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REVIEWS

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A Comparative Analysis of Various Antigenic Proteins Found in *Haemonchus contortus*—a Review[1]

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Abstract—Many innovative researches on the development and introduction of recombinant vaccines against many economically important parasites were carried out in the 20th century. Research continues to hold promise with the development of immunological and molecular approaches for control of these parasites and in this regard it has already been seen that blood-sucking parasites such as *Haemonchus contortus* and *Ostertagia ostertagi* are susceptible to control by vaccines containing "novel" or "concealed" antigens. *Haemonchus contortus* is primarily pathogenic to sheep and its blood-feeding behaviour causes effects ranging from mild anaemia to mortality in young animals. Current means of control which are dependent on repeated treatment with anthelmintics are responsible for the increasing drug resistance of this parasite. Together with the growing concern of residual chemicals in the environment and food chain, this has led to attempts to better understand the biology of the parasite with an aim to develop alternate means of control, including the development of molecular vaccines. More problematic and also important is the formulation and delivery strategy to induce expulsion of this parasite, using vaccines containing recombinant "conventional" antigens. Tremendous progress has been made in the last decade in identifying several antigens from *Haemonchus contortus* which in their native form stimulate useful levels of protective immunity. Vaccines have been developed against *H. contortus* using 'novel' gut antigens from the parasite, but variable responsiveness of the host sheep has resulted in varying degrees of protection which are stimulated by these vaccines. Computer models have also been used to simulate vaccine efficacy in worm control and have yielded good results. This review will try to summarise the protective efficacy and also the molecular properties of principal candidate antigens which are expressed by this parasite. The review will try to cover the aspirations, current success, limitations and problems faced by researchers in the control of this economically important parasite.

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INTRODUCTION

Parasitism by worms is one of the most challenging problems to livestock industries with an estimated loss of $1000 million per annum attributed to parasitism [1]. The rapid and widespread emergence of anthelmintic resistant strains of the highly pathogenic blood-feeding ovine parasitic nematode *Haemonchus contortus* and other economically important parasites has resulted in the need to develop alternative control strategies such as vaccination. Research on parasitic nematodes has suffered from a lack of genetic and genomic resources, a situation that is beginning to change with the availability of genomic and transcriptomic sequences from species such as *Haemonchus* [2–4]. Recombinant protein-based vaccines have recently been developed against the cattle tick *Boophilus microplus* [5] and the sheep cestode *Taenia ovis* [6] highlighting the importance as well as effectiveness of this approach in field conditions. In case of *H. contortus*, attention has been focused on the fractionation of protein extracts in an attempt to identify antigens that induce protective immunity and this has led to the identification of a number of promising candidates including the so-called 'hidden' antigens expressed on the microvillar surface of the gut and also the surface antigens present on the cuticle [7, 8]. Many parasitic nematodes are developing resistance to chemical treatment and the work in this aspect is on to produce commercially viable molecular vaccines. Much progress has been made with highly protective hidden antigensas well as natural antigens which has yielded promising results. Significant progress towards successful vaccination against animal parasites of veterinary importance has been made during the last two decades. Previous vaccination trials against *Ostertagia ostertagi* in cattle have demonstrated the protective capacity of a protein fraction termed ES-thiol, which is enriched for activation-associated secreted proteins

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Parasite vaccines that are presently commercially available [17]

Disease (Parasite)	Trade name
Avian coccidiosis (*Eimeria* spp.)	Paracox, Coccivax, Livacox, Immucox
Toxoplasmosis of sheep (*Toxoplasma gondii*)	Toxovax
Giardiosis of dogs (*Lamblia* spp.)	GiardiaVax
Anaplasmosis of cattle (*Anaplasma marginale*)	Anaplaz
Lungworn (*Dictyocaulidae*)	Huskvac, Dictol
Taxas fever tick (*Boophilus microplus*)	TickGard, Gavac

(ASPs) and cysteine proteases [9]. Also protease inhibitors are thought to protect intestinal parasitic nematodes from their hostile proteolytic environment. Screening of *O. ostertagi* cDNA libraries with local antibody probes of the abomasal lymph nodes and mucus has revealed an (28 kDa) aspartyl protease inhibitor (API), which is exclusively recognised by antibodies from immune calves. Preliminary analysis of the significance of genetic diversity in cysteine proteinase genes has been performed simultaneously in sheep and goats, with regard to the immunological control using these enzymes against haemonchosis. The study has suggested the possibility for exploring the mechanisms involved in natural protection against non-adapted strains, in order to develop strategies for controlling haemonchosis [10]. Veterinary parasitology has witnessed a tremendous boost of recombinant antigens for their use as vaccines against the parasites of veterinary importance.

The abomasal nematode, *H. contortus* is predominantly a sheep parasite, with the pathological effects being largely due to the worms' blood feeding behaviour. It is responsible for causing major economic loss and ill health in sheep production. Traditionally, such losses have been contained through use of anthelmintic drugs combined with pasture management and this has resulted in some serious drawbacks like anthelmintic resistance, environmental pollution, problems in the food chain etc. Therefore alternative methods for control are required. Sheep can become immune to infection with *H. contortus* by repeated experimental or natural infection, suggesting that natural immunogens may be important in effective vaccination procedures. Protease inhibitors have been thought to protect intestinal parasitic nematodes from their hostile proteolytic environment. This review will try to give a general perspective about the vaccination process that has been developed against *H. contortus* and also will try to give an idea about the potential immunogenic proteins that have been found in *H. contortus* [11, 12].

Rapid developments in molecular biology have had an enormous impact on the development of vaccines to control the major nematode and trematode infestations of livestock. Vaccine candidates continue to be purified using conventional and less effective protein chemistry techniques but the limitations imposed by the limited number of parasite material proves to be an inaccessible barrier for commercial vaccine production. The ability to purify mRNA from different life-cycle stages of the parasite and also the development of cDNA expression libraries from it has been responsible for the identification of immunogenic parasite proteins. This has helped a great deal so that the potentially protective parasite antigens can now be produced in recombinant form in a variety of vectors and this has been a key breakthrough in the production of commercial vaccines.

VACCINES

Several inter-related disciplines are needed for the efficient designing of a vaccine. First and foremost, there is a need for basic parasitological techniques to be employed and this will enable the maintenance of parasite cultures and provide material for antigen purification which will always be important in the development of an effective vaccine. Over the last decade, the anti-parasitic market has been one of the most fast growing sectors of the overall $18 billion animal health market. While drugs for the treatment of parasites of livestock continue to dominate this sector because of consumer demands for chemical-free food, there is a growing interest in the development of safe and effective vaccines [13]. There has also been an important need for effective vaccine development in the prestigious $3 billion-plus companion animal market. A combined proteomic and transcriptomic analysis has also been performed to understand the mechanisms underlying the immunomodulation induced by recombinant galectins of *H. contortus* (rHco-gal-m/f) on goat peripheral blood mononuclear cells (PBMC). The results have shown that rHco-gal-m/f could be distinguished by antisera from goats experimentally infected with *H. contortus* and bound to the surface of goat PBMC. It has thus provided insight into the interactive relationship between parasitic nematode galectins and host PBMC. It also has shed new light on the molecular mechanisms of helminthic immune evasion [14–16]. There are presently very few anti-parasite vaccines sold commercially (table) and this situation is likely to change in the future as there is a great desire to move away from the use of conventional antihelmintics. Several proteins have now been characterised from the microvillar surface of *H. contortus*. Since this parasite is a blood feeder, these proteins are exposed to host immunoglobulin in the blood meal. Most of these proteins have been found to be protective when used to immunise sheep against a challenge infection with the parasite. What is important is to draw an outline of the

salient characteristics of these proteins together with a description of their protective antigen capability.

To date, the best proof for production of antihelmintic vaccines is derived from animal-based studies involving the use of attenuated infective larval stages. Typically attenuation is achieved through ionising radiation such as X-rays, gamma-rays or even ultraviolet light. Although usage of live non-irradiated helminth larvae can also induce protection, the use of larvae attenuated by ionising radiation is generally considered to be beneficial in achieving a level of protection which will be more robust and will last longer. The fact that helminth larvae maintain their viability and the associated immune responses against larval secretory products suggest that immunodominant larval excretory/secretory (E/S) antigens could be used as vaccine targets. Many of these ES antigens are enzymes such as metalloproteases, acetylcholine esterases as well as proteases [18–20]. Other E/S products from nematodes that show promise as vaccines include the activation associated secreted proteins (ASP) [21].

Protease activity has also been reported in extracts and secretions of numerous nematode parasites of livestock and other companion animals and genes encoding proteases of different mechanistic classes have been isolated and cloned. Proteolytic enzymes have been given more attention by molecular parasitologists and immuno-parasitologists than any other protein family since they are very important for parasitic existence and help in mediating fundamental physiologic processes such as tissue invasion, feeding, embryogenesis and evasion of host immune responses [22]. In case of *H. contortus* it has been found that the sheep can be protected by immunising them with the protease-rich gut membrane extract, H-gal-GP [23]. H-gal-GP is a semi-purified, detergent-soluble protein fraction from the intestine of adult *H. contortus* that has been found to be rich in proteases of different mechanistic classes [24]. Immunisation of sheep with purified, native H-gal-GP has been found to confer high levels of protection (both antiparasite and antifecundity) against *H. contortus* and besides this at least three different protease activities have been detected in this extract. Immunisation of sheep with a cysteine protease-enriched fraction of *H. contortus* membranes (purified using thiol Sepharose) has also been found to result in 47% protection against adult worms and 77% reduction in faecal egg output [25].

Another family of aspartic proteases, the nematode pepsin-related enzymes has been identified from *H. contortus* [26] and these molecules are currently being tested as vaccine antigens. Immunisation of sheep with an aminopeptidase M, termed H11 has also been found to be highly effective against *H. contortus* inducing levels of protection of up to 90% reduction in adult worms when the native protein has been used [27]. Recent advances have been made in the field of vaccine designing in which various other agents have been employed in designing an effective vaccine against *H. contortus*. Glutathione peroxidases from *H. contortus* have been found to be potential candidates for vaccine to control haemonchosis. The results have suggested that recombinant *H. contortus* HC29 glutathione peroxidase DNA vaccine induces a partial immuneresponse and has protective potentials against caprine haemonchosis [28]. Also glyceraldehyde-3-phosphate dehydrogenase from *H. contortus* (HcGAPDH) has been found to be a potential candidate for vaccine to control haemonchosis. Recombinant HcGAPDH DNA vaccine has been found to induce a partial immuneresponse against *H. contortus* infection in goats [29]. DNA vaccines expressing *H. contortus* H11 antigen with or without interleukin (IL)-2 have also been tested for protection against *H. contortus* infection in goats and have conferred partial protectionagainst *H. contortus* infection in goats [30]. Studies have also suggested that recombinant *H. contortus* disorganized muscle family member (Dim-1) DNA vaccine induced partial immune response and has a protective potential against goat haemonchosis [31]. Studies have also suggested that recombinant *H. contortus* actin DNA vaccine can induce partial immune response and has protective potential against goat haemonchosis [32].

NATURAL ANTIGENS FOUND IN *Haemonchus contortus*

Limited natural immunity to gastrointestinal nematodes has been found to develop in older animals and with continued exposure to infective larvae, the immunity is maintained. Repeated infection with irradiated *H. contortus* larvae has been found to result in high levels of protection in older sheep but this is not a commercially viable vaccine and young lambs which are at most risk are not protected. Similarly in case of native antigens isolated from the gut of adult *H. contortus*, it has been found to induce a significant level of protection after vaccination in sheep. Worm membrane proteins such as H11, H-Gal-GP and TSBP have consistently been found to induce a reduction by 77 to 90% in faecal egg counts and a reduction by 47 to 78% in worm burdens [33]. Because commercial viability of these antigens cannot be guaranteed due to problems of recombinant protein expression, there is still a need to search for new protective antigens that will be cost effective and in this regard *Haemonchus* expressed sequence tag (EST) dataset has been found to be an excellent tool in the discovery of new genes of immediate relevance to the parasite and its lifecycle.

Antibody-secreting cell (ASC) probes isolated from abomasal lymph nodes of sheep immune to *H. contortus* have been found to specifically react with L3 antigens of approximately 44–48 kDa and a broad band of 70–83 kDa. Affinity columns prepared from ASC probes has been found to specifically purify the 70–83 kDa band from L3 homogenates and this antigen which is designated as Hc-sL3 is regulated develop-

mentally and is present on the surface of ex sheathed L3 [34, 35] from where it can be extracted and purified by size-exclusion chromatography. Adult 15 and 24 kDa E/S antigens have also been identified in *H. contortus* by comparison of serum antibody responses to a primary infection with those generated during the secondary infection of partially immune sheep [36]. This has been very important in the evaluation of adult somatic extracts enriched for the 15 and 24 kDa E/S antigens and has yielded a reduction in faecal egg content by 99.9% and in adult worm burdens by 97.6% in four out of five sheep.

HIDDEN ANTIGENS FOUND IN *Haemonchus contortus*

Considerable efforts have been madein developing strategies based on the use of hidden antigens, especially gut molecules as vaccines. Studies have shown that vaccination of cattle with a native *Ostertagia* polyprote in allergen (nOPA) in combination with Quil A adjuvant has resulted in protection against *Ostertagia* challenge infections [37]. In another study, sheep were administered intramuscularly 500 μg of thiol-purified excretory/secretory antigen along with montanide as an adjuvant on day 0, 30 and 60. On ELISA, it was observed that the mean absorbance values were significantly ($p \leq 0.01$) higher up to 20 weeks post immunization in group I (purified antigen) compared to group II (unimmunized control) [38]. A number of protective gut antigens have been described for *H. contortus*, although no one has yet reached the stage of a commercial vaccine. The first of such is a microvillar surface-associated polymer termed contortin. Vaccination with this polymer has yielded a mean reduction in worm burdens by 78% [39]. H11, a short hand designation for H110D, is another integral membrane glycoprotein which is expressed only in the parasitic stages and only on intestinal microvilli. It has been seen to have microsomal (membrane) aminopeptidase M and microsomal aminopeptidase A activities attributable to distinct isoforms [40, 41].

Another group of gut surface antigens found in *H. contortus* with potential for vaccines is called H-gal-GP (*Haemonchus* galactose-containing glycoprotein complex). The complex can be obtained from membrane preparations of whole *H. contortus* by nonionic detergents such as Triton X-100. H-gal-GP has been found to selectively bind to lectins with a specificity for N-acetylgalactosamine. The complex has been found to show aspartyl protease, neutral endopeptidase and cysteine protease activities. The H-gal-GP complex has been found to be highly effective in protecting sheep against *H. contortus*. The question as to whether *H. contortus* gut membrane glycoproteins like H11 and H-gal-GP are "hidden," i.e. not recognised by the host during infection continues to be an important subject of debate [42].

Another group of antigens called p52 and p46 antigens of molecular weights 52 kDa and 46 kDa, respectively, have been obtained from extracts of adult *H. contortus* by affinity chromatography using a carbohydrate-specific monoclonal antibody. Besides them pl00 has also been shown to induce protective immunity to challenge infections in goats [43]. Two distinct cysteine protease fractions from *H. contortus* have also been evaluated as vaccines. The first of these containing a fibrinogen-degrading complex, consisting of 35 and 55 kDa proteins of which the former has been cloned and sequenced and has been found to show homology to cathepsin B [44]. The second cysteine protease fraction that has shown promise as a vaccine candidate is obtained from detergent extracts as thiol-binding proteins [45] and comprises of three 70 kDa cathepsin B-like proteases with 70–90% sequence homology.

Monoclonal antibodies have also been used to identify and purify a group of proteins, M_r 46, 52 and 100 kDa, which collectively have been shown to induce reduction by 60 and 50% in worm and faecal egg outputs respectively in immunised goats [43]. It has also been found that immunization of sheep with a purified antigen (Hc-sL3) expressed on the surface of L3 larvae of *Haemonchus contortus* using different adjuvant and immunization routes induced protective effect [46].

VARIOUS OTHER POTENTIAL VACCINE CANDIDATES

Besides the antigens mentioned above that have shown promise as vaccine candidates, various other antigens have also been discovered which are being worked upon for their efficacy and various other parameters. The recombinant HcENO has been found to be recognized by the sera from experimentally infected goats. This indicates that HcENO has the ability to induce the host immune response [47]. A genomic copy of a gut-expressed *H. contortus* vaccine antigen, pepsinogen has been isolated using polymerase chain reaction (PCR) and this isolated sequence has been found to be 4 kb in length and to contain eight introns ranging in size from 54 to 1475 base pairs [48]. Another study involving phylogenetic analysis and protein modelling has shown that cytochrome C (CYT) proteins of *H. contortus* are structurally distinct from those of mammals and other organisms, suggesting their potential as targets for parasite intervention [49]. Studies have also shown the presence of two different classes of small nematode specific lipid-binding proteins, the nematode polyprotein allergens/antigens (NPAs) and the fatty acid- and retinol-binding (FAR) proteins in *H. contortus* and they have shown promise in devising a control strategy against this very important parasite [50]. Recently many other potential vaccine candidates have been studied and have yielded promising results [51–54]. A study has shown that par-

asitological and antibody responses of grazing weaner Merino sheep can be assessed following vaccination with gut membrane proteins prepared from adult worms of the gastrointestinal nematode, *H. contortus* and it has been concluded that if similar protective effects could be obtained with recombinant versions of the proteinspresent in either H11 or H-gal-GP, then the prospects for a commercial Haemonchus vaccine are real [55]. In yet another type of study, eight month old sheep kept on pasture were treated with anthelmintic 8 weeks before vaccination with a larval surface antigen of the nematode parasite, *H. contortus*, under a commercially acceptable protocol, i.e. 2 immunizations using a commercial adjuvant; they were then given a controlled challenge infection 4 weeks later in indoor pens. The results have confirmed the protective properties of the larval surface antigen [56].

A cDNA encoding a small heat shock protein HSP20 has been isolated from *H. contortus* which has been found to encode a predicted protein of 156 amino acids which has been found to show high sequence identity with other nematode small heat shock proteins [57]. In yet another type of study, twenty-four reproducible differentially-expressed bands have been identified in 60 pairs of dd-PCR comparisons. The first of these cloned and sequenced proteins corresponded to the *H. contortus* 60S ribosomal protein L35A. The remaining bands need to be cloned and validated and may prove useful for effective parasite control [58].

CONCLUSION

Important advances have been made in characterizing protective native antigens, particularly hidden antigens, for *H. contortus*. However progress with the other economically important nematodes of livestock lags considerably behind *H. contortus*. In case of *H. contortus* only H11 is approaching a commercial vaccine product for reasons ranging from difficulties in reproducing the effect with recombinant molecules to consolidation in the animal health industry. Nevertheless, vaccination continues to be viewed as an attractive alternative to anthelmintics, even where widespread multidrug resistance is not yet established. Although hidden versus natural antigen approaches are most often seen in competition, they could be used together with possible synergistic effects. Studies have also suggested that not only host blood, but also haemoglobin in particular is an important force in the evolution of invertebrate parasites and it is important to mention here that four phylogenetically unrelated parasite genera, namely *Plasmodium* spp., *Schistosoma* spp., *Haemonchus* spp. and *Ancylostoma/Necator* spp. have independently evolved to select haemoglobin as an important nutrient source of amino acids [59]. The observation suggests that the study of the sequence and structural organization of the active site of parasite-derived haemoglobinases will provide a basis for elucidating host-parasite specificities at the molecular level. The chemical biology of the haemoglobinase active site is most likely to prove a useful tool for developing new targets for drug and vaccine development.

Obviously the biggest single barrier to the commercialisation of a vaccine against haemonchosis is the production of recombinant proteins which will approach the efficacy of the best native antigens that have been used. More field trials are needed to optimise how and when such a vaccine would be administered, bearing in mind the age and reproductive status of the sheep as well as the likely level of parasite exposure, before the potential benefits and limitations of a vaccine can be defined.

Molecular biology has provided the means to identify antigens, to define their function, patterns of expression and most importantly a means to produce them in quantity. Advances in genomics and proteomics and determination of the patterns of antigen expression will help in the long run in the identification of many more candidate protective antigens. Although vaccines have been successfully produced against many other important parasites, the final product for *H. contortus* will need to be active against more than one species of parasite. Trials have been very encouraging and work is being done in this aspect but so far there is no field data to assist researchers to find the levels of vaccine efficacy that will be required for cost-effective reduction of parasitism in livestock and the role of vaccines in integrated worm control strategies. Therefore much more still needs to be done.

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