Diagnosing gastrointestinal nematodes in livestock

Nicole Hedley[1], **D Grant Richards**[2] and **Enzo A. Palombo**[1]

[1] Department of Chemistry and Biotechnology, Swinburne University of Technology, John Street, Hawthorn, Victoria, Australia 3122
[2] Gretals Australia, Unit 8 / 1140 Nepean Highway, Mornington VIC 3931

Gastrointestinal parasite infections of livestock are typically detected through routine testing using a variety of methods which differ in speed, cost, complexity or robustness. These methods include physical examination of animals, microscopic analysis (of tissues or faeces) and serology. As technology improves and availability increases, various methods have emerged, intended to improve one or more parameters of their predecessors. The use of molecular methods, such as polymerase chain reaction, is likely to increase in popularity with wider access and adoption. The development of methods utilising automated preparation and/or analysis has great potential for reliable, high-throughput testing.

Keywords: nematode; helminth; livestock; diagnosis

1. Introduction

Gastrointestinal nematodes (GIN) are responsible for millions of dollars of economic loss in the farming industry each year [1]. In livestock, these parasites cause numerous negative effects including reduced growth rates, productivity and product quality [2]. Untreated livestock are also at risk of malnutrition, anaemia and death [3]. Treatment with anthelmintics is used to remove or significantly reduce the worm burden faced by livestock. However, increasing drug resistance and calls to restrict usage of anthelmintics in order to extend their life span [4], have put greater emphasis on preventative and targeted treatment methods. Alternative practices are already essential to organic farming, where anthelmintic treatment is not permitted. In such an environment, efficient, low cost monitoring of livestock health is essential for timely infection detection and appropriate treatment. Interventions to minimise livestock and financial losses centre around understanding parasite life cycles, immune status and environmental interactions.

2. Detection methods

Early methods of GIN detection relied heavily on the presence of physical symptoms. The physical condition of livestock can be used as a preliminary indicator of infection, with testing of faeces (for excreted GIN eggs) and serum (for antibodies or antigens) used to confirm the diagnosis.

2.1 Physical examination

Physical inspection of livestock can identify animals most likely to be infected with a particular parasite. Multiple forms of inspection, including body scores, weight gain, productivity and other symptoms, may be used to select livestock for treatment [5, 6]. Diagnosis can be assisted using commercial products, such as FAMACHA© which uses a reference card to guide physical inspection. The check identifies small ruminants most likely to have a clinical infection with *Haemonchus*, using the internal eyelid colour to indicate the extent of anaemia [7, 8]. The test is rapid, simple, inexpensive and specific to blood sucking parasites. It is important to remember that other clinical issues may also cause anaemia in livestock, and that false positives and negatives are possible. Conflicting conclusions on the correlation between infection and the presence of diarrhoea have also been reported [2], with factors such as selective breeding, feed type and age all influencing the likelihood for diarrhoea to occur. This theme is repeated in physical symptoms in general, due to multiple factors enabling or inhibiting symptom presentation. Additionally, while such inspection methods are useful theoretically, it is necessary to consider that these methods may not be suitable depending on the animal species and farming practices. As such, physical examinations remain a highly beneficial method for early and rapid detection of clinical infections of nematodes in livestock. However, they only provide a presumptive diagnosis and typically require follow up with more technically sophisticated analytical tests.

2.2 Microscopic analysis

The only method that precisely quantifies a GIN infection is necropsy. However, for obvious reasons, it is not commonly used to monitor GIN infections. It is generally reserved for when the parasite burden results in drastic and sudden symptoms, including animal death [3]. The method allows for diagnosis of GIN infections where other symptoms have not had time to manifest due to the parasite's life cycle. When this is not the case, the use of non-invasive, rapid and inexpensive techniques has been favoured by farmers.

The faecal egg count (FEC) is a method commonly used to estimate the type and extent of GIN infections in livestock. This method is not used for exact numerical quantification, but rather relies on eggs per gram (EPG) thresholds to indicate the level of infection, namely:

- EPG < 200: Generally indicates there is no need to treat livestock
- 200< EPG <500: Requires consultation and additional advice
- 500< EPG: Indicates treatment is likely required

This approach affords the diagnostic method a degree of leniency between samples without drastically altering the result of testing, which is necessary as egg aggregation causes natural variation during sampling faeces. Other factors such as the specific species fecundity and farm history may alter decisions, with some species requiring higher sensitivity for reliable detection. The repeatability, precision and accuracy of FEC methods have been frequently scrutinised in the light of new technology and methods. Many modifications have been proposed to improve the test sensitivity and specificity including adjusting the composition of the egg flotation solution [9], introduction of filtration steps, duplicate testing, adjusting sample size and dilution factors [10-12]. For example, the direct centrifugation method uses centrifugal force and the specific gravity of the suspending medium to rapidly concentrate GIN eggs [13]. The Cornell-Wisconsin FEC is better suited to cattle due to the faecal composition, which uses filtration, flotation and centrifugation with a cover slide. One limitation to this approach is the small area of the menisci to collect eggs on the cover slide during centrifugation which is prone to spills due to human error. Compared to the McMaster method and FLOTAC (described below), this method had significantly lower yields [14]. An analysis of this method by Egwang & Slocombe [15] has also shown a percentage of eggs (31%) are retained in the straining process, partially explaining this observation despite the centrifugation concentration step. Most FEC methods require a light microscope and a counting chamber or slide, such as the McMaster and Kato-Katz apparatus. Both methods are simple, rapid and inexpensive, and require minimal training. The Kato-Katz method is generally favoured for human faecal samples as they contain less fibre. It uses a direct thick smear of a faecal sample on a glass slide for light microscopy analysis. Multiple slides may be used to improve sensitivity and no dilution is required, allowing for higher accuracy [16]. The McMaster method is better suited to ruminants, as flotation and/or filtration steps can be utilised to remove fibrous debris that would otherwise obscure eggs.

More robust forms of FEC include lectin binding, specifically peanut agglutinin lectin (PNA) conjugated with fluoroscein thiocyanate which binds to *H. contortus* [17], allowing for species quantification using microscopy [18]. While it is commercially available, the preparation is cumbersome [19] requiring incubation periods and filtration prior to analysis. There is a range of agglutinins that show binding affinity to one or more GIN species [20] indicating potential for growth in this avenue of diagnostics.

Table 1 provides a comparison of the detection and analysis methods described in this paper. In general, the lowest cost methods are more rapid but less informative. Method preference is largely influenced by availability and which of these available methods best reflects the priorities of the individual (i.e. farmer, veterinarian or researcher). Since a single method may not match an individual's priorities, methods may be performed in combination or in a sequential manner to better diagnose potential infections.

A common example of combination testing is the use of FEC together with larval culture or hatching assay to identify the causative genus or species [21] or drug resistance, respectively. Larval cultures require several days of incubation, but have a greater sensitivity to low level infections than traditional FEC testing [22]. Some bias for which species develops occurs according to the selected temperature, causing species proportions to potentially appear different to the fresh sample when compared with DNA tests, e.g. polymerase chain reaction (PCR) [23, 24].

Table 1 Comparison of gastrointestinal nematode detection methods.

Detection methods	Time[1]	Anthelmintic resistance[2]	Species identification	Quantitative	Cost[3]	Complexity[1]
Cornell-Wisconsin	++	FECRT	No	Yes	++	+
ELISA	++	-	Yes	Yes	++	++
FAMACHA©	+++	-	No	No	+++	+++
FECPAK	++	FECRT	No	Yes	++	++
FLOTAC	++	FECRT	No	Yes	+++	++
Kato-Katz	++	FECRT	No	Yes	+++	++
Larval culture	+	*	Yes	No	++	+
Lectin staining	+	-	*H. contortus*	Yes	+	+++
McMaster	++	FECRT	No	Yes	+++	++
Necropsy	+	-	Yes	Yes	+	+
PCR	++	+	Yes	Yes	++	+

[1] '+' = high, '++' = acceptable, '+++' = minimal

[2] '+' = Yes, '-' = No, 'FECRT' = Faecal egg count reduction test

[3] '+' = high cost >$10 each, '++' = acceptable cost $5 - $10 each, '+++' = low cost <$5 each, based on service costs listed by government services in Australia and testing equipment and material costs. Costs are based on the averages of commercially available costs for bulk orders for Australian State Services and online kits at the time of writing.

* May be conducted alongside an FECRT from an accompanying method

Other methods with low technology requirements have been tested for their potential to replace the Kato-Katz and McMaster methods in remote and low-income areas. The FLOTAC and FECPACK are two such methods that use their own set of equipment to conduct FEC testing. FECPAK aims to deliver an easily accessible product that utilises imaging technology to analyse samples. The preparation method involves a homogenisation step, a series of decanting steps and a filtration step which could theoretically be carried out directly by farmers. The method uses its own camera for image capture. FLOTAC utilises its own preparation device and counting chamber design; this device has a stronger focus on areas with limited access to technology. The newer Mini-FLOTAC device covers some of the initial design concerns of the FLOTAC and is more user-friendly than its predecessor. Several studies comparing FLOTAC, FECPAK and traditional McMaster methods in terms of their sensitivity, accuracy and precision have been published. FLOTAC, Mini-FLOTAC and FECPAK all boast greater precision and accuracy over the traditional McMaster method [25, 26]. This achievement was likely due, at least partially, to larger analysis volumes and longer preparation time [11, 27–28].

While even the best FEC quantification is not exact, the information from these tests is generally adequate to make an informed decision on the appropriate treatment requirements. These tests may be used to detect anthelmintic resistance through a faecal egg count reduction test (FECRT). FECRT samples taken before and several days after treatment can indicate resistance to the administered treatment. Results take several days as there is a waiting period; a more rapid result could be achieved through genetic methods such as PCR analysis for resistance genes. There are many existing reviews on global anthelmintic resistance [29-31], with a focus on determining the mode of resistance and the corresponding genes. Several methods for detecting for resistance in nematodes that may complement or replace FECRTs have also been reviewed and compared to address this concern [32, 33].

2.3 DNA amplification methods

Polymerase chain reaction (PCR) amplifies a DNA sequence(s) by orders of magnitude according to the primer pair(s) that initiate the *in vitro* polymerization reaction. Specific primers target various DNA fragments for parasite species identification or sequencing, which can identify genes associated with anthelmintic resistance [34]. Several variations of PCR techniques have been developed for species identification and quantification, including real time or quantitative PCR (qPCR), nested PCR (nPCR), multiplex PCR [35] and multiplex tandem PCR (MT-PCR) [36]. Of these, qPCR, multiplex PCR and MT-PCR are commercially available with automated procedures allowing for high throughput of samples. PCR method development is still prone to generic issues, including primer dimers and inhibitors within samples that can prevent enzymatic reactions. The numerous forms of DNA purification methods available prior to PCR analysis can alter the sensitivity. Sampling and preparation variations such as initial volumes, sedimentation steps and extraction method/kits used will have an impact. Most methods use DNA extracted from eggs or larvae collected from faecal samples. Using nematode eggs instead of larvae removes the need to incubate samples for several days. However, due to the multiplication of cells within eggs, older samples and may require an accompanying test such as an FEC to determine the age of the sample [37]. This is relatively easy to incorporate due to the manual preparations for PCR generally including a combination of filtration, flotation, sedimentation and DNA extraction.

qPCR utilises a fluorescently tagged primer in to detect and quantify the presence of helminths. This method can detect an infection approximately a week earlier from faecal samples than an FEC [38]. Multiplex PCR follows the same principles as qPCR [34], but uses multiple sets of specific primers in a single reaction. Multiplexing is subject to potential primer competition within similar target regions, limiting amplification of less abundant targets [35].

nPCR is completed in two sequential reactions to improve specificity of DNA amplification. The first reaction's primer set is non-species specific; product DNA is then purified for the second reaction whose primer set amplifies species/genus specific sequences for identification. This method has demonstrated 85% sensitivity to nematode species (*Nematodirus*, *Marshallagia* and *Teladorsagia*) tested for from larvae and 88% to singular egg samples [39].

MT-PCR utilises a combination of strategies described in the techniques above. An initial non-specific amplification as in nPCR is conducted for a short number of cycles in a single PCR, followed by several reactions in tandem with species-specific primers for identification. Correlation between larval cultures and egg based MT-PCR quantification was found to range from 68.4% - 94.7%, with 100% primer specificity [36]. MT-PCR has been automated (AusDiagnostics), and has been proposed as a replacement to larval cultures, where higher costs are offset by reduced time and labour requirements [37]. However, manual preparation of samples for the automated system is still required before high throughput processing overnight or while other samples are being prepared.

Loop-mediated isothermal amplification (LAMP) utilises isothermal reactions between 60 – 65 °C to amplify DNA. Unlike PCR, it does not require sophisticated equipment (i.e. thermocyclers) and is performed using standard laboratory equipment, such as waterbaths or heating blocks, thus reducing the equipment cost and creating the potential for onsite testing. LAMP uses four highly specific primers targeting six distinct sites within the DNA sequence and Bst DNA polymerase rather than the more common two primers and Taq polymerase used in PCR. Its sensitivity is as low as a few eggs [40] in a sample, but requires a higher number of cycles than other techniques to achieve this. A comparison of qPCR and LAMP has reported 89% and 76% sensitivity, respectively [19], with qPCR generally having a lower cycle threshold.

Preparation of samples for DNA amplification generally takes longer than for FEC. The additional time and costs associated with these methods are offset by the informative nature of the results. Hence, selection and adoption of these methods is based on personal preference and resource availability.

2.4 Serology

Serological techniques for detection and identification of GIN infections utilise a variety of biological fluids including blood, saliva, urine, excrement and milk. These analytical techniques take the form of an enzyme-linked immunosorbent assay (ELISA) targeting antigen from the parasites (direct ELISA) or host antibodies specific to the parasites (indirect ELISA). At the time of writing, most commercially available diagnostic kits were indirect ELISA tests targeting a single species or genus in a specific biofluid. Examples include *Ostertagia ostertagi* or *Fasciola hepatica* in cattle from milk (O. ostertagi-Ab, SVANOVIR®; *F. hepatica*-Ab, SVANOVIR® and Fasciolosis Verification Test, IDEXX, Hoofddorf), *Ascaris suum* in pigs from blood (A. suum-Ab ELISA, SVANOVIR®) and *Trichostrongylus* species in sheep from saliva (CARLA®, AgResearch Animal Health). The production of antibodies for direct ELISA can be considered inconvenient, due to idiosyncratic immune systems of production animals. The use of purified antigens to host antibodies is more consistent, thus more reliable for routine testing. Post infection, an ELISA can detect an infection approximately two weeks earlier than an FEC on average [38] and approaches 100% sensitivity at 8-10 weeks. ELISA screening has great potential to replace physical inspection such as liver condemnation for detection of *A. suum* and *F. hepatica*, due to its capacity to detect infection prevalence [41]. Some assays targeting nematodes such as *Ostertagia ostertagi* have demonstrated cross-reactivity with other species [42, 43], while other ELISAs lack or have low cross-reactivity [44, 45]. This is not ideal for species specific diagnostics but is not a problem when analysing for the total worm burden.

An alternative to ELISA is a dipstick method that has been developed to identify blood in faecal samples. The Haemastix® reagent sticks are used to detect faecal occult blood associated with haemonchosis [46]. However due to the nature of the test, other infections and diseases that result in blood in faeces, such as *F. hepatica*, reduce its specificity.

3. Future directions

The greatest potential to improve the throughput, consistency and cost of currently available technologies and techniques will come from automated analysis. Incorporation of automation into analysis can reduce training requirements, labour costs and allow analysts to focus on other tasks, improving the overall timeliness of testing. Many existing methods are likely to experience these benefits by incorporating automated processes into routine preparation and/or analysis steps, such as computer-based image processing and data analysis. Existing examples are the imaging device in FECPAK and the automated sample cycling and transfer management by Easy-plex PCR [36].

The use of mobile cameras as the detection device has been explored, especially in combination with fluorescence. A quantitative analysis of fluorescence of dual wavelengths has been achieved with a silver-coated glass slide, which enhanced fluorescent emission [47]. For molecular diagnostics, many ELISA techniques and agglutinin binding are specific to a limited set of GIN species. Extending the range of species targeted is an immediate area of improvement for these techniques. Simplification of the preparation process for the agglutinin binding method would improve accessibility of this method, as would a small device for viewing and/or imaging fluorescent eggs. Tags that act with similar specific binding as PNA could be used for rapid species identification and quantification during routine FEC testing.

Ongoing work to create more intuitive image recognition technology and the continual improvements in machine learning are central to accomplishing greater method robustness, and meeting high-throughput demands. High accessibility in terms of both location and resources, and providing a broader analytical range in a single method, will be key to the next generation of GIN analytical methods. This may include the use of mobile units and wireless services to improve accessibility, especially in remote regions.

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