

Genetics of *Haemonchus contortus* and risk factors for sheep flocks related to anthelmintic resistance

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Abstract

Despite the increasing economic importance of the small ruminant production, parasite resistance continues to stand out as the main constraint to this activity. *Haemonchus contortus*, the most prevalent and pathogenic nematode in tropical areas, is responsible for economic losses and health problems. The purpose of this review is to present the current knowledge about *H. contortus* genetics, anthelmintic resistance and management practices in sheep flocks associated with parasite resistance. We present the main results of our team obtained in sheep flocks from São Paulo state, Brazil, in recent years regarding efficiency assessment of five anthelmintics by the fecal egg count reduction test (FECRT); genotyping of two genes related to anthelmintic resistance (β -tubulin and P-glycoprotein); and risk factors in management practices associated with anthelmintic resistance. We aim to provide useful information to improve control, maintain the effectiveness of chemical groups, slow the emergence of anthelmintic resistance in sheep and help farmers deal with parasitic resistance.

Key words: management practices, multidrug resistance, nematodes, PCR.

Introduction

Gastrointestinal nematodes are the main obstacle to the production of small ruminants in tropical areas. Among sheep parasites, *Haemonchus contortus* is the most pathogenic and prevalent, causing large economic and production losses, recurrent need for treatment and even animal death. The fecal egg count reduction test (FECRT) is a useful test to determine anthelmintic efficiency in flocks. However, it is laborious and time-consuming since farms must be visited twice for sample collections. Moreover, it is only reliable when more than 25% of worms are resistant, which makes it difficult to revert the status of resistance. Thus, early diagnosis through molecular techniques is a promising management tool to guide the rational use of anthelmintics and prolong their effectiveness. Also, the identification of risk factors in flock management practices is a prevention tool that can guide decisions to control the establishment and progression of anthelmintic resistance.

Gastrointestinal parasites, *Haemonchus contortus*, anthelmintics and resistance

Every sheep category can be infected by helminths, whose spoliation leads to lower growth of lambs, lower production rates of adults and lower quality of meat and wool (Pinheiro, 1979). *H. contortus* is the main gastrointestinal helminth of sheep. It afflicts the abomasum of ruminants, causing anemia and occasionally death (Troell, 2006).

Infection with gastrointestinal parasites is the main problem in sheep production in tropical areas due to the significant economic losses caused (Girão et al., 1992). In Australia, South Africa and South America, the losses attributed to parasite resistance have been estimated at US\$ 222, 45 and 42 million, respectively (Waller, 2006). The

effects on production rates were evidenced in lambs treated with anthelmintics of different efficacies (40.9-48.4% versus >99% in FECRT): the efficient anthelmintic resulted in lambs with increased live-weight gain (by 9 kg), carcass weight (by 4.7 kg) and carcass value (by 10.4%) and shorter time to reach live-weight of 38 kg (by 17 days) (Miller et al., 2012).

Gastrointestinal parasites are usually controlled by anthelmintics (Charles, 1989). Thus, failure in this type of control is the first sign of the emergence of anthelmintic resistance (Sangster, 2001). In Brazil, five classes of broad-spectrum anthelmintics are commercially available: 1) benzimidazoles; 2) imidazothiazoles; 3) salicylanilides; 4) macrocyclic lactones (ivermectin and milbemycin) (Prichard, 2008); and 5) amino-acetonitrile derivatives (AADs) (monepantel), which were developed in 2008 (Kaminsky et al., 2008). Another new class of anthelmintics, the spiroindols (derquantel) (Kaminsky et al., 2013), is still not licensed for sale in Brazil.

Each class of anthelmintics acts by a different mechanism. The benzimidazol class binds to the β -tubulin and prevents tubulin polymerization in microtubules, and it also inhibits fumarate reductase and glucose transport; the imidazothiazoles and salicylanilides are acetylcholine receptor agonists that cause muscle contraction and paralysis; and the macrocyclic lactones open glutamate-gated chloride channels and cause neuromuscular paralysis, including of the pharynx (Almeida and Ayres, 1996; Coles et al., 2006). The AADs act on nematode-specific acetylcholine receptors (Kaminsky et al., 2008), and the spiroindols interfere with B-subtype nicotinic acetylcholine receptors, leading to flaccid paralysis (Epe and Kaminsky, 2013).

In the past two decades, the AADs and the spiroindols have been the only new classes of anthelmintics developed for ruminants. They are effective even in flocks with resistance to all anthelmintic classes (Kaminsky et al., 2008; Prichard and Geary, 2008;

Little et al., 2011; Geurden et al., 2012). However, for the old anthelmintic classes, resistance emerged just a few years after they were launched. For example, resistance to thiabendazol, released in 1961, was described in 1964; to levamisole (1970) in 1979; to ivermectin (1981) in 1988; and to moxidectin (1991) in 1995 (James et al., 2009). We therefore believe that the development of resistance to the new anthelmintic classes will start soon if they are used in the same way as the other anthelmintics were in the past.

Since the process of anthelmintic development is time consuming in comparison to the speed of resistance development, it is essential to slow the resistance process to assure that the existing anthelmintics are effective for longer periods. Thus the new classes of anthelmintics should be employed in very specific and controlled conditions.

Anthelmintic resistance to gastrointestinal nematodes of small ruminants has been observed worldwide (Bartley et al., 2004) and it is increasing in recent years, as noted by Kaplan and Vidyashankar (2012) and Torres-Acosta et al. (2012). Our team reported that multidrug resistance is widespread in São Paulo state, Brazil, where 100% of flocks had worms resistant to albendazole and ivermectin, 96.6% to moxidectin, 92.9% to closantel, and 53.6% to levamisole (Veríssimo et al., 2012). Anthelmintic resistance has also been reported in other Brazilian states, such as Santa Catarina (Ramos et al., 2002; Rosalinski-Moraes et al., 2007), Paraná (Thomaz-Soccol et al., 2004), Mato Grosso do Sul (Sczesny-Moraes et al., 2010), Ceará (Melo et al., 2003), Bahia (Barreto et al., 2006), and Rio de Janeiro (Cruz et al., 2010); and in other countries, such as Paraguay (Maciel et al., 1996), Uruguay (Nari et al., 1996), Argentina (Eddi et al., 1996), Malaysia (Chandrawathani et al., 1999), New Zealand (Waghorn et al., 2006), Spain (Díez-Baños et al., 2008; Martínez-Valladares et al., 2013), Trinidad (George et al., 2011), Costa Rica (Maroto et al., 2011), Norway (Domke et al., 2012), Canada (Falzon et al., 2013), and Ireland (Good et al., 2012; McMahon et al., 2013).

The situation of increasing anthelmintic resistance worldwide makes it urgent to develop more efficient diagnosis techniques and alternative measures of parasite control.

Mechanisms and diagnosis of parasite resistance to anthelmintics

The accurate diagnosis of parasitic diseases and resistance is a crucial step for prevention, survival, epidemiology and control of parasites. Molecular diagnosis of resistance can overcome some limitations observed in traditional parasitological methods (Gasser et al., 2008) and improve the detection of the emergence of parasite resistance.

Resistance is an evolutionary process that results from the exchange of resistance genes by the crossing of parasites that survive treatment (Echevarria, 1996). Since only the most adapted individuals survive treatment and reproduce (Griffiths et al., 1998), they transmit their genes to the next generation. So, after a few generations the frequency of resistant individuals in the population increases (Blackhall et al., 2008) and leads to treatment failure (Beech, 2008). The resistance is considered total when the maximum dose of the drug tolerated by the host is no longer effective to control the parasite (James et al., 2009).

Due to their easy application and affordability, anthelmintics are widely used, which has led to reduced efficiency by selection of resistant parasites (Leathwick et al., 2001; Molento, 2004). There are several mechanisms used by parasites that lead to anthelmintic resistance (Blackhall et al., 2008; Prichard, 2008; James et al., 2009; Vokřál et al., 2012), which include:

a) Variations of the target gene or protein, resulting in lower binding affinity for the drug, e.g., single nucleotide polymorphisms (SNP) in isotype 1 of the β -tubulin

gene, which confers resistance to benzimidazol; and in the glutamate-gated chloride (GluCl) gene, associated with macrocyclic lactone resistance.

- b) Differences in gene expression that can affect the drug's effect on the target protein without interfering in its binding affinity.
- c) Reduction of anthelmintic concentration in the target due to changes in transport mechanisms or cell permeability, e.g., changes in P-glycoprotein membrane protein, which exports drugs from cells, reduce the intracellular drug concentration and promote underdosing, which in turn results in multidrug resistance.
- d) Differences in processes of anthelmintic metabolism or detoxification, e.g., changes in activities of thioredoxin, glutathione-cytochrome P450, peroxidases, catalase and UPD-glucosyltransferases, which also lead to multidrug resistance.

Thus, knowledge of the mechanism that leads to resistance is crucial to prolong the effectiveness of anthelmintics and to develop molecular markers to monitor the emergence of drug resistance. It is also beneficial to the development of new chemicals and new drug targets (James et al., 2009).

Genetic resistance to anthelmintics

The molecular mechanism of resistance to benzimidazoles was the first to be elucidated. Beech et al. (1994) reported that tubulin is the target site of benzimidazoles in parasites, and that benzimidazol resistance is mediated by decreased binding affinity to tubulin and increased tolerance to the drug. The first mutation described in isotype 1 of the β -tubulin gene was a T to A transversion (codon TTC is modified to TAC), leading to the substitution of a phenylalanine by a tyrosine at position 200: the F200Y polymorphism (Kwa et al., 1994). The F167Y and E198A polymorphisms in β -tubulin gene were also reported (reviewed by Beech et al., 2011).

Several studies have evaluated the frequency of F200Y polymorphism in parasite populations and found that populations susceptible to benzimidazole present low resistance genotype frequencies, while the frequencies are highly variable in resistant populations. Our team reported F200Y resistance (r) allele frequencies from 9 to 74% and genotype frequencies from 0 to 66.7% in field isolates of *H. contortus* from São Paulo state (Niciura et al., 2012). Álvarez-Sánchez et al. (2005) found r allelic frequencies ranging from 71.3 to 86.3% in resistant isolates, while its frequencies varied from 24 to 32.3% in susceptible isolates. In flocks with suspected or clinical resistance using FECRT, the r allelic frequency was either 100% (Höglund et al., 2009), or the genotype rr distribution was present in a variable proportion (36 to 100%) in resistant isolates (Elard et al., 1999; Čudeková et al., 2010). In a multiresistant isolate of *H. contortus*, r allelic frequency was higher than 95% (Williamson et al., 2011).

The macrocyclic lactones bind to GluCl channels and enter cells, resulting in hyperpolarization of neuromuscular cells and paralysis (Blackhall et al., 2008). Thus, polymorphisms, like L256F and A169V, in the gene encoding the subunit alpha of GluCl were already associated with resistance to ivermectin and moxidectin in *H. contortus* (Blackhall et al., 2008; reviewed by Beech et al., 2011). In addition, changes in genes encoding gamma-aminobutyric acid (GABA)-gated chloride channels, such as the K169R polymorphism, have been related to resistance to macrocyclic lactones due to changes in drug target (Blackhall et al., 2008; reviewed by Beech et al., 2011).

Resistance to levamisole was associated with gene polymorphisms in the nicotinic acetylcholine receptor (nAChR) in *Ascaris suum* (James et al., 2009). The nAChR receptor is present in neuromuscular junctions, pharynx and muscles of the head and central ganglia (Prichard, 2008).

The above mentioned polymorphisms act specifically on the target of drugs, so they are not responsible for the multiple resistance to anthelmintics. On the other hand, drug concentration modulation mediated by the P-glycoprotein gene (PgP), also named ABC (ATP-binding cassette) efflux transporter (ABCB1) gene, may be a potential target to the multidrug resistance mechanism (Kerboeuf et al., 2003). The PgP are transport proteins located in cell membranes that promote drug efflux (Molento and Prichard, 1999) and have very broad substrate specificity (Schwab et al., 2003). Changes in the PgP gene were associated with multiple resistance because they affect the transport of ivermectin, benzimidazol (Blackhall et al., 2008) and imidazothiazol and reduce the amount of the drug that reaches the target (James et al., 2009). Our team recently discovered five new SNPs in the PgP gene, and one of them presented frequency differences ($P < 0.05$) between multiresistant and susceptible isolates of *H. contortus* (Mello, unpublished results).

Gene expression studies in a *H. contortus* isolate highly resistant to benzimidazol, levamisole and ivermectin revealed increased expression of the PgP-2 and PgP-9 genes and of a truncated transcript of nAChR (Williamson et al., 2011).

Risk factors of sheep flock management practices related to anthelmintic resistance

In addition to the genetic condition, the selection of resistant parasites can be influenced by farm management practices (Barger, 1997). It is difficult to determine the risk factors associated with drug resistance due to the many different management strategies, each of which imposes specific drug selection factors on the flock (Sargison et al., 2007) and affects the maintenance of refugia, i.e. nematode population not

exposed to anthelmintic treatment. Hence, it is necessary to perform studies of local conditions.

In São Paulo state, several farm management practices have been found to be associated with high frequency of F200Y polymorphism related to resistance (Niciura et al., 2012). The high resistance genotype was observed in more recently established farms and farms that do not have animal records, reflecting the lack of farmers' experience in dealing with parasite control. Among drug-based treatment procedures, the whole-flock treatment increased the resistance when compared to the use of selective treatment based on the FAMACHA method as a clinical indicator (Kenyon et al., 2009; Niciura et al., 2012).

Host immunity results in variable resistance to parasites, and although not directly related to anthelmintic resistance, the introduction of less tolerant breeds in flocks with high parasite challenge leads to the higher use of anthelmintics, which in turn can result in higher resistance. The Brazilian hair sheep breed Santa Ines, as other native sheep breeds (Baker et al., 2003), is more tolerant to helminths than wool breeds (Bueno et al., 2002; Amarante et al., 2004). An adequate diet is also important to reduce the use of anthelmintics and consequently the resistance problem. It is clear that sheep are able to respond efficiently to infection by gastrointestinal nematodes when given a protein-rich diet, as reported for the Santa Ines breed (Chagas et al., 2013; Domingues et al., 2013).

The use of rotational grazing can increase resistance (Melo et al., 2009), as can the presence of dry lands on farms, because both can reduce the number of worms in refugia due to, respectively, reduced larval pasture contamination (Colvin et al., 2012) and desiccation (Kenyon et al., 2009). The dose-and-move practice can result in low

resistance (Niciura et al., 2012) or high resistance (Molento et al., 2004) because it depends on the larval contamination (refugia) of the new pasture (Waghorn et al., 2009).

Pasture sharing with other species (as cattle or horses) can result in higher resistance (Lawrence et al., 2006; Niciura et al., 2012); while grazing in private pastures can also result in higher resistance (Calvete et al., 2012). Since alternate grazing has been associated with both increased ability of worms to infect sheep over time (Barger, 1997) and to lower EPG counts in sheep (Souza et al., 2005; Sczesny-Moraes et al., 2010), its effects still need to be elucidated in different regions and under different management conditions that affect refugia maintenance (Henrioud, 2011).

Frequent incorporation of animals into the flock leads to high resistance (Lawrence et al., 2006; Niciura et al., 2012). This management practice may have two side effects: one effect is if the new animals bring resistant helminths to the area, causing increased resistance. However, if they bring significantly more susceptible helminths, this practice would quickly dilute the existing resistance (Moussavou-Boussougou et al., 2007).

The rotation of anthelmintics after a single treatment increased the risk of resistance (Craig, 1993; Niciura et al., 2012), as does the prolonged use of only one class of anthelmintics (Kaminisky et al., 2013). Therefore, the rotation of a broad-spectrum anthelmintic group should be performed soon after its efficiency declines (Sargison et al., 2007). Another drug-based management practice is the use of drug combinations. Farms that use drug combinations presented low resistance (Niciura et al., 2012), while the efficacies of the drugs were higher than 70% (Leathwick et al., 2012). This occurs because drug combination can reduce the number of heterozygous resistant genes, prolonging the lifespan of the particular drug (Molento, 2009).

However, the use of drug combinations can lead to resistance to all the anthelmintics used in a 'low-refugia' management environment (Leathwick, 2012).

The visual estimation of the weight of animals for treatment facilitates underdosing, causing more selection pressure (Chartier et al., 1998; Sargison et al., 2007) and resulting in higher resistance (Calvete et al. 2012; Niciura et al., 2012).

Conclusions

Since accurate diagnosis of resistance is a crucial step for prevention and control of parasites and considering that changes in gene frequencies explain the outcome of anthelmintic resistance, genetic polymorphisms detected by molecular techniques can be used to monitor anthelmintic emergence.

Resistance diagnosis (molecular or by FECRT) can be used to estimate risk factors in management practices in order to formulate measures to prevent or delay the establishment of resistance and to assist farmers and technicians to enhance the control and slow the development of resistance to anthelmintics in small ruminants.

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