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Review article

Recent developments in research into the Cyathostominae and *Anoplocephala perfoliata*

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Abstract – Intestinal helminths are an important cause of equine disease. Of these parasites, the Cyathostominae are the commonest group that infect horses. These nematodes consist of a complex tribe of 51 species, although individual horses tend to harbour 10 or so common species, in addition to a few rarer species. The Cyathostominae can be extremely pathogenic, and high levels of infection result in clinical symptoms ranging from chronic weight loss to colic, diarrhoea and death. As part of their life cycle, immature cyathostomins penetrate the large intestinal wall, where they can enter a state of inhibited larval development. These larvae can exist in this state for months to years, after which they subsequently re-emerge. If larvae re-emerge in large numbers (i.e. several million), severe pathological consequences ensue. The inhibited larvae are also relatively refractory to several of the currently available anthelmintics, so that horses treated previously with anthelmintics can still carry life-threatening burdens of these parasitic stages. Little is known about the cyathostomin larvae during their mucosal phase, and current research efforts are focused on investigating the biology of these stages. Much of the research described here highlights this area of research and details studies aimed at investigating the host immune responses that the mucosal larvae invoke. As part of this research effort, molecular tools have been developed to facilitate the identification of larval and egg stages of cyathostomins. These molecular tools are now proving very useful in the investigation of the relative contributions that individual, common cyathostomin species make to the pathology and epidemiology of mixed helminth infections. At the more applied level, research is also in progress to develop an immunodiagnostic test that will allow numbers of mucosal larvae to be estimated. This test utilises antigen-specific IgG(T) serum antibody responses as markers of infection. As anthelmintic resistance will be the major constraint on the future control of the Cyathostominae, researchers are now actively investigating this area and studies aimed at elucidating the molecular mechanisms of drug resistance are described. Another parasite which has assumed a clinically important role in horses is the tapeworm, *Anoplocephala perfoliata*. This parasite is prevalent world-wide and has been shown to be a significant cause of equine colic. Because previous methods of estimating the infection intensity of tapeworm were inaccurate, recent research has been directed at developing an immunodiagnostic ELISA for these cestodes. Specific IgG(T) responses to antigens secreted by adult tapeworms have been shown to provide a reasonable indication of infection intensity. An ELISA based on these responses is now commercially available. The steps involved in the development of this ELISA are described here. In addition to these recent advances in research, this review also outlines the principle areas for future research into these important equine parasites.

equine / helminths / Cyathostominae / *Anoplocephala perfoliata* / research

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1. INTRODUCTION

World-wide, horses are exposed to complex intestinal helminth infections which can compromise their health and welfare. These parasites have a high prevalence and are an important cause of morbidity and mortality. Intestinal helminths are difficult to control, not least because there is limited information available on the basic biology of the commonest group of species, the Cyathostominae. Within this group, there are more than 50 different species and little is known about the contribution that individual species make to the pathogenesis and epidemiology of mixed infections. This article reviews recent developments in research into intestinal parasites of horses, with particular emphasis on studies into the biology of the Cyathostominae.

2. BACKGROUND TO THE CYATHOSTOMINAE

These nematodes infect virtually all grazing horses. Unlike the large strongyle (*Strongylinae*) group, anthelmintic resistance is common in the Cyathostominae, particularly with regard to benzimidazole compounds such as fenbendazole [54]. Most horses harbour burdens of tens of thousands of these parasites without developing clinical disease, but in some horses, cyathostomin infection leads to a severe inflammatory enteropathy affecting the large intestine

[18, 32]. The cyathostomins have a direct life cycle, during which the parasites undergo a period of inhibited development as early third stage larvae (EL3) in the large intestinal wall. The EL3 play a pivotal role in cyathostomin-associated disease, as large numbers of larvae can accumulate and subsequently reactivate simultaneously to cause a syndrome known as larval cyathostomiasis. The principle clinical effect of this syndrome is weight loss, but individuals may exhibit other signs including diarrhoea and/or subcutaneous oedema and/or pyrexia [32]. Clinical larval cyathostomiasis occurs more commonly in young horses in late winter/early spring, however horses have a lifelong susceptibility to infection and clinical disease can be seen in any age of horse. Inhibited larval development has been observed for periods of over two years and is thought to be favoured by factors such as negative feedback from mature luminal worms, a large larval challenge or a trickle infection [40]. Cyathostomins have also been associated with various types of equine colic [35, 36, 40, 59]. This has been attributed to the presence of large numbers of mucosal stage larvae. Currently, these stages are not detectable diagnostically and following treatment, covert, life-threatening burdens of mucosal larvae may persist [31]. Moreover, when these parasites enter inhibited larval development, they have limited susceptibility to most of the currently available anthelmintics [14, 25].

Despite their ubiquity and clinical importance, little is known about the Cyathostominae, which makes it difficult for rational treatment and control strategies to be devised [46]. As mucosal larvae are pivotal to the pathogenesis and persistence of these parasites in horses, much of the research described here investigates the biology of these stages.

3. CYATHOSTOMINAE RESEARCH

3.1. The development of species-specific DNA probes

As mentioned above, the Cyathostominae consists of over 50 individual species [29]. The detailed biology of the free-living and host stages is not yet known at the single species level. Furthermore, the role of individual species in the pathogenesis of mixed infections is unclear. Recent approaches to investigate the relative importance of individual species in terms of their basic biology, epidemiology and the role of different species in drug resistance, have employed molecular methods of species identification. Traditionally, identification relied on the examination of the morphology of the head and tail of adult parasites [26]. Prevalence studies based on parasite identification at post mortem examination, indicated that many (up to 27) species can be identified in one study [4, 17, 39, 41]. However, most reports indicate that 90% of the parasites present belong to one of several common species, for example *Cylicostephanus longibursatus*, *Cylicocyclus nassatus*, *Cyathostomum catinatum*, *Cylicostephanus minutus* and *Cylicostephanus goldi* [41]. Such studies require access to intestinal contents from experimentally infected horses or horses from an abattoir. To create tools that would enable identification of pre-parasitic stages, molecular approaches have been developed to identify egg and larval stages of the most common species. Initially, first and second internal transcribed spacer (ITS) sequences were characterized [16],

and specific oligonucleotide primers designed for some common species [22]. The method allowed the species-specific amplification of parasite DNA derived from faecal samples. Despite these promising results, the application of this test in the field has not been published. Intergenic spacer (IGS) region DNA sequences have also been used to derive species-specific molecular probes for common species [19, 24]. Sequence comparison of the IGS DNA showed low intra-specific variation and higher inter-specific sequence variation, allowing the design of probes for six common species. These probes have subsequently been developed for use in a high-throughput PCR-ELISA system [20]. This system employs biotinylated species-specific probes to immobilise digoxigenin (DIG)-labelled IGS PCR products of the homologous species onto streptavidin-coated ELISA wells. Detection is performed by incubation with an alkaline phosphatase-conjugated anti-DIG antibody followed by an alkaline phosphatase substrate. The ability of these probes to detect IGS PCR products from individual eggs, L3 and L4 has been demonstrated [19].

Clinicians have previously ascribed the variation in clinical signs of larval cyathostomiasis to the presence of particular species, the level of the mucosal larval burden or the characteristics of a host's inflammatory or immune response [34]. No experimental data had been published to prove any of these theories. There had been attempts to identify L4 in diarrhoea of clinical cases by morphology [5, 53]. In these studies, *C. longibursatus*, *C. catinatum*, *Cylicocyclus radiatus* and *Cylicocyclus insigne* L4 were observed. However, morphologically discriminating features of the L4 were found to be very limited. Recently, the PCR-ELISA system was used to identify the cyathostomin species present in populations of L4 from diarrhoeic faeces of horses with larval cyathostomiasis [20]. Analysis of the data indicated that there was no association between the species identified and the outcome of the disease or the

severity of the diarrhoea observed. These results suggest that clinical larval cyathostominosis is predominantly caused by mixed species infections. This study constitutes the first application of molecular species identification to investigate clinical disease associated with cyathostomin infection in horses. These probes are currently being used to identify the species of eggs present in faeces prior to and following treatment with benzimidazoles (Matthews, Freeman, Hodgkinson, unpublished data). This molecular approach will investigate the egg reappearance time of individual common species and the impact that these species have on the transmission of cyathostominosis.

3.2. A phylogenetic tree for cyathostomins

Previously, there was disagreement in the literature as to the phylogenetic relationships between the two subfamilies of the Strongylidae, the Strongylinae and the Cyathostominae. Previous researchers [12, 27, 28] suggested that the arbitrary separation of the Strongylinae and Cyathostominae on the basis of size and shape of the buccal capsule should be re-examined to determine whether the two families are natural groups. They considered that the genera with large subglobular buccal capsules (Strongylinae) were ancestral to those with small cylindrical buccal capsules (Cyathostominae). In contrast, other researchers [13] considered the strongylids to be monophyletic, but suggested that genera with large buccal capsules had evolved from those with small buccal capsules. To examine these interspecies relationships at the molecular level, phylogenetic trees were developed based on mitochondrial cytochrome oxidase c subunit I (COI), mitochondrial large ribosomal RNA sequences (18S rRNA) and ITS-2 sequences [37]. The COI gene was found to be less informative than the 18S rRNA and ITS-2 genes. Combined analysis of the 18S rRNA and ITS-2 genes supported a monophyletic clade of the cyathostomins with *Tridentoinfundibu-*

lum gobi, which had previously been classified as a nematode of "uncertain origin". The Strongylinae grouped consistently outside the clade containing cyathostomins and *T. gobi*. Furthermore, the molecular analysis failed to provide strong evidence for the separation of cyathostomins into the classical genera, which were previously defined based on morphological classification. Therefore, there could be an argument to support the reclassification of the Cyathostominae based on the molecular data.

3.3. Immunobiology of cyathostomin infection

It has previously been assumed that larval inhibition follows exposure of the parasites to adverse environmental conditions as third stage larvae (L3) prior to infection. This assumption is based on observations that parasites often emerge from the mucosa once the adverse period is over. No real experimental evidence has been obtained to vindicate this theory and the host immune response may play a role. Recently, local cytokine responses were assessed in the mucosal environment of horses with negative to high burdens of mucosal cyathostomin larvae. The responses in parasite-infected horses were compared with those in parasite-free horses and in those that presented with colitis histologically classified as infiltrative inflammatory bowel disease (IBD). The presence of interleukin-2 (IL-2), IL-4, IL-5, IL-10, tumour necrosis factor alpha (TNF α), interferon gamma (IFN γ) and transforming growth factor beta (TGF β) was examined by reverse transcriptase PCR (RT-PCR). IL-2 was detected at all sites, whilst IL-4 was detected at all but one site [8]. The presence of IL-10, IL-5, IFN γ and TGF β varied, with no significant differences amongst groups. Detection of TNF α was significantly higher in the group that had infiltrative IBD than those with larval cyathostominosis or those that were helminth negative. These results indicated a possible role for TNF α in the pathogenesis of equine infiltrative inflammatory bowel disease.

Subsequently, levels of IL-4, TNF α , IFN γ , TGF β and IL-10 were quantified by competitive RT-PCR (Davidson, Proudman, Matthews, unpublished data). IL-4 levels were significantly different, with highest levels in the cyathostomin-infected group, followed by the cyathostomin-negative group and lowest levels in the group classified as having IBD. Significant differences in IL-10 were detected between the parasite-infected and non-infected groups when anatomical sites were compared directly. TNF α levels were also significantly different amongst the groups, with highest levels in the IBD group. Taken together, these results imply that larvae in the intestinal wall are associated with T helper 2 type responses, as demonstrated by higher levels of IL-10 and IL-4 transcripts. These responses may down-regulate potential inflammatory responses and be overcome in heavy infections or when larvae reactivate. In horses with reactivating larvae TNF α was detected and this cytokine may be involved in the pathology associated with this syndrome.

In a separate study, mast cell and eosinophil levels were analysed in the large intestines of 42 horses collected from an abattoir [6]. Numbers of mucosal and sub-mucosal mast cells (MMC and SMMC), intraepithelial, mucosal and submucosal eosinophils (IE, ME and SME) were studied in relation to parasite burden. Numbers of larvae, adult worms and total worm burdens correlated with tissue eosinophil counts, while the percentage of EL3 was linked to mast cell densities. In horses under two years, most significant associations were found between eosinophil counts and the numbers of larvae and worms; in 2–10 year-olds, correlations were observed between all eosinophil types and total cyathostomin burdens. In contrast, in horses over 10 years, a MMC hyperplasia was observed and correlations were recorded between MMC and total numbers of adult worms or the percentage of EL3. These results indicate a role for these cell types in the response to mucosal infection. Future

experiments should investigate the cytokine response further by identifying which cell types express IL-4, IL-10 and TNF α in horses with varying parasite burdens.

3.4. Development of an immunodiagnostic assay for mucosal Cyathostominae

There is currently no specific laboratory method to diagnose pre-patent cyathostomin infection non-invasively and coprological methods only allow a crude assessment of the adult burden [60]. In fact, horses with high mucosal burdens often have low or negative faecal egg counts [44]. Animals with larval cyathostominosis often develop hypoalbuminaemia and neutrophilia, but there are no parasite-specific clinico-pathological features specific to the disease. An immunodiagnostic test for mucosal larvae would have an immediate impact upon the diagnosis of larval cyathostominosis and would allow veterinarians to identify horses that require immediate larvicidal anthelmintic treatments. Recent work has led to the identification of two antigen complexes that have diagnostic potential for estimating mucosal larval burdens. ELISA experiments using sera from experimentally infected ponies showed that increases in larval antigen-specific IgG(T) were observed by 5–7 weeks post-infection [9]. When these responses were analysed qualitatively using Western blotting, two antigen complexes of 20 and 25 kDa in mobility were observed to be bound by IgG(T) by seven weeks post-infection [9]. Both antigens were purified by electro-elution from polyacrylamide gels and ELISA and Western blotting experiments were performed using the purified antigens. Significant increases in anti-20 and -25 kDa IgG(T) levels were observed six weeks post-infection [11]. Furthermore, the antigens appeared specific to mucosal larval cyathostomins, indicating their utility as markers of pre-patent infection. When sera raised against heterologous nematode species were tested, there was no reactivity with sera raised

against *Parascaris equorum* or *Strongyloides westeri*, but low levels of binding were observed in sera raised against *S. edentatus* and *S. vulgaris* [11]. Subsequently, IgG(T) responses in experimentally and naturally infected horses were measured against both purified antigens by ELISA [10]. Sera from three cohorts of ponies were tested. One cohort consisted of three groups of ponies, which grazed either cyathostomin-contaminated pasture ("exposed ponies"), which grazed pasture not previously grazed by parasitized animals ("non-exposed ponies") or which were raised under parasite-free conditions. Anti-25 kDa complex IgG(T) responses between the groups were significantly different and corresponded to the level of exposure to cyathostomins before challenge. Furthermore, there was strong significant correlation of the anti-25 kDa IgG(T) responses with the total mucosal burden particularly to EL3 burdens. These results indicate the utility of the 25 kDa antigen complex as an excellent marker in dynamic epidemiological studies.

In a second cohort of animals, the IgG(T) responses were measured in sera obtained from infected horses ("endemically-infected") from a local abattoir [10]. The IgG(T) responses in this group were compared with those in sera from ponies which were raised helminth-free or in helminth negative animals from an abattoir. In "endemically-infected" horses, the IgG(T) responses were significantly greater than those in uninfected individuals (Wilcoxon rank-sum *P* values of 0.002 and 0.0001 for 20 and 25 kDa responses, respectively). Furthermore, there was significant correlation of the anti-25 kDa IgG(T) responses with the burdens of EL3, DL, eggs per gram of faeces, total mucosal burden, total luminal burden and total parasite burden. Only the correlation of anti-20 kDa IgG(T) levels with the total luminal burden were significant. Finally, in a third set of experiments, the IgG(T) responses to purified 20 and 25 kDa complexes were measured in horses (*n* = 28) presenting with clinical larval cyathostominosis [10]. The

antigen-specific IgG(T) levels were significantly higher in clinical cases than in helminth-naïve ponies and parasite-negative horses from an abattoir. The results indicate that an immunoassay based on the antigens present in these complexes could ultimately be used to differentially diagnose weight loss and diarrhoea cases, or used to aid in selection of the type of anthelmintic treatment. Further experiments are being performed to identify the half-life of such antibody responses. Because of the cross-reactivity observed with *S. edentatus* and *S. vulgaris* species, the components present in both complexes are being defined by of 2-dimensional gel electrophoresis and mass spectrometry, to identify those components that are cyathostomin-specific. These antigens are time-consuming to prepare and rely on a continuous source of infected colonic mucosa. Therefore, once peptide sequences have been derived for cyathostomin-specific antigens, the genes encoding them will be isolated and the antigens expressed in recombinant form.

3.5. Cyathostomin anthelmintic resistance

Cyathostomin anthelmintic resistance was first reported in the 1960s when suppressive treatment strategies originally designed to control large strongyles unfortunately led to selection of drug-resistant cyathostomins. Currently, these parasites demonstrate resistance to two of the three classes of anthelmintic, the benzimidazoles (BZ, fenbendazole, oxfendazole) and the tetrahydropyrimidines (pyrantel) [23]. Resistance to the widely used BZ class of drugs is the most prevalent and has been documented in numerous countries throughout the world [33]. The issue of cyathostomin anthelmintic resistance has been discussed extensively by Kaplan [23] and will not be addressed in detail here, however we will highlight the current situation with respect to the molecular analysis of cyathostomin anthelmintic resistance.

A recent focus of cyathostomin research has been to investigate mutations in the β -tubulin gene, as these have been shown to confer BZ resistance in nematodes of ruminants. The sequence of β -tubulin isotype I gene has been described for seven common cyathostomin species [43, 54]. One report suggested that the mutation at codon 200, which is associated with BZ resistance in ruminant nematodes, may not be responsible for BZ resistance in cyathostomins [55]. PCR was used to assess allele frequencies in single larvae (pre- and 66 days post-treatment), in susceptible and resistant BZ populations [56]. A general trend toward higher percentages of phe/tyr and tyr/tyr individuals was observed following treatment, however no statistically significant difference was found between these two genotypes and the phe/phe genotype percentages [56]. However, a positive correlation was observed between the faecal egg count reduction test results and the phe/phe percentages. However, continued research in this area suggests additional complexity in the molecular mechanisms that are responsible for BZ resistance, with the possible involvement of other mutations in the β -tubulin isotype 1 target gene or other β -tubulin isotypes. Further work is required to address the role of individual β -tubulin genes and/or isotypes in BZ resistance. We are using molecular methods to determine the sequence variation in the β -tubulin isotype I gene both within and between species (Clark, Matthews, Hodgkinson, unpublished data). Future work will use this data to perform single nucleotide polymorphism (SNP) analysis to explore the role of particular mutations in the β -tubulin isotype I gene in BZ resistant cyathostomin populations of L3 from field samples. In addition, we are investigating the presence of other β -tubulin isotypes by genomic analysis.

Despite the propensity with which cyathostomins develop resistance and over 20 years of intensive use of the macrocyclic lactones resistance has yet to be reported for this third group of anthelmintics. It is unknown why this resistance has not emerged in cyathostomins.

As these anthelmintics are now the most commonly used anthelmintics, it is widely accepted that resistance is inevitable, especially as the pharmacological properties of moxidectin, such as its persistent action and good efficacy against mucosal stages, will encourage further selection pressure [57]. There is little data on the molecular mechanisms of macrocyclic lactone action in cyathostomins. Strategies to decelerate selection for resistance, such as reduced usage and surveillance of egg reappearance period, must be implemented whenever possible, particularly, as many horse populations are dynamic, which would encourage the rapid spread of resistance. The future study of cyathostomin anthelmintic resistance requires the understanding of the molecular mechanisms of resistance in order for anthelmintic control of nematodes in horses to remain effective. In addition to the continued research into the molecular mechanisms of BZ resistance, an approach focussed on understanding the molecular basis of macrocyclic action and resistance is surely one of the foremost aims of future cyathostomin research.

4. AN IMMUNODIAGNOSTIC ASSAY FOR EQUINE TAPEWORM INFECTION

For many years, the equine tapeworm *Anoplocephala perfoliata* was thought to be relatively harmless [58]. However, a number of clinical case series and individual case reports [1–3, 7, 42] made a circumstantial association between the presence of large numbers of tapeworms and certain types of colic arising from problems at the ileo-caecal junction. Further investigation of this problem was hampered by the limitations of coprological diagnosis. Faecal flotation methods have been described for the detection of tapeworm eggs in the faeces of infected horses. Whilst these tests are inexpensive and demand no sophisticated equipment, they are time consuming, messy

and lack sensitivity. Validation studies of various coprological methods have reported sensitivities of 11–61% [38, 47].

Tapeworm infection has been demonstrated to stimulate increases in specific antibody responses in infected horses [21, 49]. Subsequently, IgG(T) responses to excretory/secretory antigens of 12/13 kDa weight have been used in the development of a diagnostic tapeworm antibody ELISA [50]. The test results from the ELISA have been shown to correlate well with the infection intensity [49], which is important clinically because the risk of tapeworm-associated colic is known to be proportional to the infection intensity. This finding is consistent with the observation that ileo-caecal pathology is proportional to the infection intensity [15, 45]. Serological diagnosis of tapeworm infection intensity has been used to investigate the epidemiology of tapeworm infection, to establish the relationship between tapeworm infection and disease and to investigate populations of horses with a history consistent with tapeworm associated colic.

Using the anti-12/13 kDa IgG(T) ELISA, it has been possible to explore the immunoepidemiology of tapeworm infection in the horse population. Blood samples were obtained from a random selection of horses in various age groups and the age-intensity profile described [51]. As with many other intestinal helminth infections, peak infection intensity occurs in younger animals (0.5–2 years old), then reaches a plateau in horses between 3 and 15 years old, rising again in older animals. The determinants of this age-intensity profile could be immunological or behavioural. The relationship between the risk of spasmodic colic and tapeworm ELISA optical density was explored in a case-control study [52]. Serum samples from horses with spasmodic colic were submitted for serological testing along with serum from an unaffected control horse on the same premises. Conditional logistic regression modelling revealed a dose-response relationship between tape-

worm infection intensity and the risk of spasmodic colic. This relationship has been further illustrated in the investigation of a “colic outbreak”. The animals with coprological and serological evidence of high tapeworm infection intensity were observed to be at increased risk of colic [48]. Treatment of the horses with an anti-cestode drug resulted in a marked decrease in anti-12/13 kDa IgG(T) levels concomitant with a marked decrease in the incidence of colic in the population. Recent work in the USA supports the role of tapeworms as a risk factor for specific types of equine colic [30]. (The tapeworm ELISA is available through “Diagnosteq”, University of Liverpool, Leahurst, Neston, Wirral, CH64 7TE, UK. Email: diagnosteq@liv.ac.uk.) The anti-12/13 kDa IgG(T) ELISA is currently available commercially in the UK (www.diagnosteq.co.uk), and samples from many countries around the world have been assayed. The assay is being used clinically to investigate horses that have suffered from colic in order to identify animals that would benefit from anti-cestode treatment. It has also proved useful as a research tool, helping to define the extent of equine tapeworm infection in different regions of the world. The equine veterinary surgeon now has a diagnostic tool that can be used to evaluate the tapeworm status of individual animals, allowing appropriate preventive measures to be instituted before infection intensity reaches a level that causes intestinal disease.

5. CONCLUSION

This review highlights that active research is ongoing in the areas of cyathostomin and *A. perfoliata* biology. Particular emphasis has been on development of serologically-based tests to detect these parasites. As indicated, a commercially available ELISA is now available, whilst development of an ELISA for cyathostomins awaits further characterisation of the larval antigens which have shown promise as markers for infection. Other research, on the immunobiology

of the Cyathostominae, is starting to shed light on the interactions that these important parasites have with the host immune response, whilst the molecular probes, developed to identify common species of cyathostomin, are providing important information on the epidemiology and pathogenesis of these infections. We are pleased to report that the molecular probes will now be used by researchers in other groups to help elucidate particular characteristics of these species in a broader geographical range. Probably, the most important future objective with regard to Cyathostominae research is to prevent the spread of anthelmintic resistance. Studies aimed at developing molecular methods for the early detection of anthelmintic resistant genotypes is now under way in several laboratories. It is imperative that this research be continued to be funded, given that, in the foreseeable future, there is no hope of new anthelmintic compounds being developed for control of these parasites.

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