

## Anthelmintic Resistance in Farm Animals

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### ABSTRACT

Anthelmintic resistance means that developing genetically transmitted lack of susceptibility to an anthelmintic which is previously known to be susceptible to a parasite population. Anthelmintic resistance has an increasing importance in recent years. The anthelmintic resistance which is developed especially due to use of unconscious anthelmintics also brings with it economic problems. Investigations have shown that resistance has developed to anthelmintics in a short period of time after launch to the market and even in some countries, several sheep and goat farms have been closed due to anthelmintic resistance. For this reason, especially in livestock breeding; The development of resistance should not be overlooked while planning of treatment and control programs and choosing anthelmintics. In this review, resistance mechanisms which is developed to anthelmintic drugs, resistance detection methods and anthelmintic resistance status of livestock in Turkey were evaluated.

**Key words:** Anthelmintics, Cattle, Goat, Resistance, Sheep

### Çiftlik Hayvanlarında Antelmintik Direnç

#### ÖZ

Bir parazit popülasyonunun daha önce duyarlı olduğu bir antelmintik karşı gelişen ve genetik yolla aktarılan duyarlılık kaybı olarak değerlendirilen antelmintik direnç, son yıllarda giderek artan bir öneme sahiptir. Özellikle bilinçsiz ilaç kullanımına bağlı olarak gelişen antelmintik direnç ekonomik problemleri de beraberinde getirmektedir. Yapılan araştırmalar bazı ilaçların piyasaya sürümünü takiben kısa süreler içinde ilaca karşı bir direnç geliştiğini hatta bazı ülkelerde sadece direnç gelişimine bağlı olarak çiftliklerin kapatıldığı göstermektedir. Bu nedenle özellikle çiftlik hayvanları yetiştiriciliğinde; antelmintik kullanılırken veya helmint enfeksiyonlarının tedavi ve kontrol programları planlanırken direnç gelişimi göz ardı edilmemelidir. Bu derlemede antelmintik ilaçlara gelişen direnç mekanizmaları ve direnç tespit yöntemleri ile Türkiye’de çiftlik hayvanlarında belirlenen antelmintik direnç hakkında özlü bilgi verilmiştir.

**Anahtar kelimeler:** Antelmintikler, Direnç, Keçi, Koyun, Sığır

## INTRODUCTION

Infections which is caused by parasites limit the welfare and yield of livestock around the world. The control of helminth infections is mostly based on the use of anthelmintic drugs (McKellar and Jackson, 2004). Anthelmintic resistance has developed in a short period of time after drug launched to the market, as a result of the intense and unconscious use of drugs. World Association for the Advancement of Veterinary Parasitology (WAAVP) published methods to detect anthelmintic resistance to draw attention to this issue in 1992 (Coles et al. 1992). Anthelmintic resistance has become a serious problem, especially in sheep nowadays. In some countries such as Australia, United Kingdom, New Zealand and South Africa, some sheep and goat farms have been closed due to multiple drug resistance (Kaplan 2004, Geary 2005).

### **Anthelmintics**

Anthelmintics constitutes the cornerstone to control helminth infections. Until recently, there were three main broad-spectrum anthelmintic groups in the market. These are benzimidazoles (BZs); imidazothiazole and tetrahydropyrimidines (I/Ts) and macrocyclic lactones (MLs). They are also classified as white, yellow and clear drug groups. Monepantel which is a member of amino-acetonitrile derivatives (AAD) was found about 30 years later than ivermectin and classified as the fourth anthelmintic group. Finally, Derquantel which is from spiroindoles group was classified as a fifth anthelmintic group and launched to the market as a combination with abamectin. Fourth group and fifth groups are shown with orange and purple respectively (Abbott et al. 2012).

### **Use of Low Dose Anthelmintics**

To ensure that the treatment is fully effective, the animals should be weighed and appropriate dose should be given by calculating. The use of low-dose drugs are caused to remain alive of more parasites and accelerates the development of anthelmintic resistance after treatment. Decreased bioavailability of the drug is related to drug administration routes and the animal species. Especially irregular topical (pour-on) applications are caused predisposition to the development of anthelmintic resistance. The pharmacokinetics of the anthelmintics are also effective with regards to the development of resistance. As a result of using long-acting or slow releasing anthelmintics, the host is exposed to low doses at the end of the elimination phase. Thus, short-acting anthelmintics are preferred (Wolstenholme et al. 2004, Sutherland and Leathwick, 2011).

### **Genetic And Biological Factors Contributing Anthelmintic Resistance**

Resistance is the heritable ability of the worms to survive a dose of anthelmintic which would normally be effective. The resistance is inherited and passed to the next generation. If a drug resistance develops to one anthelmintic in a class, other drugs in the same class will be effected and it is called as side resistance. If a drug resistance develops to two or more different anthelmintic groups, it is described as a cross or multiple resistance. Several sheep and goat farms have been closed cause of multiple drug resistance in Australia, South Africa and New Zealand (Kaplan 2004, Geary 2005, Abbott et al. 2012).

Although the development of anthelmintic resistance seems to be slow at the beginning, the resistance, it is increasingly continued after each treatment and the susceptibility is eventually lost. Once resistance has developed in the parasite population, it is not possible to sensitize this population again until now (Sangster and Dobson, 2002).

It is thought that the parasite population carries a resistant allele even before the drug is administered (Wolstenholme et al. 2004). According to another hypothesis, it is thought that the resistance occurs as a result of spontaneous and repetitive mutations (Skuce et al. 2010). If an individual carries two alleles or copies of a gene, it is called as homozygous. If it carries different alleles or copies of a gene, it is called as heterozygous. Homozygous parasites could be sensitive or resistant. Although the genetics of resistance is not fully understood, participating in a single gene for resistance leads to develop resistance faster. In case of resistance genes are dominant, resistance will be developed faster compared to recessive genes. Moreover, some parasites have various biologic features such as direct (monoxen) development without an intermediate host, short life cycle and high fertility rate, which accelerates the development of resistance in a parasite population (Sangster et al. 1998, Coles 2005).

### **Detection of Anthelmintic Resistance**

Anthelmintic resistance means that developing genetically transmitted lack of susceptibility to a drug which is previously known to be susceptible to a parasite population. Detection of anthelmintic resistance in a parasite population is very important when the frequency of alleles is low. Thus, development of anthelmintic resistance could be delayed and susceptibility of the drug could be preserved (Martin et al. 1989).

Currently, the detection of anthelmintic resistance is based on in vivo and in vitro tests. Using of these tests are limited because of taking a long time, expensive, labor-intensive and required test animals. Some of the tests which are used to detect anthelmintic resistance are only successful if the target parasite population is 25% or more resistant phenotypically (Coles et al. 1992).

### **In Vivo Methods for The Detection of Anthelmintic Resistance**

The two most commonly used methods for detecting anthelmintic resistance are; fecal egg count reduction test (FECRT) and the controlled efficacy test (CET). Although the controlled-efficacy test is the most trustable method, the fecal egg count reduction test is the most widely used test as an in vivo method (De Graef 2013).

#### **Fecal Egg Count Reduction Test (FECRT)**

Fecal egg count reduction test is the most practical in vivo method for detecting anthelmintic resistance and has been recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP). This method is based on the calculation of the difference as a percentage after counting of nematode eggs in feces for pre-treatment and post-treatment (10-14 days later). It is considered to develop resistance if the number of eggs per gram (EPG) in the stool decreases by a percentage below 95% after treatment (Coles et al. 1992).

This test is available for all the anthelmintics. If parasite population has more than 25% resistance, it can be said that this technique is reliable. Ideally, ten animals which have higher than 150 EPG are selected for each group (Coles et al. 2006).

If there are less than 50 EPG in feces, the modified McMaster technique cannot be used and this situation limits the use of the technique. In case that, the number of eggs in the stool before treatment is less than 150, it is recommended to use a more sensitive method. Another disadvantage of this method is the lack of species specificity (Coles et al. 2006, Levecke et al. 2009).

#### **Controlled Efficacy Test (CET)**

The controlled efficacy test is seen as the best method to determine the effect of the anthelmintics (Martin et al. 1989, Cook et al. 2006). In this test, the animals are experimentally infected with known resistant and susceptible L3 and then treated with different concentrations of the anthelmintic. After a certain period of time, parasites are collected from the abomasum. If the decrease in the number of parasites is less than 90% or more than 1000 parasites remain alive after treatment, it is considered to be resistant. The

disadvantages of this method are being expensive, time-consuming and labor-intensive. Also, using animals for testing possess some ethical problems (Coles et al. 1992, Taylor et al. 2002).

### **In Vitro Methods for The Detection of Anthelmintic Resistance**

The advantages of in vitro tests are being low cost, not differ from host to host and not necessary to use testing animals. Many methods have been developed to detect anthelmintic resistance using the nematode larvae. The most of these tests are not widely used practically because of reliability, reproducibility, sensitivity, and easy interpretation of tests are not at the desired level. Only Egg Hatching Test (EHT) and Larval Developmental Test (LDT) are widely used (De Graef 2013). In addition, Larval Paralysis Test, Micro-Motility Measurement Test, Larval Migration Inhibition Test and Molecular-Based Tests are used (Coles 2005, Jabbar et al. 2006, Demeler et al. 2010).

#### **Egg Hatching Test (EHT)**

The egg hatching test is only used to detect benzimidazole and levamisole resistance, but can not be used in macrocyclic lactones due to not being ovicidal. Eggs are incubated at various concentrations with anthelmintics to calculate the percentage of egg hatching after the eggs are obtained from the feces (Taylor et al. 2002, Coles 2005). The optimal dose is calculated using sensitive isolates. In the tested samples, the percentage of egg hatching is also regarded as the percentage of resistance. An advantage of this method is that only once stool collection is sufficient (Coles et al. 2006). The results of the egg hatching test are generally interpreted using values of ED50 (50% inhibition value) or ED99 (99% inhibition value). If the ED50 is used as the threshold value, resistant parasites in the population must be at least 25% to detect benzimidazole resistance. The sensitivity of the test was increased with the use of ED99 value, and it has become possible to detect resistant parasites with a low percentage in the population (Várady et al. 2007).

#### **Larval Developmental Test (LDT)**

The larval development test was developed to measure the potential of the anthelmintic drug with regards to inhibiting egg development. Trichostrongylid type eggs are incubated with tested anthelmintics in the medium containing *Escherichia coli* for 6-8 days in this method and then the ratio of developed L3 is calculated. The use of freshly collected eggs is the most important factor for working efficiently of the test (Demeler et al. 2010). The larval development test is used to determine the resistance of many anthelmintics

including macrocyclic lactones, although it is more labor-intensive and time-consuming than the egg hatching test. Larval growth test is more sensitive than FECRT and egg hatching test (EHT); it is able to detect up to 10% of parasites carrying resistance in populations (Jabbar et al. 2006).

### Larval Migration Inhibition Test

Motility and migration and tests are based on paralyzing muscles of the trichostrongylid nematodes by way of anthelmintics. The third stage larvae are incubated with anthelmintic serial dilutions for twenty-four hours and then transferred onto a mesh for another twenty-four hours. In spite of resistant L3s pass through the mesh, sensitive L3s remains on the mesh. Then the percentage of migrating larvae is calculated. Migration inhibition is determined by the curve resulting from different concentrations (Demeler et al. 2010).

### Molecular-Based Tests

DNA-based tests have been developed to identify genetic-based qualitative or quantitative changes (differences in gene expression). Low benzimidazole resistance which can not be detected by in vitro methods can be determined with the development of molecular-based tests. Molecular-based tests are more sensitive and faster than in vivo and in vitro tests, in spite of expensive equipment and materials. These tests allow individual detection of parasites which is carrying resistance genes in a population (Von Samson-Himmelstjerna 2006). Theoretically, even if the frequency of resistance is low in the population, molecular-based tests can detect resistance alleles. Polymerase Chain Reaction (PCR) based tests detect benzimidazole resistance by using of single nucleotide polymorphisms (SNPs). Subsequent to PCR, DNA is separated on agar electrophoresis and the bands revealed. Then real-time PCR and pyrosequencing techniques began to be used. So far, most of the molecular research has been conducted on benzimidazoles to detect anthelmintic resistance. The resistance mechanism of other anthelmintics is not as well known as benzimidazoles yet, but studies are still being continued (Kwa et al. 1994, Jabbar et al. 2006, Von Samson-Himmelstjerna 2006).

### Refugia

Parasites are called refugia, which have not been exposed to an anthelmintic drug in a parasite population. Refugia is the basis of the large majority of sustainable parasite control programs. Refugia constitute one of the sources of re-infection and prevent resistant parasites from becoming a majority of the population. Also, it consists of developmental stages of the parasite

from the egg to L3 in nature, the cysted larvae in the abomasal glands and the untreated parasites in the host (Abbott et al. 2012). Reducing the proportion of resistant parasites in the population and delaying the development of resistance by increasing the proportion of susceptible parasites constitutes the principle of refugia. Short treatment intervals reduce the reproduction of susceptible parasites, while also reduce the number of unexposed parasites. In addition, some animals in the herd should not be treated to maintain the presence of sensitive parasites (Sangster and Dobson, 2002).

### Mechanism of Anthelmintic Resistance

#### Benzimidazole Resistance

In genetic studies on benzimidazole-resistant gastrointestinal nematodes, several specific changes in the beta-tubulin-encoding sequence lead to point mutations, thus reducing drug susceptibility (Von Samson-Himmelstjerna et al. 2007, Dicker 2010). Genetic studies on *Teladorsagia circumcincta*, *T. colubriformis*, *H. contortus* and *Cooperia oncophora* have shown that tyrosine (resistant, TAC) is encoded instead of phenylalanine (sensitive, TTC) at codon 200 in beta-tubulin isotype 1 gene (Phe200Tyr or F200Y) which is caused point mutation (Kwa 1994, Von Samson-Himmelstjerna et al. 2007). The second, less common, benzimidazole resistance mechanism is the phenylalanine-tyrosine (Phe-Tyr) polymorphism at codon 167 which is seen especially in the nematodes of horses (Wolstenholme et al. 2004, Hodgkinson et al. 2008, Silvestre and Cabaret, 2002). Tyr (Tyrosine) was required at codon 200 for benzimidazole resistance in *Haemonchus contortus*; The homozygous phenylalanine-phenylalanine (Phe/Phe), the heterozygous phenylalanine-tyrosine (Phe / Tyr) or homozygous tyrosine-tyrosine (Tyr/Tyr) at codon 167 can cause the parasite to become resistant in *T. circumcincta*. Tyr (Tyrosine) is required at codon 200 for benzimidazole resistance in *Haemonchus contortus*. The homozygous phenylalanine-phenylalanine (Phe / Phe) at codon 200 and the heterozygous phenylalanine-tyrosine (Phe/Tyr) or homozygous tyrosine-tyrosine (Tyr/Tyr) at codon 167 in *T. circumcincta* can cause the parasite to become resistant (Silvestre and Cabaret, 2002, Von Samson-Himmelstjerna et al. 2007). Resulting from point mutation at codon 198, alanine (Ala) is encoded instead of glutamic acid (Glu) as an alternative mechanism of benzimidazole resistance which is found in *H. contortus* (Ghisi et al. 2007). Some of the studies have shown that P-glycoproteins are indirectly involved in benzimidazole resistance of nematodes. Another mechanism of resistance to benzimidazole is the deletion of  $\beta$ -tubulin isotype 2 in the *H. contortus* population. While heterozygous parasites are

advantageous compared with susceptible parasites in terms of benzimidazole resistance, although it is not completely resistant. Parasites are more likely to survive, especially after inadequate dosing of anthelmintics (Roos et al. 1995, Von Samson-Himmelstjerna 2006).

### Levamisole Resistance

There are insufficient studies on the resistance mechanisms of tetrahydropyrimidines including levamisole, imidazothiazole and pyrantel (Kopp et al. 2009). Studies on *Caenorhabditis elegans* have shown; 5 genes that encode the subunits (L-AChRs) of ionotropic acetylcholine receptors which is sensitive to levamisole resistance. These are 3  $\alpha$ -subunit genes (*lev-8*, *unc-63*, *unc-38*) and 2 non- $\alpha$  subunit genes (*lev-1*, *unc-29*) (Fleming et al. 1997, Boulin et al. 2008). In addition, the L-AChR expression is lost in muscle cells due to mutations in *ric-3*, *unc-74* and *unc-50* and resulting in loss of sensitivity to levamisole. For the development of the levamisole resistance, glycine (Gly) must be encoded instead of glutamic acid (Glu) at codon 153 in the *unc-38* gene of *C. elegans* (Rayes et al. 2004, Martin and Robertson, 2007). Lack of susceptibility to pyrantel receptors has occurred as a result of encoding glycine (Gly) instead of glutamine (Gln) at codon 57 of the *unc-63* gene of *C. elegans*. (Bartos et al. 2006). Nicotinic acetylcholine receptors consist of 5 glycoprotein subunits which are arranged around a central ion channel and each subunit gains different pharmacological properties to the nAChR (Fleming et al. 1997). Expression difference in HA17, which is a gene fragment, between levamisole-sensitive and levamisole resistant parasites was identified by cDNA-AFLP technique and aimed to be a potential marker for detection of levamisole resistance (Neveu et al. 2007). In *Ancylostoma caninum*, significant polymorphic differences were not observed in ARR-29, ARR-38 and ARR-63, which are subunits of the pyrantel, but it was proven that expression of these genes is significantly reduced in resistant parasites. These genes are ortholog with the *unc-29*, *unc-38* and *unc-69* genes which is found in *C. elegans* (Kopp et al. 2009).

### Macrocytic Lactone Resistance

The mechanism of macrocytic lactone resistance has not been fully understood yet. Glutamate-gated chloride channels and acetylcholine receptors have a similar structure and the central ion channel are constituted by the combination of 5 subunits ( $\alpha$  and  $\beta$ ). The  $\alpha$  subunits contain the glutamate binding site, while the  $\beta$  subunits contain the ivermectin binding site (Martin et al. 1997, Bartos et al. 2006). Some of the genes which are involved in ivermectin resistance include glutamate and

GABA-gated chloride channels (Gilleard, 2006). Changes in allele frequencies of glutamate and GABA chloride subunits were observed in different populations of *Haemonchus contortus*, but the changes in a single allele were not correlated with resistance (Blackhall et al. 1998, Blackhall et al. 2003). Macrocytic lactone resistance is emerged by mutation of a few glutamate-gated chloride subunit genes in *C. elegans* (McCavera et al. 2007). In order to develop a high level of ivermectin resistance in *C. elegans*, simultaneous mutation is required in all three genes (*avr-14*, *avr-15* and *glc-1*) which is encoding  $\alpha$ -subunit of the glutamate-gated chloride channel. *Avr-15* encodes GluCl2 which is expressed in the pharyngeal muscles of *C. elegans* and *Avr-14* encodes GluCl3 which is expressed in the extrapharyngeal nerve cell of *C. elegans*. One of the most important mechanisms of action of ivermectin is the inhibition of the pharyngeal pump which causes starvation of parasites (Dent et al. 2000, Cook et al. 2006). While parasitic nematodes have different GluCl subunit genes compared to *C. elegans*, there are also orthologs that reduce the sensitivity of ivermectin, such as *avr-14* in *C. oncophora* (McCavera et al. 2007). The genetic mechanism of the ivermectin resistance in Trichostrongylid parasites is not fully understood (Geary 2005, Prichard and Roulet, 2007).

Changes in the  $\gamma$ -amino butyric acid (GABA) receptor genes are thought to be responsible for the macrocytic lactone resistance (Blackhall et al. 2003).

Detoxification process of P-glycoproteins (PGP) is thought to play a role in macrocytic lactone resistance. P-glycoproteins are a member of the ATP binding cassette superfamily and provide active transport of endogenous and exogenous hydrophobic molecules across the membrane (Sangster and Dobson, 2002). P-glycoproteins are significantly localized in the digestive tract and it is expressed at high levels in the membranes of the intestinal and pharyngeal cells (Smith and Prichard, 2002). The main role of P-glycoproteins is to protect the organism by pumping toxic agents out of the cell. It has been reported that Tc-Pgp-9, which is a kind of PGP in the study on ivermectin resistant *T. circumcincta*, has increased expression at mRNA level, high level of polymorphism in sequence, and helminths may play an important role in resistance to ivermectin. It has been reported that increased expression at mRNA level and high level of polymorphism in the sequence are observed in Tc-Pgp-9 which is obtained from ivermectin resistant *T. circumcincta* of sheep and it has been determined that it may play an important role with regards to ivermectin resistance of helminths. Pgp-inhibited mice and Collie dogs with

PGP deficiency are highly susceptible to ivermectin and result in death as a result of extreme neurotoxicity (Lespine et al. 2008, Dicker et al. 2011).

Verapamil, as a calcium channel blocker, inhibits the binding site of Pgp, thereby increasing the efficiency of the anthelmintics. In vitro using of verapamil as a Pgp inhibitor has shown that macrocyclic lactone-resistant parasites become more sensitive (Demeler et al. 2013).

#### **Amino-Acetonitrile Derivatives (AAD) Resistance**

Monepantel was first used in small ruminants in 2009 with the commercial name Zolvix®, and the first resistance case was reported four years later after introduced to the market (Scott et al. 2013). Then there are different resistance reports from various parts of the world (Mederos et al. 2014, Love 2014, Cintra et al. 2016).

Monepantel which is a member of amino-acetonitrile derivatives targets nicotinic receptors as the mechanism of action. These receptors include DES-2 and ACR-23 subunits which is located in the pharyngeal muscles, between the nerves throughout nerve cord and the sensory nerves. Subunits of nicotinic acetylcholine receptors sensitive to amino-acetonitrile derivatives have a mechanism that only affects nematodes, and so it is not toxic to mammals, insects and other vertebrates. In vitro studies on *Haemonchus contortus* have shown that two genes are effective on resistance. As a result of deletions at the intron-exon border in monepantel-1 (*Hco-mptl-1*, also called *Hc-acr-23H*) gene of resistant *H. contortus*, stop codon is located before the regular site. Another mutation is occurred by 5' end insertional mutation in the *Hco-des-2H* gene and result in decreased susceptibility (Rufener et al. 2009, Kennedy and Harnett, 2013).

#### **Famacha (Faffa Malan Chart)**

Famacha is a low cost and easily applicable test which is developed by South African scientists to determine the anemia associated with haemonchosis in sheep and goats and it is aimed to avoid unnecessary use of anthelmintics. This test is widely used in Sub-saharan Africa and South America. The principle of this test is based on a comparison of the color of the eye conjunctiva of small ruminants with the Famacha card to determine the severity of the anemia (Malan et al. 2001).

#### **Alternative Control Methods of Anthelmintic Resistance**

Alternative treatment methods have been studied due to the problem of anthelmintic resistance in many regions of the world. The most common alternative treatment methods are; copper oxide wire particles, use of tannin-containing feeds, nematode-trapping fungi, vaccine, breeding for resistant animals, nutrition and using anthelmintic activities of medical plants (Fleming et al. 2006, Jabbar et al. 2006).

#### **Anthelmintic Resistance in Turkey**

Çırak et al. 2004 performed FECRT to detect the resistance status of strongylid nematodes on ten horse farms in Western Anatolia. Seven farms were found to be infected with the resistant cyathostomin population to benzimidazoles. Resistance of pyrantel embonate on five farms and macrocyclic lactone on six farms were investigated, but anthelmintic resistance was not detected.

Tınar et al. 2005 tested anthelmintic resistance in trichostrongylid nematodes of small ruminants by FECRT on twelve sheep and goat farms. Albendazole, tiabendazole, tetramisole and ivermectin resistance were tested and tetramisole resistance was detected in only one sheep farm.

Köse et al. 2007 tested albendazole, oxfendazole-oxyclozanide and ivermectin resistance by FECRT on seven sheep farms in Afyonkarahisar and found that ivermectin did not work at the desired level in five farms.

Çırak et al. 2010 found that macrocyclic lactone groups against *Parascaris equorum* in a horse farm were resistance.

Önder et al. 2016 determined the frequency of benzimidazole-sensitive and resistant alleles in the *H. contortus* population by 87.1% and 12.9%, respectively, and revealed the BZ resistance by the molecular method.

## **RESULTS**

Consequently, as World Association for the Advancement of Veterinary Parasitology (WAAVP) has also noted, anthelmintic resistance is a very important and restrictive factor especially in livestock breeding. For this reason, the development of resistance to anthelmintics should not be overlooked in the selection and implementation of treatment and control options for helminth infections.

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