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# The Identification of Haemonchus Species and Diagnosis of Haemonchosis

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CHAPTER FIVE

# The Identification of *Haemonchus* Species and Diagnosis of Haemonchosis

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## Contents



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## Abstract

Diagnosis is often equated with identification or detection when discussing parasitic diseases. Unfortunately, these are not necessarily mutually exclusive activities; diseases and infections are generally diagnosed and organisms are identified. Diagnosis is commonly predicated upon some clinical signs; in an effort to determine the causative agent, identification of genera and species is subsequently performed. Both identification and diagnosis play critical roles in managing an infection, and involve the interplay of direct and indirect methods of detection, particularly in light of the complex and expanding problem of drug-resistance in parasites. Accurate and authoritative identification that is cost- and time-effective, based on structural and molecular attributes of specimens, provides a foundation for defining parasite diversity and changing patterns of geographical distribution, host association and emergence of disease. Most techniques developed thus far have been grounded in assumptions based on strict host associations between *Haemonchus contortus* and small ruminants, that is, sheep and goats, and between *Haemonchus placei* and bovids. Current research and increasing empirical evidence of natural infections in the field demonstrates that this assumption misrepresents the host associations for these species of *Haemonchus*. Furthermore, the capacity of *H. contortus* to utilize a considerably broad spectrum of ungulate hosts is reflected in our understanding of the role of anthropogenic forcing, the 'breakdown' of ecological isolation, global introduction and host switching as determinants of distribution. Nuanced insights about distribution, host association and epidemiology have emerged over the past 30 years, coincidently with the development of increasingly robust means for parasite identification. In this review and for the sake of argument, we would like to delineate the diagnosis of haemonchosis from the identification of the specific pathogen. As a foundation for exploring host and parasite biology, we will examine the evolution of methods for distinguishing *H. contortus* from other common gastrointestinal nematodes of agriculturally significant and free-ranging wild ruminants using morphological, molecular and/or immunological methods for studies at the species and genus levels.

## 1. INTRODUCTION

The differentiation of *Haemonchus contortus* from *Haemonchus placei* has been deemed by many as inconsequential because of morphological, biochemical and biological similarities between the organisms, as well as similarities in the way they affect host physiology. Over time, two camps have emerged; those wishing to define *H. contortus* and *H. placei* as distinct species and those considering them as morphs, races or isolates of a single, widespread species. Since comparative morphological criteria were recognized, supporting their classification as distinct species (Jacquiet et al., 1995, 1997; Lichtenfels et al., 1986, 1988, 1994), studies of the

epidemiology and population genetics of these organisms have become dependent on increasingly rapid and cost-effective protocols for accurate identification. Although there is a dearth of methods currently available that allow accurate differentiation of *H. contortus* from *H. placei*, those that are available are not routinely applied. Consequently, there is ongoing confusion about the relative importance of characters, such as variation in vulval morphology, which are often explored in the absence of clear criteria for specific identification. Furthermore, there is persistence in the literature of host-based identifications for species of *Haemonchus* which are not verified relative to specimens, morphology or molecular data. It is important to be mindful that the ability to readily delineate these species should be the standard, where complete and accurate identification serves, for example, as the foundation for field-based epidemiological studies. In the absence of definitive identification, the value of such studies becomes equivocal.

The importance of our capacity to identify *Haemonchus* species to a degree parallels that for other recognized helminth pathogens. In the early 1970s and for a long time thereafter, when classification of the genus *Trichinella* was in flux, it was sufficient to present data on the epidemiology of *Trichinella* spp. without having genetically characterized the isolates. Consequently, today, most prior work on the circulation and biology of *Trichinella* is of diminished value because the context linked to differentiation among 12 currently recognized species and genotypes was not available. Whereas such an example may not have a demonstrable impact on the diagnosis of haemonchosis, it can conflate and hinder a refined understanding of the epidemiology and population genetics of these organisms.

Reviews since 2008 have holistically examined methods for diagnosing and identifying nematode parasites infecting livestock (Gasser et al., 2008; Preston et al., 2014; Roeber et al., 2013a,b). We have taken a more guided examination of these studies in the hope of teasing out efforts focussing on the genus *Haemonchus* and in particular *H. contortus*.

## 2. MORPHOLOGICAL APPROACHES FOR IDENTIFYING *HAEMONCHUS CONTORTUS*

### 2.1 Morphology; the gold standard

The genus *Haemonchus* Cobb, 1893, was established for the large stomach worms occurring globally in sheep, cattle and other free-ranging artiodactyl ungulates. Recognition of these nematode parasites has a deep

history, extending over the past 200 years, consistent with their economic and veterinary significance. Of the 12 currently recognized species, *H. contortus* (Rudolphi, 1803) was described based on abomasal parasites in sheep, although it has been considered to include morphologically variable nematodes with an otherwise exceptional range of ruminant hosts (Gibbons, 1979; Hoberg et al., 2004; chapter: Evolution and Biogeography of *Haemonchus contortus*: Linking Faunal Dynamics in Space and Time by Hoberg and Zarlenga (2016), in this volume). Species of *Haemonchus* are, for the most part, well differentiated morphologically, and delineation of adults is possible based on typical structural and meristic characteristics of male and female worms (Gibbons, 1979). Until the 1980s, however, it was not possible to provide reliable identification of *H. contortus* and *H. placei* (Place, 1893) in domesticated ruminants. Conventional wisdom in the veterinary literature often separated these species based erroneously on assumptions about host association, with the former regarded as parasites of sheep, and the latter seen as distributed only in cattle (Giudici et al., 1999; Jacquiet et al., 1997; Lichtenfels et al., 1994). Further creating potential confusion were proposals for extensive partitioning of subspecies within *H. contortus* based largely on the structure of the vulva and associated vulval fans and knobs evident in female nematodes from different hosts and geographical localities (discussed in Gibbons, 1979).

The need for efficient methods for rapid identification and separation of *H. contortus* and *H. placei* had been evident, extending into the 1950s when early observations were being assembled about the status of these proposed species (summarized in Lichtenfels et al., 1986). Paramount was the appreciation that an effective means of control and a clear understanding of epidemiological patterns, host associations and geographical distribution would emerge from an unequivocal definition of diversity for these nematodes. In mixed natural infections, morphological differentiation of *H. contortus*, *H. placei* and species hybrids is now based on multiple structural attributes, including the configuration of the spicules and bursa in males and the synlophe in males and females (system of cuticular ridges visible on the surface of most trichostrongyloid nematodes (eg, Durette-Desset, 1983)).

### *2.1.1 Identification of adult worms*

Initial development of reliable means for the morphological identification of species and primarily limited to parasites circulating in domesticated hosts emerged through studies of the synlophe. Criteria for identification included the pattern of cuticular ridges, their numbers, and the extent or distribution on the body of male and female nematodes as revealed in cross-section or

in examination of whole mounted specimens (Durette-Desset, 1983; Lichtenfels and Pilitt, 2000; Lichtenfels et al., 1986, 1994, 2002). For example, transverse sections at the level of the esophageal—intestinal junction reveal the presence of 30 ridges in *H. contortus* and 34 in *H. placei* (Lichtenfels et al., 2002). Specific patterns of distribution for ridges in the subventral and sublateral fields in the esophageal region of the body of adult nematodes are diagnostic, and provide a capacity for robust identification of individual males and females (Hoberg et al., 2002; Lichtenfels et al., 1994, 2001, 2002). Concurrently, morphometric protocols linked to discriminant analysis for spicules provided an alternative means for rapid identification; however, such protocols are limited to males of *H. contortus* and other species in domesticated ruminants (Jacquiet et al., 1997). Hybrids of *H. contortus* and *H. placei* occurring in sheep, cattle or other ungulate hosts in sympatry can also be unequivocally identified based on the intermediate range of attributes observed in adult nematodes (Lichtenfels et al., 1986, 1994).

The recurring necessity to provide authoritative and accurate identification for species of *Haemonchus* that circulate among domestic and free-ranging ungulates is emphasized by the strongly developed mosaic structure of ruminant parasite faunas (Hoberg, 2010; Hoberg et al., 2008). Translocation, introduction and successful establishment have been dominant processes since the 1500s, associated with widespread invasion and expansion of nematode faunas globally (Zarlenga et al., 2014). Dissemination and gene flow associated with recurrent introductions of parasites with small and large domestic ruminants likely extend to exchanges and trade near the time of domestication over 10,000 years ago, but may have been maximized during the time frame for extensive European colonization (Hoberg, 2010; Rosenthal, 2009). Such a history of invasion may account for founder events and considerable population structure now partitioned globally, and contrasts with current or contemporary intercontinental geographical barriers to dispersal that are evident (Giudici et al., 1999; Troell et al., 2006). Although population genetic structure is apparent on continental scales, this structure does not coincide with identifiable morphological variation (eg, vulval morphology) that had been the primary basis for subspecies designations (Gibbons, 1979). Consequently, integrated morphological and molecular evidence is consistent with *H. contortus* as a single, highly variable and widespread species with a considerable capacity to infect a broad range of ungulate hosts (Hoberg et al., 2004).

Accurate species identification and an understanding of circulation and epidemiology for nematode faunas at the intersection of managed and native or wild ecosystems remains a priority. Identification is particularly important

in habitats under accelerating environmental change linked to climate and other factors of anthropogenic forcing (Cerutti et al., 2010; Hoberg, 2010; Hoberg et al., 2008). Patterns of geographical invasion and host switching between domestic and free-ranging ruminants in this arena of perturbation are expected to influence persistence, dissemination and genetic exchange among drug-resistant populations in zones of contact or sympatry. Comparative morphological approaches provided an initial pathway for clear identification of *H. contortus* and continue to constitute relatively efficient means to explore species and faunal diversity that are at the foundations of managing and mitigating impacts associated with parasites and parasitism. Studies have shown that ante-mortem, morphological examination of third-stage larvae (L3) coincides well with PCR-derived data for differentiating *H. contortus* and *H. placei* (Santos et al., 2014a). Further, a combined parasitological and molecular barcoding assay used by Budischak et al. (2015) to examine cultured L3s from wild hosts (due to limitations imposed by postmortem analyses) accurately estimated both total and species-specific worm abundance, and exhibited similar rates of parasite species discovery as derived from postmortem analyses. Worm prevalence and community compositions were similar to those derived from lethal sampling, and all morphological analyses were corroborated by the molecular data. Consequently, morphology provides the foundation upon which other direct methods of parasite identification are predicated; namely molecular and biochemical-based technologies. In the following sections we review and explore some of the techniques that have emerged.

### 2.1.2 Identification of infective third-stage larvae

Identification of free-living, infective stages (L3) of gastrointestinal nematodes of ruminants remains an important aspect of epidemiological studies and in defining the dynamics of transmission. It is becoming increasingly critical to understand the persistence and expansion of parasitic populations in rapidly changing environments. Assessment of gastrointestinal parasite diversity has often relied on culturing larvae from eggs recovered from faeces of naturally infected hosts (MAFF, 1986). Further, determination of pasture contaminants and, thus, the potential for transmission across and within seasonally defined windows has been related to egg counts and the identification of L3s collected from rangelands populated with domestic stock.

Development of methods and criteria for identification of L3s for gastrointestinal nematodes in ruminants has an extensive history and, over time, has resulted in standardized protocols based on comparative morphological

approaches (eg, Dikmans and Andrews, 1933; MAFF, 1986; van Wyk and Mayhew, 2013). An understanding of the range of diagnostic characters that could provide differentiation among members of the Trichostrongylina and other strongylate nematodes in ruminants emerged initially from the studies of life cycles, life history and development of free-living and parasitic stages (Ransom, 1906; Veglia, 1915). Increasingly detailed descriptions of L3s focussed on overall length, number of intestinal cells, structure of the cuticular sheath, sheath length, tail length and cephalic morphology including attributes of the buccal capsule have allowed the separation of trichostrongylines such as *Haemonchus*, *Cooperia*, *Ostertagia*, *Trichostrongylus*, nematodirines including *Nematodirus* and *Nematodirella*, and other strongyles including *Chabertia* and *Oesophagostomum* (Dikmans and Andrews, 1933; Goodey, 1922; Veglia, 1926). These studies confirmed that among genera, it was usually possible to distinguish most nematodes circulating in ruminants based on relatively constant and consistent attributes (Veglia, 1926). Although often unequivocal identification could be achieved among four genera typically observed in sheep (*Cooperia*, *Haemonchus*, *Ostertagia* – now *Teladorsagia* – and *Trichostrongylus*), overlap in the tail and tail-sheath lengths obviated the use of additional attributes (Dikmans and Andrews, 1933). Further, reliable characters for the definitive identification to species have remained elusive (Dikmans and Andrews, 1933; Mönnig, 1931). Methods and criteria applied to parasite diversity among domestic ruminants also could not be generally translated to free-ranging wild hosts or to a broader understanding of parasite circulation in zones of contact or sympatry at the interface of managed and natural systems (Budischak et al., 2015).

Criteria currently applied to identification of L3s have not been modified substantially over the past 80 years and primarily relate to a series of definitive papers addressing either single species or species assemblages of nematodes in domestic ungulates (eg, Borgsteede and Hendricks, 1974; Dikmans and Andrews, 1933; Hansen and Shivnani, 1956; Keith, 1953). A standardized protocol for identification has been codified in the veterinary literature, as exemplified by diagnostic keys that reflect nematode faunal diversity among domestic ruminants on a regional and global stage (MAFF, 1986; van Wyk and Mayhew, 2013). Although comparative morphological approaches will remain a central approach in diagnostics, the capacity for accurate species-level identification can be directly linked to a range of available and developing molecular-based pathways (eg, Budishak et al., 2015).

### 2.1.3 Identification of parasitic fourth-stage larvae

Recognition that parasitic infection by an assemblage of gastrointestinal nematodes in ruminants often involved immature or larval stages suggested the importance of being able to identify genera and species that were involved. Fourth-stage larvae (L4s) could be present in the abomasum or small intestine during typical development, or could reflect the occurrence of inhibition (Michel, 1963, 1974), thus representing distinct epidemiological processes in transmission that have different consequences for infection and disease. Although considerable attention had been focussed on the identification of free-living larvae, only sporadic studies, often limited to development observed in single species, characterize available information for parasitic L3s and L4s (eg, Douvres, 1957a,b). Parasitic L3s and L4s of trichostrongylines (eg, *Cooperia*, *Haemonchus*, *Ostertagia* and *Trichostrongylus*) can be differentiated morphologically by primary attributes of the buccal capsule, tail and placement of the excretory pore, and identification is limited to separation of genera. Furthermore, prior to the separation of *H. contortus* and *H. placei* as distinct species, most infections in sheep or cattle were attributed to the former species, and definitive identification was not possible. Arrested development of *H. contortus* occurs in the early L4, and structurally these may not differ substantially, except in length and the degree of development of the genital primordium in males and females, relative to conspecific nematodes observed under typical ontogeny (Blitz and Gibbs, 1971; Veglia, 1915).

### 2.1.4 Identification of eggs

There exist other less conventional assays for the diagnosis of nematode infections and, in particular, those of the genus *Haemonchus*. In addition to the well-established FAffa MAlan CHArt (FAMACHA) (Bath et al., 1996), which today has been relegated to assessing the level of *H. contortus* infections in small ruminants, because of resultant anaemia, there have been other efforts to correlate morphometric dimensions and appearances of eggs in faeces to genus-level identification. Given improvements in computer technology and digital imaging in the last decade, morphometrics bears mentioning in the context of this chapter because state-of-the-art, technological advances have not yet been fully exploited in the direct examination of faecal eggs. Further, tests have been developed to differentiate eggs based on lectin binding, wherein *Haemonchus* specifically binds peanut agglutinin. Advancements in the last 5–10 years may provide the impetus for more common use. However, the central theme in these assays remains genus- rather than species-level identification.

Cunliffe and Crofton (1953) were among the first to systematically characterize and attempt to standardize egg measurements as a method to identify parasites, though they did not pioneer this approach (Shorb, 1939; Tetley, 1941). After examining eggs derived from dissected female worms, their data compared well with prior art, and they devised a series of equations to define each parasite group based on the norms of length and width measurements. However, given the ranges in size, they concluded that, based only on these measurements, mixed populations would be difficult to classify, even though the method was more rapid and less variable than larval culture. In 1982, Christie and Jackson used egg measurements, coupled to information on the stage of embryonic development, to identify with high accuracy, *Ostertagia* and *Trichostrongylus* species; however, they made similar conclusions regarding other species, and suggested that many of the sheep parasites, including *Haemonchus*, would require larval culture followed by the examination of L3 morphology, depending on the composition of the infection.

Georgi and McCulloch (1989) utilized an electronic digitizer and multivariate analysis to combine data from length, width, area and perimeter as well as areas and arc lengths of egg polar regions. Stepwise discriminant analysis allowed them to correctly identify *H. contortus* and *Trichostrongylus colubriformis* 85% of the time. Unlike prior data, eggs prepared from fresh faeces were no different from those immediately fixed in formalin, although eggs derived from the uterus were morphometrically distinct from those obtained from faecal material (Tetley, 1941). Kerboeuf et al. (1996) was among the first to adopt flow cytometry to analyse *Haemonchus* eggs. The investigators used native egg fluorescence and scattering pulses to generate histograms similar to cell histograms. Although they did not investigate the ability of the technique to distinguish between parasite genera, they did show a strong correlation between the level of native green fluorescence and resistance to anthelmintics (benzimidazoles). Consequently, this test may be adaptable to assessing the level of resistance within a flock or herd of animals.

About the same time that Kerboeuf et al. (1996) was testing flow cytometry, digital imaging was employed to examine morphometric parameters (Sommer, 1996) and egg texture (Sommer, 1998), as a means to differentiate common bovine nematodes. Species from five common genera (*Ostertagia*, *Cooperia*, *Haemonchus*, *Trichostrongylus* and *Oesophagostomum*) were examined. Using linear discrimination analysis, the test generated a correct classification 86% of the time when 19 of 25 measured features were evaluated. Relegating this analysis to the five most important features slightly reduced the

accuracy to 82%. In contrast to the test developed by Georgi and McCulloch (1989), this analysis did not require outlining the egg prior to taking measurements. Although the test was not evaluated for quantifying eggs in mixed populations, it, nonetheless, suggested its utility for this purpose, provided sufficient sampling was done. This same group examined egg texture including grey colour levels throughout the egg using digital imaging (Sommer, 1998). Of the 25 different texture parameters that were used, 10 had significant discriminatory power and collectively identified *Ostertagia ostertagi*, *Cooperia oncophora* and *Ostertagia radiatum* 91% of the time. When these data were combined with egg size and shape, correct identification increased to 93%. At the time that this technology was developed, methods were not available to accurately quantify mixed egg populations by PCR, to validate or refute the morphometric and digital imaging approach for examining mixed populations. However, revisiting the assay with state-of-the-art cameras and digital imaging software concomitant with real-time PCR might provide better insight into the efficacy of the technology for herd/flock-level analysis.

Nwachukwu et al. (1987) showed that egg shells from the nematode *Onchocerca gutturosa* were able to bind to peanut agglutinin (PNA) and suggested that nematode eggshells were capable of eliciting host-protective responses. It was not until 1996, however, that Palmer and McCombe (1996) demonstrated that PNA was able to specifically bind to *Haemonchus* eggs and that lectin binding corresponded well with data from larval cultures. Binding was monitored using fluorescently labelled PNA, and the data provided a good estimation of the number of *H. contortus* eggs in mixed populations.

Colditz et al. (2002) expanded on earlier work by extending the breadth of species examined and incorporating flow cytometry into the analysis, which enabled quantification of lectin staining. They showed that staining was not altered due to the developmental stage of the egg and that the prevalence of *Haemonchus* in mixed field infections compared well with that obtained from larval culture. In order to garner broader use, the method was further modified by Jurasek et al. (2010) for expediency, cost and the need for less training. In particular, the time required to purify eggs (multiple sieving and overnight flotation in saturated salt) was a major deterrent to the adaptation of this technique. These authors also showed that formalin fixation could be used to preserve the eggs, but that staining intensity was substantially diminished by 5 weeks following treatment.

Hillrichs et al. (2012) took the technology one step further and screened 19 different lectins spanning wide-ranging sugar specificities in the hope of

identifying a bank of lectins that specifically differentiated four life-cycle stages of *H. contortus* and *T. circumcincta*, including eggs, adult worms, and sheathed and exsheathed L3. As previously mentioned , PNA was indeed the preferred lectin for specifically interacting with *Haemonchus* eggs. Lectins that interacted with the other stages were less specific and depended on the age of the worm. Unfortunately, differential rather than specific interactions were routinely observed.

## 3. MOLECULAR METHODS FOR IDENTIFYING *HAEMONCHUS*

### 3.1 *Haemonchus contortus*, *Haemonchus placei* or both?

Over the years, numerous 'first-generation' molecular and biochemical methods have been developed for identifying *Haemonchus* species and for examining drug-resistant genotypes. Restriction enzyme digestion followed by agarose gel electrophoresis (Beh et al., 1989), Southern blotting (Roos et al., 1990; Zarlenga et al., 1994), repetitive DNA hybridization probes in conjunction with Southern blots or dot blots (Christensen et al., 1994a,b), and isoenzyme banding profiles (Bentounsi and Cabaret, 1999; Echevarria et al., 1992; Knox and Jones, 1992) were among the most popular examples of first-generation technologies. Sensitivity and specificity, however, were key issues that prompted the transition to PCR-based assays for developing more advanced tests. Also, DNA sequencing for differentiating closely related species has been available for many years; however this technology only gained popularity and momentum once PCR took hold and problems associated with PCR inhibitors in biological samples were addressed.

As noted earlier, the biggest misconception in the identification of *Haemonchus* species is the belief that *H. contortus* is a sheep parasite and *H. placei* is a cattle parasite. Hence, most techniques to identify *Haemonchus* species have targeted genus- rather than species-level differentiation with this limitation in mind. While years ago this assumption may have held true and may even today be appropriate in regions where cattle production is either nonexistent or very limited, today anthropogenic forcing has globalized the dissemination of these and many other parasite species (Hoberg, 2010; Zarlenga et al., 2014). As an example, *H. placei* was found in western Australian cattle; a geographical region believed not to be conducive to this species because of its predilection for more tropical and subtropical

climates (Jabbar et al., 2014). Consequently, blanket assumptions of exclusivity regarding host–pathogen associations can no longer be considered unilateral, as it relates to *H. contortus* and *H. placei*. do Amarante (2011) made special note of this issue, providing as support for differentiating the species the need to establish proper and sustainable control strategies, especially in light of drug-resistant parasites and the inability of *H. placei*–infected animals to cross-protect against challenge infection with *H. contortus* (see Santos et al., 2014b). It is also necessary to consider circulation among domestic stock and free-ranging ungulates, and the growing understanding that populations that involve multiple species of *Haemonchus* can be maintained in a broad array of wild cervids, camelids and bovids (including caprines) that occur in sympatry in particular regions of the world (eg, Cerutti et al., 2010; Hoberg et al., 2001, 2008).

Chaudhry et al. (2014) noted the presence of drug-resistant alleles in cattle-derived *H. placei* obtained from mid-western and eastern southern United States. Six of nine populations contained the characteristic P200Y (TAC) isotype-1 polymorphism indicative of β-tubulin benzimidazole resistance, albeit at low frequencies. This group also identified the presence of naturally derived hybrids in isolates of *Haemonchus* obtained from Pakistan and southern India, where numerous worms were heterozygous for fixed, species-specific single nucleotide polymorphisms (SNP) within the internal transcribed spacer 2 (ITS-2) of nuclear ribosomal DNA (rDNA) (Chaudhry et al., 2015). Among these worms, one hybrid contained the *H. contortus* isotype-1 β-tubulin benzimidazole resistance allele, suggesting not only that hybridization had occurred, but also that introgression of drug resistance loci can transpire between the two species. This finding could only have ensued from mixed infections. In this same study, these authors noted that cattle in southern India were only infected with *H. contortus*; *H. placei* was not to be found.

Other reports of mixed or dual infections have been emerging worldwide. *H. contortus* and *H. placei* have been reported as highly sympatric species in North Africa in both large and small ruminants based on morphometric parameters and PCR (Akkari et al., 2013). Results showed that >50% of all small ruminants tested had multiple infections, with numbers being slightly less in cattle. There are similar reports from West Africa (Achi et al., 2003) for small and large ruminants. In a herd in the United States, sequence analysis of faecal eggs prior to anthelmintic treatment revealed an *H. placei* infection; however, following drug treatment and a second round of egg DNA isolation and sequencing, *H. placei* infection was

expelled, but a low level of drug-resistant *H. contortus*, not originally detected by sequencing, was noticeably present in the herd (unpublished data). Although one early report identified a DNA hybridization probe (Christensen et al., 1994b) and several additional reports of species-level PCR-based assays for differentiating *H. placei* from *H contortus* are discussed in the following sections, it has become increasingly important to develop methods for the differentiation of these species, and depend less on genus-level identification.

## 3.2 Traditional PCR

Clearly, the biggest hurdles to generating quality PCR data have been in obtaining amplifiable DNA or RNA devoid of inhibitors and if possible, producing genetic material of sufficient length to allow adequate sensitivity during amplification. Efforts to perform egg-based PCR directly from faeces, to reduce processing time has met with sporadic success, owing to sensitivity issues and PCR inhibitors (Demeler et al., 2013; Roeber et al., 2012a). There are a plethora of commercial and noncommercial methods now available for isolating nucleic acids for PCR; however regardless of the method chosen, the presence of enzymatic inhibitors in biological samples must be addressed before or during PCR.

Over the years, numerous genes have been targeted for identifying parasite-specific PCR primers. Roos and Grant (1993) were among the earliest to develop a *H. contortus*—specific PCR test. In this assay, the investigators synthesized primers that amplified a region of the isotype-I β-tubulin gene spanning an intron that exhibited size variation between *H. contortus* and *T. colubriformis*. The primers chosen did not bind to other common sheep parasites; however, the sensitivity of the assay was low. At the time, it was not clear whether the low sensitivity was related to PCR contaminants, the size of the amplicons (1300—1500 bp) or to a suboptimal copy number of the gene. Consequently, the focus switched to using mitochondrial gene sequences (Blouin, 2002) and genomic spacer sequences associated with the rRNA gene repeat (for review, see Chilton, 2004) as amplifiable targets for PCR. The compelling arguments for these choices have been that both are highly abundant in all life-cycle stages and sufficiently variable among species of gastrointestinal nematodes to attain adequate sensitivity and specificity when designing an assay. Stevenson et al. (1995) were among the first to assess the second internal transcribed spacer (ITS-2) sequence for the differentiation of *H. contortus* from *H. placei*. Several SNPs were identified among the individuals chosen, which resulted

in unique restriction enzyme digestion patterns for each species after PCR amplification. One or more of these SNPs were later used to identify hybrid organisms in Pakistan (Chaudhry et al., 2015) by sequencing. Though not directly related to the delineation of species, other studies have used amplified ITS-2 sequences and denaturing gradient gel electrophoresis (DGGE) to examine sequence heterogeneity among populations of *H. contortus* (see Gasser et al., 1998).

In 1994, SNPs in the external transcribed spacers (ETS) were observed between these same species (Zarlenga et al., 1994), as well as distinct differences in the rRNA gene repeats emanating from the external nontranscribed spacer (NTS) that permitted the differentiation of *H. contortus* from *H. placei*. The SNPs were generated from cloned sequences and were not validated on larger numbers of field samples. However, Santos et al. (2014a) used and validated the existence of multiple rRNA gene repeats within *H. contortus* (cf. Zarlenga et al., 1994) by comparing PCR fragmentation patterns to morphometric data on individual *Haemonchus* worms. Their results showed that the morphology of L3s could be used as the primary method to identify and differentiate the two species. A multiplex PCR developed by Zarlenga et al. (2001) not only differentiated five major genera of gastrointestinal nematodes routinely found in cattle and sheep, but provided data wherein the chosen primers which amplified portions of the ETS were capable of differentiating *H. contortus* from *H. placei*. Such a test would work well on individual worms of *Haemonchus*; however, given the overlap in sizes between the two species, it would be problematic in the event of a mixed infection or if performed on populations of eggs. The doublet generated in this assay was produced only from *H. contortus* DNA and was the result of either multiple-sized fragments or heteroduplex formation from sequence variation among the repetitive units of the rRNA gene within *H. contortus* (see Zarlenga et al., 1994). Given that *H. contortus* was shown to have multiple and distinct repeats, the former explanation is likely correct. This proposal is further supported in studies showing substantially less genetic variability among populations of *H. placei* than among populations of *H. contortus* (see Brasil et al., 2012; Hussain et al., 2014; Jacquiet et al., 1995). Chilton (2004) reviewed the benefits of targeting ribosomal DNA markers for delineating bursate nematodes.

Blouin et al. (1997) showed that numerous fixed differences existed among the mitochondrial ND4 gene sequences from *H. contortus* and *H. placei* to allow for sequence-based or PCR-based differentiation between the two species. These haplotypic differences were used to examine genetic

variation among worms parasitizing sheep and goats in China, where nearly all 152 individual worms exhibited distinct haplotypes (Yin et al., 2013). Random amplified polymorphic DNA assays were also tested (Humbert and Cabaret, 1995; Jacquiet et al., 1995; Rabouam et al., 1999) where sufficient genetic variation was observed between the two species (Jacquiet et al., 1995). However, given the variability in the assay, the dependency on pristine DNA and amplification conditions and inconsistencies in PCR amplification using small, nonspecific primers, this technology was abandoned relatively soon after its inception.

Many assays have been developed with genus-specific rather than species-specific detection in mind. As such, linking *H. contortus* and *H. placei* in assay development has been a common and pervasive theme. Gasser et al. (1994) developed a restriction fragment length polymorphism (RFLP) linked PCR assay based on ITS-2 sequences to delineate six common trichostrongyles of ruminants, including *H. contortus*. Heise et al. (1999) sequenced the ITS-2 from eight species of gastrointestinal nematodes and later, Schnieder et al. (1999) developed a PCR assay for differentiating five major genera of gastrointestinal nematodes infecting cattle and sheep, among them, the genus *Haemonchus*; however, species-level identification was not assessed. Bisset et al. (2014) developed a multiplex PCR capable of differentiating 10 strongylid species that commonly infect small ruminants. They combined both species-specific primers and genus-specific primers to generate gel-banding profiles unique for each of the organisms. The inclusion of genus-specific primers obviates the need for PCR-positive controls (Zarlenga et al., 1999).

## 3.3 Real-time PCR

With the advent of real-time PCR, some new methodologies emerged for the identification of *Haemonchus* spp. Real-time PCR had its inception in the desire to quantify gene transcription; however, over time, many workers adapted it as a means to supplant conventional PCR for identification. Some approaches use fluorescence via resonance energy transfer between fluorophore and quencher molecules bound to a DNA-probe for added specificity (Harmon et al., 2007; Learmount et al., 2009; McNally et al., 2013; von Samson-Himmelstjerna et al., 2002; Siedek et al., 2006). Though PCR probes greatly enhance specificity, generating DNA probes that are dual-labelled for proper energy transfer and fluorescence can be cost prohibitive. Consequently, other techniques have emerged, wherein nonsequence-specific fluorescent dyes are used that intercalate and/or bind double-stranded

DNA and fluoresce either by resonance energy transfer interactions with the helix, or by stabilization of the fluorophore when bound to DNA (Dragan et al., 2012). Though substantially easier and less costly, many of the available fluorophores, including the most commonly used, SYBR I green, are inhibitory to PCR to varying degrees, and can alter the melting temperature of the DNA in a concentration-dependent manner (Gudnason et al., 2007). This influence on melting temperature can affect studies involving melting-curve analysis for identification and for quantification. For this reason, real-time techniques have emerged using fluorescent dyes other than SYBR I green (Bott et al., 2009; Roeber et al., 2011).

As the technological advances have moved towards real-time PCR, efforts have begun to focus more on application rather than mere assay development. Siedek et al. (2006) showed good correlation between probe-based real-time PCR data and coproculture, though the study focussed only on cultured larvae. Since this time, efforts have turned to ante-mortem PCR-based identification of faecal eggs, rather than culturing to L3 followed by morphological examination; a technique that has been extensively reviewed (Preston et al., 2014; Roeber et al., 2013a,b). In conjunction with performing molecular tests on faecal eggs, numerous reports have been published, in which egg isolation was not preceded by purification, and DNA isolation was performed directly on whole faeces. For instance, Sweeny et al. (2011) used the Power Soil DNA Isolation Kit (Mol-Bio, West Carlsbad, CA, United States) and were able to successfully perform nematode-specific PCR on DNA isolated directly from ovine faeces. The data coincided well with egg flotation assays where the epg $> 50$; however, the limits of egg detection were never determined and cultures were not performed on the faecal eggs in an attempt to confirm the PCR data. The same group (Sweeny et al., 2012) modified the procedure in the hope of applying real-time PCR to quantify (qPCR) larval burdens on pasture. Little correlation was observed between qPCR Ct values and log-transformed pasture larval counts, possibly due to a mixture of L3s and eggs on pasture. The qPCR data was, nonetheless, encouraging. Later, McNally et al. (2013) developed a method to extract DNA from sheep faeces that involves dehydration in ethanol, bead-beating to disrupt faecal samples, and magnetic bead—based DNA extraction, followed by genus-level multiplex qPCR to quantify eggs from *Haemonchus*, *Trichostrongylus* and *Teladorsagia*. The assay showed a sensitivity of 10 eggs per gram (epg) using this approach. Given that this test also was somewhat labour intensive and exhibited a sensitivity that was substantially less than that achievable when using purified eggs, general

laboratory practices have thus far conceded that some level of egg purification and/or DNA dilution to reduce inhibitors is in order, to maximize PCR sensitivity rather than isolating DNA from unfractionated, environmental samples (Demeler et al., 2013; Roeber et al., 2012b).

Efforts have been made to quantify faecal eggs in mixed species infections. While it is well accepted that egg output rarely coincides with adult worm burdens (except for *Haemonchus*), and bias is often generated in faecal cultures, making it difficult to apportion egg counts to worm species (Dobson et al., 1992), quantification nonetheless can provide information on pasture seeding densities. This is particularly important when considering the potential for drug-resistant worms in the flock and when establishing pasture management programmes to reduce worm burdens within the host. Conventional approaches to quantification, that is, larval culture, followed by morphological identification of L3, require a person skilled in the morphological identification of L3, and presume that the different worm species develop at the same rate and efficiency under artificial growing conditions. Some researchers would argue that molecular amplification of faecal eggs can succumb to differences in DNA content (egg stage development) and variations in target gene copy numbers among the species. Anecdotal evidence indicates that the former is not an issue, and the latter point can be addressed by properly controlling assay conditions and parameters. Also, Harmon et al. (2007) showed that the time following egg embryonation, and higher concentrations of competing DNA derived from similar nematodes could affect egg quantification; however, changes in DNA content demonstrably affecting quantification occur only within the first 6—7 h following embryonation.

von Samson-Himmelstjerna et al. (2002) was among the first to develop real-time PCR for quantification of gastrointestinal nematodes of sheep. Genus-specific probes and primers to regions within the ITS-2 were designed to encompass common nematodes of small ruminants. The assays exhibited good specificity and sensitivity over a large dynamic range using DNA derived from cultured L1 and L3 parasites. The test had the advantage of partial multiplexing due to the different labels that were chosen among subsets of nematodes. Bott et al. (2009) developed real-time PCR methodology coupled to melting curve analysis to delineate seven distinct strongylids of sheep using a single conserved reverse primer with species- and genus-specific forward primers. In order to quantify, on a relative basis, the numbers of eggs derived from any given species, standard curves were generated and used in the final analysis. The technology was later applied to naturally

acquired infections in sheep for a sample size of 470 animals (Roeber et al., 2011). The method exhibited near 98% sensitivity and 100% specificity, well supporting the overall goal to migrate from larval cultures and morphological examination of L3 to molecular-based analyses. The approach also demonstrated better efficiency in assessing drug-susceptibility/resistance in strongylid nematodes of sheep relative to more conventional approaches, such as the faecal egg count reduction test (FECRT) (Roeber et al., 2012b). Identification was further advanced by automation via the development of a robotic, high-throughput, multiplex tandem PCR to delineate key nematodes infecting sheep and goats, including *H. contortus*. This assay again developed primer sets targeting ITS-2. Results in field trials showed high levels of sensitivity and specificity, and correlated well with the more laborious larval culture techniques.

Droplet digital PCR (ddPCR) is a methodology that provides absolute quantification of PCR products without the need for generating standard curves that plague many of the real-time technologies (Hindson et al., 2011). In ddPCR, a fluorescent probe—based PCR reaction is segregated into 1-nL reverse-micelles (water-in-oil), where zero or more copies of the target DNA are randomly partitioned into nanoparticles along with all other reagents needed for amplification. Following PCR, the absolute fluorescence of each droplet is measured, and defined as negative or positive based on fluorescence intensity, which accounts for droplets containing multiple copies. The absolute number of target nucleic acid molecules is then calculated directly from the ratio of positive droplets to total droplets analysed. Some research has been advanced, demonstrating the applicability of ddPCR for quantifying protozoan parasites. Yang et al. (2014) developed such a method for identifying and quantifying *Cryptosporidium* in environmental samples, and Wilson et al. (2015) have shown that ddPCR is more sensitive and accurate than microscopy for quantifying *Babesia* spp. in blood samples. To date, however, no studies have been generated using droplet digital PCR (ddPCR) to quantify nematode eggs.

## 3.4 The next generation

### *3.4.1 Loop-mediated isothermal amplification*

Several methodologies are on the horizon for DNA-based identification of gastrointestinal nematodes and, in particular, those belonging to the genus *Haemonchus*. In addition to ddPCR, which might find application in quantifying nematode eggs in a mixed population, another technology that has been around for 15 years, but has only recently gained traction for the

identification of gastrointestinal nematodes is loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000). Typically, this technology utilizes *Bst* DNA polymerase rather than a thermostable enzyme, which makes it far less subject to inhibition by contaminants. Other benefits include high sensitivity, no requirement for expensive equipment (isothermal reaction) and visualization that can be performed by whole sample fluorescence or turbidity without gel electrophoretic analysis. These benefits bode well for adaptation to 'in-the-field' assays. Also, the use of four to six distinct primers per reaction increases specificity, making it less prone to false-positive reactions from irrelevant DNA; nonetheless, the primer selection usually requires a proprietary Primer Explorer software package. There are caveats, however, where one has less freedom to choose primer locations and product lengths, and where difficulties arise when differentiating closely related organisms and multiplexing for the detection of other nematodes. Furthermore, a high false-positive rate often results inadvertently from the high sensitivity of the assay, and displaying results via agarose gel electrophoresis is critical when examining small amounts of target PCR product amidst an excess of irrelevant genomic DNA. Still, Melville et al. (2014) developed a highly specific LAMP assay for the identification of *Haemonchus* spp. based on fluorescence using DNA from purified eggs. The sensitivity of this assay was 10 times greater than conventional PCR, and the assay enabled a calculated detection level of 2 epg. In addition, the sensitivity of the assay rivals nested PCR, and the ability to perform this test in 30 min makes it particularly appealing.

### 3.4.2 Metagenomics and pyrosequencing

Metagenomics is a technical innovation involving holistic genetic analysis of an assemblage of microorganisms recovered directly from environmental samples and obviates the need for prior culturing and/or purification. Analysis is usually performed by high-throughput, next generation, shotgun sequencing methodologies. Though diagnostic metagenomics (Pallen, 2014) has been generally relegated to discerning genomically less-complicated organisms, such and bacteria, viruses and fungi, it is finding its way into population genetic studies (Mobegi et al., 2014; Wu et al., 2011) and other organisms such as plant-parasitic (Porazinska et al., 2014) and marine nematodes (Carugati et al., 2015). One study showed the utility of pyrosequencing to examine nematode biodiversity in sylvatic rats (Tanaka et al., 2014) using 18S rDNA–based metagenomics and an Illumina MiSeq sequencer. Currently, as a test to identify gastrointestinal nematodes in faecal matter,

this approach is somewhat excessive, given the cost of supplies, equipment and algorithms needed for data analysis. However, in the future, continuing technological advancements in whole genome sequencing and data analyses are likely to replace many other forms of molecular genotyping. One example is in the adaptation of nanopore technology to high-throughput sequencing (Maitra et al., 2012) which has the potential to substantially reduce the time and cost of sample preparation. Furthermore, as costs continue to moderate and as science migrates towards the philosophy of 'One Health', we can expect that diagnostic tests based upon metagenomics will be developed to encompass multitudes of pathogens, among which gastrointestinal nematodes could be included.

## 4. IMMUNOLOGICAL METHODS FOR DIAGNOSING HAEMONCHOSIS

Serological methods for diagnosis can be highly informative for determining *Haemonchus*-specific exposure or infection. Review articles on the immunological methods used for diagnosing haemonchosis via the specific detection of IgG antibodies have been published (eg, Preston et al., 2014; Roeber et al., 2013b). In general, these assays have not received wide application because they can exhibit problems with antigen specificity and relevancy where antibody levels can remain long after the infection has cleared. In addition, the host tends to exhibit clinical signs of infection long before *Haemonchus*-specific antibody titres increase to reproducibly detectable levels. Moreover, serum antibody levels to infection can vary substantially among outbred animals. The application of bead-based technologies for immunodiagnosing nematode infections has shown promise for distinguishing cattle infected with *C. oncophora*, *Dictyocaulus viviparus* and *Fasciola hepatica* (Karanikola et al., 2015); however, like most immune-based assays including those for haemonchosis, this test is genus rather than species specific. Consequently, haematological-based methods, such as blood packed cell volume, eye-lid colouration (FAMACHA), and faecal egg counts (FECs) have been used as generic indicators of nematode infection. In combination with FAMACHA, which is a subjective assessment of host anaemia resulting from blood-feeding nematodes, such as *Haemonchus*, the other techniques are easy to use, practical and in some cases amenable to field applications.

Beyond the simple diagnosis of infection, immunological methods are important tools in research for estimating levels of exposure, population

immunity, correlating natural resistance to the level of immune response, and in identifying animals that respond poorly to *H. contortus* infection. Evaluation of population immunity and identification of poor- or nonresponders in the flock can be useful for managing infections and for strategic deworming.

Herein, we cover immune-based advances that focus on indirect, serological detection of haemonchosis, and discuss less conventional immunological assays that deviate from the detection of parasite-derived diagnostic markers. As such, the assays described here examine specific IgG and non-IgG antibodies against *Haemonchus*, as well as host responses to infection, such as eosinophilia and eosinophil peroxidase, mast cell and mastocytosis, T cell proliferation, and changes in cytokine profiles as markers of infection.

## 4.1 Antibody assays for the diagnosis of haemonchosis; ELISA and Western blotting

Antigen-specific, anti-*Haemonchus* antibodies can be detected and quantified using ELISA or Western blot. These techniques involve target antigens (whole parasite extract, secreted, purified native or recombinant proteins) being immobilized on a solid support, followed by incubation with host body fluids (eg, serum, mucus and saliva) containing antigen-specific antibodies. Detection is followed by incubation with a labelled isotype-specific secondary antibody, followed by an appropriate substrate. ELISA testing is generally prone to nonspecific interactions; consequently, specificity must often be confirmed by Western blotting. All members of sheep immunoglobulins (Ig), including IgG, IgA, IgE and IgM, can be measured by ELISA using isotype-specific antibodies.

Serum or mucous IgG and IgA are by far the most studied Ig classes in sheep infected with *H. contortus* (see Miller, 1996). Early on, serum or mucous IgG and IgA were measured by radio-immunoassay (RIA) (Duncan et al., 1978; Smith, 1977); however, this test was rapidly replaced by ELISA, which does not require the use of radioactive materials, and exhibits higher throughput and sensitivity. As noted earlier, sensitivity and specificity can pose problems where the titre of specific IgG in *Haemonchus*-infected sheep is generally low (Cuquerella et al., 1995; Smith, 1977; Duncan et al., 1978), and clinical signs normally appear before the antibody titres reach detectable levels. Since the 1990s, more reagents have become available, so that *Haemonchus* antigen—specific IgG1 and IgG2, IgA and IgM can now be monitored. Delineating subclasses has become important because IgG1 appears as the predominant antibody species elicited by *Haemonchus* infection (Schallig et al., 1995; Schallig, 2000).

Antigen-specific antibodies in body fluids can also be evaluated by Western blot, which first separates parasite antigens by SDS-PAGE before transferring them to a membrane and screening them with diluted host antibodies. Though more labour intensive and not conducive to high throughput, it has the distinct advantage of determining whether or not antibody binding is specific or cross-reactive in nature. This assay can also identify the presence of isotype-specific antibodies that unambiguously recognize known antigen(s) or particular protein bands with known molecular masses if total antigens are used. As with ELISA, this assay has not received unilateral use as a diagnostic test for haemonchosis; however, it has become an invaluable tool for discovery research on *Haemonchus* (García-Coiradas et al., 2009; Hart et al., 2012; Raleigh and Meeusen, 1996; Rathore et al., 2006; Schallig et al., 1995, 1997; Wang et al., 2014a,b; Yan et al., 2010).

## 4.2 Antibody assays as research tools to study haemonchosis

Assays to detect *Haemonchus*-specific antibodies in body fluids (serum, tears, saliva and faecal fluids from live animals) include ELISA and Western blotting. These assays have been well described (Preston et al., 2014; Roeber et al., 2013b), and few significant technological advances have been noted in the literature. These assays generally target serum IgG and can be conveniently accomplished with ELISA; however, the detection of IgE and IgA in the circulation, which is important in relation to understanding disease, and are considered to be more important than IgG when assessing levels of host protection, can be challenging due to limited availability of costly reagents. Antigen-specific IgE and IgA from infected animals can be reliably assayed in local mucosal tissues following biopsy or postmortem. Thus, these assays have great utility for laboratory research, but less for the diagnosis of haemonchosis in live animals.

The reliable detection of low levels of IgE and IgA in blood is usually achieved using capture/sandwich ELISA with high sensitivity, accuracy and reproducibility. A broadly cross-reactive IgA sandwich ELISA, which also detects ovine IgA, is commercially available (http://www.antibodies-online.com), though it seems not to have received wide use in ovine studies. The availability of commercially available antibodies against sheep IgE has permitted the measurement of this Ig subtype by ELISA (Kooyman et al., 1997; Redmond and Knox, 2004; Shaw et al., 1996). A sheep IgE capture ELISA was developed using an antisheep IgE monoclonal antibody, 2F1, generated from a chimeric IgE protein (Bendixsen et al., 2004). This

assay was used to detect total IgE in colostrum and intestinal homogenates, but not in serum. Of particular note, antigen-specific IgE appeared higher in resistant than in susceptible sheep infected with *T. colubriformis* (see Bendixsen et al., 2004). A similar trend in elevated IgE was seen in Gulf Coast Native (Native) sheep known to be more naturally resistant to *Haemonchus* infection than Suffolk lambs (Shakya et al., 2011). Also, systemic *H. contortus*—specific IgE was evident in sheep exposed to infection on pasture, as determined using this same assay, and protection in *H. contortus* antigen-vaccinated lambs correlated better with levels of IgE than with IgG1 (Kooyman et al., 2000; LeJambre et al., 2008). Inasmuch as IgE facilitates basophil activation and IL-4/IL-13 release, which in turn are essential for host protection against helminth infections in the mouse models (Schwartz et al., 2014), this information suggests the need for better and more sensitive assays to consistently measure IgE in body fluids and tissue homogenates of ruminants infected with *H. contortus*. If key *H. contortus* antigen—specific IgA and IgE can be more accurately and consistently detected in ovine blood or other body fluids, these antibodies may be useful in determining population mucosal immunity as well as in selecting animals with natural resistance to *H. contortus* infection, as mediated by high levels of IgA and IgE.

### 4.2.1 Eosinophils and eosinophil peroxidase assays

Eosinophilia is well documented in *H. contortus*—infected animals as well as in animals infected with other nematodes, and has been correlated with protection (Fawzi et al., 2014; Huang et al., 2015; Preston et al., 2014; Reinhardt et al., 2011). Traditionally, eosinophilia has been determined by counting this cell population in whole blood. However, obtaining eosinophil counts in blood and tissues from infected animals at postmortem can be difficult, and data from current enumeration assays tend to be highly variable and inconsistent. Recent advances to assess eosinophilia rely on monitoring levels of eosinophil-specific peroxidase (EPX) in serum, tissue homogenates or other body fluids using a sensitive sandwich ELISA. Eosinophil peroxidase is specific to primary and secondary granules of mammalian eosinophils. This sandwich ELISA utilizes a matched pair of monoclonal antibodies specific for EPX (Ochkur et al., 2012) and reflects not only eosinophil activation such as degranulation, but may also correlate with the magnitude of the activation (eg, number of activated eosinophils). Although a good indicator of nematode parasite-induced eosinophilia, evidence is lacking to link EPX directly to host protection (Cadman et al., 2014; Ramalingam et al., 2005).

Given that this assay can detect ruminant EXP, it should also be useful for assessing individual and population immunity and for selecting eosinophil-mediated resistant breeds.

### 4.2.2 Mast cell and mastocytosis assays

Like eosinophilia, mastocytosis is also correlated with protection in *H. contortus* and other gastrointestinal nematode infections (Hepworth et al., 2012; Schallig, 2000; Schallig et al., 1997; Shakya et al., 2011). In the mouse model, mast cell accumulation and, in particular, mast cell degranulation at early stages of infection by gastrointestinal nematode parasites are critical to priming a protective Th2 response (Hepworth et al., 2012). Tryptic peptidases (tryptases), which belong to the serine-class peptidases, are among the most abundant proteins in mast cell secretory granules and they are released externally during exocytosis. Thus, detection of local or systemic mast cell—specific markers, such as tryptase (Miller and Pemberton, 2002; Pemberton et al., 2000; Schwartz, 2006), can be useful for assessing mast cell activation/degranulation and for determining parasite susceptibility of the host. Currently, an assay for the specific detection of mast cell tryptase in sheep is not available; however, a bovine ELISA tryptase appears to have broad cross-reactivity with tryptases of other host species, including sheep and goats.

Another marker for infection is mast cell proteinase-1 (Miller and Pemberton, 2002; Pemberton et al., 2000), which is also a serine proteinase with dual chymase/tryptase activity. It is expressed in gastrointestinal mast cells and transported to the surface mucosa during nematode infections. With respect to fibrinogen cleavage and fibroblast stimulation, the sheep mast cell proteinase (SMCP) exhibits functional similarities to mast cell tryptase (Pemberton et al., 1997). ELISA targeting SMCP has been developed (Huntley et al., 1987). The presence of SMCP has been linked to protection in sheep infected with *H. contortus*, where SMCP is elevated in immune gastric mucosa compared with that in normal tissues (Huntley et al., 1987). Although, SMCP is abundant in homogenates of abomasal tissue of parasite-immune sheep, it remains low to undetectable in serum and lymph, thus limiting its application to live animals. Furthermore, sheep serum and lymph contain inhibitors that can interfere with the SMCP—antibody interactions. Consequently, the SMCP-ELISA works best with homogenates from abomasal tissue.

### 4.2.3 T cell proliferation assay

T cells are one of the major components of peripheral blood mononuclear cells (PBMC) and key players in both innate and adaptive immunity. T cells

proliferate upon activation in a recall response, which is required for protection (Haig et al., 1989; Jasmer et al., 2007; Peña et al., 2006). Antigen-specific T cell assays can be useful in determining T cell responses to *H. contortus* infection, and can be used to study parasite molecules capable of modulating host immunity (Torgerson and Lloyd, 1993). Research on isoforms of recombinant galectin (Hco-gal-m and −f) from *H. contortus*, have shown that parasite-derived galectins suppress immunity and therefore promote the infection process by binding the surface of PBMC, including T cells (Wang et al., 2014a,b) and, in particular, to the transmembrane protein 63A (Yuan et al., 2015). Consequently, T cell assays can be quite informative. However, the process of identification and characterization is quite tedious, involving $^{3}$H-thymidine, homologous host cells as antigen-presenting cells (APCs) and irradiated APCs, if T cell lines or clones are used (Tuo et al., 1999). This type of assay is useful only for research purposes, particularly in assessing vaccine efficacy and candidate vaccine discovery.

### 4.2.4 Cytokine and host alarmin assays

Cytokines are well known for their involvement in the expulsion of gastrointestinal nematodes. In preparation for the protective Th2 immunity against such nematodes, the gastrointestinal epithelial cells respond to the infection by releasing innate cytokines, such as IL-1, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). They also release tissue/cell injury-associated alarmins or danger-associated molecular pattern (DAMP) molecules, such as uric acid, ATP, high mobility group box 1 (HMGB1) and S100 proteins (reviewed by Hammad and Lambrecht, 2015). Thus, characterizing the immune state of the animal can have a demonstrable impact on delineating pathways involved in the infection process. For example, one might expect to see ATP levels increase during *Haemonchus* infection, due to cell damage; however, levels of adenosine and ADP were reported to be substantially reduced (Gressler et al., 2014). This finding aligns with the importance of adenosine and ADP in controlling platelet activation and allowing *Haemonchus* to feed on blood. In addition, extracellular HMGB1 was shown to have a dual function, where it can regulate inflammation and cellular repair, as a passively released molecule from damaged cells or as a secreted molecule from activated immune cells (Vande Walle et al., 2011).

Quantifying selected groups of cytokines, such as IL-4, IL-13,TNF-$\alpha$, IFN-$\gamma$, either directly, or by reverse-transcription PCR (RT-PCR) has aided in evaluating breeds of small ruminants that are resistant to *H. contortus* infection (Alba-Hurtado and Muñoz-Guzmán, 2013; Miller and Horohov,

2006; Zaros et al., 2014). Such assays can be critical for assessing vaccine and drug efficacies. Unfortunately, the lack of commercially available immunological assays for sheep and goats has made direct investigations of many cytokines difficult. Consequently, today RT-PCR has become the method of choice.

### 4.2.5 ELISPOT and the identification of antibody-secreting cells

Immunohistochemistry can be used as a research tool to identify the spatial localization of isotype-specific antibody-containing cells in situ. This procedure involves tissue fixation, embedding, sectioning, rehydration and probing with antiisotype antibodies labelled with a reporter enzyme, followed by detection using a substrate. For instance, Gill et al. (1992, 1993) detected IgA-, IgG1-, IgG2- and IgM-containing cells in the abomasum of *H. contortus*—infected sheep, where the most abundant cell types were test positive for IgA, IgG1 and IgM. The disadvantages of this method are that it can only assess relative numbers of isotype-specific antibody-containing cells, and that the antibodies detected are not necessarily antigen specific. There is no report of the use of the enzyme-linked immunospot assay (ELISPOT) for the detection of *H. contortus*—specific antibodies in small ruminants, although the test was successfully applied to study *T. colubriformis*—specific antibodies (Emery et al., 1999). This assay might be used for assessing the frequencies of isotype-specific secreting cells in a mixed cell population in *H. contortus*—infected small ruminants.

## 5. FINAL THOUGHTS

When one holistically examines the identification of *Haemonchus* spp. and the diagnosis of *Haemonchus* infection or haemonchosis, certain issues become apparent. First, for the most part, delineation among *Haemonchus* in domestic livestock has been relegated to host associations rather than direct methods of identification. Other than for morphological identification of adult worms and direct DNA sequencing of specific gene targets, PCR tests to define species based on worm populations are lacking. This aspect becomes problematic when performing epidemiological studies and equally important, when accessioning gene sequences to worldwide databases. Personal experience has instructed us that the sources of gene sequence data derived from earlier database submissions can at times be faulty due to nematode misidentification. In most instances, such genetic data were not accompanied by the submission of morphologically identified

voucher specimens for archival storage in museum repositories. We suggest that genetic data should be concurrently derived from specimens that have an authoritative identification and which, because of archival deposition, can be available to confirm or secondarily assess the validity of field-based observations. Voucher specimens are particularly critical in areas of sympatry for assemblages of domestic and free-ranging ungulates, where there is a considerable expectation for cross-transmission of parasites between or among animal species. In recent years, most of the sequence databases have been updated using highly inbred worm populations or laboratory strains of parasite species; however, new data from epidemiological studies can no longer rely on host associations for definitive identification/diagnosis, given anthropogenic forcing and the broad host associations of members of this genus with wild ruminants.

Second, it has become clear that antibody-based assays and other immune-related tests have not been widely used for diagnosis, although they have played insurmountable roles in understanding haemonchosis and the host immune response against *Haemonchus*. Physiological parameters have taken precedence, because of their ease of use in the field, lack of need for expensive equipment and laboratory consumables/supplies, and because the host exhibits symptoms of disease long before the serological tests become functionally beneficial to use.

Finally, rapid changes and progress in molecular and proteomic technologies will continue to advance this field of research, and at a remarkable pace. However, we need to be mindful that morphology has and will continue to be the benchmark for defining taxa in the foreseeable future, given the significant genetic diversity within and among populations that defines most nematodes of the gastrointestinal tract of ruminants.

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