1 21 April 2016
2 CVMP/EWP/573536/2013
3 Committee for Medicinal Products for Veterinary Use (CVMP)

4 Reflection paper on anthelmintic resistance

5 Draft 2

Draft agreed by CVMP Efficacy Working Party (EWP-V)	February 2014
Adopted by CVMP for release for consultation	10 April 2014
Start of public consultation	23 April 2014
End of consultation (deadline for comments)	31 July 2014
Revised draft agreed by EWP-V	24 February 2016
Revised draft adopted by CVMP for release for second consultation	21 April 2016
Start of public consultation	29 April 2016
End of consultation (deadline for comments)	31 July 2016

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Reflection paper on anthelmintic resistance

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1. Introduction

Helminth infestations are common in most animals. Usually, there is a balance between helminth infestation and the immune system of the animal and thus helminths may not lead to clinical signs of disease. However, helminth infestation may impact severely on the health status of animals that do not have sufficient immunity against worms *e.g.* young, diseased animals, or those exposed to high infestation pressure. This might subsequently impact on performance (e.g. sports horses) and production (e.g. reduced milk and weight gain in sheep). It is therefore important to ensure the availability of effective veterinary anthelmintics. However, resistance to anthelmintics in veterinary medicines is an increasing problem worldwide, especially in ruminants and horses.

The scope of this reflection paper is to describe the current resistance situation in Europe for different helminths and anthelmintic classes and to reflect current knowledge on known resistance mechanisms. For companion animals, there is currently limited knowledge about anthelminthic resistance. Thus, this reflection paper focuses mainly on food producing animals and horses. Moreover, monitoring systems and methods of detecting resistance are described, as well as the currently applied management strategies to delay resistance development. Finally, this paper provides some recommendations on measurements that might delay resistance development.

2. Definition of resistance

In line with the World Association for the Advancement of Veterinary Parasitology (WAAVP) Guideline on anthelmintic combination products targeting nematode infections of ruminants and horses (18), anthelmintic resistance can be defined accordingly: "t*he ability of parasites to survive doses of drugs that would normally kill parasites of the same species and stage"*. It is inherited and selected for, because the survivors of drug treatments pass genes for resistance on to their offspring. These resistance genes are initially rare in the population or arise as rare mutations, but, as selection continues, their proportion in the population increases as does the proportion of resistant parasites. Cross resistance can be defined as acquired resistance to an anthelmintic, not as a result of direct exposure but by exposure to another anthelmintic.

3. Current resistance situation (overview)

There is evidence for the development of anthelmintic resistance to various helminths in almost all animal species and to various classes of anthelmintics.

Several scientific reports indicate, to different extents, an increase of helminth resistance to the established classes of anthelmintics (benzimidazoles, tetrahydropyrimidines, imidazothiazoles and macrocyclic lactones) in the EU (4, 51, 52). However, isolated reports on cases of helminth resistance to the newest classes of anthelmintics have also been published (e.g. resistance of *Haemonchus contortus* to monepantel, an amino-acetonitrile derivative, 58).

Benzimidazoles are the oldest class of modern anthelmintics; thiabendazole was introduced in the 1960's. The first report of decreased efficacy of thiabendazole against *Haemonchus contortus* strains dates from 1964, just 3 years after its introduction into the market (57). Resistance has developed rapidly to other anthelmintic classes, particularly those used in sheep and horses, after their introduction into the market. For example, resistance to imidazothiazoles-tetrahydropyrimidines and avermectin-milbemycins classes developed within 3 – 9 years in sheep (26). Today, resistance to anthelmintics is a major constraint in the sheep industry in Australia but resistance is also evident in Europe. Papadopoulos *et al*. (2012) reported the widespread incidence of multidrug-resistant

populations of *Haemonchus contortus*, *Teladorsagia* and *Trichostrongylus* to benzimidazoles, imidazothiazoles and macrocyclic lactones in sheep throughout Europe (43).

In Europe, an emerging problem is the decreased efficacy of triclabendazole against liver fluke (*Fasciola hepatica*) in sheep and cattle (46). Resistant populations of *Cooperia spp* to ivermectin were observed in cattle (13). In horses, resistance was reported for *Cyathostominae* and *Parascaris equorum* to benzimidazoles, pyrantel and macrocyclic lactones (19, 40, 41). *Oesophagostomum spp* has been reported to be resistant to pyrantel in pigs (50), and resistance in ascarids and hookworm to pyrantel has been observed in dogs and cats (28, 47).

4. Mechanisms of resistance

Due to modern molecular technology, mechanisms of resistance in worms are becoming increasingly understood. As described by James et *al.* (2007) and Prichard (2001) and Wolstenholme *et al.* (2004), resistance in worms can be the result of a variety of mechanisms and can be roughly categorised as genetic changes in the drug target, changes in the drug transport (*e.g.* ATP-binding Cassette (ABC) transporters), or changes in the metabolism of the drug within the parasite.

The relationship between the above mentioned changes and resistance varies between helminth species. Whereas benzimidazole resistance in nematodes can be due to a mutation in the gene coding for the target site, the same mutation does not seem to cause resistance to triclabendazole in the trematode *Fasciola hepatica* (62). Even within a single worm species different mutations can lead to resistance to the same anthelmintic. For instance, benzimidazole resistance in *Haemonchus contortus* can be caused by the phenylalanine to tyrosine mutation at amino acid position 200 of the isotype 1β-tubulin gene (31). However, the frequency of this resistance point mutation (single nucleotide polymorphism, SNP) varies considerably and it can be low even in benzimidazole (BZ)-resistant populations (25, 20) which carry other mutations (e.g. codon 167). Although genetic selection contributes to resistance, changes in drug transport mechanisms or in the metabolism of the drug within a worm species also account for different resistance mechanisms to the same anthelmintic (3, 59).The P-glycoprotein, a cell membrane transport protein able to transport many different drugs (including ivermectin, benzimidazoles and imidazothiazole derivatives), may lead to multi drug resistance by increasing the active transport of drugs (25, 27, 65).Therefore, it can be concluded that more research is needed in order to understand the mechanisms and to develop suitable assays for detection of resistance.

5. Monitoring resistance

Investigations on resistance in helminths is a demanding task since mechanisms of resistance are complex and suitable methods of detecting and evaluating resistance are limited. A worm species which has developed resistance towards a certain anthelmintic substance might still be susceptible towards other classes of antiparasitics, implying that treatment alternatives may also be applicable for resistant species. It may be of interest to evaluate the occurrence of cross-resistance between different substance classes to inform the user on potential treatment options.

Prevalence of resistance in helminth species to different classes of anthelmintics in different target species is not systematically documented throughout Europe, and the development of resistance across Europe is therefore difficult to estimate.

5.1. Monitoring systems

There are only a few monitoring programmes running in the EU, which are mostly organised locally. It would be helpful to establish surveillance programmes to get a better insight into the evolution of resistance. It would also be useful to gather information on any academic studies being conducted in the Community.

5.2. Pharmacovigilance system

Reports of a “lack of expected efficacy” are part of the EU-wide pharmacovigilance system comprising the spontaneous adverse event reporting system and periodic safety update reports (PSURs). The currently available reports might be supportive in providing an indication of potential development of resistance to a particular active substance.

However, the system has some limitations partly because resistance might not be immediately recognisable in the field and there is underreporting of lack of efficacy and the true incidence of lack of efficacy is likely to be underestimated.

5.3. Methods of detecting resistance

There are various *in vivo* and *in vitro* methods available to assess the efficacy of anthelmintics. Furthermore, specific laboratory methods can be applied to confirm a suspicion of resistance in the field, e.g. as described in the WAAVP study recommendations and guidelines (64, 6).

5.3.1. Nematodes

5.3.1.1. Faecal egg count reduction test

Reduced efficacy, that may reflect the development of resistance, can be detected by using the Faecal Egg Count Reduction Test (FECRT). This test estimates the anthelmintic efficacy by comparing the worm eggs counted in faeces of naturally infected animals before and after treatment. This test can be used for all anthelmintic classes, which is a great advantage compared to other tests. However, the sensitivity of the FECRT may be low. For example, it detects only BZ-resistance of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* in sheep when the proportion of resistant worms is greater than 25% (39). In addition, the egg output of some helminth species varies depending on the density of the adult worm population (30). This is the case for *Ancylostoma caninum*, in dogs (29) or *Oesophagostomum dentatum* in pigs (5). In cattle, there is also no clear correlation between egg

output and worm number in cattle (8, 22, 30). This implies there is a risk for biased assessments when using the FECRT for the detection of resistance.

In general, FECRT can be used in horses, ruminants and pigs (6) for nematodes which shed their eggs in the faeces. The interval between treatment and second sampling should be shorter than the pre-patent period of the specific worm, thus the genus and (where possible) the species should be determined before testing. Nevertheless, the correct sampling interval depends on the type of anthelmintic (6, 7).

Different FECRT methodologies and thresholds for interpretation of efficacy are recommended for investigation of different anthelmintics in different target species e.g. different minimum group sizes, minimum pre-treatment faecal egg counts, presence/absence of a control group, time between anthelmintic administration and repeat egg counting. The WAAVP guideline on anthelmintic resistance (6)[1] interprets a faecal egg count reduction of less than 90% (arithmetic mean) as indicative of resistance in pigs, provided that a minimum pre-treatment individual egg count is present.

In horses, a reduction of less than 90% can be seen as indicative of resistance but there is some discussion on this point. Some prefer a 95% mean cut off value (9, 32) whereas others suggest different cut-off values for different classes of drugs e.g. 90% for pyrantel and 95% for benzimidazoles and macrocyclic lactones (10). Therefore further research is required in horses (8).

In small ruminants, the WAAVP guideline defines resistance as when the percentage reduction in egg count (arithmetic mean) is less than 95% and when the 95% confidence level is less than 90%; if only one of the two criteria is met, resistance is suspected.

Resistance is more difficult to accurately determine in cattle than in small ruminants, since the faecal egg counts tend to be lower (22). The major limitation of the FECRT is its lack of sensitivity. Another disadvantage is that it is not species-specific since eggs of different nematode species cannot be differentiated. Moreover, the interpretation of the test depends upon various factors including the detection limit of the method, the number of animals per group, the host species, and the level of egg excretion by the helminths (22).

Although FECRT can be very useful in the field, it is not sufficient to prove resistance to an active substance of a worm strain and other methods have to be used to confirm any suspected finding to support an SPC claim.

5.3.1.2. Egg reappearance period test

Egg reappearance period (ERP) is defined as the time interval between the last anthelmintic treatment and the resumption of significant helminth egg shedding (8). The ERP after dosing should be compared with the historical ERP of the veterinary medicinal product. The ERP is a more sensitive method of detecting a reduction in efficacy than the FECRT in some helminth species (1, 41).

5.3.1.3. Molecular assays

Molecular techniques, such as polymerase chain reaction (PCR) or pyrosequencing, can reveal mutations in helminth genes responsible for resistance to a certain anthelmintic class. Currently, in helminths, only resistance to benzimidazoles can be detected by PCR (31).

These methods are useful when resistance is caused by a single gene mutation (i.e. SNP), or by a small number of such mutations. The relevance of the mutation in the development of resistance to a

[1] The WAAVP anthelmintic resistance guideline is currently under revision. The date of publication of the revision is currently unknown

certain anthelmintic class can be substantiated by controlled laboratory, field studies or documented in literature.

5.3.1.4. Other methods

Other methods for detection of resistance are the egg hatch assay (EHA) and the microagar larval development assay (LDA), which have been developed for detection of resistance to benzimidazoles or levamisole in horses, pigs or small ruminants. Coles et al. (8) have described the procedure of these assays and the interpretation of the results. A novel method for detection of drug resistant helminths is based on the objective and digitalised evaluation of worm motility. Continued motility of nematodes after administration of an anthelmintic that should lead to paralysis of the parasite could indicate a lack of efficacy. This method has been described for larval *Haemonchus contortus, Strongyloides ratti*, adult hookworms and blood flukes (54).

5.3.2. Trematodes and cestodes

At present, there are no validated tests available for evaluation of resistance in trematodes and cestodes.

Coles et al. (7) propose a “dose and slaughter” trial to further substantiate suspected resistance of trematodes in the field: After artificial infestation followed by treatment with a flukicide (e.g. triclabendazole), the animals are killed and the number of flukes in the liver are counted. However, at the moment there is no agreed view on how to determine the occurrence of resistance on the basis of these counts.

FECRT has not been standardised for tapeworms or flukes. An egg hatch assay (EHA), recently developed for the detection of resistance of *Fasciola hepatica* to albendazole, still needs to be validated (49). Fairweather et al. (14) developed an EHA test for the detection of triclabendazole (TCBZ) resistance in *Fasciola hepatica.*

The coproantigen reduction test (ELISA test in faecal samples) might be a useful alternative to investigate resistance in flukes, also in the pre-patent stage, but further evaluation of interpretation criteria are still needed (15, 16, 21, 42).

PCR could potentially be used to confirm resistance suspected on the basis of these tests (49), but current literature on this topic is very scarce.

6. Management strategies to delay the development of resistance

Different management strategies are used with the purpose of preventing infestation and/or keeping infestation pressure low, i.e. pasture management and refugia. This would result in a reduced need for anthelmintics and consequently a delay in the development of resistance. In addition, when anthelmintic treatment is applied different aspects of use could influence the risk for resistance development. Some methods are well established and often reflected in the product information of authorised VMPs whereas others may require further investigation, as detailed below.

6.1. Correct use of anthelmintics

Each time helminth populations are exposed to an anthelmintic there is a risk of resistance development. Repeated underdosing and/or a too frequent use of anthelmintics belonging to the same class (53) will increase the risk for selection of resistance.

Rotation of anthelmintics classes has been recommended to delay the development of resistance.

Routine deworming leading to some unnecessary treatment can also contribute to the increase of the selection pressure.

Farming practices such as “drench -and- move” may provide a survival advantage for resistant parasites. To decrease the selection pressure, treatment and pasture management need to be implemented to maintain refugia (22).

Treatment recommendations should be based on an in-depth understanding of the helminth epidemiology (53). It is important that deworming is based on the confirmation of worm burden and that treatment with a relevant product is applied at the right time in relation to the life cycle of the parasite to obtain sufficient effect without unnecessary exposure (2, 53).

6.2. Refugia

The rate of development of anthelmintic resistance is increased by providing a survival advantage for parasites carrying mutations that reduce drug efficacy. “*Refugia*” is being advocated as an important tool to slow the progress of anthelmintic resistance (61). Parasites *in refugia* are those that have not been exposed to an anthelmintic, including those present as free-living stages in the environment, in untreated individuals, and any lifecycle stages in the host that are refractory to anthelmintic treatment (17, 61). Maintenance of a population of parasites *in refugia* is a strategy that aims to slow the rate of development of anthelmintic resistance. To decrease the selection pressure, treatment and pasture management need to be implemented to maintain *refugia* (22).

Sargison has published an extensive overview of management measures to create refugia in sheep helminths (53). Selective deworming of those animals that are predicted to be most infested by nematodes and/or to contribute most towards pasture contamination should slow the development of anthelmintic resistance but maintain a parasite population in refugia (2, 53). In horses, this practice still needs further scientific evidence, however, the underlying principle remains the same (41). According to Van Wyk *et al.*(61), the subpopulation of encysted equine cyathostomin larvae may be considered as refugia as they escape the effect of the drug and reduce the selection for resistance by eliminating the susceptible worm eggs onto pasture.

The value of maintaining a population of parasites *in refugia* to slow down the development of anthelmintic resistance has been demonstrated in a model using sheep. In this bioeconomic model, besides the number of flock treatments, the proportion of the worm population in *refugia* had a significant influence on the rate of development of anthelmintic resistance (44).

6.3. Use of multiactive anthelmintic products

A treatment strategy to delay resistance development may include the use of products (so called multiactive anthelmintic products) containing two or more substances with activity against the same target helminths but with a different mode of action. Modelling studies and some field studies have indicated that this strategy may delay the development of resistance to active classes (33, 34, 35), or delay development of anthelmintic resistance to existing anthelmintic classes (36, 37). However, there are also concerns that the use of multiactive anthelmintics could potentially lead to the selection of multiple resistance to different anthelmintic classes particularly when livestock grazes low contamination pasture with insufficient refugia population.

6.4. Other options

Other management measures aimed to decrease helminth infestations are pasture management *e.g.* removal of faeces from pasture to reduce the level of infective larvae, reducing stocking densities, preventing high degree of infestation or improving drainage of pastures to decrease the risk of liver fluke infestations (53). In addition to this, there are other biological control methods which are currently under development (23, 60, 24). One approach that has been tested to reduce helminthic burden in small ruminants is to select genetically less susceptible livestock (55).

7. Discussion

7.1. Resistance mechanisms and assessment of resistance

The development of anthelmintic resistance is a highly complex process influenced by the host, the parasite, environment and VMPs. At present, the resistance mechanisms to a number of anthelmintics/anthelmintic classes are not yet completely known. In addition, there is a lack of standardised/validated test systems for diagnosing resistance in helminths. More research is necessary in order to understand the resistance mechanisms and to develop easy detection methods, usable in practice. The commonly used FECRT is labour intensive. It provides reliable results only if more than approximately 25% of the nematode population is resistant (7, 39). Moreover, there still is some debate on which percentage reduction in the FECRT should be used to decide on resistance in some target helminths and target species. For liver flukes, the coproantigen reduction test (ELISA test in faecal samples) might be a useful alternative to necropsy or FECRT, which is not reliable for this helminth. Resistance detection tests in helminths based on molecular techniques (PCR) can be very specific but are not useful in the field.

7.2. Monitoring of resistance

Currently, there are no systematic surveillance programmes running in any EU country, to study the prevalence of anthelmintic resistance.

7.3. Treatment strategies

Current knowledge indicates that the implementation of certain strategies can delay resistance development. Management practices related to the handling of animals on pasture and stocking density as well as applying treatment only on the basis of a confirmed worm burden, correct dosing and alternation between different classes of anthelmintics are well known factors related to a delay in resistance development. To combine substances with activity against the same helminth but with different mechanisms of action (so called multiactive combinations) is proposed to delay the occurrence of resistance to the included substance classes. However, there are concerns that such practice could lead to development of simultaneous resistance to multiple anthelmintic classes. There is currently not enough information available to conclude on the benefits and risks connected to the combination of different substance classes.

Furthermore, the implementation of the refugia strategy is proposed as a means to delay resistance development.

Alternative methods, such as biological control methods and selection of genetically less susceptible livestock are under development.

Detailed recommendations regarding management practices are not provided in this document since they should be tailored to each individual situation, taking into consideration epidemiology, environment, farm demographics, housing conditions, resistance situation, available VMPs etc.

7.4. Assessment of anthelmintic product applications

Information on anthelmintic resistance needs to be provided in all marketing authorisation applications, in particular those where efficacy is claimed against helminth strains known to be resistant to other substances. However, guidance on how to assess anthelmintic resistance and how to reflect it in the SPC and product information is limited. As anthelmintic resistance is becoming more common, it is important to document if resistance has been reported in the claimed helminth species to the active substance or other substances of the same class of anthelmintic.

More guidance is needed on how to elaborate meaningful data (from laboratory and field trials) supporting treatment efficacy against helminth strains with documented resistance towards other anthelmintics. Additionally, further guidance is required on how to address specific aspects of anthelmintic resistance/susceptibility in the product literature, apart from the technical advice already given in the Guideline on the summary of product characteristics for anthelmintics (EMEA/CVMP/EWP/170208/2005). This guideline was developed to recommend standard warnings in regard to the possible development of resistance in the SPC of anthelmintics authorised for treatment of ruminants (sheep, goats and cattle) and horses. However, other animal species (pigs, companion animals, etc.) are not within the scope of that guideline, as no resistance of helminths was reported in those species when the guideline was adopted. Investigations on anthelmintic resistance in parasites of companion animals appeared only recently (29).

For applications claiming efficacy of a product against a worm that has documented resistance towards another substance it is important that efficacy is supported for the new product against this resistant strain. Different biochemical changes or genetic mutations can lead to resistance to an active substance or to a class of anthelmintics. Moreover, a helminth can have separate (concomitant) resistance mechanisms to different classes of active substances. It can, therefore, never be claimed that a certain active substance will always remain effective in a helminth known to be resistant to another anthelmintic substance (i.e. cross resistance may develop at any time). The absence of cross resistance between anthelmintics in worm strains might be mentioned for an authorised product but given that the situation may change over time it is not appropriate to include such information in the indication.

8. Conclusions

Currently, there are no EU-wide programmes that systematically monitor the occurrence of anthelmintic resistance in helminths of relevant animal species. Therefore trends in the development of anthelmintic resistance in Europe are difficult to identify when based on local programmes.

Demonstration of anthelmintic resistance is difficult. At the moment there is a lack of standardised/validated test systems for determining resistance in many helminth species. More research is necessary in order to understand the mechanisms and to develop validated and practical detection tests.

Prudent use of anthelmintics such as the avoidance of frequent use of the same class of anthelmintics and underdosing as described in the guideline on the SPC for anthelmintics (EMEA/CVMP/EWP/170208/2005) is recommended.

Other strategies may delay the development of resistance such as treatment solely based on diagnosis, targeted treatment to maintain parasite population *in refugia*, pasture management and possibly use of multiactive products.

9. Recommendations

Not all aspects related to the use of anthelmintics aimed to reduce the risk for resistance development fall within the mandate of the CVMP/EWP. For some aspects, the professional expertise/competence of other committees or institutions is needed to improve current understanding, monitoring, management practices, and prudent use of anthelmintics in order to scale down intensive treatment practices which are known to contribute to selection pressure, thus favouring emergence of anthelmintic resistance:

CVMP recommendations

- Treatment should be based on the confirmation of helminth infestation using appropriate diagnostic measures *e.g*. Faecal egg counts.
- To improve pharmacovigilance reporting, veterinarians should be encouraged to identify and report any lack of expected efficacy.
- To review the guideline on the summary of product characteristics for anthelmintics (EMEA/CVMP/EWP/170208/2005) and extend the scope to non-food species.
- To provide guidance for applicants on how to document the resistance to an anthelmintic substance (published literature and / or field data addressing the concerned regions in Europe) and on how to characterise and confirm a suspected resistance in a helminth strain (e.g. addressing methods of detection, types of studies, number of strains to be considered).

Responsibility of Member States

- The proposal of mandatory prescription of anthelmintics in food producing animals is not within the responsibility of the CVMP for nationally authorised products.
- Large package sizes may lead to unnecessary treatment. Package sizes could be assessed, keeping in mind the indication and the possibility of unnecessary treatment.

Research and education

The following topics fall outside the mandate of the CVMP and national regulatory agencies. However, they are of major importance for understanding and monitoring the development of anthelmintic resistance.

- To continue research on resistance mechanisms. To develop suitable and practical tests for detection of resistance in different parasite species. The threshold for determining resistance in endoparasite species needs to be defined for each target animal species.
- To work on the validation of tests, e.g. by carrying out inter-laboratory ring tests.
- To recommend standardised FECRT and thresholds to facilitate better comparability of resistance data collected in the field.
- To investigate resistance of helminths in companion animals.
- To survey routinely the occurrence of resistance throughout Europe.
- To continue research on management strategies that could reduce the need of anthelmintics.

- To continue research on biological alternatives that could reduce the need for anthelmintic chemicals.
- To educate and enhance awareness amongst veterinarians and animal owners of the prevalence and magnitude of anthelmintic resistance. To critically question current deworming practices and husbandry procedures.
- To further explore through appropriate scientific evaluation the benefits and risks in relation to resistance development associated with the use of multiactive anthelmintics.

10. Glossary

Cross resistance: defined as acquired resistance to an anthelmintic, not as a result of direct exposure but by exposure to another anthelmintic (slightly amended definition from Merriam Webster dictionary).

Multiactive anthelmintic products: products containing two or more substances with activity against the same target helminths but with a different mode of action.

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