Distribution of non-synonymous Vkorc1 mutations in roof rats (Rattus rattus) in France and in Spain - consequences for management

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Distribution of non-synonymous *Vkorc1* mutations in roof rats (*Rattus rattus)* in France and in Spain - Consequences for management.

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Abstract

Rodent control is mainly done using anticoagulant rodenticides leading to the death of rodents through internal bleeding by targeting the VKORC1 protein. However, mutations in VKORC1 can lead to resistance to anticoagulant rodenticides that can cause treatment failure in the field. This study provides the first insight into the distribution, frequency and characterization of *Vkorc1* mutations in roof rats (*Rattus rattus*) in France and in three administrative areas of Spain.

The roof rat is present in France while it was thought to have almost disappeared with the expansion of the brown rat. Nevertheless, it has been found mainly in maritime areas. 151 roof rats out of 219 tested presented at least one missense mutation in the coding sequences of *Vkorc1* gene (*i.e*. 69.0% of the rat). Nine *Vkorc1* genotypes were detected (Y25F, A26P, R40G, S57F, W59C, W59R, H68N, Y25F/K152T and Y25F/W59R. Biochemical characterization of the consequences of these different genotypes proved that these various genotypes did not induce severe resistance to anticoagulant rodenticides.

Even if many mutations of the *Vkorc1* gene are present in roof rat populations in France, their management may be based in a first approach, considering the low levels of resistance induced, on the use of first-generation anticoagulants less dangerous for wildlife. The use of second-generation may be considered when treatment failure is observed or when bait consumption is limited

Keywords

Rattus rattus; Anticoagulant rodenticide; VKORC1; Mutation; Resistance, VKOR activity assay

Abbreviations

AR, anticoagulant rodenticide; VKORC1, Vitamin K epoxyde reductase complex subunit 1; PCO, pest control operator

1. Introduction

Rodents, by sharing our environment, can transmit to humans, directly or indirectly, more than 40 zoonotic pathogens, such as *Yersinia pestis*, *Leptospira sp.*[1] Furthermore, the roof rat, as vector of *Yersinia pestis* is the responsible for the black plague, causing death to more than a third of Europeans in the fourteenth century. They can also cause the destruction or degradation of important quantity of crops, corresponding approximatively to 10% of the grain crops in the world.[1,2] Moreover, rodents also damage substructures and electric or electronic networks.

To manage these populations of rodents, chemical controls have been organized since 1950 by using anticoagulant rodenticides (ARs), the only effective molecules. Thus, their delayed action, with death occurring 3–7 days after bait consumption, obviates the alimentary aversion problem, which is a very important behavioral trait among rodents. Indeed, ARs, by inhibiting the VKORC1-dependent vitamin K epoxide reductase enzyme,[3,4] lead to the progressive reduction of the pool of vitamin K necessary for the activation of clotting factors II, VII, IX, and X.[5,6] Therefore, prolonged or repeated exposure to ARs lead to the death of rodents by hemorrhage. Unfortunately, quickly after the first use of ARs, resistant rodents are described in Europe[7,8] and in the United States.[9] Later, it was also described everywhere in the world, such as Canada,[10] Australia[11] and Japan[12]. The emergence of such resistance to anticoagulants belonging to the first generation (*i.e.*, warfarin, diphacinone, coumatetralyl, chlorophacinone) led to the development of new Ars belonging to the second generation (*i.e.*, bromadiolone, difenacoum, flocoumafen, brodifacoum and difethialone) in the 1970's and 1980's. Two main resistance mechanisms have since been described. The first, metabolic mechanism due to an overexpression of cytochrome P-450 3A, is essentially described in Japan.[12,13] The second, the most common resistance mechanism, is a result of single-nucleotide polymorphisms (SNPs) in the *Vkorc1* gene, leading to an enzyme that is less sensitive to the action of Ars.[3,4] This mechanism of resistance has been extensively studied in Europe and around the world in brown rats,[14–18] house mice[17–20] and humans.[21] It is thus described to be highly prevalent in brown rats and mice. However, this mechanism has been less studied in the roof rat, especially in Europe where this rodent specie is considered to have disappeared. Only few studies described mutations in the *Vkorc1* gene in the roof rat in Europe[17,22] or worldwide.[23–25] In this paper, we report the different mutations of *Rattus rattus Vkorc1* gene observed in different parts of France and Spain. Using recombinant VKORC1 protein, we thus analyzed the catalytic consequences of all the different mutations described to date in roof rat in order

to evaluate the resistant phenotype associated with these mutations. This characterization allowed us to better understand the origin of the resistance described by the pest management operator in the field.

2. Materials and Methods

2.1. Materials

Vitamin K1 (Phylloquinone-2-methyl-3-[(E,7R,11R)-3,7,11,15-tetramethylhexadec-2-enyl]naphthalene-1,4-dione) was converted to vitamin K > O according to Tishler et al. [12]. Purity was estimated by LC/MS and was higher than 99%. Sodium warfarin (3-(α-Acetonylbenzyl)-4-hydroxycoumarin sodium salt, 4-Hydroxy-3-(3-oxo-1-phenylbutyl)coumarin), difenacoum 3-(3-Biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxycoumarin, 3-(3-Biphenyl-4-yl-1,2,3,4-tetrahydro-naphthalen-1-yl)-4-hydroxychromen-2-one) and brodifacoum (3-(3-(4'-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxycoumarin) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France) with a purity higher than 98% (Figure 1). Chlorophacinone (2-[(4-chlorophenyl)phenylacetyl]-1H-indene-1,3(2H)-dione), bromadiolone (3'[3-(4'-bromobiphenyl-4-yl)-3-hydroxy-1-phenylpropyl]-4-hydroxycoumarin) and difethialone (3'[3-(4'-bro'o[1,1'-biphenyl]-4-yl)-1,2,3,4-tetrahydro-1-naphthalenyl]-4-hydroxy-2H-1-benzothiopyran-2-one) were supplied by Liphatech (Pont de CFranceFrance) with a purity higher than 98% (Figure 1). Methanol HPLC grade, and acetic acid (analysis grade) were obtained from Merck (Germany).

2.2. Rats tissue sampling*Rattus rattus* were collected from the national network of pest control operators (PCOs) in 15 out of 95 departments (French administrative areas) covering all the country and in 3 out of 47 Spanish administrative areas. This study was not considered to be an "experimental procedure" as defined by the French legislation (Rural Code, Article R214–89) and was therefore not subject to an ethical committee approval. This study complied with the ethical standards of European regulations governing the care and use of animals in research (Directive 2010/63/EU) and it did not involve any endangered or protected species, or protected areas.

Only rats captured with lethal traps were included in the study, rats found dead during chemical treatment campaigns were excluded. The tails of dead rats were cut by PCOs, and the samples were sent to the laboratory by mail in individual tubes with 70° alcohol. They were frozen at −20°C until analysis. For each tail, PCO filled a questionnaire indicating the site where the rat was trapped, details on the trapping method (use and nature of attractant, nature of the lethal trap), and if applicable,chemical methods used in the last 6 months prior to trapping.

2.3. Species determination

Two microliters of genomic DNA extract were amplified by PCR using specific primers of *cytochrome b.*[26] The sequence of the sense primer cytb-S and the antisense primer cytb-AS were ($^{5'}$-TCTCCATTTCTGGTTTACAAGAC-$^{3'}$) and ($^{5'}$-AACAATGACATGAAAAATCATCGTT-$^{3'}$), respectively. *Cytochrome b* amplification was performed using cytb-S and cytb-AS (10 pmol), GoTaq polymerase (1 unit, Promega) in a 25 µl reaction volume containing 2 µl DNA, 5 µl 5X GoTaq buffer and 200 µM of each deoxynucleotide triphosphate. The amplification was performed at 94°C for 3 min followed by 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 90 s, and a final extension step at 72°C for 10 min. The amplified product was sequenced on both strands; the resulting sequence was submitted to blast analysis.

2.4. *Vkorc1* sequencing

Two microliters of genomic DNA were amplified using specific primers of *Vkorc1* gene. In order to sequence the totality of the *Vkorc1* gene, two sets of primers were used. The sequences of the first set of primers rVKOR-S1 and rVKOR-AS1 were ($^{5'}$-GGTTCTTCCCTCTTGTGTCTG-$^{3'}$) and ($^{5'}$-GGGTCACCAAGACATGAGGTG-$^{3'}$), respectively, and were used to amplify rat *Vkorc1* gene from nucleotide -36 to nucleotide +1141 (according to *R. rattus frugivorus Vkorc1* – accession n° HM181985). The sequences of the second set of primers rVKORC1-S2 and rVKORC1-AS2 were ($^{5'}$-ACTTGGGCAAGGCTCATGTG-$^{3'}$) and ($^{5'}$-AAGAGTAGGGGACAAGGTGGC-$^{3'}$), respectively, and were used to amplify the rat *Vkorc1* gene from nucleotide +848 to nucleotide +2183. Rat *Vkorc1* amplifications were performed using rVKOR-S1 and rVKOR-AS1 or rVKOR-S2 and rVKOR-AS2 (10 pmol), Accuprime polymerase (1 units, Invitrogen)

in a 25 µl reaction volume containing 2 µl DNA, 2.5 µl 10X Accuprime buffer and 200 µM of each deoxynucleotide triphosphate. The amplification was performed at 94°C for 3 min followed by 40 cycles at 94°C for 20 s, 59°C for 20 s, 68°C for 120 s, and a final extension step at 68°C for 10 min. The amplified products were sequenced on both strands; the obtained sequences were compared to *Rattus rattus* or *Rattus norvegicus Vkorc1* sequences published in Genbank (accession number HM181985 and HM181979).

2.5. Heterologous expression of mutated *R. rattus* VKORC1

The coding sequence corresponding to the *R. rattus* VKORC1 fused with a c-myc tag *via* a flexible $(GGS)_3$ in its 3'-extremity was optimized for heterologous expression in yeast and synthetized by GenScript (Piscataway, NJ, USA). The synthetized nucleotide sequence included EcoRI and XbaI restriction sites at its 5'- and 3'- extremities, respectively. This nucleotide sequence was subcloned into pPICZ-B (Invitrogen, Cergy Pontoise, France) and sequenced on both strands.

Construction of rVKORC1 mutant was carried out using pPICZ-rVKORC1 as template with the Quickchange site directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations. Mutant was checked by sequencing and was thus expressed in *P. pastoris* as described below. Recombinant mutant VKORC1 proteins were expressed in *Pichia pastoris* as described previously by Hodroge *et al.*[14] Yeast microsomes were prepared from thawed yeast cells by differential centrifugation, as described previously.[27,28] Protein concentrations were evaluated by the method of Bradford using bovine serum albumin as a standard. Microsomes were frozen at -80 °C and used for kinetic analysis and immunoblot analysis.

2.6. Immunoblot Analysis

Expression of VKORC1 proteins in microsomal fractions was determined by Western blotting as described previously.[25]

2.7. VKOR Activity Assays and Kinetics

Microsomal vitamin K epoxide reductase (VKOR) activity was assayed as described previously.[17] K_m, and K_i values were obtained from at least three separate experiments performed on two different batches of protein. The estimation of K_m values was achieved by the incubation of at least 9 different concentrations of vit K>O (from 0.003 to 0.2 mM) to the

standard reaction. Incubations were performed in duplicate. Data were fitted by nonlinear regression to the Michaelis-Menten model using GraphPad Prism 6. In order to evaluate the inhibiting effect of warfarin on VKOR activity, K_i were determined after addition of various concentrations of anticoagulant to the standard reaction in the presence of increasing amounts of vit K>O (from 0.003 to 0.2 mM) using anticoagulant concentrations from about 0.05 to 20×K_i. Data were fitted by non-linear regression to the non-competitive inhibition model v= (V_{max}/(1+(I/Ki)))*(S/(K_m+S)) using GraphPad Prism 6.

3. RESULTS

3.1. *Vkorc1* missense mutations in French and Spanish *Rattus rattus*

A total of 219 tail samples (181 from France and 38 from Spain) were sent by PCO from 18 administrative departments (15 French and 3 Spanish administrative areas) (Figure 1) in 40 different locations (35 in France and 5 in Spain). All samples were molecularly confirmed by *cytochrome b* amplification as having been collected from *Rattus rattus*. Among the collected samples, only 68 samples presented no missense mutation in the coding sequences of *Vkorc1* gene (*i.e.* 31.0% of the rat tails sent by PCO). 151 samples presented at least one missense mutation in the coding sequences of *Vkorc1* gene (*i.e.* 69.0% of the rat tails sent by PCO). From the 151 French and Spanish rat carriers for *Vkorc1* missense mutations, 86.6% were homozygous and only 13.4% were heterozygous.

Eight different missenses mutations were found in the *Vkorc1* gene in the French and Spanish rats and led to a single mutation in the corresponding VKORC1 protein. (Figure 1 and Table 1). In exon 1, 4 mutations were detected. These mutations were located at nucleotide 74 (g.74A>T), 76 (g.76G>C), 118 (g.118C>G) and 170 (g.170C>T) and led to mutations Y25F, A26P, R40G and S57F, respectively. In exon 2, 3 missenses mutations were detected at nucleotide 982 (g.982T>A), 984 (g.984G>T) and 1009 (g.1009C>A) and led to mutations W59R, W59C and H68N, respectively. In exon 3, one mutation was detected at nucleotide 2109 (g.2109A>C) and led to the mutation K152T. The other genotypes led to *Vkorc1* with two mutations. The g.74A>T mutation (exon 1) was found to be associated with the g.982T>A mutation (exon 2) or the g.2109A>C mutation (exon 3) leading to proteins with two combined mutations, the Y25F and W59R or the Y25F and K152T, respectively.

In our sampling in France, the observed allelic frequencies of Y25F, R40G, S57F, W59C, W59R and H68N were 3.0%, 6.6%, 1.1%, 21.8%, 22.1% and 0.8%, respectively. The allelic

frequencies of these mutations were different between geographical areas. The two combined mutations Y25F and W59R were specifically found in the North of France.

In Spain, the observed allelic frequencies of A26P, W59R and Y25F were 39.5%, 31.6% and 23.7%, respectively (Table 2). The two combined mutations Y25F and K152T were found in Zaragoza (Spain province) only. However, the sampling in Spain is insufficient for giving accurate results and the investigation should be continued.

3.2. Functional consequences of Vkorc1 mutations

Functional consequences of French and Spanish single and double detected mutations on VKOR activity were characterized. The single mutation K152T was also characterized. All proteins were efficiently expressed in *P. pastoris* with the same expected molecular mass of approximately 20-kDa (Figure 2). The ability of each membrane protein to catalyze the reduction of K>O to K was determined. Four single or double mutants (*i*.e. S57F, W59C, W59R and Y25F/W59R) presented less than 2% of the VKOR activity determined for wild type VKORC1 preventing additional studies (Table 3). The other mutants were all able to reduce the vitamin K epoxide with Km similar to wild type VKORC1 (Table 3). All ARs (*i.e.*, warfarin, chlorophacinone, bromadiolone, difenacoum, difethialone or brodifacoum) were able to inhibit the VKOR activity catalyzed by the active mutants (i.e., Y25F, A26P, R40G, H68N, K152T and Y25F/K152T). Nevertheless, resistance factors, corresponding to the ratio between the K_i obtained for the mutated protein and the K_i obtained for the wild type protein, were different for each mutants towards each AR (Figure 3). Y25F, A26P, and R40G resulted in moderate resistance to FGAR with resistance factors ranging from 2 to 15. R40G and H68N resulted in slight resistance to SGAR with resistance factors of 2 to 4.

4. Discussion

4.1. Distribution of roof rats in France

This study demonstrates the presence of roof rats in France while this species was considered to have disappeared on the French territory after the expansion of brown rat in the 18th century. 181 roof rats were trapped from 15 administrative departments over a 2-year period (2014-2015). However, this sampling remains limited, suggesting a low abundance of this species in France. Furthermore, the distribution of the roof rat in France seems very

heterogeneous. Indeed, the roof rats were mainly trapped in fluvial or maritime port areas (Brest, Saint-Nazaire, La Rochelle, Dunkirk, Strasbourg) and insular areas (Islands of Marseille) whereas the sampling was planned for the whole territory. This apparent distribution could be a bias due to the sampling method based on participatory research. Indeed, the samples analyzed were supplied by PCOs from rodents trapped on their various control sites. It is nonetheless important to note that many French PCOs representative of all French regions were contacted at the beginning of the study and that the sampling of roof rats was carried out in parallel with the sampling of domestic house mice which allowed the evaluation of the prevalence of mutations in the latter in the previously published study.[17] The PCOs were requested to collect samples from both species (domestic mice and roof rats) at the same time. During the sampling period, 266 samples of mice were collected in 27 departments while the 181 samples of roof rats were collected from only 15 departments. It is therefore conceivable that PCOs that have sent in samples of mice but not roof rats, are not dealing with proliferations of roof rats even though they are more difficult to capture than mice due to their excessive neophobia and are often mistaken for brown rats. Those PCOs were in the South-East and the North of France, in and around Paris and in inland of Brittany. Despite these issues, distribution of roof rats observed in France in this study seems to be coherent with the studies published by Buckle[1] describing the presence of roof rat mainly in port areas in different countries (such as Iraq and Turkey). The use of containers on modern ships could favor the dispersion of roof rats from ports to ports and their presence in ports.

4.2. Diversity of *Vkorc1* mutations in roof rats in France and Spain

In Europe, roof rat populations are managed as the brown rat populations by using baits containing anticoagulant rodenticides. This intensive and long-standing use of ARs (ARs have been used since the 1950s) has led to a wide selection of resistant brown rat populations with predominant mutations, the Y139F mutation in France, the Y139C mutation in the East of Europe, especially Germany, the L120Q mutation in England, all leading to strong resistance to first and to some second generation ARs. For roof rats, very few studies are available in Europe, while *Vkorc1* mutations have been described in Japan,[23] New Zealand[24], Argentina[17,22], and USA[18]. Information about resistance are crucial to manage correctly roof rat populations. Indeed, it is essential to use the right molecules to reach an optimal efficacy, to avoid selection of resistant populations and to avoid secondary poisoning of wildlife

through the misuse of the most persistent and toxic molecules while roof rats are strongly present in island and agricultural environments.

Herein, we detected 9 *Vkorc1* genotypes in *Rattus rattus* in France and Spain: 7 single mutations (Y25F, A26P, R40G, S57F, W59C, W59R and H68N) and 2 double mutations (Y25F/K152T and Y25F/W59R). Among these genotypes, 3 single missense mutations and 2 double mutations have never been described: S57F, W59C, A26P, K152T, Y25F/K152T and Y25F/W59R. Y25F mutation was already described in Spain[29], New Zealand[24] and USA[18] and shown to lead to moderate resistance to first generation ARs and limited resistance to some second generation ARs (*i.e.*, bromadiolone and difenacoum)[29]. The R40G mutation was already detected in Japan, the H68N in Martinique Island and the W59R in Argentina[17] and Germany[1].

4.3. Functional consequences of *Vkorc1* mutations

The functional consequences of mutations or double mutations detected in this study were characterized after expression of the recombinant enzyme in *Pichia pastoris*. This system has already allowed to successfully characterize mutations in brown rats[14] and mice[19] with results consistent with *in vivo* results. This system has also previously allowed comparison of sensitivities to ARs in roof rats and brown rats. Based on the K_i and considering only the properties of the VKORC1 enzyme, the sensitivity of roof rats to ARs is similar to that of brown rats[29]. Nevertheless, other factors may cause differences in response, such as metabolism of ARs, but also behavior and diet. No information is available on the ability of roof rats to metabolize ARs. However, its diet and behavior are different from those of brown rats and are certainly responsible for a lower ingestion of baits.

This system allowed in this study to obtain a resistance factor for each mutant and towards each AR by calculating the ratio between inhibition constants of the mutated VKORC1 and the wild-type VKORC1. Among the identified mutations, the R40G mutation appears to induce *in vitro* the highest resistance factors with factors ranging from 2 to 15 depending on the AR. If we compare these factors with those obtained for the most frequently encountered mutations in brown rats (for Y139F, Y139C, L120Q, resistance factors are higher than 100), these factors appear rather low, suggesting that rats carrying such mutations remain sensitive to ARs in the field. Besides, previous *in vivo* laboratory tests have tended to lead to this same conclusion[23]. Nevertheless, considering that roof rats consume little or no bait in the field due to neophobia and food competition, such factors could lead to management issues. Similarly, but to a lesser extent, the H68N mutation appears to induce a slight resistance to ARs

belonging to the 4-hydroxycoumarin family. The success in the field of treatments based on such molecules is therefore certainly dependent on the palatability of baits. The Y25F, A26P, K152T mutations and the double-mutation Y25F/K152T do not lead to resistance to ARs, possibly for some (*i.e.*, Y25F and A26P) to a very slight resistance to warfarin, which is no longer used today as a rodenticide.

The other mutations (*i.e.*, W59R, S57F and W59C) seem to lead to a drastic loss of VKOR activity. When activity is lower than 2% of the VKOR activity of the wild-type enzyme, our method is not sensitive enough to be able to further characterize the VKOR activity. This result is surprising because some of these mutations are found in the homozygous state and an inactivation or severe loss of activity of the enzyme questions the recycling of vitamin K in individuals carrying such mutations in the homozygous state. Is a compensatory mechanism present? This result was acquired after characterization of the activity in the presence of dithiotreitol. The presence of such chemical reductant presence in the assay has been proposed as a potential disturbance to the measurement of activity. Nevertheless, considering the knowledge acquired on this reductant, this reducing agent would rather tend to activate an inactive VKORC1 than inhibit it. Indeed, this reducing agent could possibly directly access the catalytic site of the enzyme to activate it directly (*i.e.*, C132XXC135), whereas the normal functioning of the enzyme involves an electron transfer from the luminal loop via the C43 and C51 present in the luminal loop to the C132 and C135 of the catalytic site present in the fourth transmembrane domain. The S57F, W59F and W59R mutations are present in the luminal loop responsible for activation of the catalytic site. It is conceivable that such mutations could disrupt the folding of the luminal loop preventing activation of the catalytic site by the latter (the enzyme would then be really inactive) or possibly that their presence prevents the activation of this loop by dithiotreitol (this loss of activity would then be a bias due to the method). Another indirect method, the cell-based assay has recently been developed and would allow to elucidate this loss of activity.[30] Answering this question is crucial since some of these mutations have been detected in the homozygous state, raising the question of the viability of these rats. Indeed, the KO of the VKORC1 enzyme is lethal by hemorrhage[31] due to the lack of an alternative system to recycle the vitamin K necessary for the activation of coagulation factors II, VII, IX and X. Roof rats carrying inactivated mutations in the homozygous state would then have an alternative system, possibly an overexpression of VKORC1L1 in the liver.[32] Nevertheless, this alternative mechanism could induce possible resistance to ARs. Indeed, the W59R mutation was detected in a German village where 89% of the animals carried this mutation in the homozygous state. This

population was complex to manage with either bromadiolone or difenacoum.[1] But at the same time the authors reported a low consumption of bait which could alone explain this difficulty of treatment. Further studies are necessary to elucidate the case of the 'inactivating" mutations.

4.4. Consequences in term of rodent management

While in brown rats, 3 mutations associated with severe resistance to first-generation ARs or even some second-generation ARs are predominant and found at very high allelic frequencies in Europe[16,19,28,33–35], mutations in the *Vkorc1* gene found in roof rats in France and Spain are more diverse, seem to reach lower frequencies if considered on a broad scale (of the order of 10% or less than 10% for mutations with significant activity) and induce little or no resistance. That is surprising while the management of the roof rat has been done in the same way as that of the brown rat. In the brown rat, management has resulted in a major selection of the most resistant populations. In the roof rat, it is possible that this selection was never intense due to low bait consumption as described by Buckle.[1] Indeed, the baits marketed are baits developed mainly for the management of brown rats. However, the diet of these two species is different: the roof rat is less omnivorous than the brown rat,[36] and animal food is less significant to it. The roof rat preferred fruits, seeds and grain (51–59% of its diet). Moreover, roof rats prefer the food they are familiar with. More adapted baits could increase the success of the roof rat management. Nevertheless, the obligatory use of bait boxes in Europe could aggravate this non or low consumption and therefore this difficulty to manage because the roof rat is very reluctant to enter in these boxes. A second hypothesis to the non-selection of resistant VKORC1 mutation is the absence at the beginning of the use of ARs, of rare mutations conferring resistance to ARs. So, there was nothing to select. A third hypothesis is the presence of other resistance mechanisms in roof rats not supported by VKORC1 mutations. Metabolic resistance is conceivable as it has already been described in Japan for this species.[37,38] Diet-based resistance is also possible, as described for voles[39]. Further studies will be necessary to evaluate the distribution of such resistance in roof rats in Europe.

In view of the results obtained in this study, the use of first-generation ARs to manage roof rats in France seems entirely conceivable in view of the prevalence of mutations in the *Vkorc1* gene and their functional consequences. It may be necessary to improve the palatability of baits for this species to ensure enough bait ingestion. The use of second-generation ARs may be considered when treatment failure is observed or when bait consumption is limited.

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LEGENDS OF FIGURES

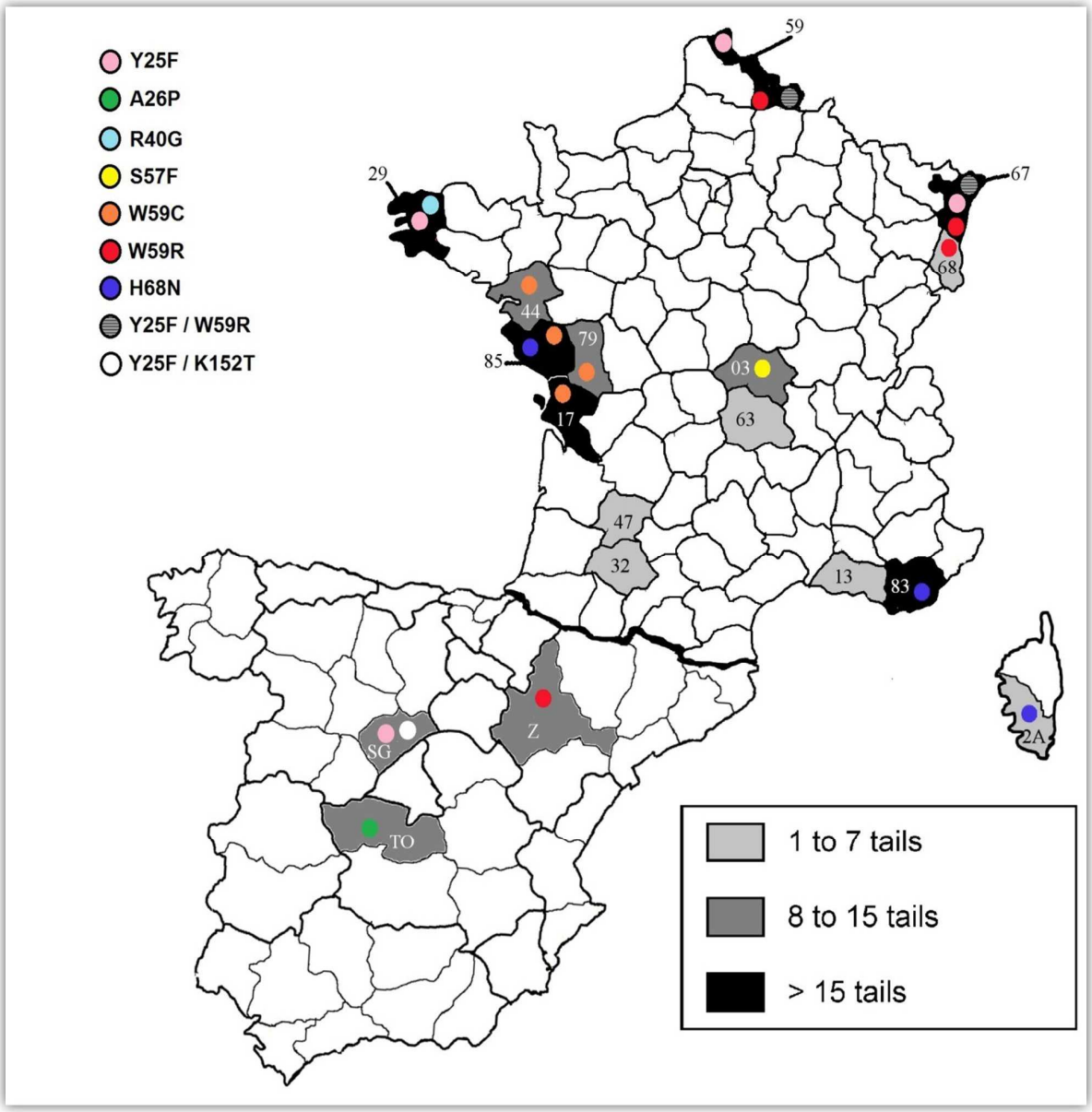
Figure 1: Map of France and Spain showing the SNPs.

Areas in white are those from where no sample was received. Numbers and letters correspond to the French and Spanish (respectively) administrative area and are correlated with Table 3.

Figure 2: Analysis of the expression of wild type or mutated rrVKORC1 proteins in yeast microsomes by Western blot.

Membrane proteins containing VKORC1-W59R (lane 1, 15 µg), VKORC1-W59C (lane 2, 15 µg), VKORC1-A26P (lane 3, 15 µg), VKORC1-Y25F/K152T (lane 4, 15 µg), VKORC1-K152T (lane 5, 15 µg), VKORC1-Y25F (lane 6, 15 µg), VKORC1-R40G (lane 7, 15 µg), VKORC1-H68N (lane 8, 15 µg), VKORC1-S57F (lane 9, 15 µg) and wild type VKORC1 (lane 10, 15 µg) were probed with primary antibody directed against the c-myc tag.

Figure 3: Inhibition effect of various anticoagulants on mutated rrVKORC1 expressed in yeast microsomes, comparatively to the *R. rattus* $VKORC1^{WT}$. Dotted lines indicate resistance factors for the mutated VKORC1, of 1, 5 and 10.


Y25F
A26P
R40G
S57F
W59C
W59R
H68N
Y25F / W59R
Y25F / K152T
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2A
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SG
TO
1 to 7 tails
8 to 15 tails
> 15 tails

Figure 1

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530 **Figure 2**

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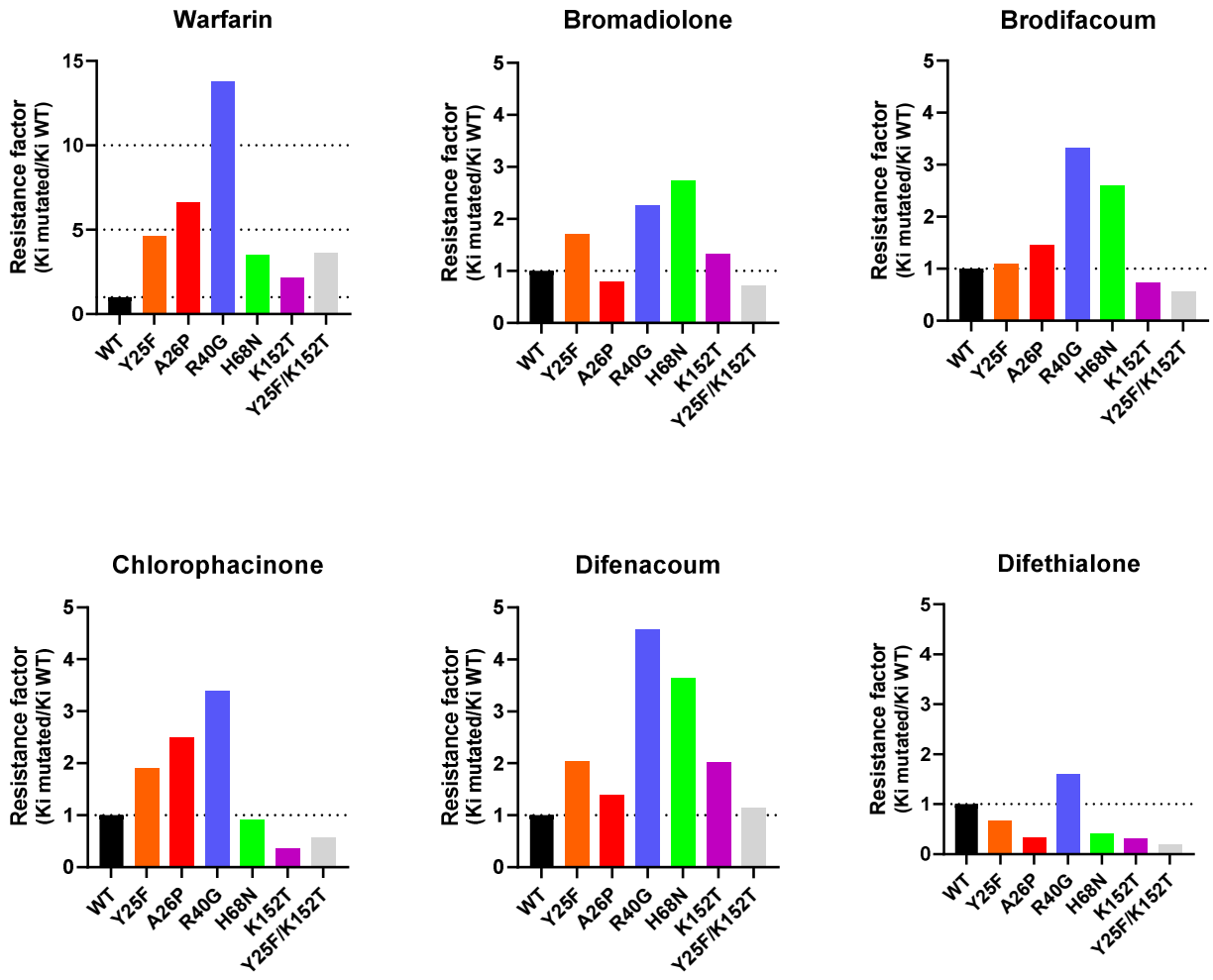

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533 **Figure 3**

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538 **Table 1: Detail of Vkorc1 mutations in Rattus rattus in France and Spain**

Country	Mutation	Position	Codon wild type	Codon mutated	Amino acid wild type	Amino acid mutated
France	Y25F	25	TAC	TTC	Tyr	Phe
	R40G	40	CGC	GGC	Arg	Gly
	S57F	57	TCT	TTT	Ser	Phe
	W59C	59	TGG	TGT	Trp	Cys
	W59R	59	TGG	AGG	Trp	Arg
	H68N	68	CAT	AAT	His	Asn
	Y25F / W59R	25 and 59	TAC and TGG	TTC and AGG	Tyr and Trp	Phe and Arg
Spain	Y25F	25	TAC	TTC	Tyr	Phe
	A26P	26	GCA	CCA	Ala	Pro
	W59R	59	TGG	AGG	Trp	Arg
	Y25F / K152T	25 and 152	TAC and AAG	TTC and ACG	Tyr and Lys	Phe and Thr

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Table 2: Detailed locations and frequencies of *R. rattus Vkorc1* mutations in France (FR) and Spain (SP)

	Area	No. of samples	Mutation	No. of mutated	% mutation/area	No. of HomoZ	No. of HeteroZ
FRANCE	2A	9	H68N	1	11	0	1
	3	5	S57F	2	40	2	0
	13	5	-	0	0	-	-
	17	20	W59C	4	20	3	1
	29	18	Y25F	1	72	0	1
			R40G	12		12	0
	32	4	-	0	0	-	-
	44	13	W59C	13	100	13	0
	47	4	-	0	0	-	-
	59	14	Y25F	5	93	2	3
			W59R	6		6	0
			Y25F / W59R	2		Y25F HeteroZ / W59R HeteroZ Y25F HeteroZ / W59R HomoZ	
	63	1	-	0	0	-	-
	67	37	Y25F	3	97	0	3
			W59R	31		31	0
			Y25F / W59R	2		Y25F HeteroZ / W59R HomoZ	
	68	3	W59R	3	100	3	0
	79	12	W59C	10	83	10	0
	83	20	H68N	1	5	0	1
	85	16	W59C	13	87	13	0
			H68N	1		0	1
SPAIN	To	15	A26P	15	100	15	0
	Z	12	W59R	12	100	12	0
	Sg	11	Y25F	9	100	9	0
			Y25F / K152T	2		Y25F HomoZ / K152T HeteroZ	
Total							
FRANCE		181		110	61		
SPAIN		38		38	100		

Each area corresponds to a French or a Spanish administrative area drawn on the map (Fig 4).

HomoZ: homozygous; HeteroZ: heterozygous.

553 **Table 3: Apparent kinetic parameters towards vit K1>O obtained for yeast microsomes**
554 **expressing wild type or mutated VKORC1**

	Km (µM)
VKORC1 *R.rattus* WT	*63.6* ± 15.3
VKORC1*A26P	82.0 ± 18.1
VKORC1*R40G	31.8 ± 18.5
VKORC1*K152T	58.2 ± 9.5
VKORC1*Y25F/K152T	35.7 ± 17.7
VKORC1*H68N	37.0 ± 22.5
VKORC1*Y25F	18.7 ± 5.7
VKORC1*S57F	VKOR activity < 2% of WT
VKORC1*W59R	VKOR activity < 2% of WT
VKORC1*W59C	VKOR activity < 2% of WT
VKORC1*Y25F/W59R	VKOR activity < 2% of WT

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556 To determine the VKOR activity, standard reactions were performed in 200 mM Hepes buffer
557 (pH 7.4) containing 150 mM KCl and 0.25 to 2 $g.l^{-1}$ of microsomal proteins expressing
558 membrane wild type or mutant VKORC1. Each data point represents the mean ± SD of three
559 individual determinations.

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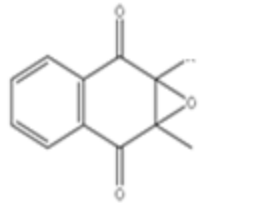
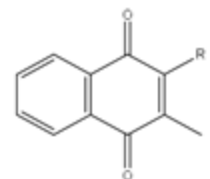


Vitamin K epoxide (K>O)

Anticoagulant rodenticides ⊣ VKORC1



Vitamin K quinone (K)

Roof rat (*Rattus rattus*)

- Y25F
- A26P
- R40G
- S57F
- W59C
- W59R
- H68N
- Y25F / W59R
- Y25F / K152T