20 ± 57·19	–	97·59

* Significant differences versus control group ($P < 0·05$).
FEC: fecal egg counts.

DISCUSSION

Random peptide phage libraries have been employed to select mimotopes, epitope mimics that regardless of their homology to the natural antigen could serve as good candidates in the development of vaccines (Demangel *et al*. 1996; Narum *et al*. 2006). The libraries can also been screened with polyclonal antisera (Tang *et al*. 2004; Wang *et al*. 2005; Wu *et al*. 2006). Here, we report on the first use of phage display technology to screen mimotopes of *F. hepatica* cathepsins L1/L2. The 7 phage clones used in this study elicited an IgG response of low but significant magnitude against the E/S, especially in the case of clones 1 and 20. Moreover, challenge induced an augmented response in these 2 groups, which suggests that the antibody levels were boosting memory B cells, and that these epitopes exist in the native molecule.

The immunization results showed that clones 1 and 20 induced significant protection levels of 47·61% and 33·91%, when they were administered separately. Likewise, a reduction in fecal egg counts ranging from 25·85 to 45·55% was observed in sheep vaccinated with phage clones. Vaccination with each cathepsin L mimotope induced unique effects on adult and egg burdens. In the first report of vaccination with native cathepsins L in sheep, fecal egg counts were reduced in 69·7%, without having an effect on adult worm burdens (Wijffels *et al*. 1994). Immunization of sheep with native cathepsin L1 or cathepsin L2 against *F. hepatica* elicited protection levels of 33% and 34% as measured by reduction of worm burdens and of 71% and 81%, reduction in egg output, respectively, while a cocktail of native cathepsins L1 and L2 induced 60% reduction in fluke burden (Piacenza *et al*. 1999). Expression of recombinant cathepsin L1 in *Pichia pastoris* for vaccination trials in sheep induced 35 to 45% protection with 50% anti-fecundity effect (McManus and Dalton, 2006). Thus, the level of protection elicited with the phage mimotopes was within the range reported with the use of other, larger and more expensive immunogens.

Flukes recovered from all vaccinated groups tended to be smaller than those collected from the control group, particularly in the case of those immunized with clone 1. Dalton *et al*. (1996) also reported that flukes recovered from cattle vaccinated with native cathepsin L and haemoglobin had a significant reduction in size, which indicated that vaccination adversely affected the development of the parasites.

In this trial, immunization with the phage clones also had an effect on the viability of eggs recovered, ranging from 58·92 to 82·11%. An anti-embryonation effect mediated by the immune response, using native cathepsin L1 was also observed in bovines, and eggs with 50·75% viability were recovered from the gall bladders (Dalton *et al*. 1996). By immunohistochemistry the presence of cathepsin L has been demonstrated in the oocytes, as well as in the Mehlis' and vitelline glands of *F. hepatica* (Wijffels *et al*. 1994).

Harmsen *et al*. (2004) immunized rats with 3 synthetic peptides of cathepsin L1 (residues 15–33) and L3 (residues 16–34) encompassing the region of the pro-peptide, and cathepsin L1 comprising 3 residues of the pro-peptide and the 17 N-terminal residues of the mature protease (residues 104–122), and found a reduction in parasite burden of 40·4 to 75·8%. The alignment of the phage clones within the amino acid sequence of cathepsins L1/L2 showed that they are located in the middle of the sequence and/or the C-terminal end. Clone 1 induced a protection level of 47·6% and aligned with amino acids 180–189 and 307–308 of mature cathepsin L1,

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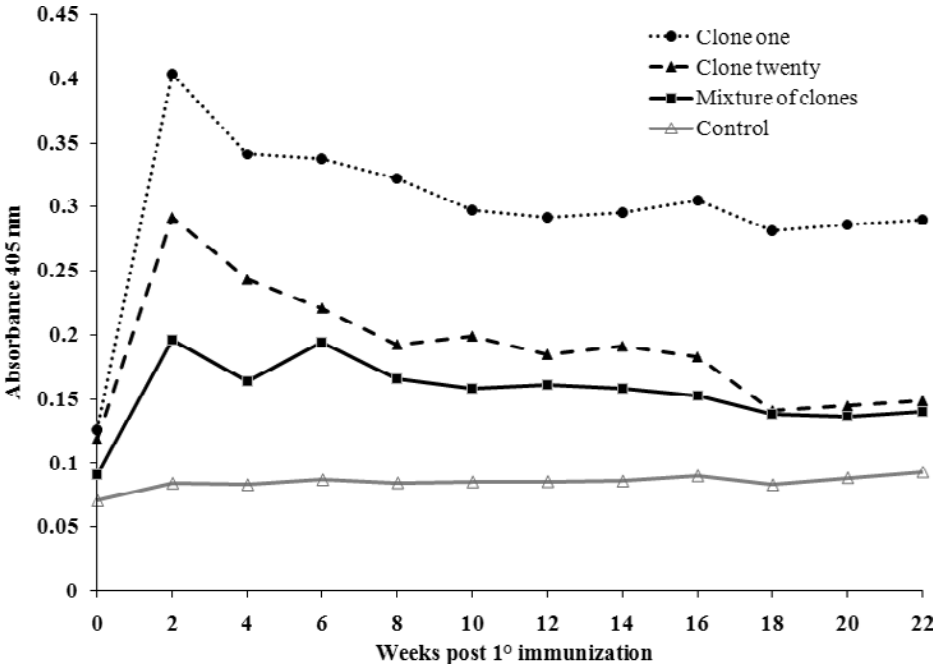

Fig. 4. IgG reactivity against selected phage clones of the sera from control and vaccinated sheep, challenged with 300 metacercariae at week 6. Sheep were given 2 immunizations at 0 and 4 weeks with 1×10^{14} pfu without adjuvant. Results are the mean of the 5 animals run in duplicate.

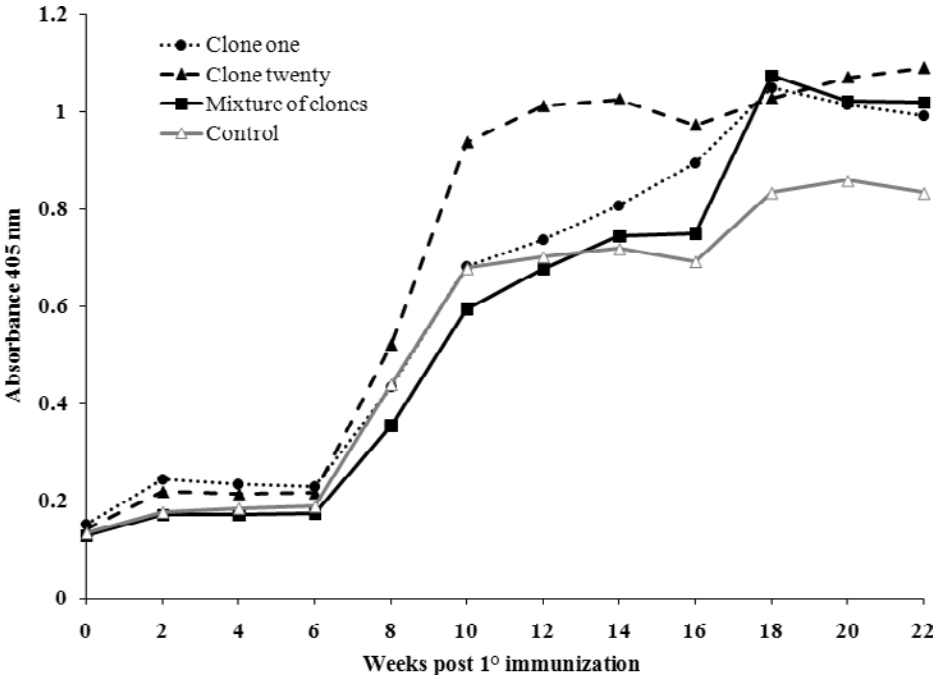

Fig. 5. IgG reactivity to E/S products in ELISA of the sera from control and vaccinated sheep, challenged with 300 metacercariae at week 6. Sheep were given 2 immunizations at 0 and 4 weeks with 1×10^{14} pfu without adjuvant. Mean OD of 1 : 400-diluted serum in duplicate wells. Results are the mean of the 5 animals run in duplicate.

suggesting this is a conformational epitope, and thus that antibodies against it may be directly playing a protective role. In this regard, it is worth mentioning that *in vitro* binding of rabbit anti-cathepsin L1 IgG to the protease results in inhibition of its ability to cleave IgG molecules that mediate eosinophil attachment to flukes (Smith *et al*. 1994).

Since clone 20 probably displayed a linear epitope (it was positive in Western blot), we aimed to compare it with clone 1, and interestingly we found induction of protection, but probably through a different way, since it induced a stronger reduction upon egg count than clone 1, but a weaker protection as measured by egg viability or adult burden and size.

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Similar to clone 1, phages 5 and 19 seemed to display conformational epitopes, as suggested by Western blot. Unfortunately, due to economical reasons, they were studied mixed with the other 3 clones. Even though no effect was observed on adult burden or size and a non-significant effect on FEC was observed, a slight but significant reduction in egg viability was demonstrated. Clearly, these clones are good candidates for individual immunoprophylaxis studies. The activity of cathepsin L and thereby miracidial development and egg output may have been blocked by the induction of antibodies.

The data presented demonstrate that the selected mimotopes of cathepsin L induced a protective immune response against infection by *Fasciola hepatica*. These results are encouraging in terms of vaccine development because of the relative simplicity of selection and the short length of the peptides. Potentiation of immune responses to the mimotope by the use of appropriate adjuvants should be further investigated. In addition, phage clones of the same or different molecules could be used in conjunction to increase the level of protection. However, reduction in worm burden and size, fecal egg count and miracidium development could lead to reduced cattle morbidity and suggests that these mimotopes of cathepsin L may represent potential vaccine candidates. A lower dissemination of eggs would have a negative effect on pasture contamination and in this way on the prevalence of fasciolosis in endemic areas (Dalton *et al*. 1996).

This study was supported by the project PAPIIT-IN208703-03 DGAPA-UNAM, and the Consejo Nacional de Ciencia y Tecnología (CONACYT), México. The authors are grateful to the staff of the Teaching and Research Centre for Animal Health and Production (CEPIPSA), of the National Autonomous University of Mexico for collecting the samples. We also thank Esther Calderón, Héctor Luna, Carmen Ortiz, Ignacio De la Mora, Alfonso Reyes, Lauro Trejo and Jorge Cruz for providing excellent technical assistance.

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