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

3

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21 **Abstract**

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

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

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

40

41 **Keywords**

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

44

45 **Abbreviations**

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

48 **1. Introduction**

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

56 To manage these populations of rodents, chemical controls have been organized since 1950
57 by using anticoagulant rodenticides (ARs), the only effective molecules. Thus, their delayed
58 action, with death occurring 3–7 days after bait consumption, obviates the alimentary aversion
59 problem, which is a very important behavioral trait among rodents. Indeed, ARs, by inhibiting
60 the VKORC1-dependent vitamin K epoxide reductase enzyme,^{3,4} lead to the progressive
61 reduction of the pool of vitamin K necessary for the activation of clotting factors II, VII, IX,
62 and X.^{5,6} Therefore, prolonged or repeated exposure to ARs lead to the death of rodents by
63 hemorrhage. Unfortunately, quickly after the first use of ARs, resistant rodents are described
64 in Europe^{7,8} and in the United States.⁹ Later, it was also described everywhere in the world,
65 such as Canada,¹⁰ Australia¹¹ and Japan¹². The emergence of such resistance to anticoagulants
66 belonging to the first generation (*i.e.*, warfarin, diphacinone, coumatetralyl, chlorophacinone)
67 led to the development of new Ars belonging to the second generation (*i.e.*, bromadiolone,
68 difenacoum, flocoumafen, brodifacoum and difethialone) in the 1970's and 1980's. Two main
69 resistance mechanisms have since been described. The first, metabolic mechanism due to an
70 overexpression of cytochrome P-450 3A, is essentially described in Japan.^{12,13} The second, the
71 most common resistance mechanism, is a result of single-nucleotide polymorphisms (SNPs)
72 in the *Vkorc1* gene, leading to an enzyme that is less sensitive to the action of Ars.^{3,4} This
73 mechanism of resistance has been extensively studied in Europe and around the world in
74 brown rats,^{14–18} house mice^{17–20} and humans.²¹ It is thus described to be highly prevalent in
75 brown rats and mice. However, this mechanism has been less studied in the roof rat,
76 especially in Europe where this rodent specie is considered to have disappeared. Only few
77 studies described mutations in the *Vkorc1* gene in the roof rat in Europe^{17,22} or worldwide.^{23–25}
78 In this paper, we report the different mutations of *Rattus rattus Vkorc1* gene observed in
79 different parts of France and Spain. Using recombinant VKORC1 protein, we thus analyzed
80 the catalytic consequences of all the different mutations described to date in roof rat in order

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

84

85 **2. Materials and Methods**

86 **2.1. Materials**

87 Vitamin K1 (Phylloquinone-2-methyl-3-[(E,7R,11R)-3,7,11,15-tetramethylhexadec-2-
88 enyl]naphthalene-1,4-dione) was converted to vitamin K₂ according to Tishler et al. [12].
89 Purity was estimated by LC/MS and was higher than 99%. Sodium warfarin (3-(α -
90 Acetylbenzyl)-4-hydroxycoumarin sodium salt, 4-Hydroxy-3-(3-oxo-1-
91 phenylbutyl)coumarin), difenacoum 3-(3-Biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-
92 hydroxycoumarin, 3-(3-Biphenyl-4-yl-1,2,3,4-tetrahydro-naphthalen-1-yl)-4-
93 hydroxychromen-2-one) and brodifacoum (3-(3-(4'-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro-
94 1-naphthyl)-4-hydroxycoumarin) were purchased from Sigma–Aldrich (Saint Quentin
95 Fallavier, France) with a purity higher than 98% (Figure 1). Chlorophacinone (2-[(4-
96 chlorophenyl)phenylacetyl]-1H-indene-1,3(2H)-dione), bromadiolone (3'[3-(4'-
97 bromobiphenyl-4-yl)-3-hydroxy-1-phenylpropyl]-4-hydroxycoumarin) and difethialone (3'[3-
98 (4'-bro'o[1,1'-biphenyl]-4-yl)-1,2,3,4-tetrahydro-1-naphthalenyl]-4-hydroxy-2H-1-
99 benzothiopyran-2-one) were supplied by Liphatech (Pont de CFranceFrance) with a purity
100 higher than 98% (Figure 1). Methanol HPLC grade, and acetic acid (analysis grade) were
101 obtained from Merck (Germany).

102

103 **2.2. Rats tissue sampling** *Rattus rattus* were collected from the national network of pest
104 control operators (PCOs) in 15 out of 95 departments (French administrative areas) covering
105 all the country and in 3 out of 47 Spanish administrative areas. This study was not considered
106 to be an “experimental procedure” as defined by the French legislation (Rural Code, Article
107 R214–89) and was therefore not subject to an ethical committee approval. This study
108 complied with the ethical standards of European regulations governing the care and use of
109 animals in research (Directive 2010/63/EU) and it did not involve any endangered or
110 protected species, or protected areas.

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

118

119 **2.3. Species determination**

120 Two microliters of genomic DNA extract were amplified by PCR using specific primers of
121 *cytochrome b*.²⁶ The sequence of the sense primer cytb-S and the antisense primer cytb-AS
122 were (5'-TCTCCATTTCTGGTTACAAGAC-3') and (5'-
123 AACAAATGACATGAAAAATCATCGTT-3'), respectively. *Cytochrome b* amplification was
124 performed using cytb-S and cytb-AS (10 pmol), GoTaq polymerase (1 unit, Promega) in a 25
125 µl reaction volume containing 2 µl DNA, 5 µl 5X GoTaq buffer and 200 µM of each
126 deoxynucleotide triphosphate. The amplification was performed at 94°C for 3 min followed
127 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
128 for 10 min. The amplified product was sequenced on both strands; the resulting sequence was
129 submitted to blast analysis.

130

131 **2.4. *Vkorc1* sequencing**

132 Two microliters of genomic DNA were amplified using specific primers of *Vkorc1* gene. In
133 order to sequence the totality of the *Vkorc1* gene, two sets of primers were used. The
134 sequences of the first set of primers rVKOR-S1 and rVKOR-AS1 were (5'-
135 GGTTCTTCCCTCTTGTGTCTG-3') and (5'-GGGTCACCAAGACATGAGGTG-3'),
136 respectively, and were used to amplify rat *Vkorc1* gene from nucleotide -36 to nucleotide
137 +1141 (according to *R. rattus frugivorus Vkorc1* – accession n° HM181985). The sequences
138 of the second set of primers rVKORC1-S2 and rVKORC1-AS2 were (5'-
139 ACTTGGGCAAGGCTCATGTG-3') and (5'-AAGAGTAGGGGACAAGGTGGC-3'),
140 respectively, and were used to amplify the rat *Vkorc1* gene from nucleotide +848 to
141 nucleotide +2183. Rat *Vkorc1* amplifications were performed using rVKOR-S1 and rVKOR-
142 AS1 or rVKOR-S2 and rVKOR-AS2 (10 pmol), Accuprime polymerase (1 units, Invitrogen)

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

149

150 **2.5. Heterologous expression of mutated *R. rattus* VKORC1**

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

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

166

167 **2.6. Immunoblot Analysis**

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

170

171 **2.7. VKOR Activity Assays and Kinetics**

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

176 standard reaction. Incubations were performed in duplicate. Data were fitted by nonlinear
177 regression to the Michaelis-Menten model using GraphPad Prism 6. In order to evaluate the
178 inhibiting effect of warfarin on VKOR activity, K_i were determined after addition of various
179 concentrations of anticoagulant to the standard reaction in the presence of increasing amounts
180 of vit K>O (from 0.003 to 0.2 mM) using anticoagulant concentrations from about 0.05 to
181 $20 \times K_i$. Data were fitted by non-linear regression to the non-competitive inhibition model $v =$
182 $(V_{max}/(1+(I/K_i)))*(S/(K_m+S))$ using GraphPad Prism 6.

183

184 **3. RESULTS**

185 **3.1. *Vkorc1* missense mutations in French and Spanish *Rattus rattus***

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

195 Eight different missenses mutations were found in the *Vkorc1* gene in the French and Spanish
196 rats and led to a single mutation in the corresponding VKORC1 protein. (Figure 1 and Table
197 1). In exon 1, 4 mutations were detected. These mutations were located at nucleotide 74
198 (g.74A>T), 76 (g.76G>C), 118 (g.118C>G) and 170 (g.170C>T) and led to mutations Y25F,
199 A26P, R40G and S57F, respectively. In exon 2, 3 missenses mutations were detected at
200 nucleotide 982 (g.982T>A), 984 (g.984G>T) and 1009 (g.1009C>A) and led to mutations
201 W59R, W59C and H68N, respectively. In exon 3, one mutation was detected at nucleotide
202 2109 (g.2109A>C) and led to the mutation K152T. The other genotypes led to *Vkorc1* with
203 two mutations. The g.74A>T mutation (exon 1) was found to be associated with the
204 g.982T>A mutation (exon 2) or the g.2109A>C mutation (exon 3) leading to proteins with
205 two combined mutations, the Y25F and W59R or the Y25F and K152T, respectively.

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

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

210 In Spain, the observed allelic frequencies of A26P, W59R and Y25F were 39.5%, 31.6% and
211 23.7%, respectively (Table 2). The two combined mutations Y25F and K152T were found in
212 Zaragoza (Spain province) only. However, the sampling in Spain is insufficient for giving
213 accurate results and the investigation should be continued.

214

215 **3.2. Functional consequences of Vkorc1 mutations**

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

231

232 **4. Discussion**

233 **4.1. Distribution of roof rats in France**

234 This study demonstrates the presence of roof rats in France while this species was considered
235 to have disappeared on the French territory after the expansion of brown rat in the 18th
236 century. 181 roof rats were trapped from 15 administrative departments over a 2-year period
237 (2014-2015). However, this sampling remains limited, suggesting a low abundance of this
238 species in France. Furthermore, the distribution of the roof rat in France seems very

239 heterogeneous. Indeed, the roof rats were mainly trapped in fluvial or maritime port areas
240 (Brest, Saint-Nazaire, La Rochelle, Dunkirk, Strasbourg) and insular areas (Islands of
241 Marseille) whereas the sampling was planned for the whole territory. This apparent
242 distribution could be a bias due to the sampling method based on participatory research.
243 Indeed, the samples analyzed were supplied by PCOs from rodents trapped on their various
244 control sites. It is nonetheless important to note that many French PCOs representative of all
245 French regions were contacted at the beginning of the study and that the sampling of roof rats
246 was carried out in parallel with the sampling of domestic house mice which allowed the
247 evaluation of the prevalence of mutations in the latter in the previously published study.¹⁷ The
248 PCOs were requested to collect samples from both species (domestic mice and roof rats) at
249 the same time. During the sampling period, 266 samples of mice were collected in 27
250 departments while the 181 samples of roof rats were collected from only 15 departments. It is
251 therefore conceivable that PCOs that have sent in samples of mice but not roof rats, are not
252 dealing with proliferations of roof rats even though they are more difficult to capture than
253 mice due to their excessive neophobia and are often mistaken for brown rats. Those PCOs
254 were in the South-East and the North of France, in and around Paris and in inland of Brittany.
255 Despite these issues, distribution of roof rats observed in France in this study seems to be
256 coherent with the studies published by Buckle¹ describing the presence of roof rat mainly in
257 port areas in different countries (such as Iraq and Turkey). The use of containers on modern
258 ships could favor the dispersion of roof rats from ports to ports and their presence in ports.

259

260 **4.2. Diversity of *Vkorc1* mutations in roof rats in France and Spain**

261 In Europe, roof rat populations are managed as the brown rat populations by using baits
262 containing anticoagulant rodenticides. This intensive and long-standing use of ARs (ARs have
263 been used since the 1950s) has led to a wide selection of resistant brown rat populations with
264 predominant mutations, the Y139F mutation in France, the Y139C mutation in the East of
265 Europe, especially Germany, the L120Q mutation in England, all leading to strong resistance
266 to first and to some second generation ARs. For roof rats, very few studies are available in
267 Europe, while *Vkorc1* mutations have been described in Japan,²³ New Zealand²⁴,
268 Argentina^{17,22}, and USA¹⁸. Information about resistance are crucial to manage correctly roof
269 rat populations. Indeed, it is essential to use the right molecules to reach an optimal efficacy,
270 to avoid selection of resistant populations and to avoid secondary poisoning of wildlife

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

273 Herein, we detected 9 *Vkorc1* genotypes in *Rattus rattus* in France and Spain: 7 single
274 mutations (Y25F, A26P, R40G, S57F, W59C, W59R and H68N) and 2 double mutations
275 (Y25F/K152T and Y25F/W59R). Among these genotypes, 3 single missense mutations and 2
276 double mutations have never been described: S57F, W59C, A26P, K152T, Y25F/K152T and
277 Y25F/W59R. Y25F mutation was already described in Spain²⁹, New Zealand²⁴ and USA¹⁸
278 and shown to lead to moderate resistance to first generation ARs and limited resistance to
279 some second generation ARs (*i.e.*, bromadiolone and difenacoum)²⁹. The R40G mutation was
280 already detected in Japan, the H68N in Martinique Island and the W59R in Argentina¹⁷ and
281 Germany¹.

282

283 **4.3. Functional consequences of *Vkorc1* mutations**

284 The functional consequences of mutations or double mutations detected in this study were
285 characterized after expression of the recombinant enzyme in *Pichia pastoris*. This system has
286 already allowed to successfully characterize mutations in brown rats¹⁴ and mice¹⁹ with results
287 consistent with *in vivo* results. This system has also previously allowed comparison of
288 sensitivities to ARs in roof rats and brown rats. Based on the K_i and considering only the
289 properties of the VKORC1 enzyme, the sensitivity of roof rats to ARs is similar to that of
290 brown rats²⁹. Nevertheless, other factors may cause differences in response, such as
291 metabolism of ARs, but also behavior and diet. No information is available on the ability of
292 roof rats to metabolize ARs. However, its diet and behavior are different from those of brown
293 rats and are certainly responsible for a lower ingestion of baits.

294 This system allowed in this study to obtain a resistance factor for each mutant and towards
295 each AR by calculating the ratio between inhibition constants of the mutated VKORC1 and
296 the wild-type VKORC1. Among the identified mutations, the R40G mutation appears to
297 induce *in vitro* the highest resistance factors with factors ranging from 2 to 15 depending on
298 the AR. If we compare these factors with those obtained for the most frequently encountered
299 mutations in brown rats (for Y139F, Y139C, L120Q, resistance factors are higher than 100),
300 these factors appear rather low, suggesting that rats carrying such mutations remain sensitive
301 to ARs in the field. Besides, previous *in vivo* laboratory tests have tended to lead to this same
302 conclusion²³. Nevertheless, considering that roof rats consume little or no bait in the field due
303 to neophobia and food competition, such factors could lead to management issues. Similarly,
304 but to a lesser extent, the H68N mutation appears to induce a slight resistance to ARs

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

310 The other mutations (*i.e.*, W59R, S57F and W59C) seem to lead to a drastic loss of VKOR
311 activity. When activity is lower than 2% of the VKOR activity of the wild-type enzyme, our
312 method is not sensitive enough to be able to further characterize the VKOR activity. This
313 result is surprising because some of these mutations are found in the homozygous state and an
314 inactivation or severe loss of activity of the enzyme questions the recycling of vitamin K in
315 individuals carrying such mutations in the homozygous state. Is a compensatory mechanism
316 present? This result was acquired after characterization of the activity in the presence of
317 dithiotreitol. The presence of such chemical reductant presence in the assay has been
318 proposed as a potential disturbance to the measurement of activity. Nevertheless, considering
319 the knowledge acquired on this reductant, this reducing agent would rather tend to activate an
320 inactive VKORC1 than inhibit it. Indeed, this reducing agent could possibly directly access
321 the catalytic site of the enzyme to activate it directly (*i.e.*, C132XXC135), whereas the normal
322 functioning of the enzyme involves an electron transfer from the luminal loop via the C43 and
323 C51 present in the luminal loop to the C132 and C135 of the catalytic site present in the
324 fourth transmembrane domain. The S57F, W59F and W59R mutations are present in the
325 luminal loop responsible for activation of the catalytic site. It is conceivable that such
326 mutations could disrupt the folding of the luminal loop preventing activation of the catalytic
327 site by the latter (the enzyme would then be really inactive) or possibly that their presence
328 prevents the activation of this loop by dithiotreitol (this loss of activity would then be a bias
329 due to the method). Another indirect method, the cell-based assay has recently been
330 developed and would allow to elucidate this loss of activity.³⁰ Answering this question is
331 crucial since some of these mutations have been detected in the homozygous state, raising the
332 question of the viability of these rats. Indeed, the KO of the VKORC1 enzyme is lethal by
333 hemorrhage³¹ due to the lack of an alternative system to recycle the vitamin K necessary for
334 the activation of coagulation factors II, VII, IX and X. Roof rats carrying inactivated
335 mutations in the homozygous state would then have an alternative system, possibly an
336 overexpression of VKORC1L1 in the liver.³² Nevertheless, this alternative mechanism could
337 induce possible resistance to ARs. Indeed, the W59R mutation was detected in a German
338 village where 89% of the animals carried this mutation in the homozygous state. This

339 population was complex to manage with either bromadiolone or difenacoum.¹ But at the same
340 time the authors reported a low consumption of bait which could alone explain this difficulty
341 of treatment. Further studies are necessary to elucidate the case of the ‘inactivating’
342 mutations.

343

344 **4.4. Consequences in term of rodent management**

345 While in brown rats, 3 mutations associated with severe resistance to first-generation ARs or
346 even some second-generation ARs are predominant and found at very high allelic frequencies
347 in Europe^{16,19,28,33–35}, mutations in the *Vkorc1* gene found in roof rats in France and Spain are
348 more diverse, seem to reach lower frequencies if considered on a broad scale (of the order of
349 10% or less than 10% for mutations with significant activity) and induce little or no
350 resistance. That is surprising while the management of the roof rat has been done in the same
351 way as that of the brown rat. In the brown rat, management has resulted in a major selection
352 of the most resistant populations. In the roof rat, it is possible that this selection was never
353 intense due to low bait consumption as described by Buckle.¹ Indeed, the baits marketed are
354 baits developed mainly for the management of brown rats. However, the diet of these two
355 species is different: the roof rat is less omnivorous than the brown rat,³⁶ and animal food is
356 less significant to it. The roof rat preferred fruits, seeds and grain (51–59% of its diet).
357 Moreover, roof rats prefer the food they are familiar with. More adapted baits could increase
358 the success of the roof rat management. Nevertheless, the obligatory use of bait boxes in
359 Europe could aggravate this non or low consumption and therefore this difficulty to manage
360 because the roof rat is very reluctant to enter in these boxes. A second hypothesis to the non-
361 selection of resistant VKORC1 mutation is the absence at the beginning of the use of ARs, of
362 rare mutations conferring resistance to ARs. So, there was nothing to select. A third
363 hypothesis is the presence of other resistance mechanisms in roof rats not supported by
364 VKORC1 mutations. Metabolic resistance is conceivable as it has already been described in
365 Japan for this species.^{37,38} Diet-based resistance is also possible, as described for voles³⁹.
366 Further studies will be necessary to evaluate the distribution of such resistance in roof rats in
367 Europe.

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

373

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503 **LEGENDS OF FIGURES**

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

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

507

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

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

515

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

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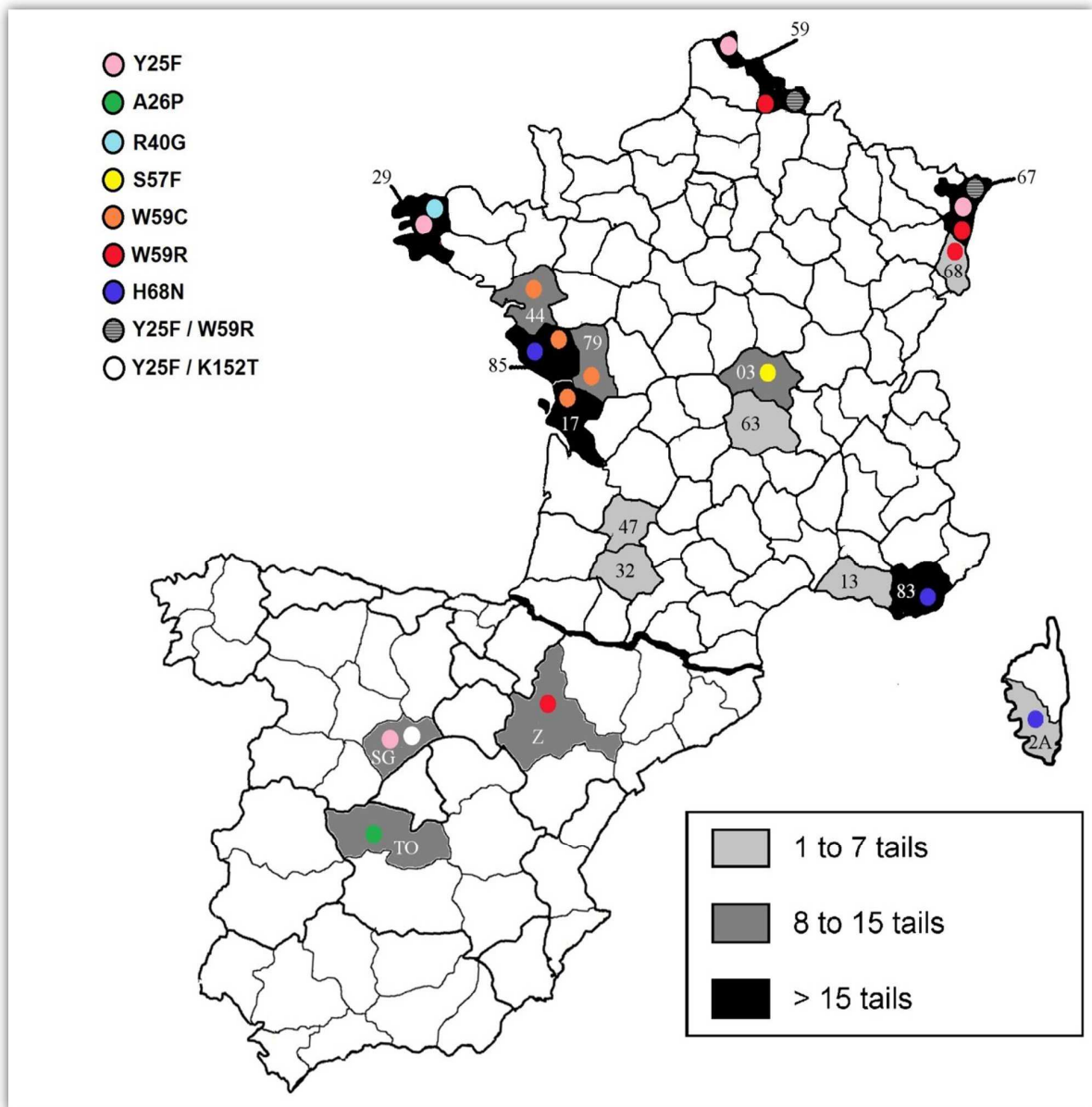
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Figure 1

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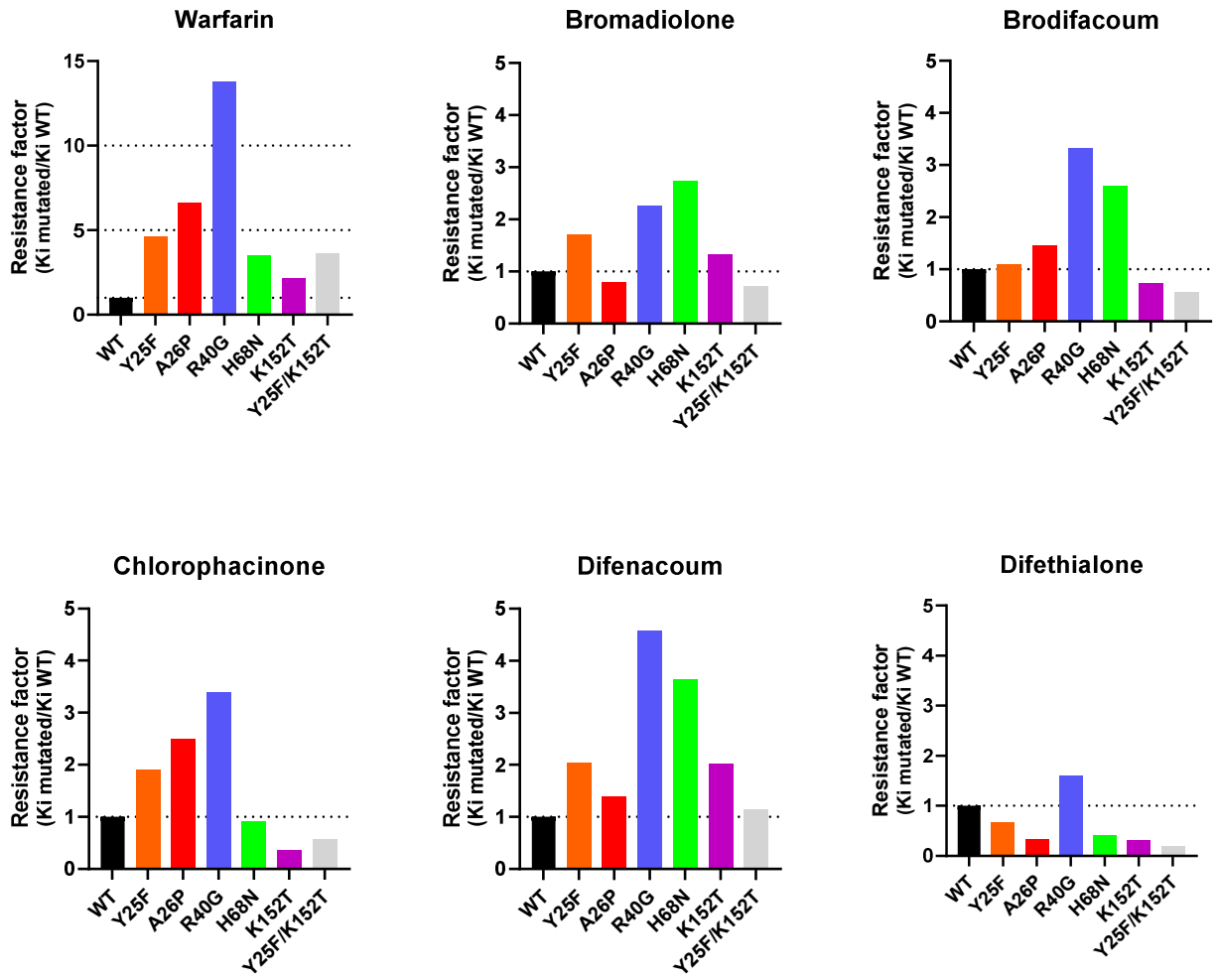
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Figure 2

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Figure 3

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

Area	No. of samples	Mutation	No. of mutated	% mutation/area	No. of HomoZ	No. of HeteroZ	
FRANCE	2A	H68N	1	11	0	1	
	3	S57F	2	40	2	0	
	13	-	0	0	-	-	
	17	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	A26P	15	100	15	0	
	Z	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			

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

551 HomoZ: homozygous; HeteroZ: heterozygous.

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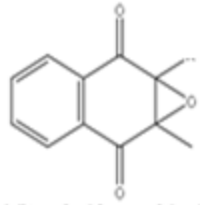
553 **Table 3: Apparent kinetic parameters towards vit K1>O obtained for yeast microsomes**
 554 **expressing wild type or mutated VKORC1**

	K_m (μM)
VKORC1 <i>R.rattus</i> 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.

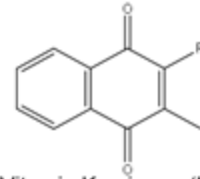
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Vitamin K epoxide (K>O)

Anticoagulant
rodenticides

VKORC1



Vitamin K quinone (K)



Roof rat (*Rattus rattus*)

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

