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Review

Studies on various types of antigenic proteins of *Fasciola* spp. as a basis for the immunodiagnosis and vaccine development: A review

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***Fasciola* spp. is responsible for huge economic losses and animal welfare issues within the agricultural sector worldwide. Protein profiling studies of the two economically important species of *Fasciola*, that is, *Fasciola hepatica* and *Fasciola gigantica* are important for identification of these species, their immunodiagnosis and also for vaccine designing. Protein profiling is expected to discover a number of target proteins for the purpose of drug designing and vaccine development by determining the function of thousands of unidentified proteins still likely to be found in the genome of *F. hepatica* and *F. gigantica*. Electrophoretic protein profiling is expected to multiply the number of known drug targets 100-fold. Different immunodiagnostic tests such as enzyme linked immunosorbant assay (ELISA), immunofluorescence and agglutination tests have been used in the early immune diagnosis of fasciolosis, but they have some disadvantages, such as cross reactions with other trematodes, leading to false positive results. In recent years, SDS-PAGE and Western blot procedures have created a new era in immunodiagnosis, and greatly reduced cross reactions. Over the last two decades, various studies to identify and characterize proteins of immunological significance have been carried out, especially the candidates for immunodiagnosis or vaccination in fasciolosis. Recent research indicates that a future prospect for the control of fasciolosis by immunological intervention appears brighter than previously thought. This paper reviews the principles of proteomics, as well as its key instruments and research applications in helminthology, including host parasite interactions, vaccine development and diagnosis of liver fluke diseases and encourage more young researchers to initiate work on the molecular aspects of these economically cosmopolitan parasites.**

Key words: *Fasciola hepatica, Fasciola gigantica,* vaccines, ELISA, SDS-PAGE, Western blot.

INTRODUCTION

Liver fluke disease or fasciolosis is an economically important disease of sheep and cattle worldwide. *Fasciola* spp. parasitize a wide spectrum of domestic and wild animals (e.g., sheep, cattle, buffaloes and deer) and cause a huge economic loss of $3 billion annually to the agriculture sector worldwide through losses of milk and meat yields (Robinson et al., 2009). In addition, fasciolosis is now recognized as an emerging human

disease. The World Health Organization (WHO, 2006) has estimated that 2.4 million people are infected with *Fasciola hepatica* and a further 180 million are at risk of infection. Adult parasites of these flukes are found in the bile ducts and the immature flukes in the liver parenchyma, of infected final hosts. The immature flukes migrate in the liver hepatic tissue after penetrating the liver capsule. This migration is usually associated with trauma, hemorrhages and necrosis, finally leading to liver cirrhosis (Ozer et al., 2003). The diagnosis of fasciolosis is very important for its control, parasitological diagnosis in this respect is however often unreliable because the parasite eggs are not found during the prepatent period (Nour Eldin et al., 2004). Even when the worms have matured, the diagnosis may still be difficult because the eggs are only intermittently released. Early diagnosis of liver fluke infection is necessary for its prompt treatment before irreparable damage of the liver occurs (Rokni et al., 2004). For these reasons, immunological and molecular techniques are the most dependable diagnostic methods. Attempts have been made to diagnose sheep fasciolosis by detecting antibodies in the serum patient suspected of being infected with the flukes (Maleewong et al., 1999). Advances in immunodiagnosis have focused on detection of parasite antigens in host body fluids and faeces; these tests have an advantage over antibody detection because antigenemia implies recent and active infection (Cornelissen et al., 1999). Similarly, somatic and excretory-secretory (E/S) antigens of liver flukes or their partially purified component are the commonest source of antigens used in protection trials and serodiagnosis (Gnen et al., 2004). Several methods have been developed for the immunological diagnosis of human fasciolosis. Enzyme linked immunosorbant assay is both a sensitive and reliable means of diagnosing the acute and chronic stages of human fasciolosis (Hillyer et al., 1992). Previously developed ELISA methods have been employed for crude somatic antigen or liver fluke excretory-secretory (E/S) products to detect anti-fluke antibodies in serum (Knobloch et al., 1985). These antigen preparations are complex and may result in reduced specificity of the assay since many parasites share similar antigens. Previous work in the laboratory led to the isolation and characterization of cathepsin LI, a major molecule of ES products. This molecule has been shown to be highly immunogenic in infected animals (Dowd et al., 1994). The diagnosis of parasite infection in domestic ruminants has recently been directed towards the detection of parasite antigens in host body fluids (Guobadia and Fagbemi, 1995). Antigen detection assays have several advantages over other methods and they can identify animals with prepatent or occult infections, which were undetected by the usual parasitological tests. Moreover, they can give a more accurate indication of current infection rather than past infection (Zheng et al., 1990). In recent years, SDS-PAGE and Western blot procedures have created a new era in immunodiagnosis, and greatly reduced cross reactions. Analysis of cross-reactivity among digenetic trematodes is of paramount importance for understanding the evolutionary conservation of antigens and for the development of sensitive and specific serodiagnostic assays. However, the extent to which these antigens cross-react with other related parasites remains to be investigated.

APPLICATION OF PROTEOMICS FOR DIAGNOSIS OF FASCIOLOSIS

Common methods for the diagnosis of fasciolosis diseases are based on old-fashioned technologies like microscopy. The microscopic detection of parasites is performed on different types of specimen, e.g. blood smears, tissue and feces. Parasitological diagnosis of fasciolosis is possible only after 13 to 14 weeks post-infection by demonstrating fluke eggs in faeces. By this time, major damages to the host hepatic system may have already occurred. Moreover, sample preparation is both time-consuming and laborious work, and efficiency of diagnosis depends on the abilities of laboratory technicians. After the recent emergence of proteomics-based approaches, biomarker discovery has continued to develop and is now being applied in the diagnosis of parasitic diseases. Protein biomarkers reveal the existence and biological state of a particular organism. Diagnosis of fasciolosis is usually confirmed during the chronic phase of the disease when worms are established in the bile ducts and started to produce eggs. Fortunately, a number of serological tests such as ELISA (Burden and Hammet, 1978) and Western blot (Duffus and Franks, 1981) are useful in detection of infection, particularly in the prepatent period. However, these tests need selection of powerful antigens that recognize the infection during the early stage of its development. The serodiagnostic methods for the detection of antibodies are quite sensitive in detecting the infection in the early stages and have been exploited for the diagnosis of fasciolosis (Sanitago et al., 1986). Serological methods also have major limitations of detecting antibodies of previous exposure to the infection in treated animals. For this reason, careful analysis of the parasite antigenic

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structure is required for accurate early diagnosis.

Candidate antigens of *Fasciola* spp. for immunodiagnosis

Studies have already been carried out on the antigenic composition of *F. hepatica*. Proteins within the molecular weight ranges of 27 and 30 kDa, are common to E/S and somatic preparations in both parasite preparations of *Fasciola* spp. (Ajanusi et al., 1993). Hence, these fractions of proteins could be essential for the purpose of diagnosis of Fasciola infection in animals. Sobhon et al. (1996) analyzed the proteins from the homogenized whole body of *F. gigantica:* it was found that there were approximately 21 detectable bands, ranging in molecular weight from 17 to 110 kDa. Eleven of the bands at 97, 86, 66, 64, 58, 54, 47, 38, 35, 19 and 17 kDa, were present in the tegument antigen which were extracted from the parasites bodies by Triton X-100. By immunoblotting analysis, Sobhon et al. (1996) found that 14 of the 21 bands of the whole body fraction were antigenic, while all 11 bands of the tegument-associated proteins were antigenic: the major antigens were 4 bands at 66, 58, 54 and 47 kDa. In comparison, there has been considerable study on *F. hepatica*. Itagaki et al. (1995) found that the major antigens of adult *Fasciola* spp. were at 64-52, 38-28, 17, 15, 13, and 12 kDa; it was also reported that the antigens at 66, 58 and 54 kDa were more species-specific, they might be possible candidates for serodiagnosis of fasciolosis in cattle.

The immunodiagnosis of animal fasciolosis by the detection of circulating antigens has not yet been thoroughly investigated. Fegbemi et al. (1995) successfully used rabbit antibodies against the 88 kDa antigen of adult *F. gigantica* for the detection of circulating antigens in experimentally-infected cattle sera. Viyanant et al. (1997) studied a monoclonal antibody specific to a 66 kDa antigen of *F. gigantica* for the detection of circulating antigens in experimentally- and naturally-infected cattle: they reported that circulating antigens could be detected as early as the second and third weeks after infection; these antigens were associated with the crude surface tegument of the parasite. A 28 kDa protein present in E/S products of adult parasites was also identified by Ruiz Navarrete et al. (1993). This protein corresponds to cysteine-L-proteinase which was identified and purified by Dalton et al. (1996) and separated from the E/S products of *F. hepatica*. Furthermore, important enzymatic components, such as glutatione-S-transferase (Hillyer et al., 1992), haemoglobinase (Coles and Rubano, 1988) and cysteine-L-proteinases of *F. hepatica* (Simth et al., 1993; Dowd et al., 1994) have been identified within the 27.5 to 29 kDa range in E/S products of the parasite. Cysteine proteinases of the E/S products of Fasciola have become the focus of research since they play important biological and immuno-modulatory functions in the juvenile and adult parasites (Chapman and Mitchell, 1982; Smith et al., 1993). Moreover, these proteinases have also been shown to induce a high protective immunity in experimentally infected cattle (Dalton et al., 1996). A protein of 12.5 kDa is also separated; it corresponds to SAP2 protein which is proved to have protective potentiality against *F. hepatica* infection (Espino and Hillyer, 2003; Hillyer, 2005). Excretory-secretory (E/S) antigens have more contact with the host immune system than somatic antigens, since the parasite excretes the content of the intestine like cathepsins and other enzymes with cytolytic activities. These enzymes degrade tissues and facilitate the invasion and migration of the parasite, and induce a stronger humoral immune response, useful for diagnostic purposes and also for protection against future infections (Dalton et al., 1989; Parkhouse et al., 1987).

Application of proteomics for vaccine development strategies against fasciolosis

The rationale of vaccine designing in *Fasciola* spp. consists of three main strategies: First to identify those antigens of the parasite that are significantly different and do not show cross reactivity with those of hosts. Secondly, the selected antigens should be able to elicit strong immunological responses in hosts, such that the migrating juveniles could be immobilized or killed when they pass into the host's tissue. And thirdly, bulk syntheses of the antigens of choice should be feasible through the application of recombinant DNA cloning techniques. Immunoproteomics is important for discovering new antigens in vaccine development. This technique combines proteomics with the immunoreaction in order to identify potential vaccine candidates. Generally, in such techniques, protein lysate of a parasite is separated by 2D-gel electrophoresis, then blotted onto a membrane. The 2D-blot is probed with infected or immunized host serum. This technique enables the discovery of novel proteins involving stimulation of the host immune system.

Identifying vaccine candidates for parasite control has historically involved vaccinating host animals with crude extracts or whole organisms before fractionating these to identify the protective components. This is a costly and lengthy method often taking decades to complete (Knox, 2010). Crude somatic extracts of parasites can be prepared in a number of ways. Proteins solubilised at each step can be loosely described as water-soluble, membrane-associated and membrane-bound, respectively (Smith et al., 2000). Proteins within the membrane-bound fraction (Triton X-100 extract) have been a rich source of protective antigens against the

blood feeding nematode, *Haemonchus contortus* (Smith et al., 2000). The highly protective H-gal-GP complex was identified within this fraction. Furthermore, proteases have, historically, made good vaccine candidates against helminth parasites. For example, the main vaccine candidates, to date, against infection with *F. hepatica* are the proteases, cathepsin L1 and L2 (Dalton et al., 1996; Mulcahy and Dalton, 2001) and leucine aminopeptidase (LAP) (Acosta et al., 2008). LAP has elicited the highest protection, to date, of a single antigen against *F. hepatica,* where immunizing sheep reduces worm burden by up to 89%, or when used in combination with the cathepsin L1 and L2, which is up to 76% (Acosta et al., 2008).

Targeting secreted antigens released by parasites has been a popular starting point for vaccine studies (Smith, 1999). This method has identified antigens which have elicited protective immunity against a number of parasites including *F. hepatica*. These antigens are termed "natural" or "conventional" antigens as they are recognized by the host during the course of a natural infection (Smith, 1999). Native intestinal antigens have been purified, characterized and shown repeatedly to reduce both egg counts and worm burdens (Knox and Smith, 2001; Knox et al., 2003; Smith et al., 1999). Targeting "hidden" antigens could prove to be a novel and successful strategy for vaccinating against *F. hepatica* as the adult liver fluke lives in a highly vascularised environment and feeds on surrounding tissue.

Candidate antigens of *Fasciola* spp. for vaccine development strategies

The vaccine development strategies against *F. hepatica* and the related parasite *F. gigantica* have shown enormous progress over the last three decades. Most vaccines may, however, not be as ideal as desired, because *Fasciola* spp. are large and complex with potential ability to tolerate any disturbance and capability to repair themselves. Even partial vaccines that could impair the penetration and migration of newly excysted juveniles or those that could reduce the fecundity of adult parasites would have beneficial effect in infected animals. Recent research indicates that future prospects for the control of fasciolosis by immunological intervention appear brighter than previously thought. Candidate antigens from F*. hepatica and F. gigantica* have shown vaccine potential during trials in cattle, sheep and rats. Interestingly, most of these vaccine candidates were first isolated as native proteins, usually from adult worm ES products, because this antigen preparation was not very complex and was easy to obtain. Several of these early antigens, including cathepsin L proteases, GST and fatty acid binding protein (FABP) induced significant protection in cattle and sheep (Toet et al., 2014), reducing not only worm burden (and egg output), but also liver pathology. Recent reports of vaccine trials in sheep with several recombinant but functional forms of these antigens such as cathepsin L1 (CL1), GST and peroxiredoxin (FhPrx) from *F. hepatica*, or Sm14 peptide from *S. mansoni*, have not only reported significant reduction of worm burdens but have shown reduced liver pathology (Zafra et al., 2009, 2013a, b; Mendes et al., 2010; Perez-Ecija et al., 2010; LaCourse et al., 2012; Toet et al., 2014). Furthermore, recent genomics/transcriptomic data reveal these proteins belong to large families with complex developmental expression patterns that need to be explored further to ensure that the appropriate members are those that are brought into vaccine trials. The observation that adult worm-derived antigens can induce protection at the level of the liver (and possibly earlier) would suggest that these antigens are either also expressed by the juvenile/immature parasite stages or that the immune response cross-reacts between the various members of the same family.

Cathepsins L and B proteases of *Fasciola*: Importance and role as potential vaccine candidates

F. hepatica relies on proteolytic activity for many of its pivotal functional activities in the host, including tissue penetration, migration, feeding and immune evasion and, hence, it is not surprising that these have been the most encouraging candidates for vaccine development for some time (Dalton et al., 2013). Adult parasites secrete an abundance of cathepsin L cysteine proteases, representing about 80% of the total protein from adult ES, that they use for digesting the protein contents of the blood, including haemoglobin, albumin and immunoglobulin, consistent with ES protein analysis (Wilson et al., 2011). This provides free amino acids required for synthesizing egg proteins (Robinson et al., 2008a). Cathepsin LI, one of the major molecules of fluke excretory-secretory product, is secreted at each stage in the development of the parasite, and has shown to be highly immunogenic in infected animals. This molecule has the ability to cleave host immunoglobulin and can inhibit *in vitro* attachment of eosinophils to newly excysted juveniles (Carmona et al., 1993). Cathepsin LI is also capable of degrading extracellular matrix and basal membrane components and thus aids in parasite migration through the tissue of the host (Berasain et al., 1997).

Native cathepsin L proteases are readily isolated from adult ES products by standard gel permeation and ion exchange chromatography into two 'homogeneous' fractions (termed FhCL1 and FhCL2) (Smith et al., 1993). The highly significant protection observed using these native preparations when delivered in Freund's complete

adjuvant was further enhanced (72.4%) when combinations were made with proteins contained in a high molecular weight haem-containing (Hb) fraction. The Hb fraction itself induced low but significant protection (43.8%) in cattle against a challenge infection and recent proteomic analysis has revealed that this mixture includes peroxiredoxin (FhPrx), the helminth defense molecule (FhHDM) and fatty acid binding protein (FABP), all of which can induce modulation of host immune responses including alterations in macrophage function (Robinson et al., 2011b, 2012; Thivierge et al., 2013; Dalton et al., 2013; Figueroa-Santiago and Espino, 2014). Our understanding of *F. hepatica* proteases, and other secreted proteins, has been greatly improved by the availability of proteome data (Jefferies et al., 2001; Morphew et al., 2007; Robinson et al., 2008b; Robinson and Dalton, 2009) and by integrating this with an analysis of the transcriptome (Robinson and Dalton, 2009). The FhCL1 and FhCL2 are not expressed and secreted by the newly emerged juveniles (NEJ) as they penetrate the liver and intestine; these early-stage parasites produce different members of the cathepsin L family, FhCL3 and FhCL4. FhCL3 is particularly abundant and secreted by NEJ while FhCL4 may play a more housekeeping role (Cancela et al., 2008; Robinson et al., 2008b, 2009). The expression of FhCL3 is down-regulated soon after the parasite enters the liver parenchyma while FhCL1, FhCL2 and another family member, FhCL5, become more predominant as the parasite migrates and prepares to enter the bile ducts. These changes in protease expression reflect the parasite's adaptation to its changing environment as it migrates through different tissues and encounters new macromolecules. Molecular and biochemical analysis of FhCL3 (Corvo et al., 2009, 2013; Robinson et al., 2011a) have shown that this enzyme possesses a constellation of residues in its active site that confer it with a unique ability to digest collagen, suggesting that this protease is critical to parasite penetration of the intestine and liver capsule (Dalton et al., 2006).

Interestingly, a similar highly regulated pattern of expression occurs with the cathepsin B proteases. Cathepsin B-like proteolytic enzyme have been shown to cleave immunoglobulins of mice, rats and sheep *in vitro* (Chapman and Mitchell, 1982). Several cathepsin B proteases (FhCB1, FhCB2 and FhCB3) show parallel expression with the FhCL3, that is, are secreted by the early NEJ but are down-regulated as the parasite migrates in the liver tissue. This would suggest that the concerted action of FhCL3/FhCBs is essential for successful intestine and liver penetration.

It has been demonstrated (Dalton and Heffeman, 1989) that immature and mature flukes secrete endo-proteinases into culture medium when maintained *in vitro.* Several functions have been suggested for the role of these enzymes including functioning in migration through host tissue (Dalton and Heffeman, 1989), the acquisition of nutrient (Smith et al., 1993) and evasion of host immune responses (Dalton and Heffeman, 1989). Two cysteine proteases were isolated and characterized as having physiochemical properties similar to the mammalian lysosomal cathepsin L proteinases (Dowd et al., 1994). The two enzymes were observed to differ in their specificities for hydrolysing peptide bonds (Dowd et al., 1994) and as a result, were termed cathepsin LI and cathepsin L2. McGonigle and Dalton (1995) isolated another antigen containing a haem group from flukes maintained in culture medium, which was shown to be the liver fluke haemoglobin (Hb) (McGonigle and Dalton, 1995). It has been also observed that animals immunized with a thiol-cathepsin-related proteinase developed antibodies to the cysteine proteinase prior to infection with metacercaria of *F. hepatica.* On completion of the trial, there was no difference in worm burden between animals which had been immunized prior to infection and that of infected animals which did not receive the proteinase. However, fecal egg counts and therefore worm fecundity was significantly decreased in the immunized animals.

There are other trials that have been carried out by various scientists which resulted in the generation of a number of potential vaccine candidates that are listed in Table 1.

CONCLUSION

In this review, the critical parameters of immunodiagnosis and vaccine development strategies against *Fasciola* spp., and the need for both scientific and organizational breakthroughs if the world is to meet the enormous challenge of eliminating this harmful parasite, were emphasized. Following the development and advancement of next-generation sequencing technologies, large sequence datasets have been generated for several parasites. Genome data sets can inform functional genomics analysis, including transcriptomics, proteomics, secretomics and epigenomics. Utilizing all available 'omics' data for a particular parasite provides an unbiased approach to understanding parasite biology, rather than focusing on a particular gene or protein. The recent progress in immunodiagnostic methods has enabled the detection of fasciolosis at early stages of infection. Different immunodiagnostic tests have been used in the early immune diagnosis of fasciolosis, but they have some disadvantages, such as cross reactions with other trematodes, leading to false positive results. In recent years, SDS-PAGE and Western blot procedures have created a new era in immunodiagnosis, and greatly reduced cross reactions. Over the last few decades, much progress has been made in the isolation,

Table 1. Vaccination trials carried out by various workers.

Trial research	Species	Antigen	Reduction fluke burden (%)
Dalton et al., 1996	Cattle	FhCL1, FhCL2	53.7
		FhCL1+FHb	72.4
		FhCL2+FHb	
Mulcahy et al., 1998	Cattle	FhCL2+FHb	72
Piacenza et al., 1999	Sheep	FhCL1, FhCL2	33
		FhCL1+FhCL2	60
Kofta et al., 2000	Rats	cDNA encoding Fh.Cysteine protease	74
Wedrychowicz et al., 2003	Calves	cDNA intranasally	54.2
	Lambs		56.5
Golden et al., 2010	Cattle	rmFhCL1	48.2

characterization and testing of a number of native and recombinant molecules as vaccines against liver fluke disease in ruminant hosts. Indeed, it is important that as we move forward within the research field, a standardized vaccine protocol is established to allow comparison of results between trials. For the same reason, as new immunological reagents become available to improve the analysis of ruminant immune responses, it is critical that we develop standard operation procedures (SOPs) for both sheep and cattle, and other species. Many vaccine trial protocols involve sacrificing animals at the chronic stage of infection when the challenge parasites are in the bile ducts and easily recovered. However, at this time point, liver damage cannot be easily graded as it contains both acute- and chronic-associated damage. Perhaps, we should consider placing a greater importance on diagnostic methods of hepatic pathology, so that protection against liver disease at early stages of infection can be quantified: such as estimating liver enzymes in serum (aspartatetransaminase: AST, alkaline phosphatase: ALP, gamma-glutamyl transferase: GGT and glutamate dehydrogenase: GLDH) or finding novel serum biomarkers, that indicates several serological markers could be used for assessing liver damage. The pursuit of a *F. hepatica* vaccine needs to focus on understanding fluke biology, specifically the proteins involved in the tissue invasion and migration within the definitive host. It is our opinion that the most effective vaccine would be one that is directed against the early migratory stages of the parasite, including surface tegumental proteins/glycoproteins and secreted molecules, with the primary aim of preventing the penetration of the liver capsule by the parasites. This presents us with the challenge of learning more about the early migratory stages of *F. hepatica*, a stage that has traditionally been neglected, particularly in ruminants, due to the difficulty of obtaining workable levels of parasite material. However, genomic, transcriptomic and proteomic methods have made the molecular dissection of this parasite stage possible and will facilitate the rational design of single and multiple antigen vaccine cocktails. It is imperative that we bolster this molecular progress with new methodologies and by combining robust immunological analysis of innate and adaptive responses with pathological analysis of the early stages of infection, to understanding how and when the parasite initiates control of host immune responses.

Conflict of Interests

The authors have not declared any conflict of interests.

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