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A novel adjuvant-free H fusion system for the production of recombinant immunogens in *Escherichia coli*

Its application to a 12 kDa antigen from *Cryptosporidium parvum*

Sofia J Costa[1,2], Pedro Silva[2,3], André Almeida[4,5,*], Antónia Conceição[3], Lucília Domingues[1], and António Castro[2]

[1]Institute for Biotechnology and Bioengineering; Centre of Biological Engineering; Universidade do Minho, Campus de Gualtar; Braga, Portugal; [2]Instituto Nacional de Saúde Dr Ricardo Jorge; Porto, Portugal; [3]Escola Superior Agrária de Coimbra; Coimbra, Portugal; [4]Instituto de Ciências e Tecnologias Agrárias e Agroalimentares da Universidade do Porto; Porto Portugal; [5]Hitag Biotechnology, Lda.; Biocant – Parque Tecnológico de Cantanhede; Cantanhede, Portugal

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Abbreviations: BB-SpG, region BB of Streptococcal protein G; CP12, 12-kDa surface adhesion antigen from *Cryptosporidium parvum*; CTB, Cholera toxin B; ELISA, enzyme-linked immunosorbent assay; Fh8, 8-kDa excreted-excreted antigen from *Fasciola hepatica*; GST, gluthathione S-transferase; H, 1-kDa fusion partner that corresponds to the 11-residue N-terminal sequence of the Fh8 antigen; MBP, maltose binding protein; O.D., optical density; SpA, Streptococcal protein A; SSNAP, sparingly soluble non-antigenic protein; TgOWP, oocyst wall protein from *Toxoplasma gondii*; Trx, thioredoxin

The production of recombinant antigens in *Escherichia coli* and specific polyclonal antibodies for diagnosis and therapy is still a challenge for world-wide researchers. Several different strategies have been explored to improve both antigen and antibody production, all of them depending on a successful expression and immunogenicity of the antigen. Gene fusion technology attempted to address these challenges: fusion partners have been applied to optimise recombinant antigen production in *E. coli*, and to increase protein immunogenicity. Taking a 12-kDa surface adhesion antigen from *Cryptosporidium parvum* (CP12) by example, the novel H fusion partner was presented in this work as an attractive option for the development of recombinant immunogens and its adjuvant-free immunisation. The H tag (of only 1 kDa) efficiently triggered a CP12-specific immune response, and it also improved the immunisation procedure without requiring co-administration of adjuvants. Moreover, polyclonal antibodies raised against the HCP12 fusion antigen detected native antigen structures displayed on the surface of *C. parvum* oocysts. The H tag proved to be an advanced strategy and promising technology for the diagnosis and therapy of *C. parvum* infections in animals and humans, allowing a rapid and simple recombinant production of the CP12 antigen.

Introduction

Antibodies are important tools in biomedical research. They allow the identification of new genes, the purification of proteins, and the study of protein properties such as structure, function and localization.[1,2] Most of these applications use polyclonal antibodies, which are produced in response to multiple epitopes of the same target protein (antigen). Polyclonal antibodies are usually raised against a specific protein by immunising an animal with the target protein in its purified form.[1]

The most effective way of obtaining the high quantities of antigenic protein required for efficient immunisation is by heterologous expression in a host cell. The bacteria *Escherichia coli* is one of the most widely used organisms for this purpose, as it is easy to manipulate, has a fast growth rate and is relatively cheap to use.[3-8] However, it also has its limitations; the recombinant protein it produces is not always correctly folded or sufficiently soluble for use in immunisation. The development of alternative strategies for protein production that overcome these drawbacks is therefore highly desirable.[5,9,10] One such strategy is the gene fusion technology, whereby the gene coding for the protein of interest is fused to a polypeptide chain, known as a fusion partner.

Fusion partners can simplify protein purification, improve protein production yield, reduce susceptibility to proteolysis and increase protein solubility.[6,11-14] They have also been reported to increase protein immunogenicity.[1,15-18]

*Correspondence to: André Almeida; Email: andre.cibp@gmail.com
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Figure 1. Amino acid and nucleotide sequences of the HCP12 codifying gene.

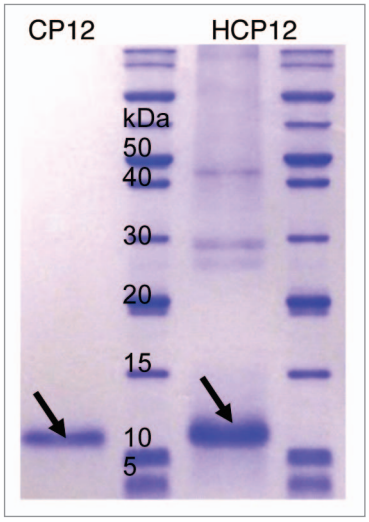

Figure 2. SDS-PAGE analysis of purified CP12 and HCP12 proteins after nickel affinity chromatography (5 μg of protein per lane).

Fusion partners, such as SpA,[2] GST,[19] BB-SpG,[18] MBP,[17] and Trx,[20] have been used to improve both antigen expression and antibody production. However, these fusion partners have also been shown to have drawbacks. In some cases, the resulting recombinant antigen is not sufficiently soluble or pure.[1,19] In others, the immune response obtained against fusion antigens is often triggered predominantly by the fusion partner itself, rather than by the target antigen.[1,2,19,21] And in others still, the fusion partners have shown inadequate immunopotentiating properties to elicit the production of sufficient quantities of the antibodies of interest. In such cases, during immunisation, it is necessary to co-administer an adjuvant. Despite being widely used for routine antibody production in animals, adjuvants are associated with non-specific immune responses and can cause several side effects and lesions at the injection site.[2,18,21,22] Therefore, the use of recombinant fusion partners for protein and antibody production has evidently much room for improvement.

This work presents the novel H fusion partner for the recombinant production in *E. coli* and subsequent adjuvant-free immunisation of the 12-kDa recombinant protein, CP12 (GenBank ID: XM625821.1), belonging to the parasite *Cryptosporidium parvum (C. parvum)*. The H partner consists of an 11 residues sequence from the N-terminal part of a calcium-binding protein (Fh8, GenBank ID: AF213970.1) excreted and secreted by the adult worm of *Fasciola hepatica*.[23] The H sequence was recently suggested as a fusion partner, and it showed to improve protein expression yields in *E. coli*, though it did not function as solubility tag.[24]

The utility of the H tag as a fusion partner for recombinant antigen and antibody production is here demonstrated by using the CP12 protein, which, as a surface adhesion protein, plays a major role in the diagnosis of *C. parvum* infections in various mammals, including humans.[25] A higher availability of this antigen and its specific polyclonal antibodies is therefore important for cryptosporidiosis prevention and therapy. In addition, the low molecular weight of the H fusion partner (1 kDa) makes it a particularly attractive option for use in antibody production.

Results

Expression and purification of CP12 in *E. coli* using the H fusion partner. The sequence of *cp12* gene cloned into pQE-30 and pQEH vectors (**Fig. 1**) matches 100% identity with the original *cp12* sequence except that it lacks the original N-terminal peptide signal and transmembrane region.

Results of protein purification were analyzed by SDS-PAGE (**Fig. 2**), and revealed that both CP12 and HCP12 proteins were obtained at the predicted 10 kDa and 11 kDa, respectively. After being purified, CP12 non fused protein achieved a production yield of 0.40 ± 0.050 mg per liter of *E. coli* culture while HCP12 fusion protein achieved a production yield of 1.5 ± 0.30 mg per liter of *E. coli* culture.

Production of polyclonal antibodies using the H tag. ELISA assays showed similar results for both CP12 and HCP12 ELISA coatings (**Figs. 3A and B**): an increased humoral immune response was observed in CP12 and HCP12-injected mice. When fused to the H partner, CP12 antigen was able to trigger an earlier immune response than the non-fused antigen: the IgG levels for HCP12-injected mice started to increase after the 14th day post injection, while IgG levels for CP12-injected mice only increased after the 28th day post injection (**Fig. 3A**). Furthermore, HCP12 mice group achieved higher polyclonal antibody titers than those obtained with CP12-injected group.

Humoral response against the H partner. For all sera analyzed, no significant IgG levels were observed in plates coated with Fh8 or HTgOWP antigens. Neither CP12-immunized nor HCP12-immunized sera reacted with HTgOWP (**Fig. 3C**) and Fh8 (**Fig. 3D**) antigens.

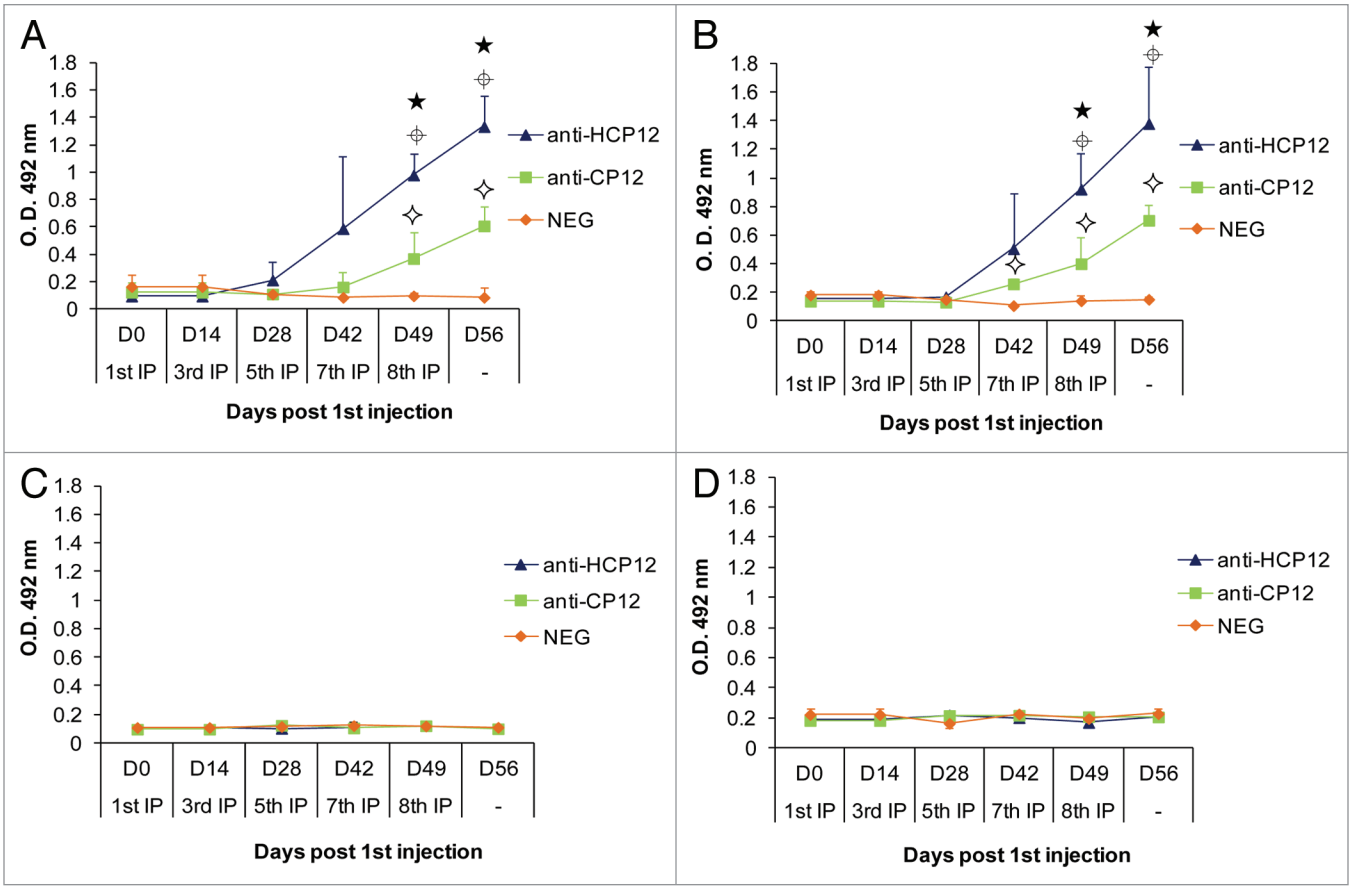

Figure 3. Evaluation of IgG production against CP12 and HCP12 antigens during the course of immunisation. ELISAs were performed with sera collected periodically from CD1 mice experimentally injected with HCP12 (here presented as anti-HCP12), with CP12 (here presented as anti-CP12), and from non-injected mice (here presented as NEG). 1st/3rd/5th/7th/8th IP, Blood collection before 1st/3rd/5th/7th/8th intraperitoneal injections. D56, Blood collection at seven days after 8th IP. Open cross circles and open diamonds, O. D. values statistically different from negative control. Closed stars, O. D. values statistically different from CP12-injected mice. (**A**) Plates coated with 10 μg.mL^{-1} of CP12. (**B**) Plates coated with 10 μg.mL^{-1} of HCP12. (**C**) Plates coated with 10 μg.mL^{-1} of HTgOWP (fusion antigen composed by the H partner and the TgOWP—oocyst wall protein of *Toxoplasma gondii*). (**D**) Plates coated with 10 μg.mL^{-1} of Fh8 (a calcium binding protein and a solubility fusion tag that contains the H partner sequence in its N-terminal).

These assays demonstrated that the H partner did not trigger itself a humoral response, since polyclonal antibodies anti-HCP12 were not capable to interact with H-containing antigens.

CP12 specific polyclonal antibodies. Western blot analyses with CP12- and HCP12-immunized sera (collected 7 d after 8th IP) revealed the formation of blots at a molecular weight that corresponds to dimer and possible oligomer forms (resistant to denaturing and reducing conditions) of Fh8CP12 antigen (36 KDa).The western blot analysis performed with the sera from the mice control group did not reveal blot formation, as expected (**Fig. 4A**, lane 3).

An immunoblotting analysis using the Fh8 recombinant antigen was also performed with HCP12-,CP12-immunized sera and sera from the mice control group, in the same conditions as above, but no blots were observed (**Fig. 4B**).

Western blot analyses showed that polyclonal antibodies produced by HCP12-injected mice group did not cross react with the H partner existent in the Fh8 protein and specifically recognized the CP12 antigen.

Recognition of native CP12 epitopes. The immunofluorescence results revealed that the control serum did not detect the *C. parvum* oocyst presented at **Figure 4C**. The fluorescence observed in **Figure 4C and D** highlights the surface of *C. parvum* oocysts, thus confirming the ability of the CP12- and HCP12-immunized serum to detect the native CP12 epitopes displayed on the surface of parasite oocysts.

Discussion

A novel approach to produce CP12 specific polyclonal antibodies is presented in this work, in which the H tag plays an important role for adjuvant-free immunisation.

Taking into account CP12 diagnostic and prophylactic relevance in the control of *C. parvum* infections in various organisms, novel strategies to improve this antigen production and its specific polyclonal antibodies availability are desired.

Following a production strategy from gene to antibody, the study conducted here reveals several advantages of using the H partner to produce polyclonal antibodies. The H partner improves

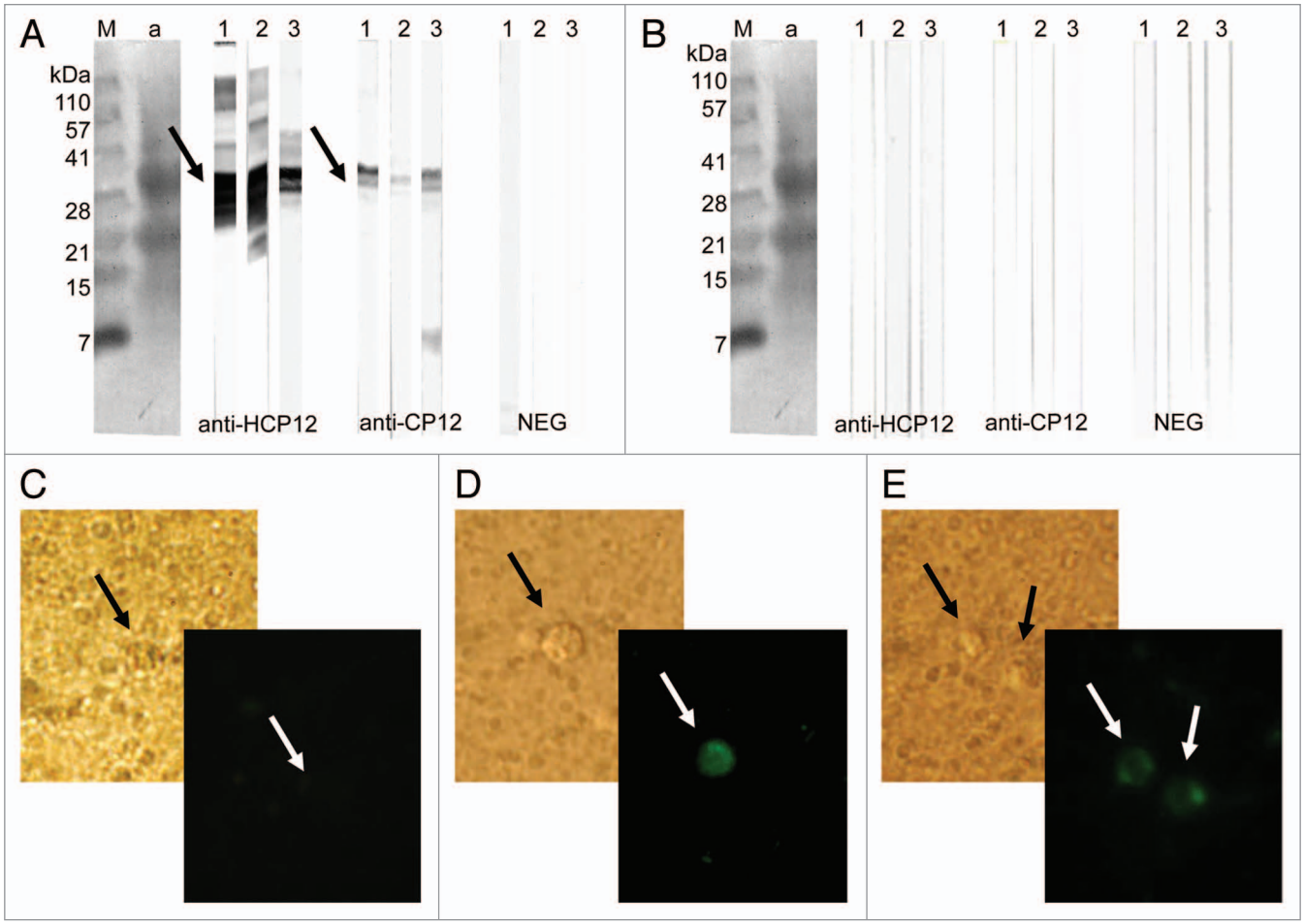

Figure 4. Evaluation of polyclonal antibodies specificity against CP12 antigen. (**A**) western blot analysis of Fh8CP12 antigen. Arrows focus the detected dimeric form of Fh8CP12 antigen. (**B**) western blot analysis of Fh8 antigen. In all images, 1, 2 and 3, sera from mice 1, 2, and 3 of each group; Anti-HCP12, Polyclonal antibodies produced by HCP12-injected mice; Anti-CP12, Polyclonal antibodies produced by CP12-injected mice; NEG, Negative sera. M, protein marker (Biorad); a, Fh8CP12-loaded antigen stained with Amido Schwartz reagent. (**C**) Immunofluorescence assay using negative serum (1000-fold total amplification). (**D**) Immunofluorescence assay using CP12-immunized serum: detection of *C. parvum* oocysts surface (1000-fold total amplification). (**E**) Immunofluorescence assay using HCP12-immunized serum: detection of *C. parvum* oocysts surface (1000-fold total amplification).

specific antibody production against CP12 antigen, resulting in an earlier immune response and in higher polyclonal antibody titers when compared with the non-fused CP12 immunisation. Moreover, HCP12-immunized sera react only with CP12 antigen but not with the H moiety contained in the N-terminal part of the Fh8 antigen as well as in the HTgOWP antigen. This CP12 specific immune response results from immunisation of HCP12 antigen without using adjuvants, whereby the immunogenicity rise may be exclusively due to the fusion of CP12 recombinant antigen with the H partner.

CP12 antigen is not very immunogenic and its low molecular weight can hinder the production of polyclonal antibodies. When producing such low molecular weight antigens, fusion partners and adjuvants are usually needed to increase the protein molecular weight and consequent immune response. However, fusion partners like GST,[19] Trx,[20] BB-SpG,[18] MBP,[17] and SpA[2] or SSNAP[21] and CTB,[26,27] which act themselves as adjuvants, are often not capable to elicit a satisfactory immune response, and adjuvant stimulations are not desirable as they can be difficult to prepare or may cause several side effects, namely, chronic inflammation response at the injection site. In addition, the immune responses obtained with large fusion partners are frequently not specific for the target antigen, resulting in polyclonal antibodies production against both fusion moiety and target antigen. For most antibody applications, extra purification steps need to be conducted, which makes this a time-consuming and expensive methodology.

Taking into account all these aspects, the ability of the H partner to effectively improve the specific immunogenicity of CP12 protein without adjuvants makes this fusion tag an innovative alternative to the traditional immunisation methods and an attractive option to the existent and commonly used fusion partners. Furthermore, the H partner has only 1 kDa, which is, to our knowledge, the lowest molecular weight partner used for protein and antibody production. Moreover, polyclonal antibodies produced by this novel strategy can be used as a diagnostic

tool for immunodetection of *C. parvum* infections in humans or animals. A small fusion partner like the H tag may not interfere negatively with target antigen conformation and, hence, polyclonal antibodies raised against antigens fused to the H partner are able to recognize native antigen structures.

The low molecular weight of H partner may also clarify the reason why this moiety does not trigger an immune response against itself when fused to a target antigen.

Materials and Methods

Fusion vector. The H fusion partner containing the restriction sites for *BamH*I and *Sac*I enzymes was introduced into pQE-30 vector (Qiagen) between the restriction sites for the same enzymes (**Fig. 1**). The resulting fusion vector is a modified pQE-30 vector with a H tag sequence site, named as pQEH, after the N-terminal polyhistidine. The H vector contains a multiple cloning site similar to pQE-30 vector, and codifying gene sequences can be cloned immediately after the fusion tag using the specific restriction site for *Sac*I enzyme. The H fusion tag has a molecular weight of only 1 kDa.

Construction of target expression vectors. The *cp12* gene used in this work (**Fig. 1**) corresponds to a truncated form of the original *cp12* gene: it lacks the original N-terminal peptide signal and transmembrane region. This truncated gene (*cp12* gene) was obtained from genomic DNA of *C. parvum* by a PCR using the forward primer: 5'-CATACTGGTA T**GAGCTC**GAA GGAGTAC-3' and the reverse primer: 5'-CATTAAAA**GG TACC**TTTCAT TATCAAG-3'. The forward primer introduced a restriction site for *Sac*I enzyme (bold) between the *C. parvum* genomic DNA sequence (corresponding to an intron) and the initial part of *cp12* gene (underlined). The reverse primer introduced a restriction site for *Kpn*I enzyme (bold) between the final part of *cp12* gene (underlined) and the *C. parvum* genomic DNA sequence. The PCR product was cloned into pGEM T-easy vector (Promega, Catalog # A1360), according to manufacturer's instructions, and used to transform *E. coli* XL1 strain. Positive clones were selected and sequenced using pQE universal primers.

pGEM plasmid containing the *cp12* gene was digested with *Sac*I and *Kpn*I restriction enzymes (Promega, Catalog # R6061 and R6341) and the *cp12* gene was then cloned into the same restriction sites of pQE-30 (Qiagen, Catalog # 32915) and pQEH (Hitag®), using T4 DNA ligase (Promega, Catalog # M1801). Following this strategy, *E. coli* M15 [pREP4] cells were transformed with pQE-30 (Qiagen, Catalog # 32915) and pQEH (Hitag®) expression vectors containing the 237 bp *cp12* gene. Positive clones were confirmed by DNA sequencing (as mentioned above) and the resulting sequence was aligned to *cp12* (GenBank ID: XM625821.1).

LB medium plates supplemented with ampicillin (100 $\mu g.mL^{-1}$) and kanamycin (50 $\mu g.mL^{-1}$) were used to select and maintain *E. coli* M15 [pREP4] transformants.

Expression and purification of CP12 proteins in *E. coli*. Cells were grown at 37 °C in LB medium supplemented with ampicillin and kanamycin. The *E. coli* culture was induced with 1 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 5 h at 37 °C. After induction, cells were harvested by centrifugation at 4000 g during 15 min at 4 °C, and the resultant pellet was lysed with 8 M urea, pH 8.0, overnight at room temperature, 150 rpm. Cell extracts were centrifuged at 10000 g, 20 min and the resulting supernatants and pellets were collected to further analyses.

The supernatant fraction was applied onto a Ni-NTA column (GE Healthcare, Catalog # 17–5268–02), pre-equilibrated with 8 M urea, pH 8.0. Ni-NTA purification was conducted according to the manufacturer's instructions, and the protein elution was performed by a pH decrease from 8.0 to 4.5.

All CP12 and HCP12 production procedures were repeated in three different periods of time to obtain biological independent replicates. Recombinant antigens (HCP12 and CP12) administrated to CD1 mice were dialysed against phosphate saline buffer 1x (PBS), prepared in apyrogenic water (B Braun, Catalog # L8502), pH 7.2, and concentrated using Centricon 3 (Millipore, Catalog # UFC800308). Pyrogens from concentrated recombinant antigen solutions were removed with Detoxi-Gel Endotoxin Removing Gel (Pierce, Catalog # 20339) according to the manufacturer's instructions. Recombinant antigen samples were recovered in pyrogen-free water (Braun, Catalog # L8502) and stored at –20 °C until use.

Protein electrophoresis and protein determination. The purity of collected fractions from Ni-NTA chromatography was analyzed by 17–4% SDS-PAGE gels stained with Coomassie brilliant blue dye. 15–4% tris-tricine gels were also used for western blot analyses, as described below. Protein samples were prepared in a buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol, and they were heated prior loading. Protein content of each collected fraction and final production yields were determined by Bradford assay.

Immunizations. Polyclonal antibodies were produced in CD1 female mice with 10 to 12 weeks old purchased from Charles River SA, Barcelona. The animals were housed and maintained with food and drink *ad libitum*.

For immunisation, groups of three mice were injected intraperitoneally (IP) at 2 week intervals with 20 μg of antigen (CP12 or HCP12) in 200 μl of inocula, without using any adjuvant. Purified recombinant antigens were diluted in pyrogen-free physiological serum (Braun, batch # 5144B15) to a concentration of 0.5 mg mL^{-1}, filtered by 0.2 μm membrane in sterile conditions, prior to administration. Control animals harbouring the same age and characteristics did not receive either protein or inocula.

Blood collection was performed for five times at 3rd IP, 5th IP, 7th IP, 8th IP at the tail vein and mice were sacrificed 1 week later. After blood collection, mice sera were separated by centrifugation at 2500 g for 10 min and then stored at -20 °C.

All animal experiments were performed in accordance to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

ELISA. The antibody titer was determined by ELISA. Briefly, 96-well microtiter plates were coated with HCP12 fusion antigen or CP12 antigen (both at 10 μg mL^{-1}) by incubating overnight at 4 °C. Bovine serum albumin (BSA) at 3% (w/v) in PBS 1x

containing 0.3% (v/v) of Tween 20 (PBST) was then used for blocking the wells. Diluted HCP12-, CP12-immunized sera or negative serum (from the non-injected group) was dispensed into each well and plates were incubated at 4 °C overnight. Protein A-Horseradish Peroxidase mixed with Protein G- Horseradish Peroxidase were used as conjugates, and o- phenylenediamine in 0.2 M phosphate buffer (pH 5.6) was used for the membrane revelation step. The reaction was stopped by adding 3 M HCl solution.

The optical density (O.D.) was measured at 492 nm using an ELISA reader. ELISA measurements were submitted to a Mann-Whitney U test with 95% confidence level.

H tag specific humoral response assay. For testing the specific humoral response against H tag, several ELISAs were performed using two different proteins as coating antigens: Fh8 and HTgOWP—a *Toxoplasma gondii* oocyst wall protein, which contain the H-tag sequence at their N-terminal, with CP12-, HCP12-immunized sera and negative sera. 96-well microtiter plates were coated with Fh8 or HTgOWP antigens (both at 10 $\mu g\ mL^{-1}$), and were subsequently treated following the above-mentioned ELISA protocol.

Western blot. In this analysis we used Fh8CP12 and Fh8 antigens to test if there were cross reactions with the H partner, which DNA sequence is part of Fh8 sequence. Tris-Tricine gel containing recombinant Fh8CP12 or Fh8 antigens (prepared in a buffer with SDS and β-mercaptoethanol and heated prior loading) were transferred to nitrocellulose membranes using a sandwich system. A strip containing the protein marker and part of the Fh8CP12-loaded antigen was cut from the nitrocellulose membrane and stained with Amido Schwartz reagent. The rest of the membrane was saturated with a 5% (w/v) PBS-Milk solution for 1h at room temperature (RT). Diluted anti-HCP12, anti-CP12 or negative group sera were added to different strips of the membrane and incubated overnight at 4 °C. Diluted Protein G-peroxidase was used as conjugate. Protein detection was performed using 4-chloro-1-naphthol in cold methanol, PBS 1x and hydrogen peroxide.

Immunofluorescence assay (IFA). Immunofluorescence assays were conducted to evaluate the potential of polyclonal antibodies produced by HCP12- and CP12-injected mice to recognize native CP12 epitopes presented on the surface of the parasite *C. parvum*. *C. parvum* oocysts were isolated from positive fecal samples of animals by using cesium chloride density gradients.[28] Isolated oocysts were counted using a Newbauer chamber, and added to the slides for testing. Oocysts were air-dried onto slides, then fixed with acetone and incubated at 37 °C in a humid atmosphere with either diluted HCP12-immunized sera, CP12-immunized sera or negative control sera. After being washed twice with PBS 1x, oocysts were incubated with diluted anti-mice IgG conjugated with FITC, according to the manufacturer's instructions. This conjugate was used to reveal the bound antibodies, which were then observed using a fluorescence microscope (photos were taken with a 1000-fold total amplification).

Disclosure of Potential Conflicts of Interest

The H tag utilization for the production of immunogens and corresponding polyclonal antibodies is covered by a worldwide patent (WO 2011071404) licensed to Hitag Biotechnology, Lda. The authors S.C., A.A., A.C. and A.Castro are co-owners of the patent and are associated with Hitag Biotechnology, Lda.

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