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Review paper

Screening and prevention of anticoagulant resistance development in house mice – A review

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SUMMARY

Unrestricted use of anticoagulants has resulted in rodents developing resistance to them. This has caused a series of problems regarding reduction in rodent populations, which has further implicated great economic losses and a serious threat to the health of people and domestic animals. The popular global trend currently is to control rodent populations by applying the least possible amounts of chemicals, which on the other hand implies that several coactive alterantive measures need to be applied in an effort to reduce rodent numbers to an acceptable economic level. On the other hand, knowledge of the genetic structure of rodent populations has become an important set of information desirable to have before setting off to apply rodenticides in practice, so as to prevent ineffective use of rodenticides and prevent further spreading of resistant rodent populations. The latest trend of using combinations of low-dose anticoagulant baits requires further research as their effects on susceptible populations are known but their impact on resistant animals is still not clear.

Keywords: *Mus musculus,* rodenticides, anticoagulants, resistance, vkorc1

INTRODUCTION

Rodents are one of the most significant groups of pests woldwide. They are known for their impressive reproductive rates and gnawing capabilities, as well as their ability to spoil food and destroy infrastructure (Meerburg et al. 2008). Rodents pose considerable risk to public health because they can be resevoirs and vectors of many diseases (Battersby et al., 2008). Considering the damage that they are able to cause to economic interests, and threat that they pose to the health of people and domestic animals, rodent control is a measure that needs to be regularly practiced.

Over the past eight decades, the use of rodenticides has been the most widely practiced and most effective way of controlling rodents. Since their discovery in 1940 to this day, anticoagulants have been the most widespread rodenticides in use for controlling rodent pest populations (Bentley, 1972; McGee et al., 2020). However, the continued and unsupervised use of rodenticides has ultimately resulted in global spreading of resistant rodent populations (Pelz et al. 2005).

Acute rodenticides were the first to be used for rodent control and they caused rapid animal death but reduced efficacy was also often noted in practice due

to neophobia in rodents (Greaves, 1994). As rodents are intelligent creatures, they communicate warnings and so make bait unacceptable to other animals in a population. Neophobia is more evident in rats than in mice. Anticoagulants were developed later to overcome the shortcomings of acute rodenticides (Link, 1944). Their slow mechanism of action and delayed death of up to 7 days following the moment of first ingestion, have prevented rodents from developing aversion to baits. The first generation of anticoagulant rodenticides (FGAR - warfarin, dicoumarin, coumachlor, coumafuryl, coumatetralyl, pindone, diphacinone and chlorophacinone) were synthesized, and warfarin had the widest application. During the 1970s and 1980s, the second generation of anticoagulants (SGAR) was developed (bromadiolone, difenacoum, brodifacoum, flocoumafen and difethialone). This group of anticoagulants was developed to overcome some deficiencies of FGARs. The main chortcoming of FGARs was that multiple feeding was necessary to cause animal death. SGAR rodenticides cause animal death after consumption of small amounts of bait (Buckle, 1994). However, due to their toxicity, and greater liposolutibility and retentivity in rodent organism, SGARs make a greater threat to nontarget organisms in the environment (RRAC, 2015; McGee et al., 2020).

FACTORS AFFECTING ANTICOAGULANT EFFICACY IN PRACTICE

Compared to rats, house mice have to some degree lower susceptibility to anticoagulant rodenticides, i.e. this group of rodenticides shows lower efficacy against susceptible house mice than against brown rats, which has been demonstrated in tests for determination of rodent anticoagulant resistance (EPPO, 2004). Feeding tests for determination of resistance in house mice last 21 days, while only 6 days are needed to achieve the same purpose for brown rats. Even though reduced efficacy of an anticoagulant is mostly indicative of the presence of resistant animals, a number of other factors may also cause a misled conclusion that resistant animals live at a location.

Inefficiency of an anticoagulant in practice is mostly attributed to resistant animals (Rowe et al., 1981; Endepols et al., 2011; Buckle et al., 2012, 2013; Endepols et al., 2013). However, reduced efficacy may not be caused by the presence of resistant animals. Several factors may be responsible for ineffectiveness of an anticoagulant in practice even though its efficacy in laboratory tests is satisfactory. Inappropriate control procedures are a frequent cause of failed control of rodent pests. Brief period of baiting, small number of bait boxes, poor assessment of area for planned control, insufficiently attractive bait or bait contamination by insects or microorganisms resulting in reduced bait attractiveness all have very negative effects on control efficacy (Timm, 1991, 1994; Blažić et al., 2017).

Immigration of new animals and availability of alternative food sources, as well as the feeding behaviour of animals, may additionally contribute to failure in conducted rodent control campaigns (Humphries et al., 1992; Quy et al., 1992; Humphries et al., 2000; Clapperton, 2006; Buckle et al., 2012, 2013; Espinosa, 2013).

Considering that green vegetables, milk products, meat and eggs are rich in vitamin K (K_1 and K_2) and the house mouse is an omnivorous species, it is possible for animals to consume an anticoagulant and an antidot simultaneously. Also, animals may avoid anticoagulant ingestion when alternative and more attractive food sources are available, which is often the case in storage facilities (Damon et al., 2005).

Inefficiency of anticoagulant rodenticides that occurs as a consequence of changed behaviour pattern has only been noted in house mice among all rodents. Problems in the consumption of grain-based baits have been observed in house mouse populations due to reduced levels or complete lack of the alpha-amylase enzyme in their digestive tract, which prevents them from ingesting grain food. This physiological difficulty is inheritable and, considering the rate of house mouse reproduction, it quickly spreads within a population. In this case, mice are susceptible to anticoagulants but survive due to their changed behaviour, in contrast to resistant rodents that consume bait containing anticoagulants but do not die (Humphries et al., 1992, 2000). However, once this alternative is eliminated, the probability that anticoagulant resistance is the cause of continued feeding activity becomes high (RRAC, 2015)

TESTING FOR RESISTANCE

Different methods have been developed to test rodent resistance, and they have distinctive advantages and flaws concerning primarily the duration and cost of testing procedures. Feeding test is a demanding and long process but it is consequently a reliable method for detecting resistance (EPPO, 2004). The method requires that house mice be fed over a period of 21 days on anticoagulant baits in no-choice feeding tests. House mice that are susceptible to an anticoagulant die within the 21 days of lethal feeding period. This test is costly and time-consuming because it requires that wild rodents be captured and put under laboratory confinement for resistance screening. Also, this test is questionable from the ethical point of view because animal mortality is required.

Alternative tests have been developed to overcome the disadvantages of feeding test. The blood clotting response (BCR) test is also costly and time-consuming because it requires the capture and laboratory confinement of wild rodents for resistance screening, but mortality is not required. Animals are considered to be resistant if their blood continues to clot when a dose of anticoagulant (discriminating dose) is administered which would otherwise prevent clotting in a given percentage, normally 99%, of susceptible rodents (RRAC, 2015).

Recent findings and our understanding of resistance mechanisms, as well as the genetic role in resistance mechanisms, have resulted in the development of rapid and cheap tests. Such tests are not deficient in ethical terms since molecular-biological techniques for identification of mutation in DNA require only small bits of rodent tisue, even from faecal pellets (Rost et al., 2004; Li et al., 2004; Pelz et al., 2005, 2012). Reliability in practice is the weak point of molecular tests, so that additional confirmation is often required by some of the other described tests (Šćepović et al., 2016).

GENETIC BASIS OF RESISTANCE

An initial discovery that resistance to anticoagulants is controlled by a gene that has an analogous position in the house mouse and brown rat were made in England in the 1970s (Wallace & MacSwiney, 1976). The study confirmed that the gene then tagged as the „resistance gene", positioned on chromosome 7 in house mice (*War*), had an analogous resistance gene in brown rats (*Rw*) on chromosome 1.

First instructions for identification of the gene encoding for VKOR enzyme were given by Kohn and Pelz (2000). Soon after, Li et al. (2004) managed to identify in human subjects the first VKOR protein, i.e. vitamin K epoxide reductase complex, subunit 1 (VKORC1). This protein is the main and possibly only component of VKOR and the chief target of anticoagulant rodenticides. The enzyme was found to be a transmembrane protein incorporated in ER membrane. The gene that encodes for the synthesis of that protein was tagged the *vkorc1* gene. In human organism, the gene for resistance to warfarin was detected on chromosome 16.

Rost et al. (2004) conducted research on brown rats and managed to identify the *vkorc1* gene using PCR methods. They noted a group of genes on human chromosome 16 positioned orthogonally to genes around the loci of *Rw* and *War* genes which were considered to be responsible for resistance to anticoagulants. They found that the entire transcript of 163 amino acids of the *vkorc1* gene, encoding for vitamin K epoxide reductase complex, contains 5126 base pairs in three distinct exones. In humans, brown rats and house mice, the *vkorc1* gene is highly conserved and shares more than 85 % sequence identity (Garcia & Reitsma, 2008; Müller et al., 2014).

Changes in the *vkorc1* gene sequence that lead to phenotypic change in rodents and humans, i.e. to expression of resistance to anticoagulants, are presented as point mutations. Changes in gene sequence occur at the nucleotide level as a base exchange. The outcome of this process is an allel with a transformed sequence (Marinković et al., 1989; Snustad et al., 1997). Mutation in the *vkorc1* gene sequence is inherited as dominant-recessive, i.e. resistance to anticoagulants occurs in all combinations containing the mutated allel, as shown in Table 1.

Table 1. Types of relations between allels of the *vkorc1* gene

Allels	Genotypes	Phenotypes
AA	homozygote	resistant
Aa	heterozygote	resistant
aa	homozygote	susceptible

A- dominant, changed allel; a- recessive allel, „wild" type

Unlike mutation, DNA polymorphism represents several versions of an allel of a single chromosome locus which may differ in their nucleotide sequence. Besides, it is believed that polymorphism is a DNA sequence variation that is frequent in populations, i.e. with frequency exceeding 1 %, while mutations are characterised by less frequent changes. The process that results in change at the level of protein, whose synthesis is encoded by a mutated gene, is known as single nucleotide polymorphism (SNP) (Schorka et al., 2000).

The changed sequence of the *vkorc1* gene encodes for the synthesis of proteins with transformed structure, which prevents anticoagulant binding to the enzyme target locus (Rost et al., 2005). However, it is believed that only changes in certain binding codons, the so called „hot spots", lead to this phenomenon (Pelz et al., 2005).

Not all nucleotide mutations of the *vkorc1* gene are associated with reduced susceptibility to anticoagulants. Also, some VKOR variants representing amino acid exchange in certain codons have no influence on animal resistance, which has been confirmed in some studies when all carriers of a particular VKOR variant died in feeding tests (Snustad et al., 1997; Schork et al., 2000; Tanaka et al., 2012). Such changes in gene sequence represent silent mutations that occur as a consequence of exchange at nucleotide level, while amino acids remain unchanged, as well as the *vkorc1* gene sequence, so that no change occurs in protein translation. Rost et al., (2009) confirmed silent mutations in the *vkorc1* gene in mice and rats, while they were confirmed not to cause resistance in humans and other rodents.

After the *vkorc1* gene was described, knowing its sequence has enabled to examine which variant of that gene could possibly have a role in expressing resistance to anticoagulants in rodents without bringing animals to the laboratory for *in vivo* experiments (Li et al., 2004; Rost et al., 2004; Pelz et al., 2005, 2007). So far, several changes in the *vkorc1* gene sequence have been detected in house mice. Three types of amino acids (VKOR variants) are associated with altered mice susceptibility to anticoagulants: Leu128Ser (leucine exchange for serine in codon 128), Tyr139Cys (tyrosine exchange for cystein in codon 139) and spretus group (a group of connected mutations - Arg12Trp/Ala26Ser/Ala48Thr/Arg61Leu) (Pelz et al., 2012). All three variants are known to carry a significant degree of reduced susceptibility to all first generation anticoagulants and some second generation anticoagulants (Pelz et al., 2012; RRAG, 2012).

Data from earlier research have shown that mice carrying the Leu128Ser VKOR variant are resistant to first generation anticoagulants, and to bromadiolone and difenacoum (Wallace & McSwiney, 1976; Rowe et al., 1981). In a study conducted by Rowe and Bradfield (1976), a single animal survived the 21-day feeding test with brodifacoum bait.

House mice carrying the Tyr139Cys VKOR variant were found to be resistant to first generation anticoagulants and bromadiolone (Prescott, 1996; Pelz et al., 2005). Brodifacoum efficacy against this variant was determined in a recent research by Blažić et. al (2018), and this anticoagulant was found to be 100% effective against animal carriers of that VKOR variant.

The spretus VKOR variant was found to give rise to resistance to first generation anticoagulants and to difenacoum to some degree (Song et al., 2011).

CURRENT STATUS OF ANTICOAGULANT RESISTANCE IN HOUSE MICE

Resistant house mouse strains are mostly not restricted to particular geographical regions, in contrast to Norway rats (RRAC, 2015). Considering their ecology and small body size, they are easy to be introduced into distant areas by commercial transport (Pelz et al., 2012).

First laboratory results on reduced susceptibility of house mice to first generation anticoagulants were confirmed in Great Britain in the 1960s. Reduced susceptibility of house mice to warfarin was confirmed in no-choice tests (Rowe & Redfern, 1964). Further research confirmed that the feature is hereditary (Rowe & Redfern, 1965). Considering the fast spreading of resistant house mice as a consequence of inheriting reduced susceptibility to anticoagulants, research was organized to find compounds that would be effective in controlling warfarin-resistant animals.

After detecting resistance in house mice in Great Britain, researchers continued to report similar results in Switzerland (Muhr, 1981), Canada (Siddiqi & Blaine, 1982), Sweden and Belgium (Lund, 1984), the United States (Jackson & Ashton, 1986), The Netherlands (Jonge, 1994), Germany, Denmark, Finland and France (Myllymäki, 1995). After the discovery of the *vkorc1* gene, house mouse resistance was confirmed in other parts of the world, mostly using molecular methods of testing, and the VKOR variant responsible for expressing resistance in different populations was determined (Table 2).

Susceptibility to anticoagulants of rodent populations in Serbia was tested on brown rat populations in the late 1980s (Kataranovski, 1988). The research consisted of six-day feeding tests with the first generation anticoagulants warfarin and coumatetralyl, and no resistance was detected in animals caught in the environs of Belgrade, Požarevac, Pirot and Korčula. Further research of rodent resistance in Serbia was discontinued until Šćepović et al. (2016) detected house mouse resistance to bromadiolone in a wide territory of Belgrade. Some more recent research has confirmed good efficacy of brodifacoum (Blažić et al. 2018) and difenacoum (under publication) to bromadiolone-resistant house mouse carriers of the variant Tyr139Cys and the combination Leu128Ser/Tyr139Cys.

Table 2. Distribution of VKOR variants based on confirmation tests for anticoagulant resistance of *Mus musculus*

Location	DNA sequence - VKOR variant				Feeding test
	L128S	T139C	VKORC1 spretus	Combination	
Azores	+	+	-	+	-
France	+	+	+	-	-
Germany	+	+	+	+	-
Ireland	+	+	-	-	-
Italy	-	+	-	-	-
Spain	-	-	+	-	-
Serbia	+	+	-	+	+
Switzerland	+	+	+	-	-
United Kingdom	+	+	-	+	-
Argentina	-	-	-	-	+

PREVENTION OF RESISTANCE SPREADING

Considering the damage that house mice are able to cause, their control has become necessary and mandatory. Resistance development to a certain rodenticide reduces the efficacy of their control. The presence of resistant wild animals is an additional danger because such animals pose a major ecotoxicological threat. There is no difference between susceptible and resistant animals in terms of the manner and degree of accumulation of anticoagulants, but the risk of secondary poisoning increases due to very significant differences in the duration of survival between these two groups of animals (Berny et al., 2011; Vein et al., 2013).

The first step after confirming resistance to some anticoagulant is to exclude it from further use and replace it with some other effective rodenticide. In Germany, brodifacoum, flocoumafen and difethialone have been suggested as a solution for controlling populations carrying some of the three known VKOR variants associated with reduced house mouse susceptibility. The application of these anticoagulants is recommended only in areas with confirmed resistance to bromadiolone or difenacoum in order to avoid a potential risk to nontarget species due to their high toxicity (Esther et al., 2014). Recent research concerning new solutions for controlling Tyr139Cys carriers associated with high resistance to bromadiolone has been conducted only with rats, and introduction of difenacoum in practice was found to ensure successful control of such animals (Buckle et al., 2013). Also, brodifacoum showed a greater efficacy than difenacoum in controlling the same group of animals (Buckle et al., 2012). The Rodenticide Resistance Action Committee (RRAC) has proposed that brodifacoum be used for controlling brown rats in populations carrying the Tyr139Cys variant (RRAG, 2012).

Grandemange et al. (2009) pointed out the fact that the use of first generation anticoagulants against populations with low resistance, i.e. characterised by the presence of heterozygous and possibly some homozygous animals, may lead to a significant increase in the frequency of resistant allels, compared to homozygous animals.

Placing restrictions on anticoagulants to which resistance has already been developed is very important for ensuring that resistant allels disappear from populations. Due to inadequacy that resistant animals, compared to susceptible animals, sustain as a consequence of vitamin K deficiency, susceptible animals could actually be privileged under natural conditions if anticoagulants to which resistance has been developed were not to be applied. The number of homozygotes would decrease to the advantage of heterozygotes, while the number of heterozygotes would decrease due to losing allels in susceptible populations. A period of 2-4 years would be sufficient for resistant allels to be lost if anticoagulant rodenticides were excluded. This is especially relevant to the very onset of resistance as the initial stages invariably correlate with rising numbers of heterozygotes (Greaves, 1994).

However, full restriction on anticoagulants for such a long period of time is mostly not economical. Also, restrain on the application of anticoagulants for the benefit of expected population „recovery", i.e. loss of its resistant allels, has failed to show the desired results in one research study as the level of resistance in a brown rat population was the same after 2 years of bromadiolone restriction (Heiberg et al., 2003). In support, Heiberg et al. (2006) also showed that heterozygous females had a greater reproductive potential than homozygous females in situations where the anticoagulant had not been applied.

The loss of resistant allels is possible but some other effective anticoagulant needs to be introduced and alternative methods applied as heterozygous animals sustain no negative biological consequences comparable to those experienced by homozygous animals that have been spared exposure to anticoagulants (Endepols et al., 2013).

A NEW INITIATIVE FOR ANTICOAGULANT RESISTANCE

Most recent rodent control research has been focused on finding new methods of rodent control that would involve as little anticoagulants as possible. Preventive methods have a positive impact on the entire rodent control system but unfortunately they are not effective sufficiently when applied independently. Those methods have been effective mostly when applied simultaneously with other rodent control measures (Smith, 1994).

One of the ways to prevent the spreading of individual rodent resistance, and so avoid anticoagulant treatment, is to apply non-anticoagulants, which are generally unrelated with physiological resistance. The negative side of these compounds is their lower efficacy due to reduced bait acceptability resulting from high rodent neophobia to baits that cause quick death and can be easily associated with their ingestion (RRAC, 2015).

The use of repellents is becoming a very popular method of protecting stored products from rodents. These are mostly environmentally-friendly formulations that do not cause negative impact on the environment and are very acceptable from the ecotoxicological aspect. Their application is often limited due to the severity and manner of manifestation of effects to them, and therefore further formulation improvements are required.

In keeping with global procedural requirements for the reduced application of anticoagulants, a method of combining two rodenticides at low active ingredient contents is now being promoted. Especially interesting from the ecotoxicological aspect is the use of rodenticides with ecofriendly compounds (Kocher & Navjot, 2013; Singla et al., 2015). Endepols et. al (2017) found that a combination of vitamin D3+coumatetralyl was effective in controlling Tyr139Cys carriers of *Rattus norvegicus*. That is an excellent alternative as it prevents secondary poisoning, which is especially relevant to predators of resistant rodents due to high residue contents and extended survival.

All current rodent control programmes in the European Union are based on environmentally friendly procedures that include limited use of anticoagulants. The recent EU Regulation 2016/1179 adopted a proposition for the production and use of rodenticides with nearly half dose of anticoagulants (>30 ppm). A recent study by Frankova et al. (2019) confirmed good effectiveness of a reduced dose of brodifacoum (0.003%) against a susceptible rodent population. However, further validation is required regarding resistant rodent populations. Reduced doses are effective exclusively in susceptible populations of house mouse, which further implicates that resistance status would have to be known in any given population prior to anticoagulant application. The available molecular methods for determining resistance ensure rapid and effective results that would serve as a good forerunner to rodenticide treatments in the field. However, there is also the issue of economic costs of such checks preceding each rodenticide treatment.

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Prepoznavanje i prevencija razvoja rezistentnosti domaćeg miša na antikoagulante

REZIME

Prekomerna i nekontrolisana primena antikoagulanata rezultirala je pojavom rezistentnosti glodara. Ovo je dovelo do niza problema u redukciji broja glodara, a što dalje implicira velike ekonomske gubitke i veliku opasnost po zdravlje ljudi i domaćih životinja. Trenutno popularan trend u suzbijanju glodara u svetu je da se brojnost glodara reguliše uz što manju primenu hemijskih mera, što sa druge strane podrazumeva primenu više udruženih alternativnih mera s ciljem da se glodari dovedu na ekonomski prihvatljiv nivo brojnosti. Takođe, poznavanje genetičke strukture populacije glodara postaje jedna od važnih informacija koju je poželjno imati na raspolaganju pre nego što se krene u primenu rodenticida na terenu kako bi se sprečila primena neefikasnih rodenticida i sprečilo dalje širenje rezistentnih populacija glodara. Novi trend primene kombinacije antikoagulanata sa smanjenom dozom zahteva potvrdu kroz buduća istraživanja, s obzirom da je njihov uticaj na osetljive populacije poznat, ali je nejasno kakav uticaj ima na rezistentne jedinke.

Ključne reči: *Mus musculus,* rodenticidi, antikoagulanti, rezistentnost, vkorc1