Anthelmintic resistance of gastrointestinal cattle nematodes

Anthelminthicumresistentie van gastro-intestinale rundernematoden

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ABSTRACT

Anthelmintic resistance of parasites in small ruminants, cattle and horses is increasing worldwide as a consequence of the over usage of the currently available products. In Belgium, *Cooperia oncophora* is the most common cattle nematode in which resistance, especially against macrocyclic lactones, occurs. Once resistance has been diagnosed, a change to another drug with a different mode of action is advised. However, effective anthelmintics will be hardly available in the near future. Therefore, it is important that farmers and veterinarians find a balance between achieving good parasite control and the sustainability of their control strategies. In this way, anthelmintic resistance may be delayed, and the effectiveness of anthelmintic drugs may be prolonged. This requires sensitive detection tools. With a sensitive detection technique, anthelmintic resistance can be diagnosed in a very early stage. Hence, the spread of resistance alleles in the parasite population may be prevented. In this review, different diagnostic assays for the detection of anthelmintic resistance are discussed, an overview is given of the current status of anthelmintic resistance in Belgian cattle, and measures are suggested to avoid or delay the development of anthelmintic resistance.

SAMENVATTING

Anthelminthicumresistentie van parasieten bij kleine herkauwers, runderen en paarden wordt wereldwijd steeds groter als gevolg van een overmatig gebruik van de beschikbare ontwormingsproducten. In België is *Cooperia oncophora* de meest voorkomende rundernematode waarbij resistentie wordt vastgesteld; voorlopig enkel tegen macrocyclische lactonen. Eens resistentie is vastgesteld, kan het nodig zijn om over te schakelen naar een product met een ander werkingsmechanisme. Er zijn echter bijna geen nieuwe doeltreffende ontwormingsproducten meer beschikbaar. Het is daarom belangrijk dat landbouwers en dierenartsen een evenwicht vinden om zowel worminfecties onder controle te krijgen als de duurzaamheid van hun behandelingsstrategie te garanderen. Op deze manier kan de ontwikkeling van anthelminthicumresistentie vertraagd worden en de doeltreffendheid van de beschikbare anthelminthica verlengd worden. Dit vereist natuurlijk een gevoelige detectietechniek om resistentie op te sporen. Aan de hand van een gevoelige detectietechniek kan de diagnose van anthelminthicumresistentie reeds in een vroeg stadium gesteld worden en kan de verspreiding van resistentieallelen in de wormpopulatie tegengehouden of vertraagd worden. In dit artikel worden de beschikbare tests voor de detectie van anthelminthicumresistentie besproken. Er wordt een stand van zaken gegeven van anthelminthicumresistentie bij runderen in België en er worden maatregelen voorgesteld om resistentie te vermijden of te vertragen.

INTRODUCTION TO ANTHELMINTIC RESISTANCE

Worldwide, infections with parasitic nematodes restrict the welfare and productivity of livestock. The control of these parasites relies heavily on the administration of anthelmintic drugs. Between 1960 and 1990, the pharmaceutical industry made major progress in developing deworming compounds with excellent broad-spectrum activity and safety (McKellar and Jackson, 2004). This led to the discovery of three major drug classes available for ruminants, each with distinct modes of action: benzimidazoles (BZs), imidothiazoles and tetrahydropyrimidines (I/Ts) and

macrocyclic lactones (MLs). Relatively shortly after their introduction into the market, the development of resistance against all anthelmintic drug classes has been reported (Table 1).

Anthelmintic resistance occurs when parasites, usually eliminated by a given dose, suddenly survive the treatment. Since resistance is inherited, the surviving worms will pass their resistance alleles to their progeny (Sangster, 1999). Resistance against drugs belonging to the same anthelmintic drug class is called side-resistance, whereas cross- and multidrug-resistance refers to resistance against two or multiple drugs belonging to different anthelmintic drug classes. Today, the problem of anthelmintic resistance is by far the most severe in small ruminants. Multidrug resistance of the major gastrointestinal nematodes has been documented worldwide in sheep and goats (e.g. *Haemonchus contortus, Teladorsagia circumcincta*, *Trichostrongylus* spp. and *Cooperia* spp.) (Van Wyk et al., 1999; Chandrawathani et al., 2003; McKenna, 2010; Sargison et al., 2010; Kaplan and Vidyashankar, 2012; Torres-Acosta et al., 2012). In South Africa, New Zealand and Australia, multidrug resistance has even forced a few farmers to stop sheep and goat farming (Kaplan, 2004; Geary, 2005).

Compared to small ruminants, few field surveys have been performed to investigate the prevalence of anthelmintic resistance of cattle parasites. Therefore, the number of cases of cattle nematodes resistant to anthelmintic drugs might be considerably underestimated. Resistance against I/Ts or BZs has been reported in most of the major gastrointestinal nematodes in cattle (e.g. *Cooperia* spp., *Haemonchus placei, Ostertagia ostertagi* and *Trichostrongylus* spp.). The prevalence of ML-resistance in cattle nematodes, especially *Cooperia* spp., is emerging in New Zealand, Argentina, Brazil, the USA and Northern Europe, including Belgium (Vermunt et al., 1996; Coles et al., 1998; Coles et al., 2001; Loveridge et al., 2003; Mejia et al., 2003; Anziani et al., 2004; Mason and McKay, 2006; Waghorn et al., 2006; Soutello et al., 2007; Suarez and Cristel, 2007; Demeler et al., 2009; Gasbarre et al., 2009; Edmonds et al., 2010; El-Abdellati et al., 2010a; El-Abdellati et al., 2010b). Table 2 summarizes the results of the few field surveys that were conducted in order to assign the extent of anthelmintic resistance of bovine nematodes. Most alarming are the reports of multidrug resistance

Table 1. Introduction of anthelmintic drugs for ruminants and the development of resistance to the drug.

Anthelmintic drug class	Mode of action	Generic drug name	Introduced on the market	Resistance reported	Reference
Heterocyclic compounds	Blocking dopaminergic transmission	Phenothiazine	1940	1957	(Leland et al., 1957)
	Agonist of the inhibitory GABA-receptor	Piperazine	1954	1966	(Drudge et al., 1988)
Benzimidazoles	Inhibiting polymerisation of microtubules	Thiabendazole	1961	1964	(Drudge et al., 1964)
		Cambendazole	1970	1975	(Berger, 1975)
		Oxibendazole	1970	1985	(Drudge et al., 1985)
		Mebendazole	1972	1975	(Berger, 1975)
		Albendazole	1972	1983	(Cawthorne and Whitehead, 1983)
		Fenbendazole	1975	1982	(Boersema and Lewing-van der Wiel, 1982)
		Oxfendazole	1976	1981	(Le Jambre et al., 1981)
		Triclabendazole	1983	1998	(Mitchell et al., 1998)
Imidazothiazoles and Tetrahydopyrimidines	Agonist of nicotinergic acetylcholine receptors	Levamisole	1970	1979	(Sangster et al., 1979)
		Pyrantel	1974	1996	(Chapman et al., 1996)
		Oxantel	1976	-	-
		Morantel	1970	1979	(Sangster et al., 1979)
Macrocyclic lactones	Allosteric modulators of the glutamate-gated chloride channels	Abamectin	Late 1970's	2001	(Wooster et al., 2001)
		Ivermectin	1981	1988	(van Wyk and Malan, 1988)
		Moxidectin	1991	1995	(Leathwick, 1995)
		Doramectin	1993	2007	(Borgsteede et al., 2007)
		Eprinomectn	1996	2003	(Loveridge et al., 2003)
Amino-acetonitrile derivative	Agonist of nicotinergic acetylcholine receptors	Monepantel	2009	-	-
Spiroindole	Antagonist of cation channels	Derquantel	2010	-	-

Table 2. Prevalence of the anthelmintic resistance of bovine nematodes. Resistance was considered if the fecal egg count reduction was below 90% (Brazil) or below 95%, with the lower confidence interval lower than 90% (all other field surveys).

Region/country and reference	Number of farms	% BZ resistance	% I/T resistance	% ML resistance	% multidrug-resistance	Nematode species involved
New Zealand (Waghorn et al., 2006)	62	76% ALB	6% LEV	92% IVM	74% ALB+IVM	*Cooperia* spp. and *Ostertagia* spp.
Brazil (Soutello et al., 2007)	25	25% ALB	8% LEV	92% IVM 24% MOX	12% ALB+IVM 8% ALB+LEV+ IVM	*Cooperia* spp., *Haemonchus* spp. and *Oesophagostomum* spp.
Argentina (Suarez and Cristel, 2007)	25	32% FEN	Not detected	60% IVM	28% FEN+IVM	*Cooperia* spp. and *Ostertagia* spp.
Belgium, Germany and Sweden (Demeler et al., 2009)	22	Not detected	Not included	74% IVM	Not detected	*Cooperia* spp. and *Ostertagia* spp.
Belgium and Germany (El-Abdellati et al., 2010a)	88	Not included	Not included	39% IVM	Not detected	*Cooperia* spp., *Ostertagia* spp., *Nematodirus* spp. and *Trichostrongylus axei*

Abbreviations: % BZ, % I/T or % ML resistance: the percentage of farms with reduced anthelmintic efficacy against benzimidazoles, imidothiazoles and tetrahydropyrimidines or macrocyclic lactones, respectively. ALB: albendazole; FEN: fenbendazole; LEV: levamisole; IVM: ivermectin; MOX: moxidectin.

against both MLs and BZs in New Zealand and South America (Waghorn et al., 2006; Soutello et al., 2007; Suarez and Cristel, 2007).

In this review, a summary is given of the principal contributors to the development of anthelmintic resistance and the detection methods to evaluate the efficacy of MLs against ruminant nematodes. Subsequently, the most recent findings about the resistance status of nematode species in cattle in Belgium are described. Finally, potential measures to delay the development of resistance are discussed.

FACTORS AFFECTING ANTHELMINTIC RESISTANCE DEVELOPMENT

The development rate of anthelmintic resistance appears to be slow at first, but once a certain level of resistance genes has been established, the following treatments result in an exponential increase of these resistance genes to a level where treatment failure occurs (Barnes et al., 1995; Sangster, 1999). The more intensively parasites are controlled with drugs, the more likely resistance will develop. Once resistance is present in a parasite population, there is no evidence of reversion or loss of resistance (Sangster and Dobson, 2002). The dynamics of the selection for anthelmintic resistance of parasites in sheep have been well studied (Leathwick et al., 2009), and some predisposing factors are likely to be similar in the nematode parasites of cattle (Sutherland and Leathwick, 2011). These factors act either independently or in an additive fashion, and may be associated with the parasite species, the infected host, drug treatment, on-farm control management or the environment.

Parasite genetics and biology

Due to their genetic diversity, parasites in a population do not respond uniformly to treatment. The high genetic diversity is linked to the huge population size and high reproduction rate of parasites (Vercruysse and Rew, 2002). It is presumed that resistance alleles already exist within the parasite population, prior to the first introduction of a drug (Wolstenholme et al., 2004). However, an alternative hypothesis suggests multiple origins of resistance by spontaneous and recurrent mutations (Skuce et al., 2010). Although the genetics of resistance are still poorly understood, resistance develops more quickly if only one gene is involved than when multiple genes are involved. Moreover, resistance develops faster if the genes are dominant rather than recessive: both heterozygote and homozygote worms will survive the treatment and contribute to the next generation (Sangster et al., 1998; Le Jambre et al., 2000; Coles, 2004). Furthermore, some parasites have biological characteristics that favor resistance alleles to build up faster in the population, such as their direct life cycles (no intermediate host), a short generation time and high fecundity. It is assumed that, if resistant parasites have enhanced fitness or if resistance is linked to other fitness genes, the spread of resistance in the population will also increase. Fitness includes all properties that enable

more worms to complete their life cycles, such as the egg-laying rate, the persistence of worms in the host (a reduced hypobiosis shortens their life cycles), survival on the pasture, the ability to migrate on herbage and their infectivity when ingested (Coles, 2005). It has also been suggested that ivermectin resistant *Cooperia oncophora* in cattle have become more pathogenic than susceptible worms (Coles et al., 2001; Wolstenholme et al., 2004).

Refugia and management factors

Refugium is the parasite population, which is not exposed to anthelmintic treatment. The larvae on pasture, the percentage of animals left untreated and the arrested larval stages not affected by treatment of the host determine the parasites in refugia. The proportion of parasites in refugia needs to be optimal in order to dilute out the resistant genes in the pool of susceptible genes. Hence, the development of anthelmintic resistance is delayed without causing clinical disease. The parasites in refugia, the frequency of anthelmintic treatment and the extent of underdosing are mainly responsible for inducing anthelmintic resistance (van Wyk, 2001). To decrease the selection pressure, it is of major importance that treatment and pasture management are fulfilled in ways that maintain refugia. Anthelmintic treatments should progress according to a strategic plan, where frequency, time of treatment and the selective treatment of first-year or infected animals are tightly followed. Short interval treatments that approach the prepatent period for the parasite, reduce the opportunities for susceptible worms to reproduce and diminish the parasites in refugia. On farms with an intensive breeding and/or grazing program, calves are given multiple treatments, and are grazed away from the adults. Hence, pasture contamination derives from worms surviving short interval treatments, which creates a selection pressure on anthelmintic resistance to develop (Kaminsky, 2003). Therefore, it is encouraged to implement an alternate grazing system, where calves are allowed to graze on pastures used by older animals the year before (Coles, 2005). It should also be avoided to treat animals and immediately moving them to a clean pasture. By doing so, contamination of the new pasture will only be attributed to a subpopulation that is resistant to treatment. In this respect, farmers should be aware that summer drought is a variable factor that clears out the free-living stages on pasture (Kaminsky, 2003). Additionally, bought-in animals should be effectively quarantine drenched before they are placed on pasture in order to dilute out the progeny of survivors of the quarantine treatment (Pomroy, 2006).

Subtherapeutic drug levels

To ensure that treatments are fully efficacious, it is important to weigh the animals first, so that the anthelmintic drug can be given at the correct therapeutic dose level. Subtherapeutic concentrations allow more worms to survive the treatment, and increase the development rate of resistance. Reduced bioavailability of the drug has been associated with the route of administration and the type of animal. Especially the inconsistent performance of topical (pour-on) applications has been questioned as a predisposing factor for resistance. Moreover, the enhanced drug metabolism of some types of animals or breeds, such as described in goats and in Belgian Blue cattle, may contribute to the selection for resistance (Vercruysse and Rew, 2002; Vercruysse et al., 2008). The selection pressure on the development of anthelmintic resistance is also affected by the pharmacokinetics of the drug. With the use of persistent (long-acting) or slow release drugs, the drug concentrations tail off slowly towards the end of their elimination phase as a result of an extended half-life. This effect has the same influence as underdosing animals. Therefore, short-acting drugs are preferably used (Bisset et al., 1990; Wolstenholme et al., 2004; Gonzalez Canga et al., 2009; El-Abdellati et al., 2010a; Sutherland and Leathwick, 2011).

THE IMPORTANCE OF DIAGNOSING ANTHELMINTIC RESISTANCE

Regular drug treatments increase the selection pressure on the resistance alleles of the parasite population. At a certain point, the anthelmintic drug is no longer useful in protecting the host against parasite infections, and a change to another drug, with a different mode of action, is necessary. It is of great importance to detect anthelmintic resistance when the frequency of the resistance alleles of the parasite population is still low. In this way, the onset of anthelmintic resistance may be delayed, and the efficacy of the currently used anthelmintic drugs could be maintained (Martin et al., 1989).

The World Association for the Advancement of Veterinary Parasitology (WAAVP) has provided guidelines on the detection of anthelmintic resistance. However, in cattle, it is still difficult to assign the correct resistance status and to compare data of different surveys. The most accepted methods are two in vivo methods: the fecal egg count reduction test (FECRT) and the controlled efficacy test (CET). Although the CET is the most reliable method, it is not feasible in commercial farm settings (Coles et al., 1992; Coles et al., 2006). In the following paragraphs the strengths and drawbacks of the available diagnostic tests for anthelmintic resistance are discussed.

Controlled efficacy test

This in vivo test is suitable for all types of anthelmintic drugs, and is the gold standard for evaluating their efficacy. The CET requires the infected host to be sacrificed. Therefore, this test is not suitable for diagnosing resistance in the field, but is ideal for dose-confirmation studies or when the confirmation of resistance is required. The percentage efficacy is determined by comparing the means of surviving parasites

in groups of treated and untreated animals after artificial infection. Resistance is confirmed when the reduction in worm counts is <90%, or when more than 1000 worms survived the treatment (Taylor et al., 2002).

Fecal egg count reduction test

This in vivo procedure is currently the most practical method for the field diagnosis of resistance against anthelmintic drugs. Based on the microscopic detection of nematode eggs in fecal samples of the infected host before and after treatment, the reduction in fecal egg counts (FECs) is calculated. At the moment, standards for the FECRT only exist for sheep. An accurate determination of resistance is more difficult in infected cattle than in infected small ruminants, since the FECs tend to be lower (Taylor et al., 2002). A population of worms is declared to be resistant if the percentage reduction is <95% and if the lower 95% confidence interval is <90%. If only one of the two criteria is met, resistance is suspected (Coles et al., 1992).

The major limitation of the FECRT is its lack of analytic sensitivity. Martin et al. (1989) demonstrated that the FECRT only detects BZ-resistance in *T. circumcincta* and *Trichostrongylus colubriformis* in sheep when the frequency of the resistance alleles is greater than 25% in the parasite population. The modified McMaster technique, with a detection limit of 50 eggs per gram feces (EPG), often fails to detect low numbers of eggs. As a consequence, an early diagnosis of resistance is impeded (Levecke et al., 2009). If pretreatment egg counts are <150 EPG, a more sensitive counting method is recommended. Recently, the commercial FECPAK counting system has been introduced, and has a detection limit of 10 EPG to test for nematode egg counts in cattle (www.fecpak.com). The FLOTAC technique, with a detection limit of 1-2 EPG, reaches the required sensitivity but loses on practicality (Cringoli, 2004). Another drawback of the FECRT is that it is not species-specific. In a mixed infection, it is impossible to differentiate microscopically the eggs of different nematode species. In order to calculate the species-specific drug efficacies, it is suggested to culture fecal samples pre- and posttreatment, from which third stage larvae can be harvested and differentiated. A third disadvantage is that the FECRT is labor intensive. Therefore, its use as a monitoring tool is limited.

The interpretation of the FECRT is affected by a complex interplay of various factors, including the detection limit of the FEC method, the number of animals per treatment group and the level of excretion and aggregation of the FECs (Levecke et al., 2012). Besides, the correlation between egg counts and worm numbers is not always clear, especially not in cattle (Eysker and Ploeger, 2000; Coles et al., 2006; Kotze and Kopp, 2008). Due to the temporary suppression of egg production caused by BZs and MLs, fecal samples should be collected 8-10 and 14-17 days after treatment with BZs or MLs, respectively (Coles et al., 2006). The variability of FECR data may also be attributed to the calculation methods (i.e. geometric means of FECs appear to overestimate the efficacy compared to arithmetic FEC means) and the multiple formulas that are available (i.e. formulas may include/exclude untreated control groups or may be based on individual FECs instead of group mean FECs) (Presidente, 1985; Dash et al., 1988; Coles et al., 1992; Wood et al., 1995; Cabaret and Berrag, 2004; Dobson et al., 2009).

The outcome of the FECRT is also prone to confounding factors, which also apply to the CET. To reduce the likelihood of false positive results (reduced anthelmintic efficacy without true anthelmintic resistance), a number of requirements should be taken into account. Weighing the animals is essential to avoid a suboptimal treatment dosage. The pharmacokinetics of the drug vary according to the route of administration (bolus, topical, oral or injectable), formulation, body condition, age and physiological status. All of these factors contribute to differences in the (persistent) activity of the anthelmintic, and may result in a lower drug efficacy if the product is eliminated from the body of the host too fast (Lifschitz et al., 2004; Vercruysse et al., 2008; Gonzalez Canga et al., 2009).

To assess the (lack of) efficacy of anthelmintic drugs that do not affect the parasite's fecundity, additional research is desired in order to optimize and validate the FECRT. The number of animals sampled and the detection limit of the test need to be better tailored to the level of infection and the aggregation of egg excretion (Levecke et al. 2012). The possibility to use pooled fecal samples should also be examined.

In vitro assays

In vitro assays have the advantages of low cost and having no inter-host variation, since no use of animals is required. Moreover, replication and standardization are possible (Sangster and Gill, 1999). Anthelmintic resistance can be detected by the following in vitro tests: larval migration inhibition assay (LMIA); micromotility meter test (MMT); larval development assay (LDA); larval feeding assay (LFA) and egg hatch assay (EHA).

Migration and motility tests are based on the drug-induced paralysis of the body musculature of trichostrongyloid nematodes. In the LMIA, ex-sheathed third stage larvae (L3) are incubated in serial dilutions of anthelmintic for 24 hours, and subsequently transferred onto a sieve for a further 24 hours. Resistant L3 are able to migrate through the sieve, while susceptible L3 remain on the mesh. Subsequently, the percentage migrated L3 is calculated. In the MMT, movements of L3 or adult worms, incubated in anthelmintic dilutions, fractionate light rays, which are measured with a photodetector. The numerical representation of this signal is termed the motility index. Active worms give higher indices than paralyzed worms (Folz et al., 1987; Demeler et al., 2010).

The LDA measures the potency of the anthelmintic as inhibitor of the development, presumably as a result

of starvation through the inhibition of feeding. In case of the LDA, trichostrongyloid eggs are incubated for 6-8 days in a growth medium with *Escherichia coli* as a food source and with the anthelmintic under test. Subsequently, the percentage developed L3 is calculated. Fresh eggs are the most crucial factor for the successful performance of the LDA (Gill et al., 1995; Demeler et al., 2010). A commercial LDA (Drenchrite®) has been developed for the detection of BZ- and levamisole resistance in sheep and goat nematodes (Tandon and Kaplan, 2004). ML-resistance may also be diagnosed with the LFA in which first stage larvae (L1) are cultured with fluorescein-5-isothiocyanate-labelled *E. coli* and serial dilutions of the anthelmintic. Under a fluorescence microscope, the ratio of fed and unfed larvae at each drug concentration is determined (Alvarez-Sanchez et al., 2005).

With the EHA, the proportion of eggs that (fail to) hatch in increasing drug concentrations is determined. Therefore, the EHA is only suitable for detecting BZ-resistance, as MLs and I/Ts are not ovicidal. Eggs are first recovered from the feces, then incubated in BZ-dilutions for 48 h at 25°C, and subsequently stopped by adding one drop of Gram's iodine. Finally, the eggs and larvae are microscopically counted. As for the LDA, fresh eggs are indispensable (von Samson-Himmelstjerna et al., 2009a).

The results of in vitro tests are interpreted using EC_{50} values, describing the concentration at which a drug is half-maximal effective (50% of the parasites is killed). As by definition, resistant isolates have higher EC_{50} values than susceptible isolates. The biggest challenges for all of the diagnostic bioassays are the establishment of reference EC_{50} values and the determination of species-specific efficacies in mixed parasite infections. Therefore, the accuracy, sensitivity, repeatability and reproducibility for different isolates and species in different laboratories still require optimization. Additionally, validation against in vivo data is required, since the pharmacology of the drug in the host-parasite system is lost in in vitro assays (Sangster and Gill, 1999).

Molecular detection techniques

The sooner anthelmintic resistance is diagnosed, the better. Therefore, a promising alternative for the in vivo FECRT and CET and the in vitro assays could be a more sensitive molecular test, which could also overcome the problem of egg suppression after treatment, for example by analysing eggs before treatment. So far, molecular markers for detecting and measuring anthelmintic resistance only exist for BZs in sheep. Therefore, the WAAVP strongly encourages further investigation of the genetic mechanisms of resistance, especially in bovine nematodes.

Theoretically, molecular tests are capable of detecting resistance alleles when the frequency of these alleles is still very low. Therefore, a genetic test for resistance requires the knowledge of the molecular basis of resistance. The identification of mutations in target genes and the detection of alterations in the expression of genes could lead to the development of probes, respectively for pyrosequencing and real-time PCR. These techniques would enable the determination of susceptible or resistant populations (Ronaghi, 2001; Gruber et al., 2002; Coles, 2005). As for in vivo and in vitro tests, the challenge still remains the correct identification of resistance in mixed parasite infections. Furthermore, tests based on the detection of one single mutation to diagnose resistance make an underestimation if the resistance results from more than one underlying mechanism (Kwa et al., 1994; Coles, 2005; Coles et al., 2006; von Samson-Himmelstjerna et al., 2009b). Once a molecular test is available, it remains to be determined at which resistance allele frequencies farmers will be recommended to stop using a drug. It can be asked whether or not it makes sense for a farmer to stop using a drug when a molecular test indicates a low level of resistance, and the anthelmintic results in 95% reduction in egg counts (Kaplan and Vidyashankar, 2012).

ANTHELMINTIC RESISTANCE ON BELGIAN CATTLE FARMS

The predominant nematode species infecting cattle in temperate climate regions are *O. ostertagi* and *C. oncophora*, with 100% prevalence on pastures grazed by cattle. In Belgium, 72% of the farms use MLs to control parasite infections, of which 27% specifically use ivermectin (IVM) (Charlier et al., 2010). *Cooperia* spp. are considered to be the dose-limiting species for MLs. This means that the recommended dose is determined based on the efficacy against these species (Vermunt et al., 1995; Vercruysse and Rew, 2002).

The first report of reduced IVM efficacy on Belgian cattle farms dates from 2006 (Demeler et al., 2009). At that time, seven farms were investigated, and on all of the seven farms, reduced efficacies were observed 21 days after IVM treatment, with FEC reductions ranging from 58-95%. After a revisit, the reduced IVM efficacy could only be confirmed on one farm, with a FEC reduction of 54% on day 21 posttreatment. On all of the farms, only *C. oncophora* was recovered from the larval cultures. Continuous monitoring of the evolution of IVM-resistance during four consecutive years on one of the previously investigated farms, showed a rapid increase of the resistance level in *C. oncophora*. After IVM treatment, reductions in FECs of 73%, 40% and 0% were recorded, respectively in 2006, 2007 and 2008. One year later, side-resistance against moxidectin (MOX) was also determined (FECR of 83%), despite the fact that MOX had never been used on this farm before. This might suggest that the use of any type of MLs is inappropriate once IVM-resistance has been detected. On the other hand, fenbendazole, belonging to the BZ drug class, was still fully (100%) effective on this farm (El-Abdellati et al., 2010b). Recently, the CET has confirmed the high resistance status of this particular *C. oncophora* field isolate, with only a reduction of

38% in worm burden after IVM treatment. Surprisingly, the failure of MOX treatment was demonstrated even more clearly with only 31% reduction in worm counts, while the FECRT in this trial only suggested borderline resistance against MOX. The discrepancy between the FECs and worm counts for *C. oncophora* was explained by the reduced fecundity after MOX treatment. Failure of the FECRT to detect MOX resistance has also been reported by other researchers (Yazwinski et al., 2013). As a consequence, the FECRT is not a reliable assay to detect MOX resistance, as some cases may be overlooked (De Graef et al., 2012; Yazwinski et al., 2013).

The rapid build-up of this resistant worm population is impressive, and has given rise to a new survey on a larger number of farms, in order to make a better estimation of the prevalence of anthelmintic resistance in Belgium and Germany (Table 2). Of 88 farms included in this study, 84 farms used MLs. A FECR <95% was observed on 33 out of the 84 farms (39%). *Cooperia* spp. were the most prevalent parasites after treatment, *O. ostertagi*, *Nematodirius* spp. and *Trichostrongylus axei* were also observed in small numbers (0.5-2.5%) on some of the farms using MLs. However, when taking into account the between-animal variation and measurement error, reduced efficacy could only statistically be confirmed on 25% of the farms. Moreover, when four farms were revisited, only on one farm, resistance against IVM could be confirmed. These results showed that reduced efficacy, observed with the FECRT, is not only caused by anthelmintic resistance, but that the detection limit of the FEC technique used and the (in)correct administration of the anthelmintic drugs are confounding factors of major importance (El-Abdellati et al., 2010a).

So far, emerging ML-resistance has only been reported for *C. oncophora* and not for the more pathogenic *O. ostertagi* on Belgian cattle farms. Since *C. oncophora* is the dose-limiting species for MLs, resistance is expected to appear first in this species. Moreover, *Cooperia* spp. are predominantly parasites of younger cattle, as immunity to *Cooperia* spp. tends to develop earlier than to for example *O. ostertagi*. Consequently, anthelmintic programs tailored to treat first-year animals are likely to preferentially select for anthelmintic resistance in *Cooperia* spp. (Vercruysse and Claerebout, 1997; Sutherland and Leathwick, 2011). On cattle farms in Sweden and Germany, ML-resistance has been suspected in *O. ostertagi*, and is also expected to occur in Belgium. However, the existing levels of resistant *O. ostertagi* in Belgium are still below the detection threshold (Demeler et al., 2009).

HOW TO PREVENT ANTHELMINTIC RESISTANCE?

Anthelmintic resistance mainly develops because of underdosing, frequent treatments and low refugia. Dosing animals according to the manufacturer's recommendations is the first requirement to reduce the development of anthelmintic resistance and to distinguish between treatment failure due to underdosing and true resistance. Secondly, farmers should integrate preventive anthelmintic treatments in their grazing management in order to reduce the number of treatments required. The main focus should be on the first-grazing season calves, since they are most susceptible to gastrointestinal nematode infections. Complete eradication of gastrointestinal parasites on the pasture is not feasible. Instead, a low level of parasitism must be tolerated to trigger a protective immune response in the host, which will protect the animals in the following grazing seasons (Claerebout et al., 1998). Measures that can be taken to reduce the larval pasture contamination and hence the number of treatments include mowing, late turnout on pasture and reduced stocking density (Charlier et al., 2010). Serum pepsinogen levels can be determined at the end of the grazing season to evaluate the applied worm prevention, which can then be optimized for the next batch of first-grazing season calves, if necessary (Charlier et al., 2011). Recently, the importance of the worm population in refugia for slowing down the development rate of anthelmintic resistance has been the focus of attention. This population is believed to be susceptible, and provides a reservoir in which resistant parasites may be diluted. Higher proportions of refugia may be achieved through a targeted selective treatment (TST) approach, where anthelmintic drugs are for example only administered to heavily infected individuals in the herd (Greer et al., 2009; Charlier et al., 2012). This strategy is based on the fact that the majority of the worms reside in the minority of the animals (Stafford et al., 2009). For the successful implementation of the TST approach, it is essential to identify those animals with the highest worm burdens. Today, most cattle farmers apply a TST strategy but only to administer additional treatments during summer to animals that show signs of clinical PGE. However, a preventive TST approach should preferably be pursued. Unfortunately, for cattle, there are no convenient diagnostics to identify the animals in the herd that should be treated. FECs can be determined two months after the turnout, or the weight gain per animal can be monitored, but both approaches are too labor-intensive to be widely used (Hoglund et al., 2009). It would be interesting if a sensitive molecular test could be integrated in a TST approach, in order to identify the most heavily infected animals and simultaneously define the resistance status of the parasites.

Another advice farmers could take into account to reduce the development rate of anthelmintic resistance, is avoiding the use of the same class of anthelmintic drugs every year. In this way, the efficacy will be maximized and the longevity of the compounds will be prolonged (Dobson et al., 2001). Recently, the WAAVP guidelines have requested the approval of anthelmintic combination products for the use in ruminant livestock and in horses (Geary et al., 2012). The use of combination products may maximize the

breadth of spectrum, may overcome species-specific resistance profiles (dose-limiting species), and may delay the development and spread of resistance when the resistance allele frequencies are still low. Moreover, research on several alternative measures that reduce the dependence on anthelmintic drugs is also ongoing. For example, nematophagous microfungi, such as *Duddingtonia flagrans*, could be given in an oral formulation. After passage through the bovine gastrointestinal tract, they reduce pasture contamination by preying on the pasture larvae (Waller et al., 1994; Assis et al., 2012). Additionally, immunologic control of worm infections through vaccination could be the answer to anthelmintic resistance. However, despite the identification of several candidate protective antigens, no vaccines against gastrointestinal nematode parasites are currently available (Claerebout et al., 2003; Vercruysse et al., 2007).

CONSIDERATIONS

Most Belgian farmers are unaware of the anthelmintic resistance status on their farms, mainly because they have not encountered any problems yet. However, it is important to be forethoughtful. In sheep nematodes, it has been demonstrated that once the frequencies of resistance alleles exceed a certain threshold, these frequencies will exponentially increase. From this stage, the used anthelmintic drugs is no longer efficacious. Although it is not known how fast the resistance to a certain anthelmintic drug develops, it is irreversible, and alternatives are scarce. Until better diagnostics are available, it remains important to routinely monitor the efficacy of anthelmintic drugs at farm level with the FECRT. Although the FECRT is not sensitive enough to detect resistance in the initial phase, it can detect resistance before clinical treatment failure occurs, which is the current 'detection threshold' for most farmers.

When reduced anthelmintic efficacy is confirmed to be ML-resistance by the FECRT, farmers are advised to change to an anthelmintic drug class with a different mode of action. Unfortunately, only few anthelmintic classes with a different mode of action are currently available as alternatives for MLs, i.e. benzimidazoles and imidazothiazoles. Recently, antiparasitic compounds with a novel mode of action, i.e. monepantel and derquantel (Table 1), have been introduced on the market, but until now, both products have only been registered for the use in sheep. When alternative drugs are advised in cases of ML-resistance, these anthelmintic drugs should also be used with caution in order to prevent the development of resistance against this drug class. Furthermore, it should be stressed that any adjustments of worm control programs are case-specific, since they depend on the treatment history and the pasture management of the farm. Decision support systems (based on computer simulations) can make it easier to improve future decision making on nematode control at farm level (Greer et al., 2009; Charlier et al., 2012).

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