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REPORT

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Finnish Safety and Chemicals Agency

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Prevalence of anticoagulant rodenticide resistance (*Vkorc1* gene polymorphism) in two commensal rodent species in Finland

Elina Koivisto[1], Alexandra Esther[2], Tuomas Aivelo[3], Sanna Koivisto[4] Otso Huitu[5]

[1]Section of Ecology, Department of Biology, FI-20014 University of Turku, Finland
[2] Institut für Pflanzenschutz in Gartenbau und Forst – Wirbeltierforschung, Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen, Toppheideweg 88, D-48161 Münster, Germany
[3]Organismal and Evolutionary Biology Research program, University of Helsinki, Helsinki, Finland
[4]Finnish Safety and Chemicals Agency Tukes, P.O. Box 66, FI-00251 Helsinki
[5]Natural Resources Institute Finland, Korkeakoulunkatu 7, FI-33720 Tampere, Finland

Summary

The most common method for rodent control worldwide is the use of anticoagulant rodenticides (ARs), which block the vitamin K cycle and thus cause death by haemorrhage. First-generation anticoagulant rodenticides (FGARs) were introduced into the pest control already in the 1940s and some of them are still in use. Second-generation anticoagulant rodenticides (SGARs), which are toxic at a lower dose, were developed after rodents began to display resistance to first generation agents. As ARs are an easy and cost-effective way to control rodents, and thus chemical control of rodents relies almost exclusively on ARs, their use is widespread, and consequently several resistant strains of rodents have emerged, especially in the brown rat (*Rattus norvegicus*) and the house mouse (*Mus musculus*). These resistant strains have evolved a modification of the VKORC1 enzyme involved in the catalytic recycling of vitamin K. Polymorphism in the *Vkorc1* gene can be identified by genetic analyses from DNA extracted from the tissue samples of rodents.

Here we report the results of a prevalence study of AR resistance (*Vkorc1* gene polymorphism) in populations of brown rat and house mouse in Finland. The brown rat and the house mouse are pest rodent species in the country. The yellow-necked mouse (*Apodemus flavicollis*), although regarded as a common pest in Finland, was not selected for this study, as pilot sequencing was unable to identify relevant *Vkorc1*-polymorphism from samples of this species. Rodent control is required as a part of own control in food and feed production sectors in Finland and ARs are commonly used in farms and urban areas. No systematic screening on the prevalence of AR resistance has been conducted in the country before. We collected tissue samples from 96 animals (48 mice and 48 rats) in both farming areas of southwestern Finland (rural environment) and in the cities of Helsinki, Turku and Pori (urban

environment) in years 2017-2019. We found evidence for *Vkorc1* mutations occurring in both species in Finland.

For mice, 65% of the sampled individuals were found to carry *Vkorc1* polymorphism. The majority of positive individuals (27 out of 31) had a type Y139C polymorphism (16 heterozygous and 8 homozygous). Three positive individuals had a type L128S polymorphism (one heterozygous and two homozygous). In addition, one individual was tested positive for both heterozygous Y139C and L128S. Type Y139C confers resistance to FGARs and of the SGARs bromadiolone and difenacoum, and L128S to FGARs and SGARS bromadiolone, difethialone and brodifacoum. The prevalence of a *Vkorc1* polymorphism ranged between study sites from 25% (Loimaa) to 100% (Jokioinen, Salo, Turku). Mice harbouring a type Y139C were found in all the sites, whereas type L128S was encountered in one location only (Salo).

In rats, however, only two sampled individuals were tested positive for a *Vkorc1* polymorphism and the type found was of a rare one (R33P). Type R33P probably confers resistance to warfarin.

These results suggest that the *Vkorc1* polymorphism type Y139C is fairly common in Finnish house mouse populations and this knowledge should be taken into account when planning control actions targeting house mice. The low number of AR-resistant rats found, and the absence of most common *Vkorc1* polymorphisms in the sampled individuals, suggests a low prevalence of resistance in rats in Finland. However, more studies are needed to gain a better picture about the prevalence of *Vkorc1* types in Finnish rat populations. In addition, to fully understand the state of resistance in Finland, especially studies on effectiveness and resistance performed on yellow-necked mouse and potentially also bank voles (*Myodes glareolus*), would be greatly needed.

Tiivistelmä

Maailmanlaajuisesti jyrsijöiden torjuntaan yleisimmin käytetty menetelmä on antikoagulanttijyrsijämyrkkyjen (AR) käyttö. Antikoagulantit vaikuttavat veren hyytymiseen K-vitamiinisyklin kautta, jolloin myrkytetty jyrsijä kuolee sisäisiin verenvuotoihin. Ensimmäisen polven ARt otettiin käyttöön jyrsijöiden torjunnassa jo 1940-luvulla ja jotkut niistä ovat edelleen käytössä. Toisen polven aineet kehitettiin, kun jyrsijöiden huomattiin tulevan vastustuskykyisiksi eli resistenteiksi ensimmäisen polven aineille. Toisen polven aineet ovat ensimmäisen polven aineita vahvempia ja siksi kuolettavia pienemmillä annoksilla. Antikoagulanttimyrkkyjen käyttö on helppo ja kustannustehokas keino torjua jyrsijöitä, mutta koska jyrsijöiden kemiallinen torjunta perustuu lähes pelkästään antikoagulantteihin, on useille jyrsijälajeille, erityisesti rotalle (*Rattus norvegicus*) ja kotihiirelle (*Mus musculus*), kehittynyt antikoagulanteille vastustuskykyisiä kantoja.

Vastustuskyky perustuu K-vitamiinin kiertoon olennaisesti liittyvän VKORC1-entsyymin mutaatioihin, jotka voidaan tunnistaa jyrsijöiden kudosnäytteistä analysoitavasta DNA:sta.

Tässä raportissa selvitämme AR-vastustuskyvyn (*Vkorc1*-geenin polymorfismin) yleisyyttä suomalaisissa rotta- ja kotihiiripopulaatioissa. Rotta ja kotihiiri ovat Suomessa maatiloilla ja kaupungeissa torjuntatoimia vaativia jyrsijälajeja. Metsähiiri (*Apodemus flavicollis*) on Suomessa huomattavasti yleisempi tuhojyrsijä kuin kotihiiri, mutta koska metsähiiren genomin sekvensointi ei onnistunut, jäi laji siksi pois tutkimuksesta. Jyrsijätorjuntaa tehdään monilla eri aloilla, kuten asuinkiinteistöissä, kaupoissa, ravintola- ja majoitusalalla, maatiloilla, elintarviketeollisuudessa ja monella muulla teollisuuden alalla, kuntien ylläpitämissä kiinteistöissä (koulut, päiväkodit, hoitolaitokset, suurkeittiöt jne.), puistoissa, kuntien keskusta-alueilla, seurakuntien kiinteistöissä mukaan lukien kirkoissa, jäteasemilla, viemäriverkostoissa, metrotunneleissa, satamissa ja lentokentillä. Suomessa ei ole aiemmin tehty systemaattista tutkimusta jyrsijöiden mahdollisesta antikoagulanttiresistenssistä. Tutkimusta varten kerättiin kudosnäytteet 96 jyrsijästä (48 rottaa ja 48 kotihiirtä) läntisen ja lounaisen Suomen maatalousalueilta sekä Helsingin, Turun ja Porin kaupunkien alueelta vuosina 2017–2019. *Vkorc1*-geenin mutaatioita löytyi molempien lajien näytteistä.

Kaikkiaan 65 % kotihiirinäytteistä löytyi *Vkorc1*-geenin mutaatio, joista suurin osa (27/31) oli genotyyppiä Y139C. Positiivisista hiiristä 16 oli mutaation suhteen heterotsygootteja eli mutaatio oli vain geenin toisessa alleelissa, ja kahdeksan homotsygootteja. Kolmella hiirellä oli genotyypin L128S mutaatio, ja näistä kolmesta yksi oli mutaation suhteen heterotsygootti ja kaksi homotsygoottia. Yhdellä hiirellä oli molempien tyyppien mutaatio, sekä Y139C että L128S. Genotyypin Y139C mutaation tiedetään aiheuttavan resistenssiä ensimmäisen polven antikoagulantteja sekä bromadiolonia ja difenakumia vastaan. *Vkorc1*-geenin mutaation esiintyvyys vaihteli tutkimusalueiden välillä 25 prosentista (Loimaa) sataan prosenttiin (Jokioinen, Salo, Turku). Genotyypin Y139C mutaatio löydettiin kaikilta alueilta, kun taas L128S-genotyyppiä löytyi vain yhdeltä alueelta (Salo).

Vain kahdesta rottanäytteestä löydettiin *Vkorc1*-geenin mutaatio, joka oli harvinaista R33P-tyyppiä. Tyypin R33P mutaation arvellaan voivan aiheuttaa resistenssiä varfariinia vastaan.

Tulokset osoittavat, että genotyypin Y139C mutaatiota esiintyy melko yleisesti suomalaisissa kotihiiripopulaatioissa, mikä tulisi ottaa huomioon torjuntatoimia suunniteltaessa. Rotilla löydetty alhainen *Vkorc1*-geenin mutaatioiden esiintyvyys ja yleisten mutaatioiden puuttuminen löydöksistä viittaa alhaiseen AR-resistenssiin, mutta lisätutkimukset ovat tarpeen kattavan tilannekuvan saamiseksi. Kokonaistilanteen selvittämisessä erityisen tärkeää olisi metsähiirellä, mahdollisesti myös metsämyyrällä (*Myodes glareolus*), tehtävä tehokkuus- ja resistenssitutkimus.

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1. Introduction

Rodents can have detrimental effects on the economy and public health of human societies in both rural and urban environments (Singleton et al. 1999), and thus methods controlling their populations have been sought and tested for a long time. Currently, the most common chemical method for rodent control worldwide is the use of anticoagulant rodenticides (ARs), which block the vitamin K cycle and cause death by haemorrhage (Laakso et al. 2010, Murphy 2018). Although ARs are an easy and a cost-effective way to control rodents, there are several

problems related to their use (Berny 2011). First, both non-target wildlife and domestic animals are exposed unintentionally to ARs either through consumption of baits meant for rodents or by consumption of poisoned rodents (Lefebvre et al. 2017, Koivisto et al. 2016). Second, because chemical control of rodents relies almost exclusively on ARs, many distinct resistant strains, especially in the brown rat (*Rattus norvegicus*) and the house mouse (*Mus musculus*) have emerged e.g., in France, Germany, and the UK (Pelz et al. 2005, Berny et al. 2014, McGee et al. 2020). The origin of resistance has been identified to specific genetic traits, namely polymorphism in the *Vkorc1* gene, which codes an enzyme involved in the catalytic recycling of vitamin K (Li et al. 2004, Rost et al. 2004). These mutations have then become favoured by natural selection after the use of ARs has become common.

The presence of resistant strains in a controlled population means that stronger ARs and/or higher quantities are needed for the control measures to be effective depending on VKORC1 polymorphism. This leads to a vicious circle of resistance becoming subsequently more prevalent in the targeted rodent population and, as a result, even heavier control measures need to be used. Thus, knowing if resistant strains exist and if they do, which type(s) they are, is essential knowledge not only for practising effective rodent control but also for reducing the risk of resistant strains becoming more common in the future because of inefficient use of ARs. Resistance and higher amounts of ARs will lead also in increased residue levels in the non-target animals.

In Finland, previous sporadic evidence exists on the occurrence of AR resistant strains in house mouse (Myllymäki 1995) but no systematic screening on the prevalence of AR resistance has been conducted in the country before this. As the use of ARs is common in Finland and resistance occurs in many European countries there was a need to clarify the resistance situation also in Finland. Here we report on results of a prevalence study of AR resistance (*Vkorc1* gene polymorphism) in populations of brown rat and house mouse in Finland.

1.1 Anticoagulant rodenticides

All anticoagulant rodenticides have a similar kind of a structural formula and the same mode of action: they act as effective blockers of the vitamin K cycle, resulting in an inability to produce essential blood-clotting factors (Berny et al. 2014, Lefebvre et al. 2017). In addition, anticoagulants cause damage to tiny blood vessels, which increases their permeability and causes diffuse internal bleeding. These effects are gradual, developing over several days. In the final phase of intoxication, the rodent collapses due to haemorrhagic shock or severe anaemia.

Anticoagulants can be divided into first- and second-generation substances. The first-generation rodenticides (FGARs) were introduced for pest control already in the 1940s and some of them, like warfarin, are still in use. First generation rodenticides are less toxic, require

multiple doses to be fatal and are eliminated within few days. Second-generation anticoagulant rodenticides (SGARs), which are toxic at a much lower dose (IPCS 1995), were developed after rodents started to exhibit resistance to first generation agents. The SGAR group includes bromadiolone, difenacoum, and the stronger substances brodifacoum, difethialone and flocoumafen. SGARs are potential PBT substances, meaning that they are Persistent, Bioaccumulative and Toxic (Commission Regulation (EU) No 253/2011). The persistent nature of SGARs is reflected in the long elimination times of these substances.

As ARs are very toxic and persistent, there are problems related to their use: the application of most toxic ARs, which is necessary due to resistance, results in higher exposure of non-target wildlife (Berny 2011). Anticoagulants have been found to transfer to non-target animals either by direct consumption of baits (primary poisoning) or more commonly by consuming contaminated rodents (secondary poisoning, Lambert et al. 2007). Because death by rodents to anticoagulants takes about a week (Laakso et al. 2010) rodents can during this time be preyed upon by predators, exposing them to the rodenticides that the rodent has consumed. Anticoagulants have been found in many non-target species around the world, most commonly in rodent-eating predators like owls, raptors, foxes, and mustelids (Berny and Gaillet 2008, Norström et al. 2009, Christensen et al. 2010, Laakso et al. 2010, NIVA 2012, Koivisto et al. 2016). The extensive use of ARs, especially the more toxic SGARs, exposes wildlife to AR poisoning and thus their use should be carefully considered. However, the existence of AR resistant rodent strains or the fear of their development can put pressure for the use stronger ARs.

1.2 Development and the genetic basis of resistance

The genetic basis of resistance lies in the *Vkorc1* gene (vitamin K epoxide reductase complex subunit 1), mutations of which render rodenticides ineffective (Rost et al. 2004, Pelz et al. 2005). Anticoagulant resistance in brown rats and house mice has been found to be linked to single nucleotide polymorphisms (SNPs) in the coding region of *Vkorc1*. *Vkorc1* polymorphisms have been identified in humans, mice, and rats (e.g., Rost et al. 2004, Pelz et al. 2012, Gryseels et al. 2015). As resistance to ARs developed so quickly after the introduction of rodenticides, it has been suggested that the resistance mediating VKORC1 polymorphisms arise from standing genetic variation. As these polymorphisms are beneficial under the use of anticoagulants, they have been selected for and their prevalence has thus increased (Lefebvre et al. 2016).

Vkorc1 resistance is co-dominant, meaning that heterozygous individuals are more susceptible to rodenticides than homozygous individuals, but less susceptible than wild type rodents without *Vkorc1* mutations (Grandemange et al. 2009, Berny et al. 2018). AR resistance is costly to the animals in terms of e.g., lower reproductive success. For example, Heiberg et al. (2006) found that homozygous resistant rats had a lower reproductive success

than what was expected, whereas heterozygous males or females had better reproductive success. Since resistance is so common, it must be a major selective advantage in a population where ARs are used despite its costs to fitness.

Quite soon after introducing the FGARs for rodent control, the first warfarin resistant rodent strains were discovered (Berny et al. 2018). The first resistance case was reported in Scotland in 1958, followed by similar reports in other areas in Europe: Wales, Denmark, the Netherlands, and Germany (Lund 1972). Following this discovery, new and stronger SGARs were introduced to overcome the resistance problem. SGARs are more toxic than FGARs and require only a single dose to be lethal (IPCS 1995), working more effectively with neophobic rodents (Berny et al. 2018).

For a while SGARs seemed to be the answer to the resistance problem. However, since all ARs have similar chemical structures and a similar mode of action, resistance to the first-generation anticoagulants brought with it a measure of cross-resistance to the second-generation compounds and soon also populations with reduced susceptibility to the more potent SGARs began to appear (Greaves et al. 1982). The resistance system has been found to be hierarchical. At the base there is warfarin (FGAR) resistance, followed by coumatetralyl (FGAR), then cross-resistance to SGARs bromadiolone over difenacoum (Pelz et al. 1995) and up to brodifacoum at the top. Resistance to difethialone or brodifacoum has, up until recent years, never become as widespread as that to the first-generation compounds (Buckle and Smith 1994, Berny et al. 2018).

1.3 Prevalence of resistance and polymorphism

Nowadays, AR resistant strains of commensal rodents, such as brown rats, roof rats (*Rattus rattus*) and house mice occur throughout the world (tables 1 and 2). There is published evidence of AR resistance in rodents from all continents but Africa (Berny et al. 2018). Most studies and reports are available from western European countries, but no published records exist for the majority of European countries, especially from eastern or southern Europe (but see Iacucci et al. 2018). This is more likely to be because resistance has not been studied in those countries than because it does not exist (Berny et al. 2018).

More than 10 and at least 15 different type of polymorphism have been found in the brown rat and the house mouse in Europe, respectively (Tables 1 and 2). Of these, specifically common are the types L128S and Y139C, which have been associated with severe resistance to FGARs and limited resistance to SGARs (Goulois et al. 2017). In many European countries, a high prevalence of *Vkorc1* polymorphism has been observed in wild-caught house mice (e.g., Pelz et al. 2011, Goulois et al. 2017, Baxter 2019, McGee et al. 2020). For example, in Germany over 90% of the house mice tested carried genetic resistance types (RRAG 2012). DNA can be

sequenced from small pieces of tissue from a tail tip and the presence of anticoagulant resistant individuals in a population can be identified.

AR resistance has been mostly studied and found in the three commensal rodent species (brown rat, black rat and house mouse). Recent studies have found anticoagulant resistance also in other rodent species, like Lesser Rice-field rat (*Rattus losea*; Wang et al. 2008), Asian house rat (*Rattus tanezumi*; Andru et al. 2013) and water voles (*Arvicola amphibius*; Vein et al. 2011), although the latter does not appear to be linked to a modification of the *Vkorc1* gene (Berny et al. 2018). The potential of *Vkorc1* polymorphism has also been recently studied in carnivores (mustelids; Stöck et al. 2019) but their full role in AR tolerance is not yet known.

Table 1. Known Vkorc1 types with the locations where they have been found and known resistance to ARs in the brown rat. (Modified from McGee et al. 2020; Table 1 and references therein.)

Amino acid change	Short name	Res to FGAR	Res to SGAR	Where found
Val12Leu	V12L			Azores
Ala21Thr	A21T			Korea
Ala26Thr	A26T			UK
Arg33Pro	R33P			UK
Arg35Pro	R35P			France, US
Tyr39Asn	Y39N			UK
Ser56Pro	S56P			Germany
Trp59Arg	W59R			Argentina
Phe63Cys	F63C			UK
Glu67Lys	E67K			Japan
Ile90Leu	I90L			Argentina, Azores, Indonesia, US
Leu120Gln	L120Q	Chlorophacinone, warfarin	Bromadiolone, difenacoum	France, Netherlands, UK
Ile123Ser	I123S			Italy
Leu128Gln	L128Q	Chlorophacinone, coumatetralyl, warfarin		France, UK
Leu128Ser	L128S			France
Tyr139Cys	Y139C	Chlorophacinone, coumatetralyl, warfarin	Bromadiolone, difenacoum	Denmark, France, Germany, Hungary, Netherlands, UK
Tyr139Ser	Y139S	Chlorophacinone, coumatetralyl, warfarin		UK
Tyr139Phe	Y139F	Chlorophacinone, coumatetralyl, warfarin	Bromadiolone, difenacoum	Belgium, France, Korea, Netherlands, UK
Ile141Val	I141V			Indonesia
Ala143Val	A143V			Indonesia, Thailand

Table 2. Known Vkorc1 types with the locations where they have been found and known resistance to ARs in the house mouse. (Modified from McGee et al. 2020; Table 1 and references therein.)

Amino acid change	Short name	Res to FGAR	Res to SGAR	Where found
Arg12Trp	R12W			France, Germany
Ala21Thr	A21T		Bromadiolone	Serbia
Ala26Ser	A26S			France
Ala26Thr	A26T		Bromadiolone, difenacoum	France
Arg35Pro	R35P			US
Glu37Gly	E37G			France
Ala48Thr	A48T			France
Arg58Gly	R58G			France
Trp59Gly	W59G	Warfarin		France, Germany
Arg61Leu	R61L			France
Leu124Met	L124M			France
Leu128Ser	L128S	Chlorophacinone, coumatetralyl, warfarin	Bromadiolone, difethialone, brodifacoum	Azores, France, Germany, Ireland, Serbia, Switzerland, UK
Tyr139Cys	Y139C	Chlorophacinone, coumatetralyl, warfarin	Bromadiolone	Azores, France, Germany, Ireland, Serbia, Switzerland, UK
Arg12Trp, Ala26Ser, Ala48Thr, Arg61Leu	R12W, A26S, A48T, R61L	Chlorophacinone, coumatetralyl	Bromadiolone, difenacoum	France, Germany, Spain, Switzerland
Ala26Thr/Leu128Ser	A26T/ L128S	Chlorophacinone, coumatetralyl	Brodifacoum, bromadiolone, difenacoum, difethialone	France
Ala26Ser/Leu128Ser	A26S/L28S	Chlorophacinone, coumatetralyl	Brodifacoum, bromadiolone, difenacoum, difethialone	France
Trp59Gly/Leu124Met	W59G/L124M			France
Trp59Gly/Leu128Ser	W59G/L128S			France
Leu128Ser/Tyr139Cys	L128S/Y139C		Bromadiolone	Serbia

1.4 Control of commensal rodents and use policies of rodenticides in Finland

From the FGAR group, coumatetralyl is allowed for rodent control in Finland. Of the SGAR substances, brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen were registered as biocidal products in Finland in 2019. The following anticoagulant rodenticides are registered in Finland: coumatetralyl (FGAR), and SGARs, brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen (https://www.kemidigi.fi/). The general public

can use rodenticides containing brodifacoum, difethialone and flocoumafen, but these products are sold only in pre-filled bait stations and they can only be used indoor for the control of mice. Since 2018, the general public has not been able to control rats. The restricted use of anticoagulant rodenticides by the general public is considered to reduce the selection pressure of resistant rodents.

Some active substances (coumatetralyl, bromadiolone, difenacoum) are only allowed for the professional pest control operators (PCOs) due the classification as toxic for reproduction (Repr. 1B; H360D: May damage the unborn child). The most potent anticoagulants are effective even below the classification limit and are therefore available also for the general public. While the use of ARs by the general public is very restricted, the pest control operators can use rodenticides both indoors and outdoors, and for the control of both rats and mice.

The professional pest control operators and pest control companies must be registered by the Finnish Safety and Chemicals Agency Tukes (https://tukes.fi/asiointi/rekisterit/biosidit) since 2017. In addition, the pest control operators need a qualification as laid down in the Finnish Chemicals Act 599/2013). Farmers are considered to have an equal qualification when they have an appropriate qualification in plant protection. A code of good practise for the professional rodent control was published in 2020 (https://tukes.fi/tietoa-tukesista/materiaalit/biosidit/jyrsijatorjunnan-hyvan-kaytannon-ohje).

Rodent control is needed in various sectors of society. Rodent control is conducted in residential buildings, shops, restaurants, hotels, farms, industry, kindergartens, schools, nursery homes, parks, churches, waste storage areas, sewers, metro tunnels, harbors and airports. The list is not exhaustive. The use of anticoagulant rodenticides is still very common, although the non-chemical alternative methods are increasingly used too.

1.5 Aims of the study

We aim to assess the prevalence of AR resistance (*Vkorc1* gene polymorphism) in populations of two species of commensal rodents (brown rat and house mouse) in Finland by using tissue samples collected from 96 animals (48 mice and 48 rats) in both farming areas of southwestern Finland (rural environment) and in the cities of Helsinki, Turku and Pori. There is no indication of a widespread resistance problem in Finland but previous evidence of the occurrence of AR resistant strains of house mouse exists (Myllymäki 1995). Nevertheless, no systematic screening of AR resistance has been conducted in Finland before. The yellow-necked mouse (*Apodemus flavicollis*), although regarded as a common pest, was not selected for this study, as pilot sequencing was unable to identify relevant *Vkorc1*-polymorphism from samples of this species.

2. Material and methods

2.1 Study species

The brown rat is a commonly controlled species of commensal rodents in Finland. The house mouse is a less frequent pest rodent. Brown rats are typically controlled in residential buildings in urban and semi-urban areas and in farms. The house mouse occurs in buildings where cereals are processed or stored, or in feed mills. Sometimes house mice are also found in cellars of old buildings.

2.2 Sample collection

Samples were collected both by PCOs and by members of a research team investigating the role of rodents as pests in cattle and pig farms. Samples were obtained in both urban and rural sites (i.e., farms) where rodent infestations were recorded. No detailed knowledge is available regarding the type of anticoagulant used, the longevity of application or the exposure history of rodents to them in these sites. Nonetheless, all sites (according to PCOs and farmers) have applied anticoagulant rodenticides. Typically, continuous bating is used on farms, while PCOs operating in urban sites more often employ pulsed baiting protocols, adhering to the instructions of use of the rodenticides and the code of good practice (https://tukes.fi/tietoa-tukesista/materiaalit/biosidit/jyrsijatorjunnan-hyvan-kaytannon-ohje).

Rodents obtained from PCOs were trapped using electronic traps and frozen whole immediately at -20˚C for further dissection and analysis. Rodents trapped for the farm study were trapped using standard lethal snap traps adhering to Finnish animal welfare legislature. Again, trapped individuals were frozen immediately whole at -20˚C for further dissection and analysis. Most sampling was carried out in cool autumn, winter and spring months when ambient temperatures were < 10˚C, which minimized potential degradation of dna material. In the laboratory, tail snippets were removed from rodents as tissue samples while still frozen, stored in ethanol in microtubes and immediately returned to -20˚C. Samples were shipped on dry ice to Germany for sequencing.

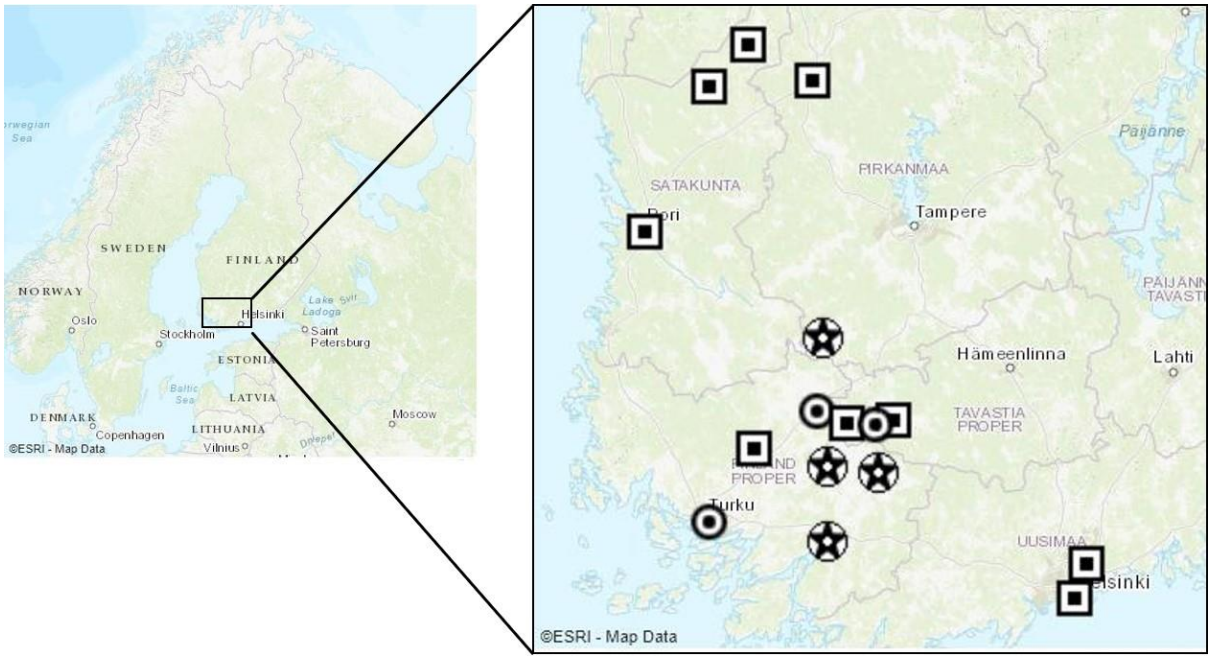

Figure 1. A map showing the study sites where the analysed samples were collected. Circles = house mice only, squares = rats only, stars = both species.

2.3. Genetic sequencing

Subsamples of tail tissue were taken, and DNA extracted using Gentra Puregene Mouse Tail Kit (Qiagen) at the Julius Kuehn Institute. Subsequent molecular work was performed at Eurofins Genomics Europe Sequencing GmbH.

All sequences were generated using BigDye terminator chemistry (version 3.1) (Thermo Fisher Scientific, Waltham, MA USA). For sequencing reactions peqStar 96 HPL (PEQLAB Biotechnologie GMBH, Erlangen, Germany) and/or GeneTouch (Biozym Scientific GmbH, Oldendorf, Germany), and/or Biometra Tadvanced (Biometra GmbH, Göttingen, Germany) thermal cyclers were used. Sequencing reaction cleanup was done on a Hamilton Starlet robotic workstation (Hamilton Robotics GmbH, Martinsried, Germany) by gel-filtration through a hydrated Sephadex matrix filled into appropriate 96well filter plates followed by a subsequent centrifugation step. Finally, all reactions were run on ABI3730xl capillary sequencers (Thermo Fisher Scientific, Waltham, MA USA) equipped with 50 cm capillaries (Thermo Fisher Scientific, Waltham, MA USA) and POP7 polymer (Thermo Fisher Scientific, Waltham, MA USA).

Sequencing data were called using the original Sequencing Analysis Software 6 (Applied Biosystems) including the KB-basecaller (Thermo Fisher Scientific, Waltham, MA USA), which assigns quality values to all called bases similar to PHRED quality score (Ewing et al. 1998).

Additional basecalling was performed using the PeakTrace basecaller from Nucleics Pty Ltd (Woollahra, AUS) to improve the single peak resolution and quality values and therefore

increase the reading lengths. The assembly was performed using the Staden Software Package (Roger Staden, LMB, Cambridge, UK/ Pregap4 version 1.6, Gap4 version 4.11.2).

For quality, clipping a sliding window of 10 nucleotides was used to average the confidence. The method starts from the point of highest average quality and then steps outwards in both directions until the average confidence within the window drops below 30. Normally both 5' and 3' sequence trace ends were quality clipped. These clips underwent a manual plausibility check and were further modified.

The assembly itself was done as a normal shotgun assembly using the quality clipped reads. The settings for the assembly were as follows: minimum initial match 20, maximum pads (gaps arising in one read due to the alignment with others) per reads 25, maximum percent mismatch 5.00. Further potential joins were searched with the find internal joins function under more relaxed conditions: minimum overlap 20 and maximum percent mismatch 30.00. These additional joins underwent a manual plausibility check.

Manual editing steps were necessary to resolve base caller errors. Comparison to the reference sequences (Genbank number NM 178 600 for house mice and *Vkorc1* GenBank accession no. NM-203 335 for brown rat) was also performed as an assembly, using the same parameters as for the single read assembly.

3. Results

3.1 Species

Some type of *Vkorc1* polymorphism was found in 31 (65%) of 48 house mouse samples. Most positive individuals (27 out of 31) had a type Y139C (16 heterozygous and 8 homozygous, Fig 2). Three positive individuals had a type L128S (one heterozygous and two homozygous). In addition, one individual was tested positive for both Y139C and L128S types.

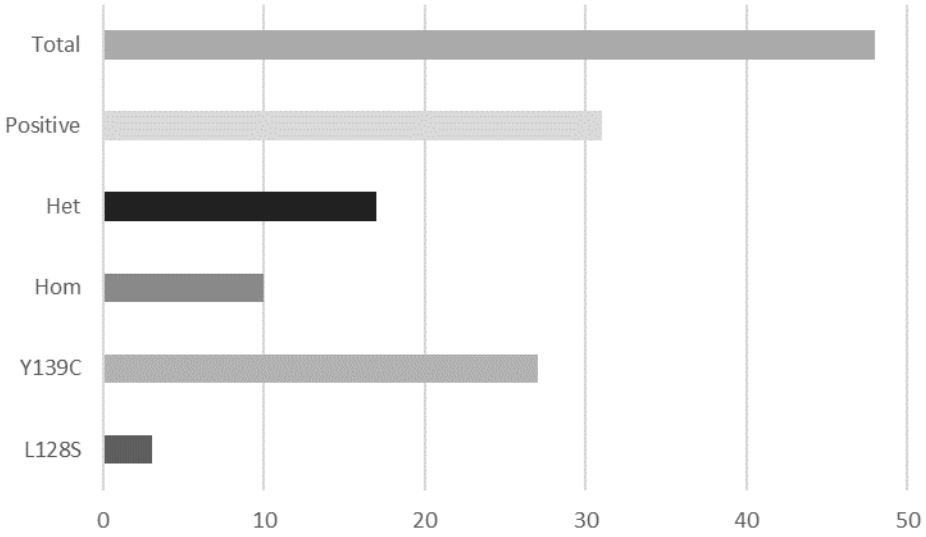

Figure 2. VKORC1 polymorphism prevalence in the analyses house mouse samples.

In rats, on the other hand, only two samples out of 48 (4%) were positive. Both individuals had a type R33P, one heterozygous and one homozygous.

3.2 Sexes

In house mice, 28 of the tested individuals were males, 16 females and in four individuals the sex was not defined. Out of 16 tested females 8 (50%) were positive (5 heterozygous, 2 homozygous and one that had two types of polymorphism, both heterozygous). Whereas in males 20 individuals out of 28 (71%) were positive (12 heterozygous, 8 homozygous). Both positive rats were females.

3.3 Study sites

The prevalence of *Vkorc1* polymorphism in house mice varied between study sites from 25% (Loimaa) to 100% (Jokioinen, Salo, Turku; Table 3). Type Y139C was found in all sites (Table 3), whereas type L128S was found in one location only (Salo).

Both positive rats originated from the same location (Koski TL).

Table 3. Sample size, prevalence, and the type of Vkorc1 *polymorphism in house mice and rats in relation to study site (het = heterozygous, hom = homozygous, ND = not defined). Asterisk (*) denotes the presence of the one mouse individual with two types (both heterozygous), classified here as heterozygous.*

Species	***Location***	***Urban/rural***	***N***	***N pos***	***prevalence***	***N het***	***N hom***	***type(s)***
Mus	Jokioinen	Rural	6	6	100%	3	3	Y139C
Mus	Koski TL	Rural	6	3	50%	3	0	Y139C
Mus	Loimaa	Rural	4	1	25%	1	0	Y139C
Mus	Punkalaidun	Rural	12	6	50%	3	3	Y139C
Mus	Salo	Rural	6	6	100%	4*	2	Y139C, L128S
Mus	Somero	Rural	11	6	55%	4	2	Y139C
Mus	Turku	Urban	3	3	100%	ND	ND	Y139C
Rattus	Forssa	Rural	2	0	0%			
Rattus	Helsinki	Urban	28	0	0%			

Rattus	Honkajoki	Rural	1	0	0%			
Rattus	Karvia	Rural	1	0	0%			
Rattus	Koski TL	Rural	4	2	50%	1	1	R33P
Rattus	Parkano	Rural	2	0	0%			
Rattus	Punkalaidun	Rural	1	0	0%			
Rattus	Pöytyä	Rural	1	0	0%			
Rattus	Salo	Rural	1	0	0%			
Rattus	Somero	Rural	4	0	0%			
Rattus	Ypäjä	Rural	1	0	0%			

4. Discussion

Here we aimed to assess the prevalence of AR resistance (*Vkorc1* gene polymorphism) in the populations of two commensal rodents (brown rat and house mouse) in Finland. We analysed tissue samples from 96 animals (48 mice and 48 rats) and found evidence for *Vkorc1* polymorphism occurring in both species in Finland. In mice, type Y139C was quite common, but a few individuals with type L128S were also found. In rats, however, only two sampled individuals were tested positive for a *Vkorc1* polymorphism and none of the types common in other parts of Europe were found. The type found in our rat samples was of a rare type (R33P), reported earlier from the UK (Rost et al. 2009) and Japan (Tanaka et al. 2013).

4.1 Species

4.1.1 House mouse

We found a 65% prevalence of *Vkorc1* polymorphism in house mice. A vast majority of positive individuals had a type Y139C, which was identified at all the sampled locations. Most individuals analysed in our study were heterozygous. As homozygotism indicates a high degree of selection for anticoagulant resistance, our results suggest that the use of rodenticides in our study sites has not been excessively persistent and heavy. Type L128S was found in a few individuals, but in only one of the study sites. One individual tested positive for both types. Double polymorphism, i.e., mice carrying two resistance types of the *Vkorc1* gene, is associated with severe resistance to all anticoagulant rodenticides (Goulois et al. 2017).

The types found in this study are the most common ones observed in house mice. Likewise, their prevalence in Finland was at similar levels compared to other studies conducted in Europe. For example, Goulois et al. (2017) trapped house mice at 65 locations throughout France and found a >70% prevalence for nine different *Vkorc1* polymorphism types. In addition, 80% of these mice had a homozygous polymorphism (Goulois et al. 2017). In the UK, Baxter (2019) found a prevalence of 46.9% for type L128S and 31.8% for Y139C polymorphism.

Also, 4.6% carried a double polymorphism (Baxter 2019). Pelz et al. (2011) found *Vkorc1* polymorphism to be common and widespread across Germany, with a high proportion of homozygous individuals.

Type Y139C was originally found in a wild population of house mice trapped around Reading, UK, in the 1990s. It causes resistance against FGARs and of SGARs bromadiolone and difenacoum (Buckle and Prescott 2012). Brodifacoum has been found to be effective against this strain of house mouse (RRAG 2012).

Regarding sexes of house mice, 50% of females had *Vkorc1* polymorphism (5 heterozygous, 2 homozygous and one individual had both types, both heterozygous). 71% of males had *Vkorc1* polymorphism (12 heterozygous, 8 homozygous). It has been suggested that female house mice could have a higher level of tolerance to anticoagulants than males. For example, Prescott (1996) found that female house mice were more likely to be homozygous resistant, whereas resistant males were more likely to be heterozygous. This resulted in male offspring having a higher level of mortality in test crosses than females (Prescott 1996). Also, Scepovic et al. (2016) reported females being more resistant than males and similar results have also been found in brown rats (Lefebvre et al. 2016). As we found a lower prevalence in female house mice compared to males, and also the proportion of homozygous polymorphism was lower in females, our results are not in line with these observations. However, we studied the *Vkorc1* polymorphism prevalence only, and not the actual level of resistance.

Although AR resistance in house mice is becoming an ever more prominent problem (Baxter 2019), the majority of AR resistance research has been done on brown rats. Rats and mice differ in their feeding behaviour, which has led to differences in the evolution of resistance (Baxter 2019). Rats mainly eat from one food source and thereby consume a lot of anticoagulant bait whereas mice eat from many food sources, therefore diluting the bait with other food sources (Lefebvre et al. 2016). More research is needed specifically to address factors leading to AR resistance in house mice under varying environmental conditions and regimes of AR application. In Finland, it would be also important to study efficacy and resistance in yellow-necked field mouse which is far more common pest rodent compared to the house mouse.

4.1.2 Brown rat

The observed AR prevalence in rats (4%) is very low compared to what has been observed in many other countries. For example, Desvars-Larrive et al. (2017) found a high prevalence (55.8%) of the type Y139F in rats sampled in France. On the other hand, Mooney et al. (2018) found no genetic evidence for the occurrence of resistance in 65 brown rat samples analysed, indicative of an absence, or low prevalence, of resistance in rats in at least the Eastern region of the island of Ireland.

The Y139C and Y139F types prevail in Europe (Iacucci et al. 2018), and they are found for example in Denmark, Germany, France, UK, Netherlands, and Hungary. In our samples, however, no Y139C, or any of the other commonly observed types, were detected. Instead, we found two individuals with the R33P polymorphism of *Vkorc1*, which has been found to confer resistance to warfarin in the laboratory. Type R33P is known from rats in Nottinghamshire (UK; Prescott and Coan 2018) and Japan (Tanaka et al. 2013).

Likewise, Iacucci et al. (2018) found none of the common types in their study conducted in rats in Italy but instead they found a new type of *Vkorc1* gene polymorphism, I123S. It is not known if this type confers anticoagulant resistance in rats, but since it is located in the same position as type I123N, responsible for anticoagulant resistance in humans (Oldenburg et al. 2014), Iacucci et al. (2018) suggest that it could be involved in resistance development in rats too. Iacucci et al. (2018) propose that the total absence in the Italian samples of known polymorphisms associated with resistance may be attributable to the different pattern of use of anticoagulant rodenticides compared to that of many other European countries (e.g., UK), where a much stricter regulation does not allow the use of more potent SGARs (i.e., brodifacoum and flocoumafen) in outdoor areas. According to Iacucci et al. (2018) in Italy, there is often routine use of the most potent anticoagulants.

Cowan et al. (2017) studied resistance in *Rattus* species in New Zealand and found three new polymorphism types but none of the most common ones. The lack of common types in New Zealand rats could however be an outcome of a founder effect, which is an unlikely explanation for our results.

The absence of the common *Vkorc1* types in our dataset does not confirm their absence in Finland but does indicate a low prevalence at least in southwestern part of the country. However, the sample size per location was quite low, especially in the rural locations. We are unsure of the origin of the R33P type present in our samples. The single location in which the resistant rats were trapped was a farm in southwestern Finland, which largely rules out the possibility of an incidental introduction of individuals (via e.g., ships) carrying an exotic resistance type. The observed R33P polymorphism found in our samples could be a result of a spontaneous polymorphism occurring in our study population. It might also be possible that type R33P could be more common than currently observed if the testing of *Vkorc1* polymorphism has been primarily focused on searching for the most common polymorphisms, as their presence plays a more important role in terms of pest management. Resampling and analysing the study populations are needed to gain more information about the prevalence of *Vkorc1* types in Finnish rat populations and to avoid spread of resistance.

Runge et al. (2013) studied the distribution of rodenticide resistance in urban and rural rat populations in Germany and found homozygous polymorphism in urban areas only. They hypothesised that these results could reflect differing selection pressures due to rodent control between urban and rural areas, in that AR use might be more intensive in the former sites. However, the results could also be due to sampling bias, as rural samples were primarily

bycatch from sites not experiencing a heavy rat infestation, while the urban samples came from selected sites with more serious rat control problems. Due to low prevalence of AR resistance, we are unable to address this topic in this current study.

4.2 Conclusions/recommendations

Our results suggest that the type Y139C polymorphism of *Vkorc1* is common in Finnish house mouse populations and this knowledge should be considered when planning professional control actions against the species. Mechanical and/or electronic traps should be favoured for the control of house mouse and if anticoagulant rodenticides are used, it would be preferable to use the most potent active substances (brodifacoum, difethialone, flocoumafen). Private use is not considered as a major risk for the development and spread of resistance as the general public can only use the most potent anticoagulants for which no resistance impairing rodent control has been observed yet. In addition, Finnish Safety and Chemicals Agency (Tukes) recommends the use of mechanical traps in first place for the control of mice in private houses and cottages.

In UK, for example, bromadiolone is not recommended to be used against house mice due to the presence of the Y139C type. Also, difenacoum products should not be used, as mice carrying the Y139C polymorphism have a natural level of resistance to difenacoum (Buckle and Prescott 2012). Brodifacoum and flocoumafen are both effective against these resistant strains of house mice (RRAG 2012, Baxter 2019).

The low number of AR resistant rats found and the absence of the most common *Vkorc1* polymorphism in the sampled individuals suggests a low prevalence of resistance in rats in Finland. However, more studies and increased sample sizes are needed to obtain a more thorough understanding on the prevalence of *Vkorc1* types in Finnish rat populations and to avoid the spread of resistance. In addition, to fully understand the state of resistance in Finland, especially studies on effectiveness and resistance performed on yellow-necked mouse and potentially also bank voles (*Myodes glareolus*), would be greatly needed.

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Turvallisuus- ja kemikaalivirasto

HELSINKI PL 66 (Opastinsilta 12 B), 00521 Helsinki

TAMPERE Yliopistonkatu 38, 33100 Tampere

ROVANIEMI Valtakatu 2, 96100 Rovaniemi

VAIHDE 029 5052 000 | www.tukes.fi