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Review

Review on anthelmintic resistances and its detection method on gastrointestinal nematodes of small ruminants

Mekonnen Gebrekrustos Haftu*, Desalegn Meresa, Etsay Kebede and Birhanu Hadush

Department of Tropical Veterinary Medicine, Mekelle University College of Veterinary Science, Ethiopia.

*Corresponding author. Email: mekonnen.gebrekrustos@mu.edu.et; Tel: +251920427145.

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ABSTRACT: Antihelmintic resistance is a genetic change in the ability of an individual parasite to survive the recommended therapeutic dose which is caused by factors like under-dosing, frequent drug exposures at short intervals, continuous use of drugs with a similar mode of action and treatment when parasites have small refugee. The objective of this review article is to fill the gap of knowledge on methods of detection against antihelmintic resistance of gastrointestinal nematodes of small ruminants by using different approaches like: *In vivo* (fecal egg count reduction test, controlled efficacy test), *in vitro* (egg hatch assay, larval paralysis, larval motility assays, larval development assay, adult development assay, larval feeding inhibition assay, biochemical assays) and molecular assays (PCR assays). Their main comparison between *in vivo* and *in vitro* tests are, *in vivo* tests do involve slaughtering the animals for the test, sensibility, specify, reputability and easiness to develop. The molecular and genetic probes capable of determining individual susceptibility and complimentary use of some of them along with their use on a suspect would allow for an increase of sensitivity were finally recommended.

Keyword: Anthelmintic resistances, nematodes, small ruminants.

INTRODUCTION

Gastrointestinal nematodes are a significant threat facing today's small ruminant producer because of its irreversible damage or even death to the animal, reduced performance and economic loss for the producer. The most common gastrointestinal nematodes that affect small ruminants in Ethiopia are Haemonchus, Trichostrongylus, Ostertagia, Cooperia, Bunostomum, Oesophagostomum, Chabertia, and Nematodirus due to the availability of suitable agroclimatic factors for diversified vertebrate hosts (Seyoum et al., 2018). Prevention and control of the parasites that infect sheep and goats are becoming increasingly difficult due to increased resistance by parasites to common anthelmintics (Taylor et al., 2016). Antihelmintic resistance is a genetic change in the ability of an individual parasite to survive the recommended therapeutic dose. Anthelmintic resistance or multi-drug resistance (MDR) has been made in every livestock host and to every anthelmintic class which is caused by factors like under-dosing, frequent drug exposures at short intervals, continuous use of drugs with a similar mode of action and treatment when parasites have small refugee (Kaplan, 2004).

The utilization of anthelmintics started in the middle of the 20th century with the introduction of phenothiazine and piperazine. However, currently, there are 3 classes of anthelmintics commonly used in small ruminants: benzimidazoles (including albendazole, fenbendazole), cholinergic agonists (including levamisole/morantel), and the macrocyclic lactones or avermectins and milbemycins (including ivermectin, moxidectin) (Sangster and Gill, 1999), and resistance to the major classes of anthelmintics has been recorded in Ethiopia (Seyoum et al., 2017), Canada (Falzon et al., 2013), North America (Uhlinger et al., 1992), Latin America (Eddi et al., 1996), Europe (Lambertz et al., 2019; Coles et al., 2006; Mickiewicz et al., 2017; Geurden et al., 2012; Chartier et al., 2001; Sutherland et al., 2008; Peña-Espinoza et al., 2014), Australia (Rashid et al., 2018; Roeber et al., 2013), Asia

(Sani et al., 2004b).

The current financial and agriculture losses caused by parasites have a substantial impact on farm profitability. For example, the annual cost associated with parasitic diseases in sheep and cattle in Australia has been estimated at 1 billion dollars (Roeber et al., 2013). The estimated treatment cost alone for *H. contortus* per year in Kenya, Nigeria, South Africa (Van Wyk et al., 1999), and India is estimated to be the US $26, US$ 40, $46 and $103 million, respectively (Sani et al., 2004a). In Ethiopia, a loss of US$ 81.8 million is reported annually due to helminth parasites of all animals (Biffa et al., 2006) and losses are proposed to be tens of billions of dollars worldwide, according to the sales of anti-parasitic compounds by pharmaceutical companies, excluding production losses. Thus, besides these losses, the massive use of anthelmintics is a paradox that increases, the impact on the economic gain of small ruminants. In spite of many insights into the epidemiology and ecology of parasitic nematodes, there are still significant gaps of knowledge in anthelmintic resistance for nematodes, mainly due to a lack of accurate and precise diagnostic tools in the past because of this there is a need to review a wide variety of parasitological (*in vivo and in vitro*), biochemical and molecular techniques available for the detection of anthelmintic resistance on small ruminants.

GENERAL PRE-TREATMENT EXAMINATION FOR GASTROINTESTINAL NEMATODE

As a pre-treatment, gastrointestinal nematode examination is normally conducted by taking a fecal sample directly from the rectum of the individual animal and put in icebox before treatment and day 14 post-treatment. The fecal examination follows the standard procedure described by Coles et al. (2006). The identification of species of nematodes are conducted through copro culture, microscopic studies of their eggs and counted EPG assessed by a modified McMaster technique (Coles et al., 2006). Peanut Agglutinin Staining (PNA) are perform to detect Haemonchus contortus eggs and from all herds with a mean FEC ≥ 300 EPG (Holm et al., 2014).

In vivo detection methods of anthelmintic resistance

Fecal egg count reduction test (FECRT)

The test relies on the relationship between the egg count and the worm burden. The fundamental principle of this test is to compare mean egg counts in a group of animals before and after treatment with an anthelmintic. It is now considered important to also undertake pooled larval cultures of each group before and after treatment (McKenna, 1997). The efficacy can be calculated by a variety of different approaches (Miller et al., 2006) but the more commonly used method is to compare the mean of egg counts 7 to 14 days post-treatment (Dangolla et al., 1997). Resistance occurs when there is a percentage of reduction in egg count is less than 95% and, the lower limit for its 95% confidence interval is equal or below 90% (Coles et al., 2006). Fecal egg count reduction test is particularly suitable for field surveys and a number of groups can be increased if appropriate, to test the efficacy of a range of broad or narrow spectrum anthelmintics at one time except with one limitation of lack of a common criterion for interpreting the results (Levecke et al., 2018). Consequently, it is difficult to compare results from different studies (McKenna, 1997).

Controlled efficacy test (CET)

The controlled efficacy test is the gold standard and fundamental test for the efficacy of a product (Wood et al., 1982). However, the test requires a large number of animals to be killed in order to test for resistance (Le Jambre et al., 1976). Animals are usually grouped into a control and a test group, with at least six animals per group. Animals are infected with L_3 about 21 to 28 days before they are treated with the anthelmintic whilst the control group is not treated. However, alternatively naturally infected animals can be used. Animals should be held in a facility where they cannot become re-infected after treatment. Animals in each group are slaughtered 10 to 14 days after treatment and the worm burden is estimated. The formula (Control -Treatment)/Control x 100 are used, where Control and Treatment are the arithmetic mean of the counts from untreated and treated groups respectively (Peña-Espinoza et al., 2014). Basically, the procedure compares the worm burdens of animals artificially infected with susceptible or suspected resistant isolates of nematodes. The parasitized animals are randomly separated into medicated and non-medicated groups and at a suitable interval after treatment (10 to 15 days), a necropsy is carried out and the parasites are recovered, identified and counted. This test is not extensively used, except in cases of special interest or when confirmation of resistance is required at the species level, and for evaluation of the effect on larval stages (Salisbury and Arundel, 1970).

In vitro detection methods of anthelmintic resistance

Several different *in vitro* tests are available but the majority is almost exclusively used for research purposes (Demeler et al., 2010). Tests can be used to quantify the level of resistance but they require considerable technical expertise and, in some cases, expensive laboratory equipment. Ideally, these tests require mono-specific infections because there can be difficulties in the interpre-

tation of results with field infections, which usually consist of multiple parasite species. The maintenance of standard laboratory strains, both drug-susceptible and resistant is necessary for comparative purposes (d'Assonville et al., 1996).

Egg hatch assay (EHA)

Egg hatch test has been developed to differentiate between resistant and susceptible strains of gastrointestinal nematodes for the benzimidazoles and for the levamisoles. It provides an accurate method for assessing the susceptibility of mixed nematode populations, and it is comparatively more rapid and economic to conduct than the Faecal egg count reduction test. It is based on the determination of the proportion of eggs that fail to hatch in solutions of increasing drug concentration in relation to the control wells, enabling the user of the test to develop a dose-response line plotted against the drug concentration (Demeler et al., 2012). To obtain meaningful data, eggs for the egg hatch test must be fresh and should be used within three hours of being shed from the host, as sensitivity to some benzimidazoles decreases as it is embryonated. The test has only been shown to work on nematode species in which eggs hatch rapidly. Due to difficulties in the interpretation of the results this assay is not widely used for field surveys (Dobson et al., 1986).

The test is based on the ovicidal properties of anthelmint. The principle is that the resistant genotypes can be embryonated and hatch in higher concentrations of the drug compared to the susceptible genotypes. The test is to incubate eggs at 26°C for 24hours, in serially diluted solutions of anthelmint, together with control as a correcting factor to establish the natural hatch rate of the eggs (Le Jambre, 1976). The limitation to EHA is that it is only suitable for the benzimidazoles and the eggs need to be as fresh as possible, preferably from feces collected directly from the rectum. An important idea, in this case, is that eggs should be used not more than one hour after they have been collected (Hall et al., 1978) and that they have not commenced aerobic development. However, various authors have described other situations under which an exception to the use of fresh eggs applies (Calvete et al., 2014).

Larval paralysis and motility assay (LPAMA)

Larval paralysis and motility assays measure the ability of anthelmintics to paralyze the infective third larval stage. Therefore, the overall principle of these assays relies on the assessment of larval motility. A variety of methods for larval paralysis and motility assays have been described. The first assays described were for levamisole and morantel (Martin and Jambre, 1979). The procedures for larval paralysis assays involve recovering the larvae from fecal cultures and incubating them in a serial dilution of anthelmintics for 24 hours. The larvae are then examined using a microscope at 100x magnification, to establish whether they are normal (moving) or paralyzed (not moving). The percentage of paralyzed larvae is then estimated and LD_{50} values determined (Várady and Čorba, 1999).

Despite the potential usefulness of these approaches to measuring motility to test for resistance, in all the methods, there is the limitation that too few larvae produce insufficient movement or significant effect, whilst too many larvae also move the dead ones and lead to exaggerated results. It is relatively easy to carry out, stocks of infective larvae are readily obtained and it is reported that there is a fairly good reproducibility of the test, any differences in repeatability being attributed to the age of larvae (Geerts et al., 1989). However, the interpretation is complicated by the fact that if the antihelmintic is added to the egg suspension too early, the development has not proceeded far enough; if it is added too late the drug has no effect. A modification of the technique was developed using the micro-motility meter, an instrument for measuring the motility of larval and adult nematodes after incubation with benzimidazole and levamisole. A further modification of the larval paralysis assay has been made in order to apply it for the detection of thiabendazole resistance (Martin and Jambre, 1979).

Tubulin binding assay (TBA)

This test is based on the mode of action of the drugs. The mechanism of benzimidazole resistance appears to be associated with a reduced affinity of tubulin for the anthelmintics. The test is based on the differential binding of benzimidazoles to tubulin, an intracellular structural protein from susceptible and resistant nematodes. The test involves the incubation of a crude tubulin extract from adult parasites, infective larvae or eggs, with a tritiated benzimidazole until equilibrium is reached. The free, unbound drug in test suspension after incubation is removed using charcoal, and the tubulin-bound label is sampled and counted by liquid scintillation spectrophotometry (Johansen and Waller, 1989). Tubulin extracts from resistant parasites bind substantially less strongly than do those from susceptible parasites. The test is claimed to be rapid, robust, highly reproducible and sensitive to minor changes in the resistance status of parasite populations, but it requires relatively large numbers of larvae, making it unsuitable for routine field assays. Moreover, it requires access to expensive laboratory apparatus for high-performance liquid chromatography (HPLC) estimations and a source of a radiolabeled drug (Taylor et al., 2002).

Larval development assay (LDA)

The larval development tests are the only ones that allow the detection of resistance against all the drugs, irrespective of their mode of action. Several methods have been described, but reproducibility, the linearity of the dose-response and susceptibility differ. The LDA is an *in vitro* assay for the detection of resistance to benzimidazole, levamisole, combinations of benzimidazole and levamisole, and avermectin and milbemycindrenches in the major gastrointestinal nematode parasites of sheep, *H. contortus*, *T. colubriformis*, and *O. circumcincta*. In this test, nematode eggs isolated from fecal samples submitted by producers are applied to the wells of a microtiter plate and larvae hatch and develop to the L_3 stage in the presence of anthelmintic. The concentration of anthelmintic required to block development is related to an anticipated *in vivo* efficacy (Amarante et al., 1997). The basic principle of the LDA test is to culture eggs through to the L_3 stage in the presence of serially diluted anthelmintics. The actual time of sampling in this test is not essential, although fresh eggs could give better results. The proportions of larvae, which successfully develop to the L_3 stage, are plotted against the $\log_{10}$ of the concentration of anthelmintic and a dose-response curve is then developed. The LD_{50} values of anthelmintics can then be estimated and used for interpretation of the results (like minimum inhibitory concentration (MIC)- minimum drug concentration required to completely inhibiting larval development) (Levine, 1968).

Larval feeding inhibition assay (LFIA)

It has been used to detect resistance to Macrocyclic lactones (ML) and Levamisole (LV) in sheep trichostrongylids and it relies on the ability of ML and LV to paralyzes the pharyngeal muscles and thus prevent feeding. This test is based on the study of the reduction of food ingested by the first larval stage (L_1). Hatched by incubation at 22^0C for 24 hours and then incubated at 25°C for a further 18 hours in serial dilutions of anthelmintic together with fluorescein-labeled *E. coil* (Jackson, 1993). Larvae are then transferred to a standard microscope slide and examined under a fluorescent microscope to see if fluorescein-labeled *E. coil* is visible in the intestine, and if seen, this indicates the larvae still feeding. Control wells containing no anthelmintics are used for comparison. The proportion of non-feeding larvae estimated can then use to develop a dose-response curve of larval feeding inhibition against the log concentration of the anthelmintic. The concentration inhibiting feeding by 50% (IC_{50}) can then be estimated, the disadvantages of using LFIA are difficult in Identifying the L_3 the cultures since larvae are not left to develop all through to larvae (which is used for morphological identification) and that this assay requires access to a fully susceptible strain for comparison (Álvarez et al., 2005a).

Adult development assay (ADA)

Unlike most *in vitro* assays, the adult development assay involves culturing eggs completely through to the adult stage, instead of only to the infective larvae. There are several reports that *H. contortus* has been cultured through to the sexually mature stage (Small and Coles, 1993). Later, Taylor et al. (2007) successfully cultured the larvae of other nematodes to the adult stage. However, only minor progress has been achieved in trying to develop this method to test for resistance in nematodes. Failure in this can be generally related to the complexity of the culture technique which suggests a limitation to further development of this method. The adult development assay for detecting benzimidazole resistance in trichostrongylid nematodes has advanced significantly and *Haemonchus contortus* has been cultured through to the adult egg-laying stages, although this test is mainly for research purposes (Jackson and Coop, 2000).

Molecular tests

Benzimidazole (BZ) resistance in nematodes seems to be best understood at the molecular level, whilst much less is known about resistances against other classes of anthelmintics (Taylor et al., 2007). Originally, a single nucleotide polymorphism (SNP) at codon 200 of the beta-tubulin isotype 1 was believed to be linked to BZ resistance (Wolstenholme et al., 2004; Driscoll et al., 1989) and has been demonstrated in resistant strains of *H. contortus*, *T. colubriformis*, and *O. circumcincta* (Elard and Humbert, 1999) in sheep. At least two more SNPs at codons 167 and 198 have been detected, but appear to be less common in different species of trichostrongylid nematodes (Palcy et al., 2010; Ghisi et al., 2007; Wolstenholme et al., 2004). Recent work has also suggested a link to the drug transporter P-glycoprotein, proposed to play a role in the transport of an anthelmintic away from its site of action and may also select for resistance to Macrocyclic lactones (Beech et al., 2011; Blackhall et al., 2008).

Allele-specific PCRs were developed to determine the BZ-resistance genotype of adult worms of *H. contortus* and *O. circumcincta*. This work was extended by combining the previously described PCR assays with an RFLP method (Silvestre and Humbert, 2000): which allowed the phonetic characterization and identification of L_3s of *H. contortus*, *T. colubriformis*, and *O. Circumcincta* designed a real-time PCR (RT-PCR) assay to assess the frequency of the beta-tubulin isotype 1 allele (linked to codon 200) in nematode samples (Álvarez et al., 2005b). Flow cytometry could be applied to the analysis of nematode populations. Forward-scatter emission can be used as a discriminating parameter

for egg size. The hatching rate and side scatter emission have a significantly positive relationship. The rate of resistance to the anthelmintic can be observed as a significant regression on the native green- fluorescence pulses that might reflect the state of oxidation of associated Flavin molecules (Sargison et al., 2019).

Research and development of new tests lately, gene probes, allele frequencies, transmembrane functional analysis, PCR, and flow cytometry have been investigated as tools for the determination of anthelmintic resistance. Even though, there is a limitation in the use of these procedures are exclusively for research purposes (Levecke et al., 2018).

CONCLUSION AND RECOMMENDATION

Anthelmintic resistance of gastrointestinal nematodes of a small ruminant is becoming a problem worldwide. Most of the nematodes developed resistance for almost all kinds of antinematodal drugs. Lack of best control strategies and lack of knowledge of method of early detection by *in vivo* (fecal egg count reduction test, controlled efficacy test), *in vitro* (egg hatch assay, larval paralysis, larval motility assays, larval development assay, adult development assay, larval feeding inhibition assay, biochemical assays) and molecular assays (PCR assays) made the easy occurrence of anthelmintic resistance. Even though most of the methods described in this paper lack sensibility and have a different mechanism (making hangry, paralyzed, non-motile and inhabit reproduction), they are easy to develop. To detect suspect, it is advisable to monitor the efficiency of anthelmintics after each treatment. This is an easy task as only the sampling of a random sample of animals before and after the anthelmintic treatment is needed.

If a parasite within a population does not respond uniformly to commonly used anthelmintics as observed, the following recommendations made:

1. An appropriate method of detection for a drug with a different mechanism of action should have to be implemented.
2. The complementary use of some of them along with their use on a suspect would allow for an increase of sensitivity.
3. The development of molecular and genetic probes capable of determining individual susceptibility to a drug should have to be developed.
4. Use a fecal egg count reduction test for field surveys.
5. Use larval development tests for the detection of resistance against all the drugs because it works irrespective of their mode of action.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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