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Review article

Anthelmintic resistance: Management of parasite refugia for *Haemonchus contortus* through the replacement of resistant with susceptible populations

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ABSTRACT

Sheep production in tropical and temperate regions is hampered by the presence of *Haemonchus contortus*, the blood-sucking nematode that is the major cause of economic losses in small ruminant enterprises. The most limiting factor in the control of this parasitic disease is the steady progress of anthelmintic resistance worldwide. The search for control strategies that minimise the use of anthelmintics is therefore central to various efforts worldwide. One strategy is the introduction of susceptible parasites in refugia when these refugia are at low levels. This strategy could lead to a renewed possibility anthelmintics being effective. At farm level, this management practice could recover the use of anthelmintics in flocks with high levels of resistance. This review explores the possibility of replacing resistant *H. contortus* populations with susceptible ones through refugia management and. highlights the experiences of on-farm research attempts carried out in different geographical areas, reaching various degrees of success.

1. Introduction

Gastrointestinal nematodes are one of the main diseases that economically restrict g sheep production anywhere in the world (Miller et al., 2012). The disease is generally characterised by the simultaneous presence of different nematode genera which differ in their pathogenicity and, therefore, the clinical presentation of affected animals is an expression of several effects caused by those individual genera on the gastrointestinal tract. The most common and economically important genera described for grazing sheep in different parts of the world are *Haemonchus, Teladorsagia, Ostertagia, Trichostrongylus, Cooperia, Nematodirus, Oesophagostomum* and *Chabertia* (Sutherland and Scott, 2010; Abbott et al., 2012; Castells et al., 2013b; Olaechea, 2013;). Sheep are susceptible to gastrointestinal nematodes at any age although lambs and periparturient ewes are the categories most affected and epidemiologically relevant (Besier et al., 2016a). Since sheep production in tropical, sub-tropical and many temperate regions is greatly hampered by the presence of *Haemonchus contortus* (Besier et al., 2016a), this review will focus on this nematode species.

A major limitation on sheep production throughout the world is the widespread anthelmintic resistance to most chemical groups (Kaplan, 2004). Anthelmintic resistance can be defined as “the significant increase in a population of individual nematodes able to tolerate drug doses that have been proved to be lethal for the majority of individuals of the same species” (Nari Henrioud, 1987). The worldwide extend of anthelmintic resistance in sheep nematodes is of major concern; as demonstrated by the data from the largest exporting countries of sheep meat (Food and Agriculture Organization of the United Nations, 2013). A nationwide survey in New Zealand revealed that 64% of the farms showed anthelmintic resistance and that 50% of sheep farms presented evidence of resistance to macrocyclic lactones (ML) (Waghorn et al., 2006). Likewise, resistance to benzimidazoles (BZD) for all nematode genera identified was placed at 37–83% (McKenna, 2010). The situation in Australia is not better, a national study showed 96% prevalence of resistance to BZD and levamisole (LVM), and 54% prevalence of resistance to ML (Playford et al., 2014). Reports from the UK show 82% of resistance to either BZD, LVM or ML in Welsh sheep farms (Mitchell et al., 2006), more than 80% of resistance to BZD in Scottish flocks (Bartley, 2008), while in Northern Ireland 81% of flocks showed resistance to BZD, 62% to moxidectin (MOX), 50% to IVM and 14% to LVM (McMahon et al., 2013). Resistance in Ireland reached 69% to BZD, 48% to LVM, 38% to avermectins (AVM), and 16% to MOX

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(Keegan et al., 2017). These authors refer actually to 'anthelmintic treatment failure' rather than 'anthelmintic resistance', but they also acknowledge that the latter was largely responsible for the former.

In another sheep-rearing region of the world, such as the Southern Cone of South America, 62% of the surveyed farms in Argentina had flocks with resistance to any of the tested anthelmintics (BZD, LVM, IVM), 50% of those showed multiple resistance; while in a smaller survey, resistance to closantel was found in 80% of the flocks (Caracostantógolo et al., 2013). Resistance to ABZ and IVM in certain parts of Brazil was 100%, and 54% of tested farms presented multiple resistance to five anthelmintics evaluated (ABZ, LVM, IVM, moxidectin and closantel) (Veríssimo et al., 2012). The situation in Uruguay is also increasing in severity, to the point that genera such as *H. contortus* are resistant to most anthelmintic classes (Castells et al., 2013a), including the organophosphates naphthalophos and trichlorfon (Mederos et al., 2016), and the prevalence of resistance to LVM, closantel, moxidectin and BZD are > 90%–100% (Mederos et al., 2016).

Three surveys in South Africa – another major sheep producing area – showed an average of 79% resistance to BZD, 73% to IVM, 23% to LVM, and 89% to the salicylanilide rafoxanide (Van Wyk et al., 1999). The same authors reported that *H. contortus* was 99% resistant to at least one of the tested anthelmintic groups and 65% resistant to three or all four anthelmintic groups.

The increase of multiple resistance to the three main groups of broad-spectrum anthelmintics, i.e. BZD, imidazothiazoles, and ML, is currently threatening the viability of sheep production (Kaplan, 2004). This situation is further exacerbated by recent reports of resistance to the latest group of anthelmintics reaching the market, the amino-acetonitrile derivatives (AADs), recorded in New Zealand (Leathwick et al., 2013; Scott et al., 2013), Uruguay (Mederos et al., 2014), Australia (Love, 2014; Constantinoiu and de Cat, 2015; Sales and Love, 2016), The Netherlands (Van den Brom et al., 2015) and Brazil (Cintra et al., 2016).

The data above emphasise the idea that the control of parasitic gastroenteritis must rely on more than one strategy within an integrated parasite management approach in order to minimise the use of anthelmintics that remain effective, using also faecal egg counts as a monitoring tool to justify treatments (Anziani and Fiel, 2015). These other control strategies, either currently in practice or under research, are related to grazing management, immune response of the host, bioactive forages and biological control (Castells et al., 2013c); however, this paper does not intend to review any of these.

2. Diagnosis of anthelmintic resistance

There are two types of available methods to detect anthelmintic resistance, *in vivo* and *in vitro* (Coles et al., 1992). The controlled efficacy test (CET) and the faecal egg count reduction test (FECRT) are the two *in vivo* methods. The CET compares the actual number of nematodes in the gastrointestinal tract at necropsy between treated and non-treated, control animals. It is the most reliable method as it has a high sensitivity and allows the identification of the parasites to species level. Its disadvantages are that it is costly and labour-intensive (Caracostantógolo et al., 2013). The FECRT compares pre- and post-treatment faecal egg counts; despite its limitations (Woodgate et al., 2017), it is the method used worldwide to diagnose anthelmintic resistance at farm level. It is an easy-to-use tool, does not require the slaughter of animals, and allows for the identification of parasite genera at each sampling time by means of faecal cultures (Coles et al., 1992).

Within the *in vitro* methods, the ones most used are the egg hatch assay (EHA), which is specific for BZD-resistance (Kotze and Prichard, 2016) and the larval development assay (LDA) that detects resistance to BZD, LVM, ML (Kotze and Prichard, 2016) and AAD (Woodgate et al., 2017). Other available methods are the larval migration inhibition assay (LMIA) and the larval feeding inhibition assay (LFIA). The former detects resistance to ML (Kotze et al., 2006), LVM, closantel and thiabendazole (Woodgate et al., 2017), while the latter detects resistance to ML and LVM (Álvarez-Sánchez et al., 2005). All these techniques require qualified personnel as well as having susceptible and resistant reference strains, and therefore they are being used for research purposes rather than for on-farm diagnosis so far (Cutullé et al., 2002).

Molecular-based techniques to identify the DNA mutations present in resistant nematodes also exist, and although not used for on-farm routine diagnosis as yet, they seem to have a promising future (Barrère et al., 2013; Roeber et al., 2013). So far, only resistance to BZD can be reliably detected. The most commonly used molecular techniques to detect genome mutations in BZD-resistant *H. contortus* are allele-specific PCR, restriction fragment length polymorphism-PCR, real-time PCR and pyrosequencing (Kotze and Prichard, 2016). The main advantages of using molecular techniques is their high sensitivity and specificity, given that they can detect mutations at 1% level of the parasite population (Elard et al., 1999). This represents a great advantage compared to the classic parasitological techniques because they can detect resistant parasites at a very early stage. Additionally, the DNA material can be extracted not only from adults but from L3 (Roos and Grant, 1993; Bisset et al., 2014), as well as eggs (Roeber et al., 2012; Demeler et al., 2013), and thus slaughtering animals to obtain adult parasites is avoided. On the other hand, similarly to the *in vitro* techniques, sophisticated equipment and qualified personnel are needed.

3. Management of parasite refugia through the replacement of resistant with susceptible populations

A parasite population in refugia is composed of all free-living stages in the environment prior to an anthelmintic treatment, plus those parasitic stages in animals not exposed to anthelmintics (Van Wyk, 2001; Besier, 2012). This concept is today an undeniably key component of any sustainable parasite control programme (Pech et al., 2009; Sutherland, 2015; Besier et al., 2016b). The work by Martin et al. (1981) is one of the first publications pointing out the importance of the population size in refugia to accelerate or delay the development of anthelmintic resistance. The authors experimentally showed that anthelmintic resistance rapidly increases when the population of susceptible parasites in refugia is scarce or nil, while resistance is delayed when this population is large.

Parasite control strategies involving the use of anthelmintics such as targeted selective treatments and drug combinations take into consideration the maintenance of susceptible parasites in refugia (Kenyon et al., 2009; Bartram, 2013). These are good approaches to delay the onset of anthelmintic resistance, even though there are already reports of emerging resistance using them (de Albuquerque et al., 2017; Hodgson and Mulvaney, 2017). Leathwick (2013) recently challenged the general view that reversion of anthelmintic resistance does not occur even if the selecting drug ceases to be used. However, the fact remains that current alternatives rely on minimising the number of treatments so as to extend the effective use of the drugs, using combinations of two or more drugs with different mechanism of action, and maintaining a large proportion of the parasite population in refugia.

The issue is what do to when the population in refugia is already resistant to most – if not, all – drug classes, as previously demonstrated (Van Wyk and Malan, 1988; Howell et al., 2008; Martínez-Valladares et al., 2015). Replacement of resistant by susceptible parasite populations seems to be an alternative to recover the efficacy of anthelmintics in areas where these drugs are needed due to the economic importance of sheep production and the prevalence of very pathogenic parasites. The overall idea of this concept is to take advantage of the time when resistant populations in refugia are at their lowest to introduce a new population of susceptible parasites onto the pasture (Van Wyk and Van Schalkwyk, 1990). There is only a handful of attempts of parasite population replacement in small ruminants described in the literature (Table 1), and only one in cattle (Fiel et al., 2017), with varying degrees

Table 1
Studies on population replacement of resistant gastrointestinal nematodes in small ruminants.

Nematode species (hosts)	ATH[a] involved in resistance	Reduction of resistant population in refugia[e]	Introduction of susceptible population	Resistance detection methods	Evaluation of replacement success[n]	Post-replacement efficacy (%)	References
Haemonchus contortus (sheep)	BZD[b]	Autumn 1: PR[f], 8 wk[g] Autumn 2: PR, 10 wk Spring 1, Spring 2, Summer: none	Autumn (two pastures) Spring (two pastures) Summer	CET[j] EHA[k]	Autumn 1:15 mo Autumn 2:14 mo Spring 1:9 mo Spring 2:8 mo Summer: 6 mo	Autumn 1:52.7 Autumn 2:96.1 Spring 1:96 Spring 2:99.6 Summer:47.4	Van Wyk and Van Schalkwyk (1990)
Haemonchus spp. *Teladorsagia* spp. *Trichostrongylus* spp. (sheep)	LVM[c] (BZD suspected for *Trichostrongylus*)	Suppressive ATH treatments[h]	Summer	FECRT[l]	12 mo	99–100	Bird et al. (2001)
Haemonchus contortus *Trichostrongylus colubriformis (sheep)*	BZD (*H. contortus*) LVM (*T. colubriformis*)	Suppressive ATH treatments PR, 6 mo[i] Pasture mowing + chemical weed control	No data	FECRT	4 and 13 mo	BZD: < 95 LVM: > 95	Aumont et al. (2002)
Haemonchus contortus *Trichostrongylus spp. (goats)*	IVM[d], BZD, tetramisole	PR, 6 mo Pasture mowing Cattle grazing, 3 mo	End of long rain season	FECRT	4 mo	BZD: 95 IVM: 96	Sissay et al. (2006)
Teladorsagia circumcincta (sheep)	BZD	PR, 6 mo Pasture mowing	Summer	FECRT EHA CET multiplex PCR	4 mo	FECRT: 99.7–100 CET: 97–99	Moussavou-Boussougou et al. (2007)
Haemonchus contortus (sheep)	BZD, IVM, LVM	Move to clean pasture	Autumn	FECRT LDA[m] Pyrosequencing Micro satellite genotyping	18 and 30 mo	FECRT: LVM = 98.8[o]/40.7[p] BZD = −55.68[o]/−18.5[p] IVM = −200[o]/ nd[q]	Miller et al. (2015)
Haemonchus contortus (sheep)	BZD	Suppressive ATH treatments PR, 2 wk Pasture mowing	Summer	FECRT CET	16 mo	FECRT: 95 CET: 97.8	Muchiut et al. (2016)

[a] ATH: anthelmintics.
[b] BZD: benzimidazole.
[c] LVM: levamisole.
[d] IVM: ivermectin.
[e] Before introduction of susceptible population.
[f] PR: pasture resting.
[g] wk: weeks.
[h] Using effective ATH.
[i] mo: months.
[j] CET: control efficacy test.
[k] EHA: egg hatch assay.
[l] FECRT: faecal egg count reduction test.
[m] LDA: larval development assay.
[n] Time from introduction of susceptible population.
[o] Efficacy at 18 months post-introduction.
[p] Efficacy at 30 months post-introduction.
[q] nd: no data.

of success.

The first part of the process to replace the resistant population is to reduce the resistant population in refugia – although this was not carried out in all studies presented in Table 1. It can be carried out by means of anthelmintic treatments with effective drugs (Bird et al., 2001) or by pasture resting (Van Wyk and Van Schalkwyk, 1990; Moussavou-Boussougou et al., 2007), or both (Aumont et al., 2002; Sissay et al., 2006; Muchiut et al., 2016). Pasture mowing can be added to help in cleaning the pasture. Anthelmintic treatments with effective drugs at regular intervals aim to avoid contamination of the pasture while allowing the remaining free-living stages to die off. Pasture resting with nil grazing aims to diminish the resistant population in refugia over time. It is extremely important that pasture resting takes place at the most damaging time for the free-living stages on pasture, which will vary according to geographical area and parasite genera. Other alternative for this first step would be to use clean pastures (Miller et al., 2015), should this be possible; a clean pasture (e.g. newly sown pasture) would then mean that there are no resistant parasites in refugia.

The second part of the population replacement process is the introduction of susceptible parasites so a new, susceptible population can be "implanted". This can be achieved by infecting parasite-free seeder animals with susceptible L3 and turning them out on pasture (Van Wyk and Van Schalkwyk, 1990; Bird et al., 2001; Aumont et al., 2002; Moussavou-Boussougou et al., 2007; Muchiut et al., 2016). An alternative approach is bringing in animals already carrying susceptible parasites from previously implanted populations (Bird et al., 2001) or from natural infections (Sissay et al., 2006). Either way, steps must be taken previously to ensure without doubt that the parasite population to be introduced is fully susceptible, preferably by means of a CET – which remains the gold standard to detect resistance/susceptibility – or, if costs are a limiting factor, by molecular assays.

Moussavou-Boussougou et al. (2007), Miller et al. (2015), Muchiut et al. (2016) keeping in mind that so far such assays are only reliable to detect resistance to BZD. The FECRT has been used in the population replacement attempts found in the literature. However, its sole use to test the susceptibility of the new population to be introduced would be highly discouraged. Its lack of precision in the detection of resistance (Martin et al., 1989) could mean that, in practice, a certain level of resistance might be already present in the new susceptible population at the time of its introduction (Bird et al., 2001).

A seemingly key factor at this stage is that the new susceptible population must be deployed on pasture before levels of pasture infectivity start to rise, so as to maximise the chances of establishment of the susceptible parasites (Van Wyk and Van Schalkwyk, 1990).

The results obtained from previous studies (Table 1) range from no recovery of susceptible status at all (Van Wyk and Van Schalkwyk, 1990; Miller et al., 2015) or recovery of susceptibility to only one class of anthelmintic used (Aumont et al., 2002), to full recovery of susceptibility (Van Wyk and Van Schalkwyk, 1990; Bird et al., 2001; Sissay et al., 2006; Moussavou-Boussougou et al., 2007; Muchiut et al., 2016). The study of Van Wyk and Van Schalkwyk (1990) was conducted in South Africa and attempted to introduce a new parasite population on five pastures in different seasons (two attempts in autumn, two in spring and one in summer), and the results (Table 1) were quite striking in that population replacement was successful in three of the five attempts – one in autumn and two in spring – and that only one of the autumn attempts succeeded. The authors hypothesized that the failure in establishing a susceptible *H. contortus* population in summer was due to the large size of the resistant refugia, while an explanation for the failure on the pasture rested for 10 weeks before introducing the susceptible strain in autumn could have been the self-cure experienced by the animals infected with the susceptible strain, and that those same animals became infected later on with the resistant L3 still remaining on pasture (Van Wyk and Van Schalkwyk, 1990).

Bird et al. (2001) accomplished the introduction of a susceptible population in an interesting design involving two neighbouring pastures implanted one at a time with the parasite population. The introduction of the susceptible population commenced in summer on one pasture, and in mid-spring of the following year on the other. The susceptible isolate used for the experimental infections, comprising mainly of *Haemonchus* spp. but also *Teladorsagia* spp. and *Trichostrongylus* spp., showed some lack of efficacy of LVM against *Trichostrongylus* spp.. However, the numbers of L3 other than *Haemonchus* spp. in faecal cultures were too low to establish reliably the proportion of each nematode genera as well as the anthelmintic efficacy against each one of them; this would most probably be related to the fact that the different biotic potential of gastrointestinal nematodes affects the number of L3 obtained in faecal cultures.

Sissay et al. (2006) showed that it is possible to recover anthelmintic efficacy under field conditions by grazing animals - in this case, goats - on pastures with naturally-occurring susceptible parasites, and “transporting” them to a pasture in which the resistant population in refugia had been previously reduced by three actions (i.e. pasture resting, pasture mowing and grazing by another livestock species (cattle)). One aspect to consider about this study is that the anthelmintic treatments to goats were administered at dose rates for sheep, and it is known that goats metabolise anthelmintics differently than sheep so they need larger doses of avermectins and BZD (Silvestre et al., 2002; Anziani et al., 2010).

The study carried out by Moussavou-Boussougou et al. (2007) in France is one of the most complete experiments to date as it used *in vivo*, *in vitro* and molecular methods to determine resistance to BZD, which permits the direct comparison between methods. The success of the population replacement was confirmed at the end of the trial by FECRT, CET, EHT and multiplex PCR, prompting the authors to conclude that it is possible to substitute BZD-resistant nematodes such as *T. circumcincta* in temperate areas. Although more studies comparing the different methods are needed, this represents an advance in itself because the veterinary practitioners could in future count on more than one alternative to diagnose anthelmintic resistance and choose to use one or another according to sensitivity, practical needs, or availability.

Of all the successful cases, the studies carried out in South Africa and Argentina are the ones with longer periods between the introduction of the susceptible population and the evaluation of the replacement strategy (fourteen and sixteen months, respectively). The only long-term study so far has lasted over three years (Miller et al., 2015). The authors used a fully susceptible laboratory strain of *H. contortus* to replace a population resistant to BZD and ML, and borderline resistant to LVM. Then they moved the flock to a clean pasture and controlled parasitism through a TST strategy based on FAMACHA, using albendazole first and LVM later. However, re-emergence of resistance to albendazole and LVM was recorded at eighteen and thirty months post-replacement, respectively. It is noticeable that resistance to ML, also returned at eighteen months post-replacement, even though the flock had not been treated with that anthelmintic class. A molecular analysis using microsatellite markers showed that the post-replacement resistant population was genetically more similar to the pre-replacement population than to the susceptible laboratory strain used. The authors suggest that the susceptible strain might have had an adaptation deficit that allowed a few residual resistant worms that were possibly present in the flock to flourish, since the same animals that harboured resistant parasites where used to introduce the susceptible strain onto pasture (Miller et al., 2015).

The sustainability of the population replacement strategy when challenged by anthelmintic treatments needs to be checked taking care to avoid using the problem drug/s for a minimum of two years (at least in the case of BZD) in order for the parasite population to get rid of any remaining resistant alleles (Moussavou-Boussougou et al., 2007). This is exemplified by the study by Muchiut et al.(2016) whose objective was the return of efficacy of BZD against *H. contortus*. Based on FAMACHA and FEC, each time that the TST strategy indicated the need of individual treatments during the phase of establishment of the BZD-susceptible population, effective drug classes other than BZD were used.

It is obvious that for the population replacement strategy to be of practical use, the rate of reversion to susceptibility must be fully tested under actual farming conditions. Additionally, a new parasite control system (and, probably, a new mindset from farmers and practitioners alike) must be put in place once the susceptible population has established itself. This new control system should use as well other non-anthelmintic strategies in order to maintain a viable control programme (Besier, 2012; Sutherland, 2015). If these rules are not observed, anthelmintic resistance will, invariably, re-emerge.

4. Conclusions

The relentless advance and spread of anthelmintic resistance has become the most limiting health factor that threatens sheep production anywhere where *H. contortus* develops and thrives. Chemical drugs are still the most widely-used way of controlling gastrointestinal nematodes, and other control strategies have a slow take-up by farmers or are yet to be transferred to farm level. Against this background, the current recommendation is not only the rational use of anthelmintics but also the monitoring of their efficacy. Since the introduction a few decades ago of the refugia concept and its importance in the development/delay of anthelmintic resistance, studies have been conducted with the common aim of managing the populations in refugia in an attempt to re-establish a susceptible parasite population to recover anthelmintic efficacy.

From the investigations highlighted above it can be noted that despite using different approaches, the post-replacement efficacy of the compromised anthelmintic (as measured by FECRT and/or CET) was satisfactory in most cases, which leads to the conclusion that parasite population replacement could be a viable practice.

It remains to be proven which of the diagnostic techniques (parasitological and molecular) would be sufficiently and reproducibly sensitive and practical for routine detection of resistance in the future, and which one (or ones) would be most suitable to corroborate the successful establishment of a susceptible parasite population. Although FECRT and CET are commonly used under field and experimental conditions, respectively, they provide little information on the genetic composition of the parasite population under study.

Another aspect to take into consideration is the minimum post-replacement time that needs to elapse before evaluating the actual success of the population replacement. If this time is too short, the results could be biased towards the recently-introduced susceptible population. Time should therefore be given for this new population to adapt to the field environment where it is introduced and increase the proportion of its individuals in refugia. Long-term monitoring of the introduced susceptible populations would provide valuable data on its sustainability, since this information is very scarce at the moment.

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