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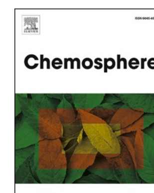

VKORC1 single nucleotide polymorphisms in rodents in Spain

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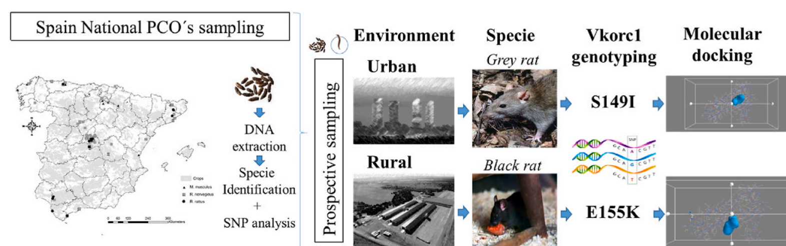
HIGHLIGHTS

- Assessment of AR genetic resistance in Spanish populations of pest rodents.
- A minimum number of DNA form faecal samples per area must be used.
- Specie-specific polymorphic nucleotide positions were found.
- Intensive land use may determine AR resistance.
- Pest control requires continuous evaluation of genetic resistance.

GRAPHICAL ABSTRACT



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ABSTRACT

Rodents are considered one of the animal pests with the greatest impact on agricultural production and public health. Anticoagulant rodenticides (ARs), used as one of the most effective ways to control rodent populations worldwide, inhibit the vitamin K 2,3-epoxide reductase (VKORC1) enzyme involved in blood coagulation. Resistances to ARs are mainly associated with mutations or single nucleotide polymorphisms (SNPs) in the *vkorc1* gene. Since the information on this subject is scarce in Spain, we monitored and discovered rodent SNPs that could favour genetic resistance in its populations. For that, more than 200 samples of stools and tails from brown rat (*Rattus norvegicus*), black rat (*Rattus rattus*) and mouse (*Mus musculus*) were collected from 12 Spanish regions previously identified with low AR efficacy in coordination with the National Association of Environmental Sanitation Companies (ANECPLA) and the managing entities of four locations. We then sequenced their *vkorc1* exon 3 corresponding genomic DNA. We identified genotypic *vkorc1* variations corresponding to amino acid changes at the VKORC1 protein at the S149I – S149T and the E155K - E155Q mutations, depending on the rodent species. Computational analysis of binding predictions found out that the brown rat S149I mutation predicted a high reduction of the binding affinity of chlorophacinone and brodifacoum ARs while, the black rat S149T, E155K and E155Q mutations slightly reduced bromadiolone AR binding. These results suggest that these mutations may be one of the causes of the increased resistance to those ARs.

1. Introduction

Rodents are considered one of the animal pests with the greatest impact on agricultural production and public health, especially the brown or Norway rat (*Rattus norvegicus*), the black or roof rat (*Rattus rattus*) and the house mouse (*Mus musculus*). Its control is an increasing

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problem worldwide. The intensification of agricultural production methods as well as the increase in merchandise transport to sustain growing populations is leading to an increase in waste production causing the growth of these rodent populations. The estimated losses in crop production caused by rodents range from between 5% and 90% (Stenseth et al., 2003) and this can cause problems in food security during harvesting (Belmain et al., 2015). Other negative impacts result from some rodent species living very close to human environments that can have a direct influence not only on human health through potential transmission of gastroenteric diseases and zoonosis to householders but also on domestic livestock. Therefore, rodent pest control is crucial and nowadays, the only effective control method available is the use of anticoagulant rodenticides (ARs).

ARs are so named because they interfere with the blood coagulation processes. The processes of activating various coagulation factors depends on the amount of vitamin K in its reduced form that exists in the organism. ARs inhibit the enzyme vitamin K 2,3-epoxide reductase (VKORC1) that is responsible for reducing vitamin K and maintaining the balance between its oxidized and reduced forms. The inhibition of VKORC1 prevents the activation of the coagulation factors resulting in animal death by internal bleeding. However, the intensive use of ARs can cause rodents to lose their susceptibility and become resistant to them. Genetic resistances to ARs are mainly associated with mutations or single nucleotide polymorphisms (SNPs) in the gene that codes for VKORC1 (*vkorc1*), causing amino acid substitutions in the VKORC1 protein (Pelz et al., 2005). There are studies on this topic in several countries of central and northern Europe detecting rodent populations resistant to AR. Currently, there are at least 13 mutations mainly located in the exon 3 of the *vkorc1* gene described in various countries of the European Union that confer resistance to specific ARs (Berny et al., 2014; Goulois et al., 2017). In Eastern and Southern European countries, the information on the incidence of resistances to rodenticides is scarce, and it is becoming increasingly important to generate information on this subject (Berny et al., 2014). In Spain, a mice population at the coastal countryside showing an adaptive introgression between house mouse and Algerian mouse that confers anticoagulant resistance has been described (Song et al., 2011). While recently, four VKORC1 mutations in black rat were found in Toledo, Segovia and Zaragoza (Goulois et al., 2016; Damin-Pernik et al., 2022). Any increase in resistant in rodent populations would lead to pest control issues that may causing serious agricultural, farming and public health problems.

Scientific advances have revolutionized the study of anticoagulant resistances in terms of understanding their genetic basis, physiological mechanisms and geographical distribution. The techniques based on the extraction and partial sequencing of genomic DNA allow a fast and precise monitoring of possible genetic resistances. Most of these tests involve laboratory studies using live rodents or blood samples taken from animals in the field. However, the improvement of DNA extraction techniques now allows the analysis of faecal samples (stool), increasing the number of samples that can be taken without the need for sampling by trapping or the management of dead animals (Meerburg et al., 2014). The importance of initial detection of genetic resistances due to mutations is crucial. The hypothesis of work, presenting it as a null hypothesis, is that there will be no rodent mutations in the *vkorc1* gene in Spain. In order to contrast this null hypothesis, we monitored for the presence of SNPs that could favour resistance in rodent populations in Spain by means of polymerase chain reaction (PCR) of the exon3 of the *vkorc1* gene. Furthermore, we characterized the binding predictions of several ARs used to control rodent populations in Spain to VKORC1 with new SNPs by *in silico* or computational analysis. We received samples from the species that were considered as the most common pests in Spain, including brown rat, black rat and mouse. In coordination with the National Association of Environmental Sanitation Companies (ANECPLA), we designed a kit and a questionnaire for identification of places where the lack of efficacy of the rodenticides has been previously reported despite the good consumption of AR poisoned bait. Additionally, the managing entities of two cities, Madrid and Leganés and two companies of agrarian locations with specific interest in this project sent us their prospective samples.

2. Material and methods

2.1. Survey of Spanish rodent populations

A survey of rodent populations, with the final aim of detecting possible mutants correlating with resistances to rodenticides, was carried out in Spain form June 2018 to November 2020. The Spanish pest controller companies concentrated their efforts on those places where a number of rodent sightings were reported. The survey was designed in coordination with "ANECPLA", the Spanish professional organization of Pest Control Operators (PCOs) to screen for resistance mutants in the rodent species, brown rat (*Rattus norvegicus*), black rat (*Rattus rattus*) and mouse (*Mus musculus*). For this survey, a questionnaire with information about the location of the stool samples was associated with a unique barcode. This barcode was used to track the samples for correct traceability during analysis. The questionnaire was included in a kit consisting of material for the collection of stool samples (a sterile cryogenic vial with the unique barcode, gloves, spatula and instructions to collect the sample). The kit was prepared in our laboratory and distributed to PCOs.

2.2. Sampling of rodent stools and tails

Rodent stools samples or pieces of tails of dead rats were collected by the PCO in places were an intensive rodenticide treatment was applied due to infestation or to citizen notice. In order to ensure traceability and individuality of the samples, barcoded tubes from one specific location were used. In the case of collection in the agricultural environment (farm, crop, etc.), collection of at least 5 samples from a single establishment/premises distributed in the different dependencies was performed in such a way as to ensure that it is not the same individual. The samples received from the technical services of Madrid and Leganés City Council were collected in accessible sewage systems. A distance of 20–25 m was established for collection of sample tubes from the first one to the next one.

The tails of dead rats were cut and collected in individual tubes with ethanol (70%). Samples were sent to the author's laboratory by mail and upon arrival frozen at −80 °C until analysis.

2.3. Homogenization of stools and tail tissue and DNA extraction

Only one stool sample (200 mg approximately) was homogenized for each location. Each sample was homogenized twice in a 2 ml Eppendorf safe-lock containing 1 ml of sterile PBS and one steel ball of 5 mm (Werfen Ref.: BAI5-1000) for 2 min at 30 frequency with the Tissue Lyser II (QUIAGEN, Hilden, Germany). Later, a first centrifugation step was performed to discard excessive non-degradable matter at 10,000×*g* for 30 s (standard rotor F45–24–11, Eppendorf centrifuge 5415R). The supernatant was collected and added to 900 μl of fresh PBS and vortexed. A second centrifugation was carried out at 4000×*g* for 15 min to obtain the pellet for DNA extraction. The pellet was re-suspended in 200 μl of BT1 buffer with 25 μl of proteinase K and incubated overnight at 56 °C for DNA isolation.

A piece of tail tissue (0.5 cm) was homogenized in 180 μl of BT1 buffer with 25 μl of proteinase K in a 2 ml Eppendorf safe-lock containing and one steel ball of 5 mm for 2 min at 30 frequency with the Tissue Lyser II (QUIAGEN) and incubated overnight at 56 °C. After a centrifugation step at 11,000×*g* for 5 min, 200 f μl of supernatant were used for lysis and DNA isolation.

DNA isolation was carried out with the SPEEDTOOLS DNA extraction kit (BIOTOOLS, Madrid, Spain) according to the manufacturer's instructions. DNA yield of all samples was 10–500 ng/μl with 260 and 280

nm UV absorbance ratios (A260/280) of 1.9–2.1.

2.4. Cytochrome b gene amplification by PCR

Rodent species were identified by analysis of the *cytb* gene in DNA extracted from faecal stools or tail tissue samples. Partial DNA sequences coding for *cytb* were PCR amplified (a heating cycle at 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s) with the DreamTaq Hot Start Green PCR Master Mix (Thermofisher scientific, Waltham, MA, US) in a final volume of 50 μl. Forward 5′-TCTCCATTTCTGGTTTACAAGAC-3′ and reverse 5′-AACAATGA-CATGAAAAATCATCGTT-3′ primers were previously designed from the available literature (Pages et al., 2010).

A BLAST search strategy was used to corroborate the identity of amplified *cytb* products. The amino acid identity with proteins of either brown rat (KY356141), black rat (FJ355927) or mouse (NP_904340) sequences was used as reference for species identification.

2.5. Vkorc1gene amplification by PCR

In the European Union, most of the 13 mutations that were described to confer resistance to specific ARs are located in the exon 3 of the vkorc1 gene (Berny et al., 2014; Goulois et al., 2017; Grandemange et al., 2009b). Therefore, amplification was only performed on exon 3 of *vkorc*1 gene as the exon with most previously described mutations in rats (Grandemange et al., 2009b). Briefly, specific primers of rat *vkorc*1exon 3 (GenBank accession no. NM_203335) are exon3-forward (5′-TTTCACCAGAAGCACCTGCTGCC-3′) and exon3-reverse primer (5′-ACACTTGGGCAAGGCTCATGTG-3′). Amplification was performed using DreamTaq Green DNA Polymerase (Thermo Scientific) at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 63 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The size of the amplified fragment was 354 bp. All amplifications were systematically verified on a QIAxcel Advanced System (QIAGEN).

Amplified PCR products were purified with the Illustra Exo ProStar kit (Sigma Aldrich, Madrid, Spain) according to the manufacturer's instructions. Products were sequenced by the deoxy chain termination method (ABI PRISM dRhodamine terminator cycle sequencing kit, Perkin–Elmer, Wellesley, MA, USA). The sequences from faeces stools samples were of different quality, with short-medium and long contiguous read length.

2.6. Vkorc1 SNP analysis

Sequencing files were analysed, aligned with ClustalX included in the MEGA7 software (Kumar et al., 2016) and homozygous mutations were detected using on line blast searches (NCBI). Heterozygous mutations were detected by examination of the sequencing chromatogram with Sequence Scanner version 2 (Applied Biosystems). All mutations found in one sample were analysed twice for confirmation.

2.7. Computational binding to mutants by molecular docking

A list of eight rodenticide anticoagulants were obtained by a search in PubMed (https://pubchem.ncbi.nlm.nih.gov) by providing the compound identification number (CID). The corresponding sdf files were downloaded.

The amino acid sequences of wild brown rat VKORC1 (Genebank Accession number NM_023335), black rat (XM_032892517) and VKORC1 mutant rat models with the most common European mutation (Y139F) and each of the mutations found in the present study in brown rat (S149I) and in black rat (S149I, S149T, E155Q, E155K and the double mutant S149T&E155K) were used to predict their corresponding tridimensional models of the whole VKORC1 proteins, respectively. Both brown and black rat VKORC1 sequences exhibited a 99% identity and 100% similarity to the whole protein, and a 100% identity at the exon 3. These rat amino acid sequences were submitted to the SWISS-MODEL homology modelling (https://swissmodel.expasy.org/interactive) which automatically selected templates with the closest sequence identity to obtain the corresponding pdb files. The template used for building protein models was the Human VKOR (6wv3.1.A) with an identity of 85.71% and a global model quality estimation of 0.85. Modelled structures were visualized in PyMOL (https://www.pymol.org/).

The AutoDockVina program (Trott and Olson, 2010) included into the PyRx 0.9.8. Package (https://pyrx.sourceforge.io/) was used in e7 64-desk computers, using grids including the whole VKORC1 molecules (blind docking). The sdf files were converted to pdbqt files after energy minimization (Open Babel included into the PyRx package). The AR-VKORC1 complexes with the lowest binding-scores or higher binding affinities (Kcal/mol) were used for analysis. The predicted structures were visualized in PyRx.), as previously described (Bello-Perez et al., 2019; Shityakov and Förster, 2014; Bermejo-Nogales et al., 2021).

Taking into account that brown rat sequence alignments showed a 99% identity with black rat and 91% identity with mouse VKORC1 proteins, we considered to include comparisons among species. We converted the affinity for vitamin K and AR to the protein VKORC1 from mouse, black and brown rat to predict possible degrees of toxicity. We transformed the docking scores as Gibbs free energy of binding (ΔG) to the predicted inhibition constants (Ki) values in molar concentrations (M). The Ki parameters for the docked poses were calculated from the ΔG values as follows [$Ki = \exp([\Delta G*1000]/[R*T])$], where R (gas constant) is 1.98 cal(mol*K)−1, and T (room temperature) is 298.15 K.

2.8. Statistics

Non-parametric Kruskal–Wallis tests were used to find any statistical significant differences among species according to total mutation frequencies (S149T, E155K and E155Q mutations). We did statistical analyses with XLSTAT (version 2012.2.02 of Addinsoft).

3. Results

3.1. Survey

A total of 203 samples from several locations in Spain were collected from the National network of PCOs. We received 96 samples from 12 out of 17 autonomous communities (Spanish administrative areas) covering rural and urban areas, from port areas, farms, industrial estates and protected areas (see Fig. 1 and Table 1). Additionally, prospective locations from two urban areas and two agrarian areas were included for sampling. The first location was within the city of Madrid, where the Department of Vector Control of the City Council provided 26 stool samples collected from the sewer network. The area of study corresponds to Madrid Centro- Embajadores. The second location and a total of 28 samples of tails were provided by the Department of Environment and Pests of Leganés from the sewer network. Samples in agrarian areas were collected from PCO's in a farm at Toledo (30 tail samples) and from an animal facility (23 tail samples) at Alicante (Table 1). The rest of the PCO's usually send us from one to three samples except the PCOs of a farm at Barcelona (seven samples) and another from the port of Cadiz (14 samples).

3.2. Species identification and location

BLAST analysis was done in either tail-tissue or stool samples to reveal the species (Table 1). The identity percentages for complete forward and reverse sample sequences with the reference *cytb* sequence for brown rat, black rat and mouse range from 90% to 100%. Environmental location of the samples obtained from the three species of rodent was heterogeneous (Table 1 and Fig. 1). For instance, mouse samples were mainly received from the North of Spain. Samples were found in urban

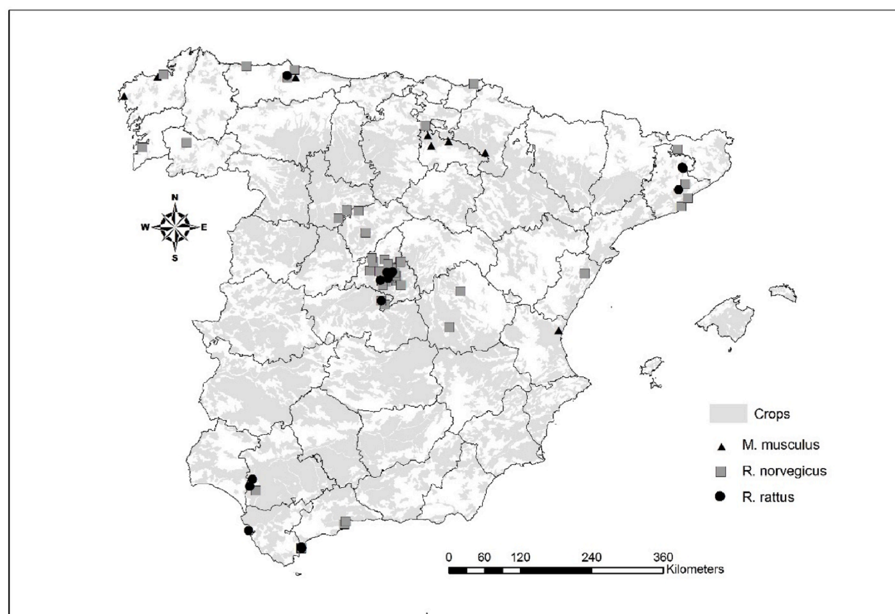


Fig. 1. Location of the samples (N = 96) received in Spain. Three species were identified, brown rat (*Rattus norvegicus*, grey square), black rat (*Rattus rattus*, black circle) and house mouse (*Mus musculus*, black triangle). Distribution of crops is highlighted in light green.

and rural areas with either coastal or agrarian environments. The most representative species was brown rat with 105 samples received, although ubiquitous, the highest number of samples came from locations in central Spain associated with the urban sewage system. Although some samples also came from crops and coastal environments. Samples from black rat (64) were located in coastal provinces, and associated to animal farm installations when found in the Iberian plateau. Only samples received from Madrid (6) were from urban origin. One sample sent from Guadalajara was found to be from a European wood mouse (*Apodemus sylvaticus*, *cytb* Accession number APT68923) and was associated to a forest environment.

3.3. Vkorc1 genotyping

The SNPs found in exon 3 of brown and black rat populations are summarised in Tables 1 and 2. In the brown rat populations three different missense mutations were found in the exon 3 of *vkorc1*. Only one of them led to a single mutation in the corresponding VKORC1. The mutation was located at amino acid position 149 (wild type G > T mutant) leading to mutation S149I. Different frequencies of S149I were found depending on the area and the sampling effort (Fig. 2A). For instance, we received two samples from Guipuzkoa with a mutation frequency of 0.5 and we received 30 samples with a frequency of 0.03 from Madrid. In the prospective sampling at the sewage system of Madrid, we found a 0.21 frequency, located in the districts of Madrid Centro, Salamanca and Legazpi.

In the black rat, four polymorphic positions were found (Table 2A and Fig. 2B). A new haplotype was found at the 143 amino acid position (G > A), two at 149 and two at 155. The S149I is the same as in the brown rat. It was found in the peri-urban area of the port of Cádiz, with a frequency of 0.14. The other SNP at the same 149 amino acid position (G > C) that led to S149T was found in Barcelona, Toledo and Valladolid. The other polymorphic position was at the 155 amino acid position, leading to two new mutations causing changes at the protein sequence, E155Q (G > C) and E155K (G > A). The E155Q mutation was found in the animal facilities at Alicante while the E155K was found in farms at Barcelona and Toledo.

The most marked differences in the total mutation frequencies were observed between the three analysed species (Table 2B). Only black rats showed a higher frequency of mutation with statistically significance.

3.4. Molecular docking of ARs to VKORC1 Spanish mutants

We conducted molecular docking simulations to investigate if the mutations found in the VKORC1 protein of Spanish rat populations could change the affinity of First Generation Anticoagulant Rodenticides (FGAR) and Second Generation AR (SGAR). In brown rats, we compared the binding-score values of the wild type with the S149I mutation and with one of the most common mutations found in Europe (Y139F). None of the mutations caused any change in the binding affinity to vitamin K (maintaining the same value (−10.6 kcal/mol)). In contrast, S149I reduced the binding affinities (increasing its binding-scores) with respect to the wild type sequence to the FGAR chlorophacinone (from −12.7 to −10.7 kcal/mol) and to the SGAR brodifacoum (from −14.7 to −9.2 kcal/mol) (Table 3A). These reductions of binding affinities correlated with those predicted by Y139F for chlorophacinone (from −12.7 to −11.9 kcal/mol) and for brodifacoum (from −14.7 to −9.1 kcal/mol). In the case of warfarin, S149I did not predict any change in the binding affinity (−11.3 kcal/mol) with respect to the wild type protein in contrast to Y139F (from −11.3 to −10.3). The rest of the ARs tested did not predict any changes with the Y139F nor with the S149I mutations (Table 3A).

Compared to the brown rat in the black rat there was a general reduction in the binding affinities to vitamin K (−6.2 to 7.0 kcal/mol) and the ARs tested (range from −6.4 to −9.6 kcal/mol) independently of the wild type or mutant sequences. Reductions in the binding affinity higher than 0.5 kcal/mol were predicted for bromadiolone (SGAR) in S149T (from −8.6 to −7.2 kcal/mol), E155K (to −7.9 kcal/mol) and E155Q (to −8.0 kcal/mol), and for flocoumafen (SGAR) (from −9.1 to −8.5 kcal/mol) (Table 3A). To note that E155K predicted a small increase in the binding affinity to vitamin K (from −6.5 to −7.0 kcal/mol).

Comparisons among species of the calculated inhibition constants (Ki) showed that the black rat needs the highest concentrations of all the ARs (Table 3B). Interestingly, the Ki value of brodifacoum was the highest for the black rat (0.9852 mM) followed by mouse (0.9829) and is the lowest for the brown rat (0.9754 mM). The same pattern also occurs for flocoumafen (0.9847 mM, 0.9800 mM, 0.9757 mM) and warfarin (0.9892 mM, 0.9832 mM, 0.9810 mM).

Table 1
Geographical origin and species of collected samples. (N total samples = 203).

Province	Nº	Mutation	Frequency	Environment
Apodemus sylvaticus				
Guadalajara	1			Forest
Mus musculus				
A Coruña	3			Coast
Asturias	2			Rural
Barcelona	1			Urban
Navarra	2			Rural
La Rioja	2			Rural
Valencia	1			Crops
Total (6 regions)	*11*			
Rattus norvegicus				
A Coruña	1			Coast
Asturias	3			Urban, riverine
Barcelona	2			Rural
Burgos	1			Rural-riverine
Castellón	1			Crops
Gerona	2			Crops
Gipuzkoa	2	S149I	0.5	Urban
Madrid community	30	S149I	0.03	Urban
Madrid city	26	S149I	0.21	Sewage
Leganés city	28			Sewage
Málaga	5			Urban
Navarra	1			Crops
Ourense	1			Urban
Pontevedra	1			Urban
Sevilla	1			Rice crop
Total (13 regions)	*105*			
Rattus rattus				
Alicante	23	E155Q	0.25	Animal facilities
Asturias	1			Rural
Barcelona	7	E155K	0.28	Farm
		S149T + E155K	0.14	
Cádiz	14	S149I	0.14	Peri-urban
Madrid	6			Urban
Segovia	2			Farm
Sevilla	2			Farm rural
Toledo	30	S149T	0.03	Farm
		E155K	0.22	
		S149T + E155K	0.32	
Vallalodid	2	S149T	0.5	Farm
Total (9 regions)	*87*			

Nº, number of samples per region. Mutations, amino acid in single letter code, amino acid in wild type, position on the VKORC1 protein and amino acid in the mutant. Frequency per region = number of samples with mutations/total number of samples.

Table 2
A) Detail of SNPs and mutations found in Spain. WT, wild type; AA, amino acid. B) Differences among total mutation frequencies obtained by Steel-Dwass-Critchlow-Fligner (Kruskal-Wallis test).

A) Mutation	Position	Codon WT	Codon mutated	AA WT	AA mutated
Rattus norvegicus					
S149I	149	TAG	TAT	Ser	Ile
Haplotype 1	107	ATC	ATA	Ile	Ile
Haplotype 2	137	ACC	ACT	Thr	Thr
Rattus rattus					
S149T	149	TAG	TAC	Ser	Thr
E155Q	155	CAG	CAC	Glu	Gln
E155K	155	CAG	CAA	Glu	Lys
Haplotype a	143	GCG	GCA	Ala	Ala

B) Specie	Nº zones	Sum of range	Mean range	Groups	
Mus musculus	6	66.00	11.000	A	
Rattus norvegicus	15	205.50	13.700	A	
Rattus rattus	11	256.50	23.318		B

Fig. 3 shows the 3D structures of the predicted bound complexes between brown rat VKORC1 protein with S149I and brodifacoum (SGAR) and chlorophacinone (FGAR), and the two AR with significant changes in binding affinity. Brodifacoum predicted a high binding affinity to the wild VKORC1 protein (−14.7 kcal/mol) while the VKORC1 protein with the S149I mutation predicted a lower binding affinity (−9.2 kcal/mol). These changes in the binding affinity correlate with different relative binding targets in the protein. Brodifacoum is completely embedded into the wild type VKORC1 while it is more at the surface in S149I. In contrast, the chlorophacinone binding location does not significatively change with mutated variation in the relative binding position to the protein (Supplementary data).

Fig. 4 shows 3D structures of the predicted bound complexes of black rat VKORC1 protein with S149T, E155T and E155Q to bromadiolone. Small variations in the binding affinities were predicted (range −7.2 to 8.6 kcal/mol) in location with bromadiolone at the surface of the black rat VKORC1. This can be clearly seen by comparing with the binding position of bromadiolone to the brown rat wild VKORC1 with a binding affinity higher that −9.0 (10.5 kcal/mol).

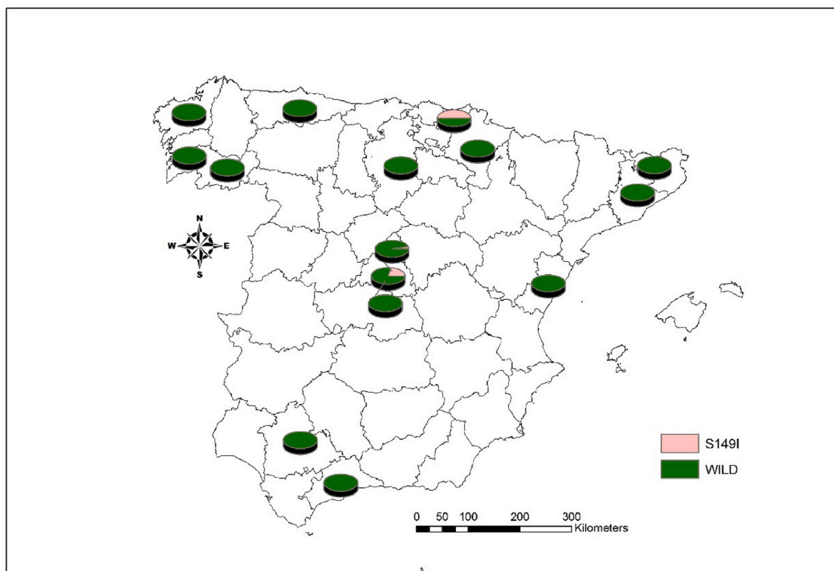
4. Discussion

ARs belong to the product type pest control PT14 according to the Regulation of Biocides (Regulation (EU) 2012) and are classified according to their killing potency. First Generation Anticoagulant Rodenticides (FGAR) (for example warfarin, chlorophencinone and coumatetralyl) require several days of feeding to be fully active. Second Generation Anticoagulant Rodenticides (SGAR) (such as bromadiolone, difenacum, brodifacum, flocoumafen and difethialone) are more active and effective after one day of feeding. Strategies based on an integrated pest management try to implement an effective reduction in the use of pesticides to decrease the possible adverse effects on wild fauna and the likelihood of the appearance of pesticide resistance. Therefore, for a correct management of the use of ARs, it is important to identify possible resistances and the susceptibility of rodent populations.

4.1. Location of rodent samples received from PCOs in Spain

This is the first study of *Vkorc1* rodenticide genetic resistance in Spain with a national coverage of 12 out of the 17 autonomous communities (Spanish administrative areas). In the present survey two priorities were assigned to the national PCO's to send us samples, i) samples where they detected possible resistances to anticoagulant rodenticides (AR) and ii) samples from different geographical locations. This information could cover rural and urban areas, from ports, farms, industrial estates and protected locations and land uses. Studies of rodent mutations at the National level are subject to the efforts of the PCOs, authorities and general public with variable sampling results. In a survey of AR resistance in USA they analysed in 557 house mouse, 216 brown rat and 49 black rat (Díaz and Kohn, 2021). Another study of the distribution of AR resistance in brown rat in the Netherlands obtain 66 rat tail samples and 361 stool samples in a research campaign covered by television, by national and regional radio stations and by newspapers (Meerburg et al., 2014). In Ireland, the PCOs provided tails of 65 brown rats and 50 house mice (Mooney et al., 2018). A study of patients in Spanish hospitals suggest that the low number of samples represent some clear limitations in finding new polymorphisms but should be enough to detect variants in VKORC1 in a cohort (Cullell et al., 2020). In the present study, the number of samples at the national level has been lower than expected, since the study has been carried out during the pandemic period and companies of the sector have dedicated their priority efforts to the disinfection of public spaces. This has been compensated by prospective studies in areas of special interest. Additionally, determination of species by visual evaluation of faeces by PCOs sometimes is difficult especially when juvenile individuals predominate. To solve this problem, we used the PCR detection of the *cytb* gene

A) Brown rat SNPs



B) Black rat SNPs

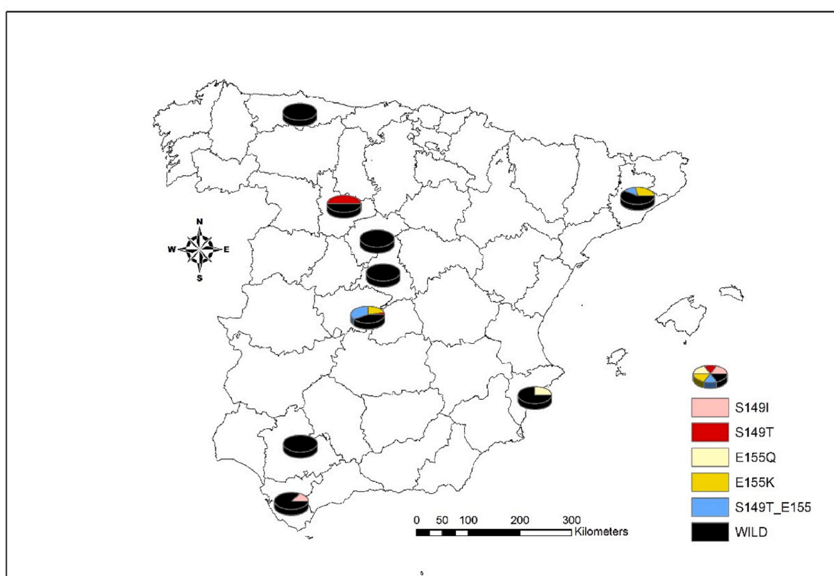


Fig. 2. Geographical location of SNPs detected during the Vkorc1 exon 3 DNA analysis of A) brown rat and B) black rat in Spain. Each SNP is colour coded (legend). The samples that carried the reference sequence (wild type genotype) are shown in green (brown rat) or black (black rat). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

method for species identification (Bermejo-Nogales and Navas, 2021). Our results show that this type of analysis is necessary for a correct species and strain identification in cases where rodent coexistence could be present. In Spain, the current rodent species distribution is based on model projections that predict contractions in the potential distribution of black rat while increasing the brown rat abundances in Central Spain (Araújo et al., 2011). Thus, although the aim of the present study is not abundance estimations we received the greatest number of samples of brown rat that came from locations in central Spain associated with the urban sewage system. Nevertheless, we detected an expansion in black rat population that was mainly located in the coast and associated with animal farm installations, when found in the Iberian Plateau. A recent study also placed new black rat populations in the Iberian Plateau but did not specify or give any more details on the type of environment (Damin-Pernik et al., 2022). The samples identified as mouse faeces were located in the north and coast of Spain.

4.2. *Vkorc1 mutations*

In mouse, we have not found any *vkorc1* SNP in the samples received. A previous work in a rural area of Barcelona province described AR resistance in this species (Song et al., 2011). In this case, mutations have been associated with an introgression in the exon 1 and 2 of the *vkorc1* that may explain the differences with the present work that focused on the study in exon 3.

Regarding rat species AR resistance, they have been usually named according to their origin and resistance properties. The first case of AR resistance in Norway rats was described in 1958 in the United Kingdom

Table 3

A) Predicted highest binding affinity to VKORC1 proteins and anticoagulant rodenticides (ligands). B) Calculated Inhibition constant (Ki) to VKORC1 proteins of mouse (*Mus musculus*), brown rat (*Rattus norvegicus*) and black rat (*Rattus rattus*).

Compound identification number (PubChem CID): Vitamin K (5280483), Bromadiolone (54680085), Difenacum (54676884), Brodifacoum (54680676), Difethialone (91,771), Flocoumafen (54698175), Chlorophacinone (19402), Coumatetralyl (54678504) and Warfarin (54678486).

A) Binding affinities of brown rat and black rat

Anticoagulant Rodenticide	Binding affinity (Kcal/mol)							
	Brown rat			Black rat				
	wild-type	Y139F	S149I	wild-type	S149T	E155K	S149I + E155K	E155Q
Vitamin K	−10.6	−10.6	−10.6	−6.5	−6.5	−7.0	−6.2	−6.4
Bromadiolone	−10.5	−11.5	−10.6	−8.6	−7.2	−7.9	−8.6	−8.0
Difenacum	−14.5	−14.5	−14.5	−9.4	−9.4	−9.4	−9.3	−9.3
Brodifacoum	−14.7	−9.1	−9.2	−8.8	−8.8	−8.4	−8.8	−8.9
Difethialone	−9.1	−9.1	−9.1	−9.6	−9.6	−9.6	−9.6	−9.6
Flocoumafen	−14.5	−14.3	−14.7	−9.1	−8.5	−9.1	−9.1	−9.1
Chlorophacinone	−12.7	−11.9	−10.7	−8.1	−7.8	−7.9	−7.8	−7.7
Coumatetralyl	−11.7	−11	−11.7	−7.1	−7.0	−7.0	−6.9	−7.0
Warfarin	−11.3	−10.3	−11.3	−6.4	−6.5	−6.5	−6.5	−6.5

B) Calculated Inhibition constant (Ki) to VKORC1 wild-type species.

Anticoagulant Rodenticide	Inhibition constant (mM)		
	Brown rat	Black rat	Mouse
Vitamin K	0.9822	0.9890	0.9822
Bromadiolone	0.9824	0.9855	0.9822
Difenacum	0.9757	0.9842	0.9757
Brodifacoum	0.9754	0.9852	0.9829
Difethialone	0.9847	0.9839	0.9829
Flocoumafen	0.9757	0.9847	0.9800
Chlorophacinone	0.9787	0.9864	0.9837
Coumatetralyl	0.9804	0.9880	0.9804
Warfarin	0.9810	0.9892	0.9832

Brown rat: wild protein (Accession number: NM_023335). Common European mutation (Y139F). Spanish mutation (S149I).
Black rat: wild protein (XP_032748408). Spanish mutations (S149T. E155K. S149I + E155K and E155Q).
Mouse: wild protein (NP_848715).
Grey background. Mutations reducing the affinity of the anticoagulant rodenticide. Estimated error of Vina ±2 kcal/mol.
[**Ki** = exp([ΔG*1000]/[R*T])].
The higher intensity of grey in boxes represents the increasing concentration for inhibition.

(Boyle, 1960). Several geographically distinct AR resistant rodent strains were described later in the UK, Denmark, Germany and the USA. These were originally designated Scottish-, Welsh-, Hampshire-, Muensterland/Westphalia-, Jutland- and Chicago-type resistance, thus identifying different resistance alleles in geographically distinct Norway rat populations. In UK, a resistance map was developed to be applied for the large-scale control of these animals. Unfortunately, the pest controllers did not use this information appropriately and in time, so that nowadays it is the country where the greatest number of AR resistances have been found (Prescott et al., 2017). The main reason for the spreading of these AR resistances has been related to an inappropriate use of ARs. Since, heterozygous resistant animals are more easily controlled by ineffective ARs than homozygous resistant animals; the continued use of these rodenticides eliminates susceptible animals, increasing the proportion of homozygous resistant animals in the population, which were thereafter more difficult to control. In our current work, we are in close contact with ANECPLA and related regulatory institutions so that the transfer of information is direct. Indeed, as we use DNA extraction techniques in faecal (stool) samples we could increase the number of samples that can be taken without the need for sampling by trapping or by managing of dead animals (Meerburg et al., 2014). Indeed, as faeces are easier to collect than trapping rodents, the ease of availability of samples for analysis with this method prompted the PCOs to send us more samples. The result was that we first discovered in Madrid samples of brown rat with the S149I mutation, a finding that was rapidly communicated to the local pest control services so they could change the control strategy accordingly. This mutation was not previously known in rats but was also recently detected in water voles with low frequencies (0.5–1%) in France (Khalil et al., 2021). Interestingly, the authors of that work did not find sensitivity of this mutation to bromadiolone or *cis*--bromadiolone, the only ARs tested. In the samples from black rat, we did not find the K152T mutation recently described in the exon 3 for other areas in Spain (Damin-Pernik et al., 2022). Instead, we first identified mutations at the polymorphic 155 position with two single point mutations, the E155K and the E155Q. We also identified a new S149T mutation so that was a new polymorphic position. Other polymorphic positions described with various mutations in European wild rat populations, such as the position 120 (L120Q and L128Q) and the position 139 (Y139F, Y139C, Y139S) that were associated with AR genetic resistance (Grandemange et al., 2009a; Hodroge et al., 2011), were not detected in this work.

4.3. Molecular docking

Molecular docking is a computational procedure that attempts to predict noncovalent binding of macromolecules and ligands in order to predict the best bound conformations and their corresponding binding affinities (Trott and Olson, 2010). We aimed to explain if the mutations found in the Spanish rodent populations could lead to changes in the binding affinities of the VKORC1 protein that may explain some of their resistance. Docking analysis previously demonstrated that human VKORC1 mutations associated to oral anticoagulant resistance affect putative warfarin binding interfaces (Czogalla et al., 2013). The present work analysed the binding affinities and best conformations of eight AR and vitamin K to brown and black rat wild type and VKORC1 mutants. In brown rat, we found that the S149I mutation exhibited a decreased affinity for brodifacoum at a similar level than the Y139F mutation. This mutation has been associated with AR resistance in other rodent

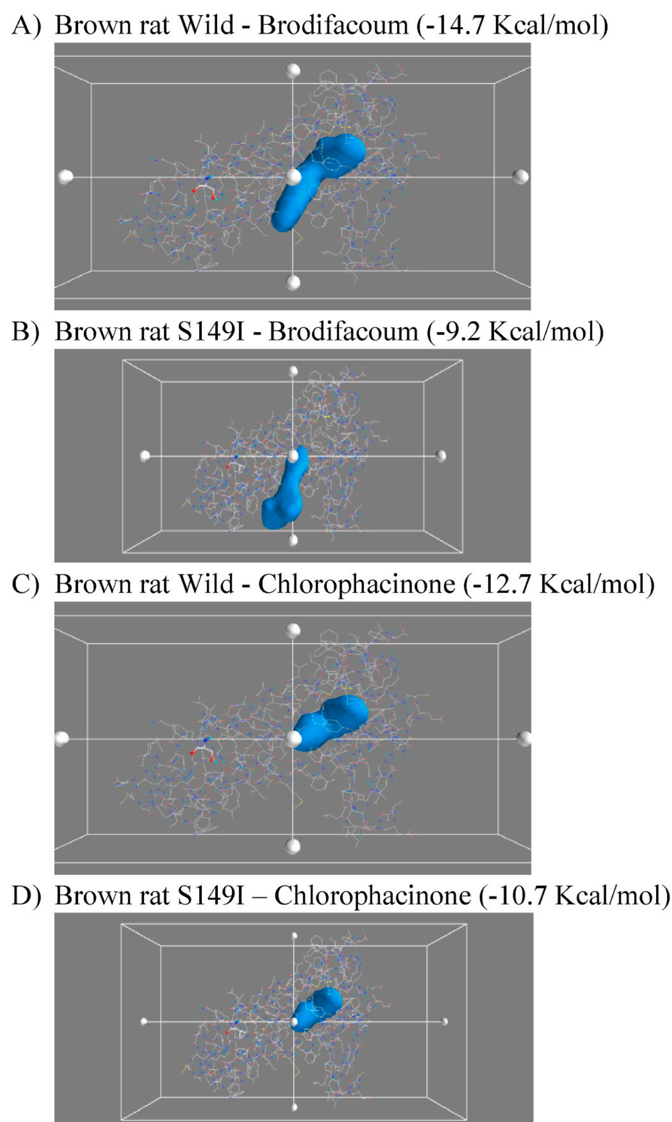


Fig. 3. View of the 3D scene along the Z-axis of the binding of wild brown rat VKORC1 and S149I mutant showing the variation of the binding affinities of anticoagulant rodenticides (AR). A-B) Brodifacoum. C-D) Chlorophacinone. The VKORC1 protein is represented as lines. The Ile amino acid at position 149 is highlighted as balls and sticks. The AR appear as molecular surface in blue colour. Number in brackets indicate binding affinity (Kcal/mol) of AR to VKORC1-S149I mutant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

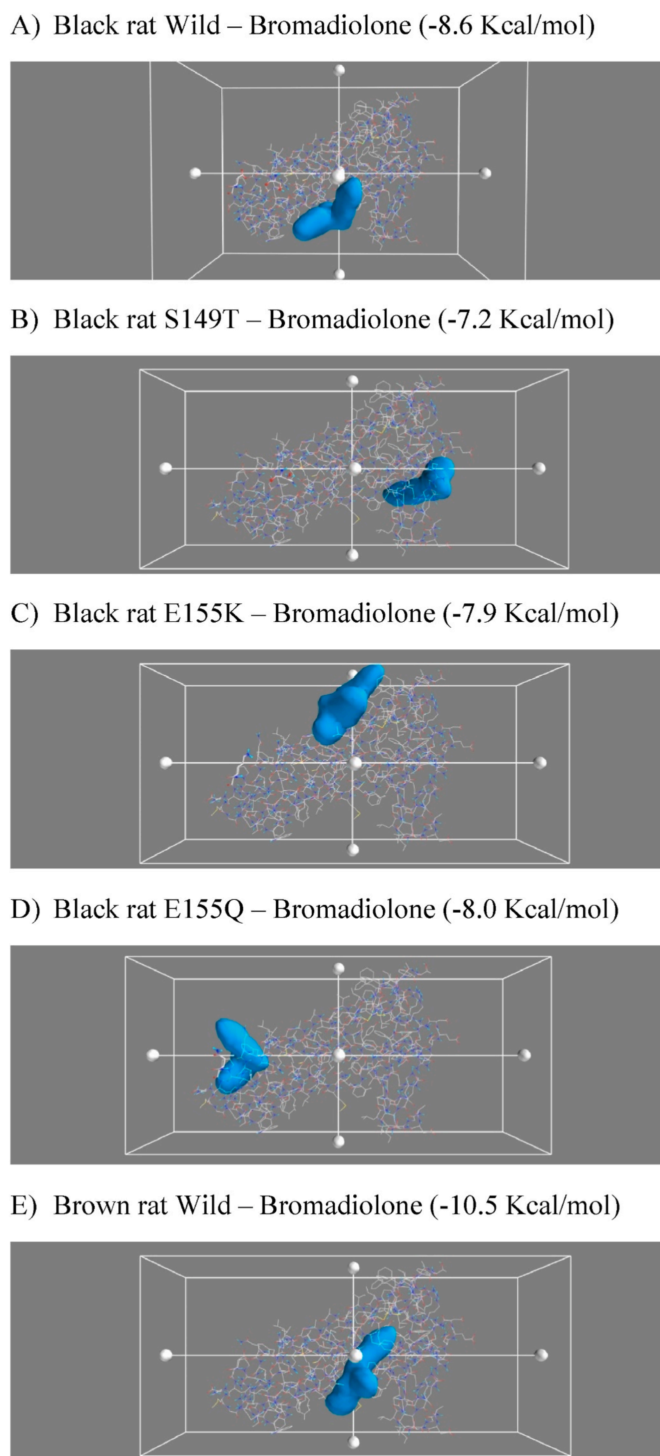


Fig. 4. View of the 3D scene along the Z-axis of the binding of black rat VKORC1-wild and mutants to bromadiolone. A) Black rat wild, B) S149T, C) E155K, D) E155Q and E) brown rat VKORC1-wild. The VKORC1 protein is represented as lines. The amino acid at mutated position is highlighted as balls and sticks. The bromadiolone appear as molecular surface in blue colour. Number in brackets indicate binding affinity (Kcal/mol) of bromadiolone to VKORC1 proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

populations (Grandemange et al., 2009a). Notably, the S149I mutation also cause a weaker binding to chlorophacinone. Our docking analysis found no significant differences in sensitivity to bromadiolone of S149I as has been previously described in the water vole measuring human VKOR activity and inhibition constants (Khalil et al., 2021).

In black rat compared to brown rat, we found a unique amino acid change at I90L in the VKORC1 wild type. The corresponding binding affinities of vitamin K and AR were lower in the black rat. Indeed, the black rat has the highest Ki for all the ARs followed by mouse and the brown rat. This higher sensitivity of brown rat compared to black rat is in agreement with lethal feeding tests showing higher daily intake of warfarin in black rats (15.62–19.2 mg/kg) than in brown rat (4.57–4.82 mg/kg) (Zhelev et al., 2019). Among the mutant populations found in the present study, the S149T mutation showed the lowest binding followed by the E155K and E155Q mutations to bromadiolone. Indeed conformational changes in these mutants showed a binding to this ligand out of the binding site of vitamin K. Previous studies have described L76P and Y25F black rat mutations associated AR resistance to conformational changes in VKORC1 (Goulois et al., 2016; Takeda et al., 2021).

In conclusion, the present study demonstrates the existence of novel VKORC1 mutations in brown and black rat populations in Span detected by analysis of their faecal or tail samples. Although a more accurate estimation of mutant frequencies would need many more samples, the polymorphic genomic positions detected showed variations with the rat species. A first estimation of possible correlations of the mutations detected with AR resistance was also obtained by studying the effects of mutations in the binding of ARs by computational binding predictions. Our results represent the first description targeting genetic resistance in rodent populations in Spain. A more accurate pest control management system may be best served by following resistance evolution over time by monitoring the genetics of *vkorc1* in the rodent populations.

Author contributions statement

Azucena Bermejo-Nogales: Conceptualization; Funding acquisition; Investigation; Methodology; Resources; Data curation; Formal analysis; Supervision; Validation; Visualization; Project administration; Roles/ Writing - original draft; Writing - review & editing. José A. Rodríguez Martín: Formal analysis, visualization. Julio Coll: Writing - review & editing. José M. Navas: Conceptualization; Funding acquisition; Project administration; Writing - review & editing.

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Availability of data and material

The data will be available upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136021.

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