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6 Plathylemimth fatty acid binding proteins as candidate vaccines

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Abstract

Parasitic platyhelminth diseases are an unresolved health issue in the world causing significant human and economic losses, particularly in developing countries. Included among platyhelminth proteins being considered as candidate vaccines are the fatty acid binding proteins (FABPs). These proteins were first isolated by virtue of their naturally immunogenic and host-protective characteristics, and thus quickly became a focus for potential use as vaccines. Most subsequent study with platyhelminth FABPs was developed with those of Schistosoma

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mansoni (Sm14) and Fasciola hepatica (Fh12/Fh15) and to a lesser extent with proteins isolated from Schistosoma japonicum (SjFABPc), Fasciola gigantica (FgFABP) and Echinococcus granulosus (EgFABP1). Studies using these FABPs have been extensive and have involved different vaccination strategies using the native protein, recombinant forms and peptides, and DNA as vaccines in which different doses, adjuvants and animal models were used. The results have been hopeful in that an average percentage of protection of 60% has been achieved. Although significant progress has been made, we consider that a better understanding of the molecular mechanisms underlying the biological roles and in particular in how FABPs elicit an immune response, can provide needed insight for further development and for refining the effectiveness of FABP-based vaccines.

1. Introduction

Platyhelminthes (from the Greek platy, meaning "flat" and helminth, meaning worm) is a phylum of relatively simple soft-bodied invertebrate animals. Most flatworms are free-living, but many are parasitic. There are four classes: Trematoda (flukes), Cestoda (tapeworms), Monogenea (ectoparasites of the skin or gills of fish, amphibians, reptiles, cetaceans and cephalopods) and Turbellaria (planaria). In this chapter we shall refer to members of the parasitic forms, the Trematoda: *Schistosoma mansoni*, *Shistosoma japonicum*, *Fasciola hepatica*, *Fasciola gigantica* and the Cestoda: *Echinococcus granulosus* [1]. Members of the first class are the causative agents of schistosomiasis, of the second, fascioliasis and of the third, hydatidosis. These diseases are severe public health and livestock problems included within the most important parasitic diseases of the world, and mainly in developing countries.

Schistosomiasis (also known as bilharzia, bilharziosis or snail fever) is a major cause of human morbidity and mortality ranking with malaria and tuberculosis in this regard. It is estimated that at least 200 million people are currently infected in tropical regions of the world, with one of five *Schistosoma* species, and with an additional 600 million individuals at risk [2, 3]. This disease is principally caused by *S. mansoni*, *S. hematobium* and *S. japonicum* species, and is most commonly found in Asia, Africa, and South America. A common route of infection involves contaminated water in areas with freshwater snails that may carry the parasite. Larval forms of the parasites, which are released by freshwater snails, penetrate the skin of persons in water. In the body, the larvae develop into adult schistosomes which live in the blood vessels. The females release eggs, some of which are

passed out of the body in urine or faeces. Others remain trapped in various body tissues, causing immune reactions.

Fascioliasis is an infectious disease of herbivores primarily caused by liver flukes *Fasciola hepatica* and *Fasciola gigantica*. The former has a worldwide distribution found mainly in temperate climates, whereas the latter is primarily of tropical climates in Africa and Asia. This disease occurs most often in ruminants with increasing evidence of human infections worldwide. Disease transmission is usually associated with ingestion of vegetation contaminated with metacercariae, a larval form of the fluke. Adult worms may then be found localized to the liver, where they can cause extensive damage. Since 1991, the number of reported human infections increased yearly, *e.g.*, from 1997 through 2001 some 500 new cases were identified [4].

Hydatidosis (cystic or alveolar) is an economically significant disease, particularly in developing countries [5, 6], but it also important in European Mediterranean countries (Spain, Italy) and in Great Britain [7]. The causative agent is the tapeworm *Echinococcus granulosus* (cystic hydatidosis) and *Echinococcus multilocularis* (alveolar hydatidosis). The adult stage of these parasites is found in the small intestine of carnivores, the definitive host, and produces eggs that are passed with the host faeces. The usual intermediate hosts become infected by grazing on vegetation contaminated with eggs shed by adult worms. Clinical signs in intermediate hosts, such as sheep, cattle, camels and other ungulates, are caused by the presence of the larval stage of the parasite migrating through internal organs, especially in the liver and lungs. Humans are also found to be an intermediate host in significant numbers; in Central Asia the annual rate of necessary surgical intervention is 25 cases/100,000 habitants yearly and in Europe, 4-8/100,000 habitants per year [8, 9, 10].

Although antiparasitic drugs have been effective in controlling these diseases in the past, renewed interest in developing better control methods have been proposed in response to the increasing occurrence of strains resistant to the commonly used drugs (triclabendazole, praziquantel and salicylaniides), and those with cross-resistances to drugs used for control of parasitic nematodes [11-14]. The demonstration that natural hosts develop an adaptive immune response that protects against secondary infection provided a framework for the development of effective component vaccines [8, 11, 13]. Vaccination is an important alternative for control and reduction of morbidity incited by these parasites in livestock and other herbivores, which should in turn serve to increase the efficiency of dairy and meat animal production. Significantly, a highly-effective vaccination program could provide a long-term solution for schistosomiasis, fasciolosis and hydatidosis [15, 16].

2. Platyhelminth FABPs

Many features make parasite platyhelminth FABPs interesting molecules for study. The parasites, *e.g.*, are incapable of *de novo* synthesis of most of their own lipids, including long chain fatty acids and cholesterol [17 and references therein], and depend largely on acquisition and utilization of host FAs during infection in order to survive. In this respect, FABPs are predicted to play an important role in facilitating the incorporation and intracellular transport of host fatty acids. The second feature is their highly immunogenic character that serves to confer significant levels of protection against challenge infections establishing FABPs as vaccine candidates.

The first platyhelminth FABP described was isolated from the parasite *S. mansoni* and designated Sm14, indicating its apparent molecular mass [18]. Homologous proteins from *Schistosoma japonicum*, Sj-FABPc [19], *Schistosoma bovis*, SbFABP [20], *Fasciola hepatica*, Fh15 [21], *Fasciola gigantica*, FgFABP [22], *Echinoccocus granulosus*, EgFABP1 and EgFABP2, [23, 24], *Mesocestoides vogae*, MvFABPa and MvFABPb [25], and *Taenia solium*, TsFABP, [26], were subsequently isolated and characterized. At the encoded protein sequence level Trematoda and Cestoda FABPs display low identity and similarity and no extensive shared identical sequence motifs (Fig 1) [27]. However, solved or predicted 3D structures are similar with the typical β-barrel structure previously described for vertebrate FABP counterparts [28, 29]. Interestingly, rEgFABP1 and rSm14 accommodates fatty acid ligands in a similar U-shaped conformation as generally observed in H-FABPs [28, 29] (Fig. 2). As shown in Fig 1. partially or fully conserved amino acids are located in β-barrel turns, at α helix 1 and at the end of α helix 2 when compared with characterized FABP structures. Residues implicated in binding the ligand carboxylate group described for vertebrate FABPs (R^{106}, R^{127}, Y^{129}) are also conserved with the exception of *Fasciola* FABPs. Platyhelminth sequences appear to be more closely related to those of the vertebrate heart FABPs subfamily than to those from intestine or liver [27, 30].

Some experimental approaches have been undertaken aimed at characterizing the role of platyhelminth FABPs during infection. Expression studies have been done to demonstrate the relationship between developmental events and localization of expression [23]. In an attempt to elucidate whether helminth proteins are involved in the uptake of host lipids, the process by which SjFAPc transfers its ligand to membrane acceptor(s) was investigated [31]. It was found that this protein displayed a "collision mechanism" of fatty acid transfer similar to that described for human heart

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                        β1        αI          αII         β2
H-FABP(Hs)      MVDAFLGTWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIEKNGD-I 49
Sm14(Sm)        -MSSFLGKWKLSESHNFDAVMSKLGVSWATRQIGNTVTPTVTFTMDGD-K 48
ShFABP(Sh)      -MSSFLGKWKLSESHNFDAVMSKLGVSWATRQIGNTVTPTVTFTMDGD-K 48
SbFABP(Sb)      -MSTFLGKWKLSESHNFDAVMSKLGVSWATRQIGNTVTPTVTFTMDGD-K 48
SjFABPc(Sj)     -MSSFLGKWKLSESHNFDAVMSKLGVSWATRQIGNTVTPTVTFTMDGD-T 48
Fh15(Fh)        -MANFVGSWKLEQSENMDAVLQKLGINVIKRKLITSSKPEITFTLEGN-K 48
FgFABP(Fg)      -------SWKYGDSENMEAYLKKLGISSDMVDKILNAKPEFTFTLEGN-Q 42
EgFABP1(Eg)     -MEAFLVTWKMEKSEGFDKIMERLGVDFVTRKMGNLVKPNLIVTDLGGGK 49
EgFABP2(Eg)     -MEPFIGTWKMEKSEGFDKIMERLGVDYFTRKMGNMMKPNLIISDLGDGR 49
TsFABP(Ts)      -MEPFIGTWRMEKSEGFDKIMERLGVDFVTRKMGNLMKPSLIVSDLGDGK 49
MvFABPb(Mv)     -----------SKSEGFDEVMRHLGVNFIARKAGNTLKPTVTITSVGDGR 39
MvFABPa(Mv)     -----------EKSEHFDEVMQKLGVGFLTRQAGKLAKPTLIVSCLGDGK 39
Consensus                  .*. ::  :  **:.    .     .*   .   *.

                    β3        β4        β5          β6          β7
H-FABP(Hs)      LTLKTHSTFKNTEISFKLGVEFDETTADDRKVKSIVTLDG-GKLVHLQKW 98
Sm14(Sm)        MTMLTESTFKNLSCTFKFGEEFDEKTSDGRNVKSVVEKNSESKLTQTQVD 98
ShFABP(Sh)      MTMLTESTFKNLSCTFKFGEEFEEKTSDGRNVKSVVEKNSESKLTQTQVD 98
SbFABP(Sb)      MTMLTESTFKNLSCTFKFGEEFEEKTSDGRNVKSVVEKNSESKLTQTQVD 98
SjFABPc(Sj)     MTMLTESTFKNLSVTFKFGEEFDEKTSDGRSVKSVVTKDSESKITHTQKD 98
Fh15(Fh)        MTMKTVSALKTTVISFTFGEEFKEETADGRTVMTTFTKDSDSKISQVQKC 98
FgFABP(Fg)      MTIKMVSSLKTKITTFTFGEEFKEETADGKTAMTTVTKDSESKMTQVTTG 92
EgFABP1(Eg)     YKMRSESTFKTTECSFKLGEKFKEVTRFTRGHFFMITVEN-GVMKHEQDD 98
EgFABP2(Eg)     YNMRSESKFKTSEFSFKLGEQFKEVTPDSREVMSMLTVED-GVLKQEQVG 98
TsFABP(Ts)      YSMRSESKFKTTEFTFKLGEKFKETTPDSREVTSLITVEN-GVMKQEQVG 98
MvFABPb(Mv)     YHMKLESTFKNTEFTFKLGEECDEVTADGRKVKSTITMDG-STMKHVQVG 88
MvFABPa(Mv)     YKMRSESTFKNTEFEFKLGEEFKEETPDGRTVQSTITLDG-DTLKQVQVG 88
Consensus         :   * :*.    *.:* : .* *   :     .  :. . : :

                      β8        β9         β10
H-FABP(Hs)      DGQETTLVRELIDGKLILTLTHGTAVCTRTYEKEA- 133
Sm14(Sm)        PKNTTVIVREVDGDTMKTTVTVGDVTAIRNYKRLS- 133
ShFABP(Sh)      PKNTTVIVREVDGDTMKTTVTVGDVTAIRNYKRLP- 133
SbFABP(Sb)      PKNTTVIVREVDGDTMKTTVTVGDVTAIRNYKRLS- 133
SjFABPc(Sj)     SKNTTVIVREIVGDTMKTTVTVDDVTAIRNYKRL-- 132
Fh15(Fh)        PENTTHVVREVTGGKMIATVTVGDVKAVNNYHKV-- 132
FgFABP(Fg)      PEYTTHVVREVVGDKMIATWTVGDVKAVTTLLKA-- 126
EgFABP1(Eg)     KTKVTYIERVVEGNELKATVKVDEVVCVRTYYSKVA 134
EgFABP2(Eg)     KDKTTYIDRVVDGNELRATVKADELVCVRTYSRGM- 133
TsFABP(Ts)      KGKTTYIDRVIEGNELKTTVKVDELVCVRTYVKAA- 133
MvFABPb(Mv)     -EKTTHIERVIEGDKMLTTVTVDDLVSKREYTRC-- 121
MvFABPa(Mv)     -EKTTYIDRVIEGNELKTIVKVDDVVSTRIYVKI-- 121
Consensus           * : * : .. :    . .   .
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Figure 1. Alignment of platyhelminth FABPs sequences and comparison with human H-FABP. The following sequences have been aligned, respectively: H-FABP, heart FABP from *Homo sapiens* (Hs); Sm14 from *S. mansoni* (Sm); SjFABPc from *S. japonicum* (Sj); SbFABP from *S. bovis* (Sb); ShFABP from *S. hematobium*; Fh15 from *F. hepatica* (Fh); FgFABP from *F. gigantica* (Fg); EgFABP1 and EgFABP2 from *E. granulosus* (Eg); TsFABP from *T. solium* (Ts) and MvFABPa and MvFABPb from *M. vogae* (Mv). Secondary structure referring to H-FABP (Hs) (1G5W.pdb) is indicated: α, α helix, β, β-strand. Numbers at the right indicate each sequence length. Alignment was done using the ClustalX algorithm and the Gonnet Pam 250 matrix. The consensus sequence is indicated at the bottom of each block: (*) identity; (:) high similarity, (.) low similarity.

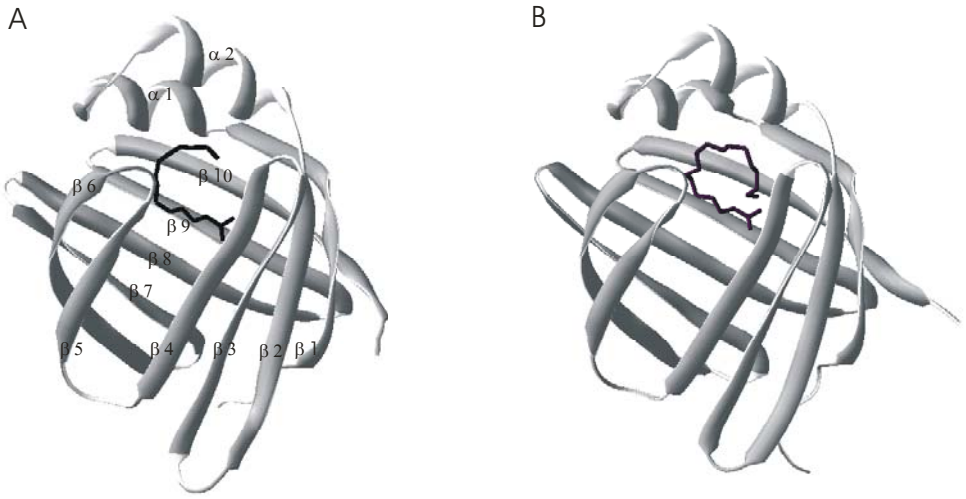

Figure 2. FABPs 3D structures. A) rEgFABP1 (108v) with palmitic acid; B) rSm14 (1VYF) with oleic acid.

and intestinal FABPs. The emergence of specific functions in accordance with particular needs of parasites was also considered, in this sense it was further suggested that platyhelminth FABPs may play a role in drug sequestration [32, 33].

3. FABPs as vaccines

3.1. Sm14

Sm14 was identified from a *S. mansoni* expression library using antisera raised against an extract of adult worms that was also shown to provide host-protective immunity in mice upon challenge with infectious larva (cercaria) [18]. These observations caused platyhelminth FABPs to become a major focus of study for parasite antigens capable of eliciting an effective immunological response to the parasite, and in which Brazilian researchers have made much progress. A recombinant protein was soon developed and assayed as a protective antigen not only against *S. mansoni* but also for *F. hepatica* infection [34]. The Sm14 polypeptide sequence was cloned in an appropriate vector (pGEMEX-1, Promega) and used to transform *Escherichia coli* strain (BL21-DE3). The recombinant protein (rSm14) was specifically and strongly recognized by antibodies from mice and rabbits infected with *S. mansoni* or by humans from endemic areas. Antibodies against rSm14 did not label any cell of normal rabbit tissues. Immunization experiments were performed with New Zealand White rabbits and outbred Swiss mice immunized with two intradermal/subcutaneous doses of a saline extract of adult *S. mansoni* or rSm14 in presence or absence of Freund's complete

adjuvant and infected with *S. mansoni* or *F. hepatica* larvae. The protective activity of the recombinant protein was confirmed with the observation of high levels of protective immunity in rabbits (89%), or mice (66%) challenged with the *S. mansoni* infective form. Infected animals that had been vaccinated showed a lesser degree of the typical immunopathological reactions compared with those not vaccinated. Protection of 100% of Swiss mice immunized with rSm14 infected with *F. hepatica* led the investigators to propose rSm14 as a potential dual purpose anti-helminth vaccine [34]. As such, this antigen was included among the vaccine antigens endorsed by the World Health Organization for phase I/II clinical trials [35].

After these first experiments, the vaccine candidate was improved with the use of an *E. coli* expression system more suitable for scale-up and downstream processing. Specifically, 6XHis-tagged fusions (pET3-His-Sm14, Promega; pRSETA-His-Sm14, Invitrogen) were expressed and tested with anti-Sm14 antibodies [34] and also with antiserum against adult *S. mansoni* soluble secreted/excreted proteins characterized by Western blot analysis [36]. Protection against *S. mansoni* cercariae infection was to the same extent (40-55% burden inhibition) as the previously reported pGEMEX-1 system expressing rSm14 as vaccine antigen [34].

S. mansoni FABP was also expressed in *Mycobacterium bovis* BCG (bacillus Calmette-Guérin), a live vector system for the delivery of foreign antigens to the immune system [3]. The construct was stable when expressed in bacteria (BCG Pasteur 1172P2 cells). Evaluation of infectivity, persistence, and plasmid maintenance in mice, in both murine and bovine monocytes cultures showed low infectivity (5-10% of a bacterial inoculum was taken up by the monocytes) and there was a gradual decline in persistence of the number of BCG or rBCG recovered from cells. BALB/c immune response induced by rBCG expressing Sm14 showed a good cellular response but no humoral response. Finally, when protection using rBCG-Sm14 was evaluated against cercaria infections about 48% protection was found for Swiss mice that was independent of the number of doses administered, and was comparable to that achieved with rSm14 [34] and pRSETA-Sm14 [36].

As noted above, different approaches could be taken to improve or refine the Sm14 or rSm14 vaccine, and initial results with one different recombinant vaccine approach, discussed above, did not appear to affect protection levels versus Sm14. One alternative approach would be to optimize codon usage in order to adapt the codon usage of *Sm14* gene to more closely match that of the expression host, taking into account the differences in G + C content and codon usage. A synthetic Sm14 gene was prepared by PCR, and evaluated in terms of stability, immune response and vaccination effectiveness [37]. The

quantity of antigen produced by transformed bacteria increased with respect to the previous construction, but parameters related to infectivity, persistence, maintenance and immune response were the same as previous found for rSM14 [36].

Fusion of Sm14 with tetanus toxin fragment C was also considered to be another alternative for the further development of the vaccine against schistosomiasis [38]. Tetanus toxin fragment C is the non-toxic portion of tetanus toxin (TTFC) [39] with ability to trigger a protective immune response against tetanus toxin challenge [40]. Antibody response and protection against tetanus toxin and *S. mansoni* cercariae was encouraging with this construction. With respect to antibody response to tetanus toxin antigen, IgG1 and IgG2a were produced while IgG1 and IgG2b were produced against Sm14, indicating a Th2 response. Mice challenged with *S. mansoni* cercariae showed high levels of protection (51.2%) when immunized with the fusion, but was again similar to results observed with animals immunized with native antigen (50.6%) [38].

Several attempts have been made to develop peptide vaccines based on *S. mansoni* derived antigens [41-45]. Potential advantages of this option include production cost, chemical stability and safety. With these considerations in mind, bioinformatics provided the tools to predict putative epitopes from Sm14 to enable the design of potential peptide vaccine. In accordance with the numerous reports for cross-protective immune reactivity between FABPs of *S. mansoni* and *F. hepatica,* a bivalent peptide vaccine against schistosomiasis and fascioliasis was considered for development. With this dual purpose vaccine in mind, sequence comparisons between both proteins and nine human FABP sequences, using a 21 residue-sliding window were done by M. Magno Vilar and co-workers to design peptides to use in a vaccine [46]. Spatial proximity in the overall three-dimensional structure was also considered in these modelling studies. Regions of the FABP molecule that included turns (between β strands 9-10, 6-7 and 8-9) and α helix 1 were selected as candidate peptide segments. Since few of the predicted residues were present in continuous segments of the primary amino acid sequence, a design strategy was used to incorporate more than one continuous segment into a single peptide. Fusions both with or without a di-glycine peptide between candidate peptide segments were prepared. Selected peptides with the predicted epitopic residues were mostly derived from the carboxy terminal region (Ct) of the protein, which was also the least conserved. Nine peptides were assayed in different vaccination trials, and showed a large degree of discrimination, selectivity and protection in subsequent tests. The smallest peptide tested giving rise to protective levels of 50% corresponded to residues 118-125 which consisted of the turn between β-strands 9 and 10

(VTVGDVTA). A similar level of protection was obtained with a synthetic peptide obtained by fusion of residues 15-24 that included α 1 helix (NFDAVMSKLG), previously discussed. A third peptide with high protective activity included residues 85-94 located in the turn between β strands 6 and 7 (EKNSESKLTQ) (Fig. 3). The percentage of protection obtained with these peptides was similar to that of the complete recombinant protein (rSm14) [34] suggesting that it may be possible to generate the same level of Sm14-protective immunity with a molecule with less than 10% of its mass [46].

Peptides selected from the Sm14 sequence that proved to be effective against *S. mansoni* infection were also used to vaccinate inbred Swiss mice infected with three *F. hepatica* metacercariae. The peptide of residues 85-94 (EKNSESKLTQ) was able to protect 100% of mice when used as a vaccine, which was limited by the number of metacercariae that could be used as challenge. Similar results were obtained when mice were vaccinated with rSm14 with the same protocol [46]. In these experiments inoculated control animals were either dead or were infected 30 days after challenge infection, when infected with one adult worm.

Since most of the data reported was derived from vaccination experiments using laboratory animal models, vaccination results obtained by Almeida *et al*. (2003) using a natural host of *F. hepatica*, the sheep, provided very useful information [47]. They performed two independent

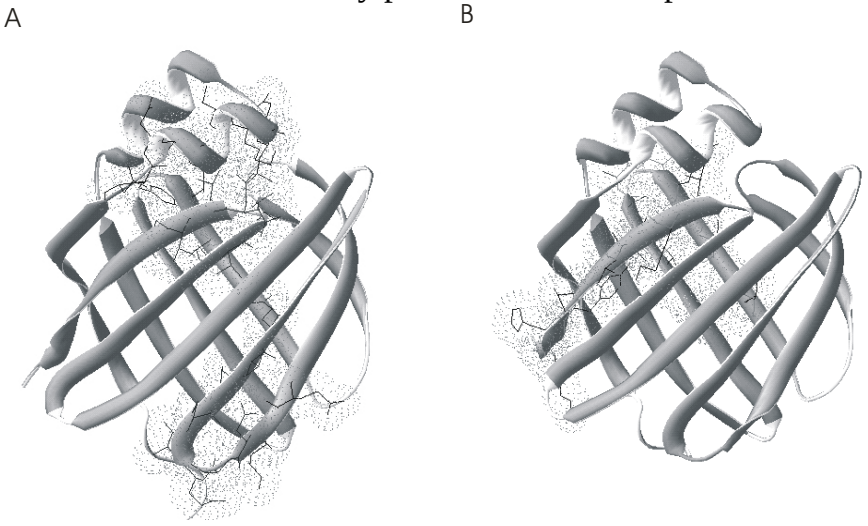

Figure 3. Ribbon diagram of 3D structures showing peptides selected for vaccination trials. A) rSm14 (1YVF), B) Fh15 (modelled through “Swiss Model facilities” using Sm14 as template). Residues predicted to participate as epitopes are illustrated within dotted-regions.

experiments with Sm14 in 6-12 month old lambs (three and four lambs in each group) challenged with metacercariae of the parasite. Despite the reduced number of animals in each treated group a significan level of protection was observed, considering the number of recovered flukes or extent of liver lesions.

A different approach based on immune protection conferred by nucleic acid based-vaccines was also attempted, as these vaccines can induce both the humoral and cellular arms of the immune system [48]. This approach has been studied using different animal models and for different parasites, such as, *Schistosoma* [49], *Plasmodium* [50], *Leishmania* [51] and for many other pathogens [52] and for immune diseases, *e.g.*, encephalomyelitis [53]. Nascimento and co-workers evaluated the immune protection levels of DNA encoding the Sm14 protein as a vaccine [54]. Plasmid (pVAX) maintaining the recombinant sequences was purified from *Escherichia coli* DH5α strains was purified and used for BALB/c mice vaccination. The animals were challenged with live cercariae similarly as noted above with proteins as antigens. Reduction of parasitic load was of 51% [54] again confirming the host-protective immunity of Sm14, even when administrated as a DNA vaccine. These protection levels were again similar to results using the native, recombinant or peptide vaccines and the humoral response was also very low.

A promising result of Fonseca and co-workers [55] showing the ability of exogenous IL-12 to enhance protection induced by recombinant Sm14 (rSm14) from 25% to 42%, encouraged the team to test the co-administration of plasmid expressing IL-12 with Sm14 protein cDNA. Unfortunately, despite an alteration of immune response profiles was observed, the combination failed to enhance protection induced by the Sm14 DNA vaccine alone [56].

3.2. SjFABP

Gene cloning, overproduction and purification of a functionally active cytoplasmic fatty acid-binding protein (Sj-FABPc) from the human blood fluke *Schistosoma japonicum* was undertaken by the Mc.Manus team, and was reported in 1994 [57]. Very little work has been reported related to the immune response and protective properties of this FABP. A study including epitope mapping was published in 1998, [58], on molecular and immunological characterisation in 2000 [52] and a study involving the development of a vaccine in 2004 [59]. The latter study showed that epitopes recognised during vaccination with functionally active rSjFABP, at least in the murine model, differed from those recognised during a natural infection. While sera from mice immunised with rSjFABP reacted predominantly with

peptides encoding amino acids in the central portion of the molecule, sera from experimentally infected mice reacted predominantly with peptides with amino acids in the C-terminal region of the molecule. In vaccination tests using a recombinant protein, all vaccinated groups (mice, rats and sheep) had an IgG antibody response against the antigen after being challenged when compared with control animals. Worm recovery differed among the various animal models challenged, and may have been influenced by the specific vaccination protocol used. The highest levels of protection (59%) were obtained using sheep, with the number of faecal eggs reduced 42.9% in vaccinated sheep [60].

3.3. Fh15

Recognition of Fh15 as host protective antigen was first shown by Hillyer and co-workers during the seventies [61-62]. This group purified a subset of adult *F. hepatica* using their reactivity with antibodies raised against *S. mansoni.* Partially purified material, termed Fh/SmIII complex, was able to protect both mice up to 78% and calves up to 55% compared with controls [63], against challenge infection with *F. hepatica* [64]. The complex was also demonstrated to provide protection against schistosomiasis [61-62, 65]. As a result of subsequent improvements in purification of protective material, an antigen of 12 kDa was identified as a major component of the protein complex.[66] This *F. hepatica* antigen was later renamed Fh12 in accordance with its estimated apparent molecular mass [66].

After screening a cDNA library from *F. hepatica* adult worms with an anti-Fh12 monospecific polyclonal antibody, the same group isolated the coding region of the gene [21]. The cDNA was sequenced and the predicted amino acid sequence revealed an open reading frame encoding a 132-amino-acid protein with a predicted molecular weight of 14,700 Da and high similarity with fatty acid binding proteins. The isolated fragment was used to produce a recombinant *F. hepatica* FABP, now termed Fh15.

The native (Fh12) and the recombinant (Fh15) forms have been shown to induce significant levels of resistance to challenge infections with *F. hepatica* in different animal models [63-64]. However, the recombinant vaccine induced lower levels of protection than did the native form; furthermore antibodies against Fh15 appeared much later after inoculation than did those in response to Fh12 [67-68]. These results suggested that Fh15 and Fh12 may have slightly differences either in immunogenicity, which might involve some differences in structure including differences in 3D configuration. Casanueva *et al.* [69] determined that the differences in protection observed were likely to be due to the time interval between the

administration of the antigens and challenge infection. These authors found a percentage of worm burden reductions of 43-76% as well as a decrease in liver lesions in rabbits vaccinated with Fh15. Circulating antibodies against the recombinant protein were detected as early as 2 weeks after a single immunization, with higher levels when animals were challenged 20 weeks post-immunization. The protective role of Fh15 was also reflected in the percentage of immature worms present in that a 66%-84% reduction was found relative to controls. A complementary explanation was found upon analysis of the composition of the active native form Fh12 isolated from *F. hepatica* adult worms. Fh12 was indeed found to consist of "complex" involving at least 8 isoforms with identical apparent molecular mass but different isoelectric points, and as well different immunological properties. Four acidic isoforms had more immunological similarity with Fh15 than with Fh12, and in addition had poor reactivity with sera from rabbits 2-4 weeks post-infection. The remaining forms, two acidic and two neutral/basic forms, showed more immunological similarity with the native Fh12 molecule than with the recombinant Fh15. These results suggested that Fh15 could be one of the acidic forms of the Fh12 complex or the less immunogenic or immune-protective member, or both, of the native Fh12 protein complex [68].

In order to improve this vaccine and to adapt it for use in routine field conditions, a system denoted ADAD was developed using the native *F. hepatica* FABP form as antigen [70], and consisted of two subcutaneous injections. The first injection called “adaptation” contained saponin (Qs) and/or a hydro-alcoholic extract of *P. leucotomos* (PAL) emulsified in non-mineral oil, but without the antigen. The second injection was administrated 5 days after adaptation and contained the antigen with Qs and/or PAL in the emulsion oil. The experimental group selected to evaluate this protocol (BALB/c and CD-1 mice), had a survival rate of around 40% at 42 days post-infection as compared with the control group vaccinated with only the protein who had 10% survival rate over the same period. Unvaccinated control groups died [70]. In sheep, the same protocol was used to test the recombinant protein with similar results [71]. Interestingly, in this experiment a reduction in the number of worms and their ability to mature was observed.

Like similar efforts for Sm14 vaccine development, potentially protective peptides within the sequence of Fh15 molecule were investigated. Peptides within Fh15 predicted to be recognized by T cells, using the “Peptidestructure” application (GCG Computer package; Accelrys GCG Accelrys Software, Inc., Madison, Wisconsin), were evaluated and their putative protective role described [4]. Two peptides were selected for

evaluation in vaccination schedules using the ADAD protocol. One peptide was from the amino terminal region (Nt) of the protein and the other from the Ct, (51-IKMVSSLKTKIT-62; 123-VKAVTTLLKA-132) (Fig. 3). The corresponding synthetic oligonucleotides were cloned in an appropriate vector (pGEX-2TK) and expressed in *E. coli*. Two kinds of peptides were obtained as GST fusions or as GST-free peptides after thrombin cleavage. The peptides alone did not give any protection when rabbits were used as models, and no evaluation was performed with mice. The GST-fusion with the Nt peptide gave the best results in which the following survival levels were found: 40% in BALB/c, 43% in CD-1, and 59.1% in rabbits. With respect to worm maturation, those rabbits vaccinated with the Fh15-Nt peptide had the highest level of worm maturation inhibition (46%) and with less hepatic damage [71]. These levels of protection/disease development were comparable with those previously obtained with native or recombinant *F. hepatica* FABPs.

As previously mentioned, the use of laboratory animals as experimental models instead of a natural host requires conducting some experiments with a natural host such as sheep. Such experiments were done with sheep immunized using Fh12 and Fh15 as antigens and infected with liver flukes. These experiments were carried using thirty lambs submitted to different patterns of immunization and times of infections. Upon completion of the trial there was no difference in fluke burden between groups vaccinated with either of the antigens and non-immunised controls, but worm size and faecal egg counts were significantly diminished in the sheep vaccinated with either of the antigens, suggesting anti-fecundity [72].

Fh12 and Fh15 were also used as antigen to vaccinate against *Schistosoma bovis* [73-74]. Different strains of mice (BALB/c, NMRI, C57/BL) were each immunized with the above-mentioned antigens and challenged peri-cutaneously with *Schistosoma bovis* cercariae. C57/BL mice immunized with Fh12 or Fh15 had significant reductions in worm burden recoveries (87%/96% and 72% reduction, respectively, over controls). When using NMRI mice or BALB/c no significant protection was observed with either of the antigens. In C57/BL mice, antibodies to the IgG1 and IgG2a isotypes increased with Fh12 and only IgG2 with Fh15. Regarding cytokine production by spleen mononuclear cells, C57/BL mice vaccinated with Fh12 had an increase of IFN-gamma production with Concanavalin A, but no increase of IL-4 in similarly stimulated cells. These results suggested that protection obtained in mice was mediated by a Th1 immune response.

3.4. FgFABP

Vaccine trials for evaluation of the efficacy of both recombinant and native FABP purified from adult *F. gigantica* flukes for use against tropical fasciolosis were conducted in Brahman cross cattle by Estuningsih and co-workers (1997) [22]. Antibody titres were found to be dependent on the use of adjuvant. Low but significant reductions in fluke burden (31%) and fluke wet weight (36%) were observed only in cattle vaccinated with the native FABP in Freund's adjuvant. There was no correlation between total antibody titre to FABP and protection of the animals tested. The protection observed in cattle vaccinated with native FABP of *F. gigantica* supports the notion that this class of proteins is a useful target for protection of animals against *Fasciola* and extends the efficacy of FABPs to the tropical liver fluke. This report was also the first in which purified FABP was used in vaccination against *F. gigantica* in cattle.

3.5. EgFABP

Isolation of an *E. granulosus* FABP gene, *EgFABP1*, was first accomplished after differential screening of a larval stage (protoscolex) cDNA library [23]. Three putative epitopic or solvent exposed surface regions of the protein identified by molecular modelling, were located at positions 9-14 (KMEKSE), 68-73 (GEKFKE) and 95-101 (EQDDKTK). In accordance with *in silico* predicted solvent accessibility, hydrophilic pattern and mobility properties, the authors suggested the 95-101 (EQDDKTK) peptide as having the highest probability of serving as an epitope [75].

The first experimental work on the immunological properties of EgFABP1 (formerly named EgDf1) was reported by Chabalogoity and co-workers using a *Salmonella tryphimurium* based vaccine delivery strain [76]. This vaccine delivery system involves expression of heterologous antigens in *Salmonellae* as Ct fusions to tetanus toxin fragment C (TetC) using expression vector pTECH2 [77], driven by the anaerobically inducible promoter *nirB* [76-78]. This system allows for the expression of guest antigens either as a full-length protein or as multiple tandem copies of a relevant peptide [77]. Similar multivalent vaccines have been developed and improved with bacterial, viral and parasite antigens using this basic system [76, 79]. The authors reported that a single oral dose of recombinant bacteria expressing EgFABP1 was able to trigger a strong antibody response against the parasite as detected by the reactivity of mouse sera with sections of protoscoleces, a larval form of the parasite. *In vitro* measurement of cytokine production indicated cellular responses against the bacteria and the expressed antigens. Mice orally immunized with one of the vaccine strains tested, *i.e.*,

(SL3261 strain carring the pTECH-EgDf1construct), elicited antibodies to the antigen between 4-6 weeks. Analysis of the isotype distribution of the anti-EgFABP1 antibody response showed that most of the mice developed specific IgG1 and IgG2a antibodies. In addition, specific IgA antibodies were detected in all the mice that previously had an IgG anti EgFABP1 response. Clear cytokine responses were evident in that significant levels of IFN-γ, IL-2, and IL-5 were produced by cells of all immunized groups. Of particular interest was the induction of cells to produce IL-5 upon stimulation with the corresponding antigens, indicating a Th2 component to the mainly Th1 response observed.

The next step was to improve the system for use in dogs, the target species. Humans are an accidental host of this parasite, usually becoming infected through contact with infected dogs in endemic areas. Vaccination of dogs, therefore, could be a cost-effective way to control hydatid disease, since it would cut off the transmission cycle by disruption of the production of infectious eggs. For these studies, live attenuated derivatives of dog-isolated *Salmonella tryphimurium* (P228067) were selected [79]. In the initial study, the work was focused on analysis of the immune response to this system, but not protection against *E. granulosus* infection. A null *aroC Salmonella* mutant (LVR01) transformed with the previous EgFABP1 construction (pTECH-EgDf1) [76], was then constructed and used to orally vaccinate (seven) six month old dogs. Two groups of immunized dogs received doses of $5x10^{10}$ bacteria and two boosters at different intervals after priming with a good tolerance and complete absence of symptoms. This experiment demonstrated that presentation of heterologous antigens to dog's immune system via the oral route is an effective strategy since both humoral and cellular responses were observed in response to the antigens tested (TetC and EgFABP1). However, the IgG response against EgFABP1 was evident in only two out of three and three out of four dogs (in tests involving boosters early on) and in only one of three dogs (the group who received boosters 6 and 7 weeks after priming). All immunized dogs showed a clear T cell response upon *in vitro* stimulation with EgFABP1, a very different response compared with that of mice. In another study, EgFABP1 together with other vaccine candidate with predicted capability of eliciting an immune response was selected to propose an alternative model to animal experimentation: a co-culture system of healthy human leukocytes and enterocyte-like Caco-2 cells [80]. The study evaluated the Th1/Th2 profile showing that the antigen mixture generated an immunogenic response associated with a mixed Th1/Th2 cytokine.

4. Concluding remarks

The FABP host protective mechanism of action has not been clarified, but current thinking is that it relates to an inability of the worms to obtain nutrients by specifically blocking the binding and transport of fatty acids. Native and recombinant proteins, peptides or DNA have been proposed as the basis of different vaccination protocols using FABPs as antigens, and experimental work has shown that modification of vaccination protocol can result in different levels of protection. However, despite modifications introduced in terms of doses, adjuvant employed, nature of antigen and its presentation, the levels of protection were not improved in animal models. Since Hillyer's work of 1988 or that of Moser in 1992 to the present, protection levels remain around 60%. Moreover, some of the experiments done illustrated the distance between animal models and natural host. New strategies must be considered, and probably important steps can be made with increased efforts for understanding the molecular basis of protective action of FABPs as well as their function in these parasites.

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References

1. Whitfield, P.J. 1993, In Modern Parasitology, F.E.G. Cox (Ed). Blackwell Scientific Publications, 24.
2. Chitsulo, L., Engels, D., Montreso, R., and Savioli, L. 2000, Acta Trop., 77, 41.
3. Varaldo, P.B., Leite, L.C., Dias, W.O., Miyaji, E. N., Torres, F.I., Gebara, V.C., Armôa, G.R., Campos, A.S., Matos, D.C., Winter, N., Gicquel, B., Vilar, M.M., McFadden, J., Almeida, M.S., Tendler, M., and McIntosh, D. 2004, Infect. Immun., 72, 33.
4. Hillyer, G. V. 2005, J. Helmintyol., 79, 241.
5. Budke, C.M., Deplazes, P., Torgerson, P.R. 2006. Emerg. Infect. Dis., 12, 296.
6. Schantz, P.P., Chai. J., Craig, P.S., Eckert, J., and Jenkins, D.J. 1995, In *Echinococcus* and Hydatid disease. Thompson, R.C.A., and Lymbery, A.J. (Eds.). Wallingford: CAB International, 233.
7. Eckert, K., Schantz, P.M., Gasser, R.B., Torgerson, P.R., Bessonov, and Movessian, S.O. 2001, Geographic distribution and prevalence. Eckert, J., Gemmell, M., Mslin, F.X. and Pawlowski, Z.S. (Eds),WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern.Paris: Office International des Epizzoties,100.
8. Heath, D.D. 1995, *Echinococcus* and Hydatid disease Thompson, R.C.A., and Lymbery, A.J, (Eds.). Wallingford: CAB International, 183.
9. Torgerson, P.R., Oguljahan, B., Muminov, A.E., Karaeva, R.R., Kuttubaev, O.T, Aminnjanov, M., and Shaikenov, B. 2006, Parasitol. Int., 55, S207.

10. Romig, T., Dinkel, A., and Mackenstedt, U. 2006, Parasitol. Int. 55, S187.
11. Haroun, E.T.M., and Hillyer, G.V. 1986, Vet. Parasitol. 20.
12. Fairweather, I., and Boray, J.C. 1999, Vet. J. 158, 81.
13. Ismail, M., Botros, S., Metwally, A., William, S., Farghally, A., Tao, L.F., and Day, T.A., Bennett, J.L. 1999, Am. J. Trop. Med. Hyg., 60, 932.
14. Capron, A., Riveau, G.J. Bartley, P.B., and Mc.Manus, D.P. 2002, Curr. Drug Targets Immune Endocrine Metabol. Disord., 2, 281.
15. Berquist, N.R. Prospects for schistosomiasis vaccine development. WHO TDRnews 71, 2004, http://www.who.int/tdr/publications /tdrnews/news71/schisto.htm
16. Gottstein, B. 2005, Ther. Umsch., 62, 685.
17. McManus, D.P., and Bryant, C. In *Echinococcus* and Hydatid disease. Thompson, R.C.A., and Lymbery, A.J. (Eds.). Wallingford: CAB International, 135.
18. Moser, D., Tendler, M., Griffiths, G., and Klinkert, M.Q. 1991, J. Biol. Chem., 266, 8447.
19. Becker, M., Kalinna, B.H., Waine, G. J., and McManus, D.P. 1994, Gene, 148, 321.
20. Vicente-Santiago, B., Muro, A., Vieira-Lista, C. and Siles-Lucas, M. Direct submission to GenBank Accesion Number:AY6 15730.
21. Rodríguez-Pérez, J., Rodrigues-Medina, J.R., García-Blanco, M.A., and Hillyer, G.V. 1992, Exp. Parasitol., 74, 400.
22. Estuningsih, S.E., Smooker, P.M., Wiedosari, E., Widjajanti, S., Vaiano, S., Partoutomo, S., and Spithill, T.W. 1997, Int. J. Parasitol., 27, 1419.
23. Esteves, A., Dallagiovanna, B., and Ehrlich, R. 1993, Mol. Biochem. Parasitol., 58, 215.
24. Esteves, A., Portillo, V., and Ehrlich, R. 2003., Biochem. Biophys. Acta., 1631, 26.
25. Alvite, G., Canclini, L., Corvo, I., and Esteves, A. 2008, Febs J., 275, 107.
26. Bae,Y.-A., Lee,E.-G. and Kong,Y. Direct submission, Genebank AN ABB76135
27. Esteves, A., Joseph, L., Paulino, M., and Ehrlich, R. 1997, Int. J. Parasitol., 27, 1013.
28. Jakobsson, E., Alvite, G., Bergfors, T., Esteves, A. and Kleywegt, G. 2003, I. Biochem. Biophys. Acta, 1649, 40.
29. Angelucci, F.,Johnson, K.A.,Baiocco, P.,Miele, A.E.,Brunori, M.,Valle, C.,Vigorosi, F., Troiani, A.R., Liberti, P., Cioli, D., Klinkert, M.Q., and Bellelli, A. 2004, Biochemistry 43, 13000.
30. Esteves, A., and Ehrlich, R. 2006, Comp. Biochem. Physiol. 142 C, 262.
31. Mc Dermott, L., Kennedy, M.W., McManus, D.P., Bradley, J.E., Cooper, A., and Storch J. 2002, Biochemistry, 41, 6706.
32. Gobert, G.N., 1998, Microsc. Res. Tech., 42, 176.
33. Erasmus, D.A. 1975, Exp. Parasitol. 38, 240.
34. Tendler, M., Brito C.A., Vilar, M.M., Serra-Freire, N., Diogo, C.M., Almeida, M.S., Delbem, A.C., Da Silva, J.F., Savino, W., Garratt, R.C., Katz, N., and Simpson, A.S. 1996, Proc. Natl. Acad. Sci., 93, 269.
35. Bergquist, N.R., and Colley, D.G.1998, Parasitol Today, 14, 99.

36. Romero Ramos, C.R., Magno Vilar, M., Tabet Oller Nascimento, A.L., Lee Ho, P., Taumaturgo, N., Edelenyi, R., Malmeida M., de Oliveira Dias, W., Diogo, C. M., and Tendler, M. 2001, Mem Inst Oswaldo Cruz, 96, 135.
37. Varaldo, P.B., Miyaji, E.N., Vilar, M.M., Campos, A.S., Dias, W.O., Armôa, G.R., Tendler, M., Leite, L.C., and McIntosh, D. 2006, FEMS Immunol. Med. Microbiol., 48, 132.
38. Abreu, P.A., Miyasato, P. A., Vilar, M.M., Dias, W.O., Ho, P.L., Tendler, M., Nascimento, A.L. 2004, Infect. Immun., 72, 5931.
39. Turton, K., Chaddock J.A., and Archarya, R. 2002, Trends Biochem. Sci., 27, 552.
40. Chabalgoity, J.A, Khan, C.M.A., Nash, A.A., and Hormaeche, C.E. 1996, Mol. Microbiol., 19, 791.
41. Reynolds, S.R., Dahl., C.E., and Harn, D.A.1994, J. Immunol.,152, 193.
42. Tarrab,-Hazdai, R., Schechtman, D., and Arnon, R.,1998, INFECT. Immune, 66, 4526.
43. Ben-Yedida, T., Tarrab-Hazday, R., Schechtman, D., and Arnon, R. 1999, Infect. Immun., 67, 4360.
44. Arnon, R., Tarrab-Hazdai, R., Steward, M.A. 2000, Immunology, 101,555.
45. EL Ridi, R., Mahrous, A., Afifi, A., Montash, M., Velek, J., and Jezek, J., 2001, Scand J. Immunol. 54, 477.
46. Magno Vilar, M., Barrientos, F., Almeida, M., Taumaturgo, N., Simpson, A., Garratt, R., and Tendler, M. 2003, Vacccine, 22, 137.
47. Almeida, M.S., Torloni, H., Lee-Ho., Vilar, M.M., Taumaturgo, N., Simpson, A.J., and Tendler, M. 2003., Parasite Immunol., 25, 135.
48. Kallina, B.H. 1997, Immunol. Cell. Biol., 75, 370.
49. Mohamed, M.M., Shalaby, K.A., LoVerde, P.T., and Karim, A.M. 1998, Mol. Biochem. Parasitol., 96, 15.
50. Grifantini, R., Finco, O., Bartolini, E., Draghi, M., Del Giudice, G., Kocken, C., Thomas, A., Abrignani, S., and Grandi, G. 1998, Eur. J. Immunol., 28, 1225.
51. Xu, D., McSorley, S.J., Chatfield, S.N., Dougan, G., Liew, F.Y. 1995, Immunology, 85,1.
52. Morris, S., Kelley, C., Howard, A., Li, Z., and Collins, F. 2000, Vaccine. 18, 2155.
53. Weissert, R., Lobell, A., de Graaf, K.L., Eltayeb, S.Y., Andersson, R., Olsson, T., and Wigzell, H. 2000, Proc. Natl. Acad. Sci. USA., 97, 1689.
54. Nascimento, E., Leão, I.C., Pereira, V.R., Gomes, Y.M., Chikhlikar, P., August, T., Marques, E., and Lucena-Silva, N. 2002, Mem. Inst. Oswaldo Cruz, 97, 105.
55. Fonseca, C.T., Brito, C.F., Alves, J.B., and Oliveira, S.C. 2004, Vaccine, 22, 503.
56. Fonseca, C.T., Pacífico, L.G., Barsante, M.M., Rassi, T., Cassali, G.D., and Oliveira, S.C. 2006, Microbes Infect., 8, 2509.
57. Becker, M.M., Kalinna, B.H., Waine, G. J., and McManus, D.P. 1994, Gene. 148, 321.
58. Waine, G.J., Scott, J.C., Mazzer, D., and McManus, D.P. 1998, Int. J. Parasitol., 28, 303.

59. Scott, J.C., Kennedy, M.W., and McManus, D.P. 2000, Biochim. Biophys. Acta, 1517, 53.
60. Liu, J.M., Cai, X.Z., Lin, J.J., Fu, Z.Q., Yang, G.Z., Shi, F.H., Cai, Y.M., Shen, W., Taylor, M.G., and Wu, X.F. 2004, Parasite Immunol., 26, 351.
61. Hillyer, G.V., del Llano de Diaz, A., and Garcia-Blanco, M. 1975, Bol. Asoc. Med. P.R., 67, 381.
62. Hillyer, G.V. 1976, Fed. Proc. 35, 2568.
63. Hillyer, G.V. 1985, Am. J. Trop. Med. Hyg. 34, 1127.
64. Hillyer, G.V., Haroun, E.T., Hernandez, A., and de Galanes, M. S. 1987, Am. J. Trop. Med. Hyg., 37, 363.
65. Hillyer, G.V., and Sagramoso de Ateca, L. 1979, Infect Immun., 26, 802.
66. Hillyer, G.V., García, Rosa, M.I., Alicea, H., and Hernandez, A. 1988, Am. J. Trop. Med. Hyg., 38, 103.
67. Muro, A., Ramajo, V., López, J., Simón, F., and Hillyer, G.V. 1997, Vet. Parasitol., 69, 219.
68. Espino, A.M., Rodríguez Medina, J.R., and Hillyer, G.V. 2001, J. Parasitol., 87, 1028.
69. Casanueva, R., Hillyer, G.V., Ramajo, V., Oleaga, A., Espinoza, E.Y., and Muro, A. 2001, J. Parasitol., 87, 697.
70. Martínez-Fernández. A.R., Nogal-Ruiz, J.J., López-Abán, J., Manga-González, Y., Hillyer, G.V., and Muro, A. 2004, Vet. Parasitol., 126, 287.
71. Muro, A., Casanueva, P., López Abán, J., Ramajo, V., Martínez-Fernández, A., and Hillyer, G. V. 2007, J. Parasitol., 93, 817.
72. Ramajo, V., Oleaga, A., Casanueva, P., Hillyer, G.V., and Muro, A. 2001, Vet Parasitol. 97, 35.
73. López Abán, J., Ramajo, V., Arellano, J.L., Oleaga, A., Hillyer, G.V., Muro, A. 1999, Vet. Parasitol. 83, 107.
74. López-Abán, J., Oleaga, A., Ramajo, V., Casanueva, P., Perez Arellano, J.L., Hillyer, G.V. and Muro, A. 2000, Veterinary Parasitol. 91, 33.
75. Paulino, M., Esteves, A., Vega, M., Tabarez, G., Ehrlich, R., and Tapia, O. 1998, J. Comput-Aided Mol. Des., 12, 351.
76. Chabalgoity, J.A., Harrison, J.A., Esteves, A., Demarco de Hormaeche, R., Ehrlich, R., Anjam Khan, C.M., and Hormaeche., C.E. 1997, Infect. Immun., 65, 2402.
77. Khan, C.M.A., Villarreal-Ramos, B., Pierce, R.J., Riveau, G., Demarco de Hormaeche, R., McNeill, H., Ali, T., Fairweather N., Chatfield, S.N., Capron, A, Dougan, G., and Hormaeche, C.E. 1994, Proc. Natl. Acad. Sci. USA 91, 11261.
78. Chabalgoity, J.A, Villareal-Ramos, B., Khan, C.M.A., Chatfield, S.N., Demarco de Hormaeche, R., and. Hormaeche, C.E. 1995, Infect. Immun., 63, 2564.
79. Chabalgoity, J.A., Moreno, M., Carol, H., Dougan, G., Hormaeche, C.E. 2001, Vaccine, 19, 460.
80. Fraize, M., Sarciron, M.E., Saboulard, D., Azzouz, S., Debard, A.L., Bosquet, G., Petavy, A.F. 2004, Parasitol. Res., 92, 506.