Invited lecture/Review

Phenotypic and Genotypic Analysis of Anthelmintic Resistance

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Abstract:
A growing issue on a global scale is the emergence of helminth species and populations that are resistant to one or more anthelmintics. The majority of currently available anthelmintics used to control parasitic nematodes of cattle and sheep belong to only three main groups, benzimidazoles, imidazothiazoles and avermectins/milbemycins. The availability of reliable and precise techniques for its identification and monitoring is a critical component of the success of helminth control programs intended to prevent the spread of resistance in nematode populations. *In vivo* method like fecal egg count reduction test and *in vitro* methods such as egg hatch assays, larval motility test, larval development test and polymerase chain reaction (PCR) can be used for the detection of anthelmintic resistance although each has some reliability, repeatability, sensitivity, and ease of interpretation issues. The genetic basis of resistance to the majority of anthelmintics are still not well understood. Thanks to recent developments in high-throughput sequencing, it is now possible to define features such as drug resistance using genome-wide techniques.

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1. Introduction

Helminths are a group of worms which cause a major health problem to humans and animals worldwide. These health impacts include significant production losses and death in livestock, as well as weight loss, anaemia and death in companion animals, and morbidity in humans.
Even though controlling pastures for domestic animals could reduce the impact of parasites, these techniques are not adequate to get rid of these parasites (Shalaby, 2013). Currently, anthelmintic drugs are the basis for management of infection caused by helminths, and will probably remain so in the future due to the general lack of antiparasitic vaccines (Workye et al., 2021). Due to its extraordinary effectiveness—which has resulted in a parasite reduction of over 95%—generally acceptable safety margins, broad-spectrum nature, and affordable pricing of anthelmintics, anthelmintics has had great success over the past 50 years (Potârniche et al., 2021). Unfortunately, the extensive administration of anthelmintic medications has resulted in a major and dramatic development of anthelmintic resistance (AR), primarily in the gastrointestinal nematodes (GIN) of cattle, sheep, goats, and horses. Resistance to anthelmintics is also increasingly reported in canines (Kopp et al., 2007).

At least hundreds of millions of sheep, goats, and cattle receive anthelmintic treatments every year to treat or prevent illness. Chemicals from the same major drug classes are utilised across all three areas; benzimidazole drugs (e.g. albendazole) are widely used for the control of GIN parasites of livestock, hookworms in companion animals, and human soil-transmitted helminths (STH). Macrocyclic lactones (avermectins, e.g. ivermectin, and milbemycins, e.g. moxidectin) are used for GIN and ectoparasite control in livestock, as well as heartworm prevention in companion animals, and onchocerciasis, lymphatic filariasis, strongyloides and scabies in humans. Pyrimidines (e.g. pyrantel) are commonly used for the control of GIN in companion animals and occasionally in humans for control of STH. Levamisole (an imidazothiazole) is used for GIN control in livestock. For almost all anthelmintic classes, AR has been reported.

The host, the parasite, animal management, and climatic characteristics all play a role in the highly complex process of AR development. The development of efficient alternative strategies to control helminth infection has not yet been discovered, despite the fact that AR is occasionally developing and becoming a significant concern due to the widespread use of anthelmintics. Therefore, it is crucial to regularly detect AR and comprehend its risk factors and mechanisms to stop the spread of resistant parasites.

2. Factors Contributing for Development of Anthelmintic Resistance

Several factors determine how quickly AR develops, the frequency of treatments is the most important (Jabbar et al., 2006). It has been indicated that providing mass prophylactic treatment contributed to the development of AR in helminths. However, it is possible to delay the development of resistance by treating about 80% of the flock (Jabbar et al., 2006).

Anthelmintic dose rates is one of the main factors that may lead to the development of AR and refers to the administration of an improper and inappropriate anthelmintic dose. The most popular approach in veterinary medicine to calculate the dosage rate of a drug, specifically an anthelmintic, is visual weight estimation. In turn, this underdosing allows heterozygous resistant worms' survival and contributes to selection of resistant strains (Nielsen et al., 2010). The introduction and continuous use of an anthelmintic give resistant worms a survival advantage. This allows them to reproduce faster than susceptible worms, resulting in an increase in the frequency of worms with a resistance phenotype within the population.

3. Methods for Detection of Anthelmintic Resistance

The detection of AR in helminths of veterinary importance has been reviewed on several occasions for each of the different species by the World Association for the Advancement of Veterinary Parasitology (WAAVP). The majority of approaches for AR detection have limitations, either in terms of cost, application and interpretation, or repeatability of results.

Tests for AR fall into three general categories: *In vivo*, *in vitro* and molecular diagnostic tests.

3.1. In vivo methods

In vivo tests measure the impact of drug treatment on the parasite population within the animal or human host. These tests generally rely on indirect measures of parasite burden before and after drug treatment in order to quantify the impact of the drug, and hence, determine if its effectiveness is reduced by drug resistance in the worm population.

3.1.1. Fecal egg count reduction test (FECRT)

This test is widely used across GIN parasites of livestock and is currently used for assessment of drug efficacy in terms of egg reduction rate (ERR) for human STH. FECRT has undergone extensive standardization, enabling its widespread usage.

Because nematode egg output does not usually correspond well with real worm populations and the test mainly evaluates effects on egg production by adult worms, test findings may not appropriately represent anthelmintic efficacy. For *Haemonchus contortus*, there has been a strong link between faecal egg counts and worm counts, but not for *Trichostrongylus colubriformis* or *Ostertagia circumcincta*. Egg production may be inhibited if there is less than a 10-day break between treatments, which might lead to an overestimation of the effectiveness of benzimidazole anthelmintics. For this reason, it is recommended to collect feces samples 10 to 14 days after therapy. It was demonstrated in a study using goats infected with *O. circumcincta* that, when treated with ivermectin, egg production suppression may occur during the 10–14 day interval, resulting in a false negative result (Papadopoulos et al., 2012). Due to the development of immature nematode stages, the FECRT, when used to assess the presence or absence of levamisole resistance, produced false positive results when based on faecal egg counts obtained 11 or more days after treatment (Papadopoulos et al., 2012).

FECRT is very insensitive, and hence, is not suitable for detecting low levels of resistance (<25%).

3.2. In vitro methods

Measure the sensitivity of helminth eggs, larvae or occasionally adult worms, to drug exposure in laboratory-based assays. Such assays detect the phenotypic effects of drugs on various aspects of worm development, activity or viability.

3.2.1. Egg hatch assay (EHA)

Benzimidazoles prevent embryonation and hatching of the eggs of nematode parasites. Resistance to this class of anthelmintics has been identified using EHA. Tetrahydropyrimidines, imidazothiazoles, and macrocyclic lactones cannot be used in the test since they are not ovicidal. After 48 hours of incubation at 27°C, fresh eggs are inserted in each well of a 24-well plate. Several concentrations (0.5, 1, 2, 3, 5 ppm) of the benzimidazoles are then added. The remaining eggs and hatched larvae are then counted, and the LD50 values are calculated (Robles-Pérez et al., 2014).

3.2.2. Larval development test (LDT)

LDT is used for benzimidazole and levamisole. This test is determined by how long larvae can live and grow in different anthelmintic drug concentrations. Using this method, AR against the main anthelmintic families is found. The timing of infection has been shown to affect the LD50 in this assay, particularly when macrocyclic lactones (ML) are used (Fissiha and Kinde, 2021).

3.2.3. Larval Motility Test (LMT)

Larvae are incubated at 25°C for 24 h in various concentrations of drugs while in the dark. Then they are exposed to light for 20 min to stimulate those not paralyzed. The number of nonmotile larvae is then estimated as a percentage of all larvae present at each drug concentration (Kohler, 2001).

3.3. Molecular diagnostic tests

Molecular diagnostic test may aim to detect "causal" genetic differences within genes coding for drug receptors or various processes within the nematode that act to regulate the amount of drug that reaches the receptor (for example, genes involved in drug detoxification, drug efflux, or amphidial drug uptake) (Kotze et al., 2014; Kotze et al., 2020). Alternatively, a molecular diagnostic test may aim to characterise sequence polymorphisms that are genetically "linked" to the causal variants within functionally relevant genes, and so act as genetic markers for resistance (Kotze et al., 2020).
Molecular tests can generally be performed within 2 days, and can be automated, allowing for examination of many samples in a short period of time, also, can provide accurate measurements of resistance alleles even at low frequencies.

3.3.1. Polymerase Chain Reaction (PCR)

The genotyping of resistant (rr) or susceptible (rS and SS) adult worms or larvae is possible using PCR. Worms can be genotyped for the mutation on β-tubulin residue 200 (phenylalanine to tyrosine), which is implicated in BZ resistance, by employing four primers in the same reaction mixture (Elard et al., 1999; Álvarez-Sánchez et al., 2005).

4. Conclusion

The development of AR, is a highly complex process and it is a result of the intensive use of anthelmintics for the control of helminths in livestock. The FECRT is the only diagnostic currently used in the field, however, it suffers from a lack of sensitivity, high costs, and labour-intensive sampling procedures, and hence is not used widely. *In vitro* phenotypic tests remain as laboratory tools only and currently lack utility across different drug classes and parasite species. Although molecular tests are currently used as research tools, they offer significant advantages in terms of sensitivity, cost, sampling procedures and speed that make them ideal for use in diagnosing resistance in field settings.

Conflicts of Interest: The authors declare no conflict of interest.

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