



# CHALLENGES TO THE DEVELOPMENT OF NEW TESTS FOR DIAGNOSIS OF INFECTION AND PREDICTION OF RESISTANCE OF SHEEP TO GASTROINTESTINAL NEMATODES

I.G. COLDITZ

*Cooperative Research Centre for Sheep Industry Innovation,  
University of New England, Armidale, NSW 2351,  
Australia and CSIRO Livestock Industries, F.D. McMaster Laboratory,  
Locked Bag 1, Post Office, Armidale, NSW 2350, Australia  
Email: [ian.colditz@csiro.au](mailto:ian.colditz@csiro.au)*

**Abstract.** Strategies for control of gastrointestinal nematode (GIN) infections in sheep require information on the severity of infection and species (or genus) of parasite present. Tests for diagnosis of GIN fall into 3 classes. Current tests measure either: 1) the presence of eggs, worm antigens or worms themselves; 2) components of host immunity (e.g. antibodies, eosinophils, other immune mediators); or 3) components of host pathology (e.g. wool growth, body growth, appetite, blood loss, digestive enzymes, anaemia, hypoproteinaemia, odours). To offer advantages over current diagnostic methods, new tests need to be more informative, more accurate, more timely, cheaper, technically easier, or suitable for use in the field. To improve sheep management, a new test needs to determine severity or predict the onset and severity of infection. This is a technically more difficult challenge than qualitative diagnosis of the presence of infection and creates a substantial obstacle to the development of new diagnostic methods. Estimation of the performance characteristics of a test including its sensitivity, specificity and predictive value is important before widespread adoption. This paper reviews current diagnostic tests for GIN, and opportunities for new tests that aid management of infections or that inform the estimated breeding value of animals for use in programs that breed sheep for resistance to GIN. Gene marker and biomarker tests for resistance to GIN infection or disease will require validation in the population in which they are to be used and may require revalidation as the genetic background of the population changes over time. Estimation of the specificity, sensitivity, and predictive value of gene markers and biomarkers for GIN infection may help inform the value of these markers as selection criteria for use in breeding programs.

## INTRODUCTION

Gastrointestinal nematode (GIN) infection is the most important infectious disease limiting production by grazing sheep in Australia (Sachett *et al.*, 2006) and worldwide (Waller, 2006). Diagnosis of the severity of infection and the species or genus of parasites present is the cornerstone of managing parasite

infections. This paper reviews the strengths and limitations of current methods for diagnosing strongyle nematode infections in small ruminants and identifies some of the challenges that face the development of new diagnostic tests.

Information gained from diagnostic tests for GIN infections can be used for 1) quarantine purposes; 2) management of sheep

health and production, including differential diagnosis of GIN infections from other diseases; 3) pasture management to limit contamination with larvae; and 4) identification of the genetic merit of individuals when breeding for parasite resistance. Information sought through diagnosis can include the species present, their distribution and abundance both in the host population and in the environment, and the anthelmintic resistance status of the parasite. In research settings, information gained through diagnosis is used for many purposes, principally focused on gaining new knowledge of host and pathogen biology.

GIN are endemic in essentially all populations of sheep grazing at pasture. With the emergence of resistance to anthelmintics, management and control of the severity of infection has replaced elimination of infection as the goal of parasite control (Waller, 2006). In contrast to parasite control in humans and some companion animals where a decision to treat can be based solely on the suspected or confirmed presence of infection, in sheep diagnosis needs to estimate the severity of infection and the real or potential cost to production that infection poses. This is a more demanding diagnostic goal than the qualitative goal of determining the infection status (infected or not infected). The need to predict the impact of infection on production and health of sheep has important implications for the type of tests used for diagnosis of GIN and for the challenges faced by new tests under development. Typically, the diagnostic test is used as a surrogate for worm burden estimation, which is in turn used as a predictor of risk of production loss and host pathology.

Diagnostic tests for GIN fall into three classes. Tests measure either:

1. The presence of eggs, worm antigens or worms themselves;
2. Components of host immune responses to infection (eg antibodies, eosinophils, other immune mediators) or
3. Components of host pathology (wool growth, body growth, appetite, blood loss, digestive enzymes, anaemia, hypoproteinaemia, odours, etc).

Tests within each of these classes will be considered in turn.

## TESTS THAT DETECT PARASITES AND THEIR PRODUCTS

### Worm eggs in faeces

The modified McMaster method (Whitlock, 1948) for counting strongyle worm eggs in faeces (WEC) is the gold standard method for quantifying the concentration of GIN eggs in faeces and is a surrogate measure of the burden of GIN in sheep. It is generally not possible to differentiate between the species of trichostrongylid eggs although a number of parasite species or genera can be identified by this method. WEC can be measured either on samples from individual animals or on pooled samples from a group of animals (Nicholls & Obendorf, 1994). Guidelines for sample size are provided by Nicholls & Obendorf (1994) and Morgan *et al.* (2005). Refinements to the procedure continue to be made (Cringoli *et al.*, 2004).

Real and potential sources of error in the WEC method include:

1. Parasite species – egg laying capacity differs between parasite species, and may differ between field isolates within a species (Peter Hunt, personal communication). According to Gordon (1967), the approximate egg laying potential of worms is:
  - a. *Haemonchus* – 5,000 to 10,000 per day
  - b. *Oesophagostomum* and *Chabertia* – 3,000 to 5,000 per day
  - c. *Trichostrongylus* and *Ostertagia* – 100 to 200 per day
  - d. *Nematodirus* – 50 per day.

Thus in mixed infections reliance on WEC in the absence of information on the parasite genera contributing to infection can make it difficult to estimate the severity of infection and the risk from infection to host health and production.

2. Interactions between parasite species – some parasite species affect the egg laying

capacity of co-infecting parasites, probably by modifying host immune responses. For example, infection with *Haemonchus contortus* increases the rate of egg production by *Trichostrongylus colubriformis* during co-infection with the two parasites (J. Lello and S.J. McClure, personal communication).

3. Host immune status – immunity of the host can suppress egg laying especially by *Trichostrongylus spp.* In addition, damage to eggs by immune effector mechanisms of the host can reduce the buoyant density of eggs thus leading to underestimation of egg numbers in a sample (L.F. Le Jambre, personal communication).
4. Recent exposure to anthelmintics – some members of a parasite population that has a degree of resistance to an anthelmintic will survive exposure to the anthelmintic but reduce or cease their egg production during the recovery phase following anthelmintic exposure. The interval between drenching and re-testing used in the Faecal Egg Count Reduction Test for assessing anthelmintic resistance is chosen to accommodate this transient arrest in egg laying by anthelmintic resistant worms.
5. Faecal moisture – moisture content of faeces can dilute the concentration of eggs in faeces (expressed as eggs per gram (wet weight) of faeces). The correction factors proposed by Gordon (1967) for adjusting WEC to a common faecal moisture content have recently been confirmed (Le Jambre *et al.*, 2007).
6. Circadian variations in egg output by parasites.
7. Circadian variations in digesta passage within the host gut.
8. Variation between faecal pellets in egg concentration and distribution.
9. Deterioration of eggs in transit from the field to the laboratory and during storage before test affecting their buoyant density and hence recovery in the lab.
10. Number of eggs in the sample. The WEC method counts the number of eggs in a

fixed dilution of faeces. Sampling theory indicates that the number of eggs estimated to be in samples with a low real number of eggs will be less accurate than the number estimated to be in a sample with a high real number of eggs (Morgan *et al.*, 2005).

11. Technical sources of error in labs, eg. between operator errors. When samples were allocated at random to laboratory staff within a lab, the repeatability of WEC counts performed on duplicate aliquots from a single egg dilution in salt solution was found to be between 0.80 and 0.91 (S.J. Eady, personal communication). These estimates of repeatability included both within-sample variation and between-operator variation.

#### **Speciation of larvae**

Faecal culture and larval differentiation on morphological criteria of infective larvae are the standard methods for identifying the genus or species of GIN present in a faecal sample. The sample may be from an individual or a group. Sources of error include:

1. Loss of viability of eggs in transit from property to lab
2. Competition between species during larval culture
3. Differential sensitivity of species to development at culture temperature
4. Differential development time between species – eg *Nematodirus spp* take at least 8 days to develop whereas *Haemonchus spp*, *Ostertagia spp* and *Trichostrongylus spp* develop to infective larvae within 7 days.
5. Technical sources of error in laboratories, eg between-operator errors

#### **Identification of the genus of parasite eggs**

A method for the identification of the genus of parasite eggs on the basis of their lectin staining characteristics was described by Palmer & McCombe (1996) and further developed by Colditz *et al.* (2002). The genus classification of eggs can be performed on the day a faecal sample arrives in the lab and has

the potential to be more accurate than larval differentiation due to retention of lectin staining characteristics by eggs that have lost viability following collection.

Research by Peter Hunt in The Australian Sheep Industry CRC showed the potential for quantitative real time PCR to quantify the number and genus of larvae on pasture and of eggs in faeces. Similarly, identification of the genus of gastrointestinal nematode eggs by PCR has been demonstrated in cattle (Zarlenga *et al.*, 2001).

#### **Post mortem examination of sheep**

Slaughter of sheep and inspection of the gastrointestinal tract for semi-quantitative or quantitative enumeration of parasite burdens and the genera present is the gold standard method for estimation of worm burden (and a prerequisite test to demonstrate anthelmintic activity of all available anthelmintics) and has been used by farmers and veterinarians for many years. An excellent guide to the method is provided by Gordon (1967).

#### **Worm antigens in faeces**

The detection of worm antigens in faeces has been explored as a method for diagnosing parasite infections for around 20 years. Assays have been developed to detect *H. contortus* (Ellis *et al.*, 1993) and *Ostertagia circumcincta* (Johnson *et al.*, 2004) in sheep, and a large number of parasites and other pathogens in other species. Assays for GIN copro-antigens in sheep do not appear to have been applied yet as routine diagnostic procedures although research on this approach continues (Colditz *et al.* 2006).

### **MEASUREMENT OF THE HOST RESPONSE TO PARASITE INFECTION**

#### **Antibody to parasite antigens**

The presence of antibody in serum, milk or faeces has been used as a diagnostic test, at least in experimental settings, for most GIN infections and for liver fluke infections in sheep. An ELISA for fluke antibodies is commercially available (Molloy *et al.*, 2005) and a test for an antibody to *Trichostrongylus spp* has been used to estimate breeding values

for resistance to gastrointestinal nematodes in New Zealand; however this test is no longer offered commercially. ELISAs for diagnosis of *Haemonchus* infections have been described (Schallig *et al.*, 1995; Gomez-Munoz *et al.*, 1996), as has an ELISA for *Ostertagia* infections (Johnson *et al.*, 2004).

#### **Circulating eosinophil count**

The number of eosinophils in peripheral blood has been observed to increase during the expression of acquired immunity to *T. colubriformis* and *H. contortus* in sheep (Dawkins *et al.*, 1989); however, circulating eosinophil numbers were found to be less accurate than WEC as a selection criterion for resistance to these parasites (Woolaston *et al.*, 1996).

### **MEASUREMENT OF HOST PATHOLOGY**

#### **Gastrointestinal enzymes**

Change in permeability of abomasal and duodenal mucosae to digestive enzymes has been used as a measure of the severity of *Ostertagia* infection in sheep and cattle. Both plasma gastrin and pepsinogen have been used as indicators of GIN infection (Berghen *et al.*, 1993).

#### **Anaemia**

Anaemia and the presence of bottle jaw (dependent oedema) due to hypoproteinaemia have been used as indicators of liver fluke and *H. contortus* infections in sheep by veterinary practitioners and graziers for many years (Gordon, 1967). A five point scale for scoring anaemia on the basis of the colour of the conjunctiva membranes has been standardised as the FAMACHA test and is used by sheep and goat owners in South Africa and the United States (Vatta *et al.*, 2001). Sensitivity, specificity and predictive value of the test have been estimated under field conditions in the United States (Kaplan *et al.*, 2004); however test performance may differ in other regions due to differences in prevalence of anaemia from causes other than *H. contortus* infection.

Haematocrit has proved more highly heritable than WEC as a selection criterion for

resistance to *Haemonchus* infections in some studies (Albers *et al.*, 1987)

### **Faecal blood**

Measurement of blood in faeces for diagnosis of the presence of blood feeding gut parasites has been described in several host species. A faecal occult blood test developed in the Australian Sheep Industry CRC employs this principle and can detect infection around 10 days before eggs appear in faeces (Colditz *et al.*, 2006; Colditz & Le Jambre, unpublished). A limitation of the test procedure is the need to heat samples to remove interference from non-haem peroxidases. False positives due to non-haem peroxidases could be avoided by use of an antibody to haem, as recently demonstrated in horses (www.SucceedFBT.com accessed 3<sup>rd</sup> November 2007). Field validation of the faecal occult blood test in commercial environments is required and in progress.

### **Weight change**

The advent of equipment for automated weighing of sheep with frequencies as high as once per day has renewed interest in the use of weight change as an diagnostic indicator of parasitism both for treatment decisions (Colditz *et al.*, 2006) and for use in breeding programs for resilience to parasite infection (Bisset *et al.*, 2001). Measures of diagnostic performance including sensitivity and specificity are yet to be estimated. This indirect measure of GIN infection will be influenced by the quality and quantity of available feed.

### **Estimation of genetic merit for phenotypic resistance to gastrointestinal nematodes**

WEC is the most widely employed measure of phenotypic resistance to gastrointestinal nematodes used for estimating breeding values of sheep in genetic selection programs for parasite resistance. As noted above, serum antibody, haematocrit, circulating eosinophil counts and body weight have been examined as selection criteria for resistance. A comment on some of the criteria new indirect selection markers for resistance need to satisfy is provided below. Accurate and predictive gene markers have been the goal of research on host

resistance of nearly 2 decades. On 23<sup>rd</sup> October 2007, a gene marker diagnostic test for identifying animals with resistance to internal parasites in sheep was released by Catapult Genetics ([http://www.catapultsystems.co.nz/products/55\\_wormstar.cfm](http://www.catapultsystems.co.nz/products/55_wormstar.cfm), accessed 2<sup>nd</sup> November 2007). An important aspect of gene marker tests is their independence from worm challenge at the time prediction of the resistance phenotype of animals or their offspring is made.

### **Differential diagnosis of anaemia in sheep**

Parasite infections need to be differentiated from a number of other diseases which can present with similar signs. A potential guide for on-farm diagnosis of gastrointestinal parasitism and anaemia for sheep grazing at pasture in Australia is presented in Table 1.

## **OPPORTUNITIES TO IMPROVE DIAGNOSIS OF GIN INFECTIONS**

The sources of inaccuracy in WEC noted above, together with the time delays associated with faecal culture followed by larval differentiation have created strong interest in new methods for diagnosis of GIN infections. Desirable features of new diagnostic tests include:

1. Similar or lower price than current tests
2. Faster provision of information to farmers
3. Suitable for implementation by farmers on-farm
4. At least as accurate as WECs and faecal cultures for larval differentiation
5. At least as precise as WECs and faecal cultures for larval differentiation
6. Applicable to other hosts including cattle and humans

As noted above, most diagnostic tests used in human and veterinary medicine aim to detect the presence or absence of infection. In contrast, diagnostic tests for GIN in sheep need to determine the severity infection and in some instances the also identify of genera present. In most instances, current sheep management practices aim not to eliminate internal parasites

**Table 1.** A guide to differential diagnosis of conditions causing anaemia in sheep grazing at pasture in Australia

Diagnostic indicator	<i>Haemonchus</i>	Liver Fluke	Other GIN	<i>Mycoplasma ovis</i>	Coccidiosis & salmonellosis	Anaemia trace element deficiency
Bottle jaw	+	+	-			?
FAMACHA (Pale ocular membranes)	+	+	-	+	?	+
Faecal occult blood test	+	+	-	-	- or +	-
Failure to walk 100 m	+	+	+ or -	+		+
Diarrhoea	-	-	+ or -	-	+	-
Bloody faeces	-	-	-	-	+ or -	-
Distribution	Summer rainfall / irrigation	Temperate	Temperate high rainfall	Widespread	High rainfall, high stocking rates, housed	Regional
Prevalence	Very common	Locally prevalent	Very common	Common in weaners	Common in weaners	Local history

but to treat animals when infections reach a level that threatens or reduces their productivity. Thus tests need accuracy and precision around the decision points associated with risk of production loss. Validating a new test designed to detect the severity of infection is a more demanding task than validating a test designed only to detect the presence or absence of infection. A further challenge is the lack of uniformity in decision points and treatment goals for minimising the production losses associated with GIN infections. Thus a new test for GIN would typically need to be validated against a suite of decision points (or test goals) rather than a single test objective, infected or not infected. Decision points may differ for stage of infection, parasite species, breed of sheep, class of sheep, physiological status of sheep, gender of sheep, resistance status of sheep, climatic region, feed type, body condition and so on. Validation of a new

test for each of these variables could be necessary.

Tests that measure the host antibody response to infection are not attractive for making decisions to treat or not treat sheep for GIN infections because the half-life of antibody (around 20 days; (Watson, 1992)) does not match the dynamics of parasite infection and re-infection after treatment. In addition, ingested larvae may stimulate antibody production in some infections but fail to mature into adults. In contrast, assessment of the presence of liver fluke, which is typically based on monitoring and treatment twice per year in temperate environments, is well suited to antibody testing.

Measures of parasite burden such as eggs or worm antigens are in principle attractive because they quantitatively reflect the presence of worms, and when eggs are counted, the presence of mature worms. Reliance on egg



counts is not desirable when pathology (or the risk of pathology) is associated with prepatent stages of infection such as severe *Haemonchus* infections where pathology and even death can be caused by immature adults feeding on blood, and with the hypobiosis phase of infection, e.g. Ostertagiasis in cattle and *Haemonchus* infections in sheep. Tests such as the *Ostertagia* ELISA in cattle that can predict the impending severity of infection during the hypobiosis period are therefore attractive. A strength of the new faecal occult blood test for *Haemonchus* infections in sheep is its potential capacity to predict the severity of subsequent mature infection (Colditz & Le Jambre, unpublished). This test is thus a predictive or “leading” indicator of the severity of *Haemonchus* infection.

A weakness of measures of pathology, such as weight loss, anaemia, FAMACHA and hypoproteinemia is the fact that production losses are incurred before positive test results are obtained. These measures are thus “lagging” indicators of the severity of infection and therefore are less attractive than leading indicators.

## VALIDATION OF NEW TESTS

While the accuracy and performance of WEC and GIN species classification based on larval morphology have been established from many years of application, new tests need formal validation against diagnostic test performance criteria. (Greiner & Gardner, 2000) provide a valuable guide to the validation of diagnostic tests. Validation of the FAMACHA test illustrates application of these criteria to a new test for GIN infection (Kaplan *et al.*, 2004). FAMACHA appears to be the only diagnostic test for GIN in sheep to have been formally validated.

## DIRECT AND INDIRECT MARKERS OF GENETIC MERIT FOR RESISTANCE TO GIN

An important application of diagnostic tests for GIN is the estimation of the genetic merit of individuals for use in programs breeding sheep

with resistance to GIN infections. Phenotypic measures used to identify resistance to GIN include WEC, antibody to GIN, eosinophil concentrations in peripheral blood, haematocrit, and body weight change during GIN infection. The resistance trait selected by each marker is likely to differ and selection for each trait addresses a different breeding objective, with potentially different consequences for parasite epidemiology. A clear conception of the breeding objective is essential to the design of a breeding program employing each trait. Identification of new biomarkers and indirect selection markers could lead to further fragmentation of components of the host response into new traits such as:

1. selective grazing to avoid pasture contaminated by larvae
2. resistance to establishment of infection
3. resistance to the induction of anorexia by infection
4. suppression of egg production by mature females
5. resilience to the pathology of GIN infection

and so on. Before application in breeding programs, it is desirable to establish for such novel correlates of resistance, in addition to conventional genetic parameters;

1. repeatability during primary and secondary infections
2. sensitivity to stage of a single infection
3. specificity or generality for single and mixed infections, and
4. performance in each of the classes of animals noted above for new diagnostic tests.

An important observation from studies of the use of insulin-like growth factor-1 (IGF-1) as a biomarker for net feed efficiency in cattle has been the absence of a significant association between the traits in commercial environments despite the association seen in research settings (Moore *et al.*, 2005). Similarly it has been observed that the association between gene markers and the phenotypic traits of foot rot in sheep (Hickford *et al.*, 2006) and intramuscular fat in cattle (Van Eenennaam *et*

*al.*, 2007) differ between populations of animals. These discrepancies indicate that both gene markers and biomarkers need validation in the target population in which they are to be applied, and the markers may furthermore need frequent revalidation as the genetic background of the populations change with time. Criteria for the validation of biomarkers and gene markers as selection tools for phenotypic traits including resistance to GIN do not appear to have been formalized. Some guidance to the standardisation of performance criteria may be provided by the criteria for validation of diagnostic tests (Greiner & Gardner, 2000). Thus estimation of the specificity, sensitivity, positive and negative predictive values for gene markers and biomarkers may provide important additional information to that derived from genetic parameters about the relative value of these novel diagnostic markers as selection criteria for use in breeding programs.

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