

# Advances in the diagnosis of the gastrointestinal nematode infections in ruminants

## *Avanços no diagnóstico das infecções por nematódeos gastrintestinais em ruminantes*

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### Abstract

Enumeration of nematode eggs in fecal samples using the McMaster technique and morphological identification of third stage larvae from fecal cultures have been extensively used with satisfactory results in the diagnosis of the gastrointestinal nematode infections in ruminants. In order to improve sensitivity and accuracy, other approaches for quantification of eggs have been employed, like the FLOTAC and Mini-FLOTAC techniques. Results obtained in different studies indicate that fecal egg counts are a reliable measure of the size of the worm burden. However, the immunological status of the animals should be taken into consideration to interpret the results of the fecal examination. Molecular techniques have also been useful in the diagnosis of parasitic diseases. The ultimate in diagnosis has been the development of robotic platforms that enable separation of eggs from feces. Because manipulation is minimal, good quality DNA from eggs is obtained, which is used for amplification, and finally, produces a result indicating the degree of the infection by the different parasite species in mix infections. The ideal method should be reliable, friendly to non-experts and quick to perform. With the advance in robotics, bioinformatics and molecular biology, methods with such characteristics are expected to become available and affordable to be used in laboratories for the routine diagnosis of gastrointestinal nematodes of ruminants.

**Keywords:** *Haemonchus*. *Cooperia*. EPG. PCR.

### Resumo

A quantificação de ovos de nematoides em amostras de fezes e a identificação morfológica das larvas de terceiro estágio a partir de culturas de fezes têm sido amplamente utilizadas, com resultados satisfatórios para o diagnóstico das infecções por nematoides gastrintestinais em ruminantes. Com o intuito de melhorar a sensibilidade na detecção dos ovos, outras técnicas, tais como Flotac e Miniflotac, têm sido empregadas. Resultados obtidos em diferentes estudos indicam que a contagem de ovos nas fezes é uma medida confiável e representativa da magnitude da carga parasitária. No entanto, o estado imunológico dos animais deve ser considerado na interpretação dos resultados coproparasitológicos. As técnicas moleculares também têm sido úteis no diagnóstico das enfermidades parasitárias. Os maiores avanços no diagnóstico têm sido propiciados pelo desenvolvimento de plataformas robóticas, que separam os ovos das fezes com pouca manipulação, propiciando a obtenção de DNA de ovos de boa qualidade, o que resulta em uma amplificação que possibilita indicar o grau da infecção pelas diferentes espécies de parasitas em infecções mistas. O método ideal deve ser preciso, rápido e de fácil execução por não-especialistas. Com o avanço da robótica, bioinformática e biologia molecular, espera-se que equipamentos com tais características se tornem disponíveis e acessíveis para serem utilizados na rotina laboratorial para o diagnóstico de nematoides gastrintestinais de ruminantes.

**Palavras-chave:** *Haemonchus*. *Cooperia*. EPG. PCR.

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### Introduction

Gastrointestinal nematode infections are an important cause of reduction in the profitability of the ruminant industry due to impairment in body weight gain, reduction in milk and wool production. Cases of massive infection are relatively common and are an important cause of mortality, especially in young animals.

Nematodes are the major parasites with several species infecting cattle and small ruminants. In Tropical and Sub-Tropical areas of the world, strongyles are the most important group of parasites. The main species in cattle are *Haemonchus placei*, *Cooperia* spp. and *Oesophagostomum radiatum* (BRICARELLO et al., 2007; NEVES et al., 2014), while in small ruminants *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Oesophagostomum columbianum* are the most important parasites (AMARANTE, 2014). Several other species can also occur in ruminants with particular relevance of *Strongyloides* spp. and *Trichuris* spp., very common nematodes that present a worldwide distribution. In addition, other species like *Ostertagia ostertagi* and *Teladorsagia* (= *Ostertagia*) *cicumcincta*, respectively, in cattle and small ruminants are very important in temperate areas of the world (KNIGHT; BISSET, 2015).

Fecal examination has been the most important way to diagnose parasitic gastroenteritis in ruminants and also to check the efficacy of anthelmintic treatments. In recent decades, molecular methods have also been developed to be used in these diagnoses. This review addresses advances in the use of techniques to detect eggs in feces and also the most recent methodologies for diagnosis of parasitic infection based on detection of nematode's DNA in fecal samples of ruminants.

### Simple flotation for egg detection

A big step in the microscopic diagnosis of gastrointestinal nematodes was the observation that eggs of *Ancylostoma* floated in a salt solution of sufficient specific gravity (s.g.) and adhered to a glass surface (WILLIS, 1921). This approach allowed concentrating and separating the eggs from fecal debris and, in comparison with smears of feces, this procedure was rapid to perform and significantly increased the chance of finding eggs in fecal samples, especially if the individuals were harboring light infections. This flotation technique known as "Willis

technique", presents several modifications, and is still useful to detect infection in ruminants harboring light infections (BASSETTO et al., 2011; SANTOS et al., 2014b).

### Nematode fecal egg counts

#### *McMaster Technique*

Because the simple flotation technique just indicates the presence or absence of nematode's eggs in fecal samples, methodologies have been developed in order to allow the enumeration of the eggs per gram of feces (EPG). The most successful technique was developed in Australia by Gordon and Whitlock (1939). It is also based in the flotation of eggs in saturate solution of NaCl and presented as an innovation the McMaster chamber. This chamber, with a known volume capacity, allowed estimation of the number of eggs per gram of feces. The McMaster technique, as it is worldwide known, presents several modifications. In Brazil, most laboratories adopt the protocol found in the Ueno and Gonçalves's manual (UENO; GONÇALVES, 1998) that recommends mixing 4 g of feces with 56 ml of saturate solution (NaCl; s.g. = 1.2) for cattle samples or 2 g of feces plus 58 ml of NaCl solution for small ruminant samples. In this case, each egg found in cattle and small ruminant samples correspond, respectively, to 50 EPG and 100 EPG.

Obviously, the counting of eggs is justifiable only when it correlates with the total number of worms present in the gastrointestinal tract of the animal. Table 1 presents the correlation coefficients between worm counts and EPG using the McMaster technique. The data was obtained in trials carried out in different areas of the world using sheep naturally or experimentally infected by gastrointestinal nematodes. In most of the studies, a significant association was found between worm burden x EPG, meaning that fecal egg counts in most of the situations is a good indicator of the level of infection by gastrointestinal nematodes in sheep.

Table 1 – Correlation coefficients ( $r$ ) between numbers of eggs per gram of faeces (EPG) and worm burden in sheep

| <u>Major Parasites</u>  | <u>Type of infection</u>     | <u><math>r</math></u>   | <u>Reference</u>            |
|---|------------------------------|---|-----------------------------|
| <i>H. contortus</i>   | Serial artificial infections | 0.68 <sup>s</sup>   | Bricarello et al. (2005)    |
| <i>H. contortus</i> ; <i>T. colubriformis</i>   | Natural                      | 0.63 <sup>s</sup> in Santa Ines and 0.89 <sup>s</sup> in Ile de France breeds | Amarante et al. (2004)      |
| <i>H. contortus</i>   | Natural                      | 0.72 <sup>s</sup> in Corriedale and 0.68 <sup>s</sup> in Crioula Lanada breed | Bricarello et al. (2004)    |
| <i>H. contortus</i>   | Single artificial infection  | 0.70 <sup>s</sup>   | Amarante et al. (1999)      |
| <i>H. contortus</i>   | Natural                      | 0.83 <sup>+</sup>   | Roberts and Swan (1981)     |
| <i>H. contortus</i> ; <i>Ostertagia</i> ; <i>Trichostrongylus</i> ; <i>Nematodirus</i> ; <i>C. curticei</i>       | Natural                      | 0.82 <sup>#</sup>   | Douch et al. (1984)         |
| <i>O. circumcincta</i>  | Single artificial infection  | 0.63 <sup>+</sup>   | Stear et al. (1995)         |
| <i>T. circumcincta</i>  | Serial artificial infections | 0.83 <sup>#</sup>   | Beraldi et al. (2008)       |
| <i>T. colubriformis</i>   | Serial artificial infections | 0.79 <sup>+</sup>   | Berajaya and Copeman (2006) |
| <i>H. contortus</i> ; <i>O. circumcincta</i> ; <i>Trichostrongylus</i> ; <i>N. spathiger</i> ; <i>C. curticei</i> | Natural                      | 0.91 <sup>#</sup> and 0.85 <sup>#</sup> in each year of the study             | Bisset et al. (1996)        |

<sup>#</sup>Spearman's rank correlation coefficient; <sup>s</sup>Pearson's correlation coefficient; <sup>+</sup>Pearson's correlation coefficient calculated with log transformed data.

The studies with cattle also indicate that fecal egg counts are a reliable measure of the size of the worm burden in animals with mix infections (BYRAN; KERR, 1989). Bricarello et al. (2007) and Condi et al. (2009) reported a moderate association between worm burden and EPG in dairy calves ( $r = 0.65$ ) and Nellore young bulls ( $r = 0.47$ ), respectively. In these studies, cattle had a mix natural infection predominantly by *Cooperia* spp., *H. placei* and *O. radiatum*.

Fecal examination can present some limitations. Frequently, it did not indicate the presence of *Trichuris* spp. eggs in fecal samples of cattle, which, however, proved to be infected with this nematode at the post mortem examination of the large intestine

(CONDI et al., 2009; BASSETTO et al., 2014). Two reasons can be related to this problem of *Trichuris* spp. diagnosis: (1) animals shed a small number of eggs that is not detected by the McMaster technique; or (2) most of the eggs do not float in NaCl solution (s.g. = 1.2). Recently, while processing fecal samples of cattle using the FLOTAC method, José H. da Neves (personal information) observed that *Trichuris* eggs were not detected using NaCl solution (s.g. = 1.2). However, when he used zinc sulphate solution (ZnSO<sub>4</sub>; s.g. = 1.35) in order to detect *Dictyocaulus* larvae, a large proportion of animals presented *Trichuris* eggs. A similar problem might occur in infection by *Oesophagostomum columbianum*. This parasite

induces anorexia and diarrhea in sheep, both of which influence the numbers of parasite eggs per gram of feces. Moreover, *O. columbianum* appears to be sporadic in its egg laying habits, causing erratic daily fecal egg counts (DOBSON, 1974).

### **FLOTAC and Mini-FLOTAC Techniques**

In order to develop a technique with higher sensitivity and accuracy, CRINGOLI et al. (2010) developed multivalent techniques, denominated FLOTAC, for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. The FLOTAC apparatus consists of a cylindrical device with two 5-ml flotation chambers, which allows up to 1 g of stool to be prepared for microscopic analysis, i.e., it allows the quantification of 1 egg per gram of feces. The other advantage of the technique is that, besides the diagnosis of gastrointestinal parasites, it also allows the diagnosis of lungworm larvae (*Dictyocaulus* spp.) and trematode eggs (*Fasciola hepatica*). In the case of these last parasites, it is possible to simultaneously diagnose lungworm larvae and *Fasciola* eggs using a flotation solution with higher specific gravity, like zinc sulphate (s.g. = 1.35).

FLOTAC technique proved to present advantageous in comparison with the McMaster technique in a survey of anthelmintic resistance in cattle, because it allowed the use of animals with mild infections, shedding less than 50 EPG (NEVES et al., 2014). However, in comparison with the McMaster technique, FLOTAC is more time consuming and requires a centrifuge for plates. Taking these factors into consideration, a friendlier technique, Mini-FLOTAC, was developed by the same researchers (CRINGOLI et al., 2013). It does not require centrifugation and it has a good sensitivity, allowing detection of 10 eggs per gram of feces. The device includes two 1-ml flotation chambers designed for examination of fecal sample suspensions (total volume = 2 ml).

### **Fecal cultures for production of third stage larvae**

Different from the eggs of *Trichuris* spp., *Strongyloides* spp. and *Nematodirus* spp. that are easily identified due to their peculiar morphology, eggs of the majority of strongyles (*Haemonchus*, *Ostertagia*, *Trichostrongylus*, *Cooperia* and *Oesophagostomum*) present similar morphology (UENO; GONÇALVES, 1998). For this reason, the best way to interpret results of fecal examination is associating the values of fecal egg counts of the strongyles with the identification of third stage larvae produced in fecal cultures. Using this approach, it is possible to determine the level of infection by each nematode genus (AMARANTE et al., 2004).

Dikmans and Andrews (1933) and Keith (1953) presented, respectively, detailed descriptions about differentiation of the infective larvae of nematode parasites of sheep and cattle. Several other publications with details about the larvae morphology were published later. More recently Wyk et al. (2004) presented details summarizing the identification of nematodes infective larvae of cattle and small ruminants. In Brazil, the manual of Ueno and Gonçalves (1998) is the most used guide for making fecal cultures in order to produce third stage larvae as well as their identification.

There are also reports about the possibility of differentiation of *Haemonchus* species based on the morphology of the third stage larvae (AMARANTE, 2011). According to Santos et al. (2014a), the differentiation of *H. contortus* from *H. placei* based on morphometrics of the third stage larvae sheath tail is more precise than the morphological analysis of the adult males based on the morphometrics of spicules.

The usual recommendation is the identification of 100 third stage larvae of the strongyles with results being expressed in percentage. A frequent mistake is the inclusion of *Strongyloides papillosus* larvae identification among the results of the strongyles larval identification. The level of *S. papillosus* infection must be evaluated during the fecal egg counts, because it is

possible to differentiate its small embryonated egg from the morulated strongyle eggs. It is very important to take into consideration that *S. papillosus* presents a generation of free living adults that rapidly produce eggs that results in infective larvae in fecal cultures. For this reason, even cultures made with samples with small number of *S. papillosus* eggs may result in production of large amount of *S. papillosus* third stage larvae. Therefore, *S. papillosus* do not need to be included in the percentage of nematode larvae identified in fecal cultures (UENO; GONÇALVES, 1998). Similarly, the big *Nematodirus* eggs can be easily enumerated during the fecal egg counts as well as the *Trichuris* eggs. In conclusion, only the percentage of strongyle larvae should appear in results of fecal cultures.

### ***Influence of the rate of infection and the immune response on the fecal egg counts***

The parasite-host relationship mediated by the immune response is dynamic and has major implications in fecal egg counts and in the outcome of parasitic diseases. Young animals are the most susceptible category and when exposed to high rate of infection, they usually present clinical signs of parasitism associated with high number of EPG. If the animals recover, due to development of the immune response, a reduction in EPG follows. Continuous infection with small number of larvae may induce the development of an efficient immune response, especially when the animals are kept in good nutritional conditions, which leads to low values of EPG. However, malnutrition is also frequently associated with animals displaying high FEC in Brazil.

There are some animals, even adults, that are unable to respond efficiently against the nematode infections. For this reason, parasite burdens present an over dispersed distribution: most hosts carry few parasites, while a few heavily infected hosts harbor a large proportion of the total parasite population. This aggregated distribution (negative binomial distribution) has been demonstrated in grazing sheep

and cattle (BARGER, 1985; BRICARELLO et al., 2007) and also in sheep that received serial experimental infections with *T. colubriformis* (CARDIA et al., 2011) or *H. contortus* (BRICARELLO et al., 2005). Therefore, it is expected that in a group of ruminants, most of them display low FEC while a few show high values.

Due to the immune response, the infective larvae ingested may be eliminated as well as the adult worms established in the gastrointestinal tract. In addition, worms may present arrested development and may display reduction in size and reduction in egg production (CARVALHO et al., 2015). All these events can result in low fecal egg output with several animals in the herd showing “zero” EPG in the McMaster technique. However, with the use of a more sensitive technique, it can be demonstrated that most of this “resistant animals” shed eggs in feces, i.e., they are infected (NEVES et al., 2014). Any stressful situation can result in an increased susceptibility that may result in high FEC values. Due to relaxation of the immune response, cows and ewes usually present a sharp increase in fecal egg counts at the periparturient period (GENNARI et al., 2002; ROCHA et al., 2004).

Usually animals displaying clinical signs of hemonchosis (anemia and bottle jaw) present high FEC. However, in some circumstances, animals with high fecal egg counting (> 5000 EPG) do not present clinical signs of parasitism. This happens with resilient animals kept in good nutritional conditions. The losses caused by the parasites are compensated and an equilibrium in the host x parasite is established. For this reason, high fecal egg counts and/or worm burden sometimes are not associated with clinical signs of parasitic gastroenteritis. However, it is important to note that under stressful situations, like shortage of food supply or during the peripartum period, the intensity of the infection might be exacerbated with consequent clinical manifestation of the parasitic infection. Therefore, the interpretation of the results of the fecal examinations should also take into

consideration the age, breed, nutritional condition and physiological status of the females.

### ***Molecular techniques***

Molecular techniques have played an important role in biological studies allowing the understanding of the molecular and genetic processes involved in the host – parasite interaction. Logically, it has been also useful in the diagnosis of parasitic diseases. In the case of the gastrointestinal nematode infections, the advances of the methodology over time are presented in Table 2. The first approaches tested resembled the first techniques employed to detect eggs in feces (like the Willis technique), indicating only the presence or absence of the parasite DNA in a sample.

The main molecular approaches used for detection of genetic variation, identification and/or diagnosis in nematodes of livestock involved methods based on PCR and sequencing (GASSER, 2006; GASSER et al., 2008). Additionally, genus- or species-specific PCR primer pairs have been used to detect polymorphisms (SCHNIEDER et al., 1999; WIMMER et al., 2004).

Nuclear and mitochondrial genomes are important sources of DNA markers that have been used for nematode identification of species or strains. Because mitochondrial DNA (mtDNA) evolves rapidly, it was observed that the difference among closely related species of nematodes is in the 10–20% range. Then, mtDNA has been successfully used to identify members of the same interbreeding populations and cryptic species (BLOUIN et al., 1998). In contrast, DNA sequences of the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) or external transcribed spacer (ETS) of nuclear ribosomal DNA (rDNA) have been used extensively for nematodes identification given their abundance within all genomes (ZARLENGA et al., 2001; SAMSON-HIMMELSTJERNA et al., 2002; GASSER et al., 2008; AMARANTE et al., 2014). ITS-1 and ITS-2 often present less sequence variation among individuals within a population and between populations.

According to Gasser et al. (2008), the magnitude of sequence variation in both rDNA regions within a species is considerably less (usually < 1.5%) than the levels of sequence differences among species. That trait allows the specific identification of strongylids and consequent diagnosis of infections of livestock, including species of *Haemonchus*, *Teladorsagia*, *Ostertagia*, *Trichostrongylus*, *Cooperia*, *Nematodirus*, *Bunostomum*, *Oesophagostomum* and *Chabertia*, (reviewed by GASSER, 2006; GASSER et al., 2008).

Different from the diagnosis in tissue parasites, in which only the parasite and the host DNA are expected to be present in a sample, in the case of the gastrointestinal nematodes, the eggs are in the feces, which presents heavy “contamination” with DNA of an immense bacteria flora. In addition, feces might contain several inhibitor substances of the PCR reactions. For this reason, most of the procedures employed DNA extracted from eggs or larvae, i.e., some parasitological technique had to be used in order to obtain “clean eggs” or to produce larvae in cultures (Table 2). However, with the use of specific kits for extraction of DNA from feces, *Strongyloides* infection in rats was diagnosed by PCR (MARRA et al., 2010).

The development of the multiplex PCR gave the opportunity to diagnose in a single reaction different nematode genera and/or species (ZARLENGA et al., 2001). Such approach, associated with the development of the Real-time PCR assays, created the opportunity to refine the diagnosis incorporating quantification of the DNA in a given sample, i.e., indicating the proportion of each genus or species in each sample (SAMSON-HIMMELSTJERNA et al., 2002; BOTT et al., 2009). In this case, one problem to take into consideration is the development of the eggs in the fecal samples. If not properly preserved, the eggs develop and the cell multiplication will result in an increase of the DNA contents, which will have influence in a quantitative PCR (SCHNIEDER et al., 1999).

The most recent advances have been the development of robotic platforms that allow

processing the stool sample with minimum manipulation. An automated diagnostic platform for veterinary pathogens, denominated Easy-Plex™ multiplex tandem PCR kits has been available ([www.ausdiagnostics.com](http://www.ausdiagnostics.com)), which allows the diagnosis of the major nematodes of sheep and cattle.

The ultimate in diagnosis has been the development of robotic platforms that make possible separation of eggs from feces, obtaining good quality DNA from eggs for amplification, and finally, produce a result indicating the degree of the infection by the different parasite species that commonly cause mix infection (ROEBER et al., 2012). The ideal method should be reliable, friendly to non-experts, quick to perform and affordable. With the advance in robotics, bioinformatics and molecular biology, methods with such characteristics are expected to become available and affordable to be used in laboratories for the routine diagnosis of gastrointestinal nematodes of ruminants.

## Final Comments

Enumeration of eggs in fecal samples and morphological identification of third stage larvae from fecal cultures have been extensively used for several decades with satisfactory results. There is an association between the FEC and worm burden. However, the immunological status of the animals should be taken into consideration to interpret the results of the fecal examination. Recent advancements in molecular biology, bioinformatics and robotics will possibly refine the diagnosis of gastrointestinal nematodes of ruminants, with reduction in the labor involved in such activities. Based on the technological advancements in other areas, it is possible to predict that such methodologies will progressively improve and result in reduced costs.

Table 2 – Molecular methods developed to diagnosis gastrointestinal nematodes of ruminants

| Methodology   | Source of parasitic DNA for diagnosis              | Species diagnosed                    | Comments   | Reference                          |
|---|--|--------------------------------------|--|------------------------------------|
| PCR   | eggs or larvae                                     | Ost, Coop, Nema, Haem, Tricho        | Single eggs or larvae could be differentiated to genus level without previous DNA extraction | Schnieder et al. (1999)            |
| PCR-based assay using species-specific primer pairs | eggs and cultured larvae                           | Bt, Co, Df, Nb, Nf, Ta, Tc, Tv, To   | Qualitative  | Wimmer et al. (2004)               |
|   | adult worm   | Cc                                   | Qualitative  | Amarante et al. (2014)             |
| Multiplex PCR test                                  | eggs   | Oo, Hp, Or, Co, Tc                   | Qualitative  | Zarlenga et al. (2001)             |
| Real-time PCR (RT PCR)                              | first stage larvae derived from overnight cultures | Hc, Ol, Tcol, Cc                     | Quantitative assays based on genus-specific primer and probe combinations                    | Samson-Himmelstjerna et al. (2002) |
| RT PCR and melting-curve analysis                   | eggs   | Hc, Tc, Tricho spp., Co, Oc, Ov, Co  | Semi-quantitation of parasite DNA in faeces  | Bott et al. (2009)                 |
| Semi-automated, multiplexed-tandem PCR platform     | Undeveloped eggs                                   | Hc, Tc, Tricho spp., Co, Ov          | Estimate the proportion of eggs of the different species/genera in a sample                  | Roeber et al. (2012)               |
| RT PCR  | Eggs from crude faecal egg preparations            | Trichostrongylids                    | For identification of species or resistance alleles, using different post PCR methods        | Demeler et al. (2013)              |
| Loop-mediated isothermal amplification              | Amplification from relatively crude samples        | Hc                                   | Allows detection of <i>Haemonchus</i> in a faecal samples containing two eggs per gram       | Melville et al. (2014)             |
| A closed-tube RT PCR                                | L3 larvae  | Hc, Tc, Tcol, Ns, Ov, Ta, Tv, Cc, Co | Identification of individual strongylid nematode larvae                                      | Knight and Bisset (2015)           |

Bt, *Bunostomum trigonocephalum*; Cc, *Cooperia curticei*; Co, *Chabertia ovina*; Co, *C. oncophora*; Df, *Dictyocaulus filiaris*; Hc, *Haemonchus contortus*; Hp, *H. placei*; Nb, *Nematodirus battus*; Nf, *Nematodirus filicollis*; Ns, *Nematodirus spathiger*; Oo, *O. ostertagi*; Oc, *Oesophagostomum columbianum*; Or, *O. radiatum*; Ov, *Oesophagostomum venulosum*; Ol, *Ostertagia leptospicularis*; Ta, *Trichostrongylus axei*; Tc, *Teladorsagia circumcincta*; Tcol, *Trichostrongylus colubriformis*; Tv, *Trichostrongylus vitrinus*; To, *Trichuris ovis*.



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