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MUS MUSCULUS DOMESTICUS CARRYING VKORC1^{SPR} AND SUSCEPTIBILITY TO RODENTICIDES

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Abstract This paper describes the control of a mice infestation of a bakery in Lyon (France). Where other anticoagulant molecules used for the treatment of the situation failed, the use of difethialone grain showed a great efficacy in the control of this population. Live mice captured on the site where genetically tested and appeared to be *M. musculus domesticus* species with a *M. spretus Vkorc1* gene naturally introgressed. Susceptibility to AVKs of VKORC1^{spr} was *in vitro* evaluated by expressing recombinant enzymes in yeasts. The use of this *in vitro* yeast system is of great interest for monitoring the anticoagulant activity of VKORC1 in AVK resistant rodents and for the development and the evaluation of new anticoagulants molecules.
Key words Anticoagulant rodenticides, vitamin K.

INTRODUCTION

Rodent population management (rats and mice) should be based on different rules such as hygiene, natural behavior, environment structure… but also implies to use chemicals. Anti Vitamin K anticoagulants (AVKs) have been, for more than 50 years in Western Europe, the principal molecules used. Rodent populations adaptability and natural selection pressure involved animal's natural adaptation, and the development of resistance strategies, making these molecules less and less efficient. Among resistance mechanisms, the one called target resistance is the most frequent. The AVKs are inhibitors of the Vitamin K cycle by inhibiting the mechanism of recycling Vitamin K through their action on the Vitamin K epoxyde reductase.The gene responsible for this enzyme synthesis, *Vkorc1*, and the corresponding protein were described in 2004 (Rost et al.. 2004). AVKs are strong inhibitors of the enzyme activity of VKORC1 protein and as a consequence of the Vitamin K cycle. Inhibiting this cycle that ensure the production of clotting factors (IX, X, VII and II) involves lethal bleeding.

Mutations of this gene were found on resistant rodents (Pelz et al. 2005), but the *in vivo* proof of a *Vkorc1* mutation for the resistant phenotype was demonstrated by introgressing the Y139F mutation in wild strain of rodents (Grandemange et al 2009) which led to rodents with a resistant phenotype. Different mutations of this gene were reported on mice (Pelz et al.. 2012; Pelz et al., 2005; Rost et al., 2009). Three mutations or mutation groups seems to be particularly frequent: L128S, Y139C and a set of 3 or 4 mutations Arg12Trp / Ala26Ser / Ala48Thr and / or Arg61Leu. This set of 3 or 4 mutations seems to be the consequence of the adaptive introgression of *Vkorc1* gene from *Mus spretus* into the genome of *Mus musculus* (Song et al., 2011). An initial crossing between these two mice species might have occurred in their cohabitation area (North Africa or Spain). Descendants should have mated with *Mus musculus* and acquired the *Vkorc1* gene of the *Mus spretus* strain (*Vkorc1*^{spr}). These mice (*Mus*

musculus with *Vkorc1*^{spr}) were found in Spain and Germany, but not in Italy or Greece. Concerning France that is half way between Spain and Germany, no information is available concerning the existence of this genotype.

This study describes the presence of such this type of mice in the Lyon (France) area. An efficacy field trial of difethialone on a site where only homozygote animals were found was set up. It was interesting to describe more in details the resistance induced by this genotype by expressing the recombinant VKORC1^{spr} in yeasts and analyzing this specific protein catalytic properties toward the action of AVKs used for mice pest control.

MATERIALS AND METHODS

Sequencing of *Vkorc1* and *Cyp b* Gene

Mice were trapped from various sites located in Lyon (France) or around Lyon. Genomic DNA was extracted from mice tails using Nucleospin Tissue kit (Macherey Nagel) according to the manufacturer's recommendations. *Vkorc1* gene was amplified by PCR using two specific primers sets: F1 $^{5'}$GATTCTTCCCTCCTGTCC$^{3'}$ and R1727 $^{5'}$AGACCCTGTCTCAAAACCTA$^{3'}$, and F1252 $^{5'}$GAAAGCAGAACACTTAGCAGG$^{3'}$ and R2512 $^{5'}$AACCAACAGCAGAATGCAGCC$^{3'}$.

PCR products were sequenced (Biofidal, Vaulx en Velin, France). *Cyt b* gene was amplified by PCR using specific forward $^{5'}$TCTCCATTTCTGGTTTACAAGAC$^{3'}$ and reverse $^{5'}$AACAATGACATGAAAAATCATC GTT$^{3'}$ primers.

In vitro Characterization of Resistance

Recombinant VKORC1 enzymes were expressed in *Pichia pastoris* as described previously by Hodroge et al. (2011, 2012). Microsomal fractions containing recombinant hVKORC1 proteins prepared as described previously (Hodroge et al., 2011, 2012) were used to determine the susceptibility of VKORC1 proteins to rodenticides. VKOR activity and reaction products analysis by liquid chromatography-mass spectrometry were performed as reported (Hodroge et al., 2011, 2012).

Figure 1. Live mice trapping localization in the neighborhood of Lyon

Field Trial

The field trial was performed in a bakery invaded by mice which is localized in the sixth district of Lyon. Thirty baiting boxes containing wheat (with difethialone 25 ppm) were distributed in the bakery. Consumption was evaluated every 3 days and dead mice were collected for genetic analysis. The baiting boxes were refilled every 3 days until the consumption was null. The field trial lasted 23 days.

RESULTS AND DISCUSSION

Trapping of Mice

Alive and dead mice were caught in Lyon and around (Fig. 1). In Lyon, 12 mice were captured in the "Parc de la Tête d'Or" and 18 mice were captured from the above mentioned bakery of the sixth Lyon's district. In the Lyon neighborhood (i.e. in a 50 km radius from downtown Lyon), 9 mice were trapped in Savigny (in 40 km on the West of Lyon); 5 mice were trapped in Saint-Cyr-le-Chatoux (in 50 km on the northwest of Lyon); 6 mice were trapped in Pontcharra-sur-Turdine (in 40 km on the northwest of Lyon).

Characterization of the Species

It's quite impossible to precisely determine the mice species on the basis of morphological analysis. Although the Algerian mouse *Mus spretus* known to be endemic in the South of France is considered as a small mouse with a short tail, it is very easy to confuse an Algerian mouse with a house mouse *M musculus domesticus*. In order to determine the species of the mice trapped in Lyon area, *Cyt b* gene was amplified by PCR from the mice tail-extracted genomic DNA and sequenced. *Cyt b* mitochondrial DNA gene is frequently used for phylogenetic analysis of organisms. The comparison of the sequences of the PCR products with databases allowed us to conclude that all the 49 mice trapped in and around Lyon were all mice belonging to the sub-specie *M. musculus domesticus*. While numerous pest control operators reported the presence in Lyon of small mice, the result obtained herein suggests that in spite of the global warming the Algerian mouse *Mus spretus* distribution remains limited to the South of France.

Sequencing of *Vkorc1* Gene

In order to detect a possible resistance to rodenticides due to VKORC1 mutations, the *Vkorc1* gene was amplified by PCR from the mice tail-extracted genomic DNA and sequenced. Mice captured in Pontcharra showed the previously described L128S and Y139C mutations (Pelz et al., 2005; Rost et al., 2009). The sequence analysis of all three coding exons from mice captured in Lyon ("Parc de la Tête d'Or" and Bakery), Savigny and Saint-Cyr-le-Chatoux, revealed the presence of mutations in five nucleotides positions as compared to the published *M. musculus domesticus Vkorc1* gene. Four mutations are located in exon 1 (C34T, G76T, A111G, G142A) and one is located in exon 2 (G182T). The A111G mutation did not result in amino acid substitution (silent mutation E37E); the 3 others led to amino acid substitutions. Arginine-12 was found to be substituted by tryptophane (Arg12Trp), alanine-26 by serine (Ala26Ser), alanine-48 by threonine (Ala48Thr) and arginine-61 by leucine (Arg61Leu). Out the 44 mice carrying these four mutations, 41 were homozygous for all these mutations and only three, from Saint-Cyr-le-Chatoux, were heterozygous for all these five mutations. These mutations were reported by Pelz et al. (2005) and Rost et al. (2009). Shortly after, Song et al. (2011) described that these four non-synonymous mutations were introduced into the *M. musculus domesticus* genome by an adaptive introgressed hybridization with *M. spretus* (*i.e.*, the naturally occurring process including inter-specific hybridation followed by generations of backcrossing and selection of introgressed alleles). Indeed, *M. spretus* VKORC1 contained tryptophane, serine, threonine and leucine in amino acid positions 12, 26,

48 and 61 respectively. *M. musculus domesticus* carrying the complete or partial *Vkorc1* allele of *M. spretus* (*Vkorc1*^{spr}) were observed in Spain and Germany (Song et al., 2011).

Consequences of the Susceptibility to Rodenticides

To evaluate the consequences of the introduction of the four mutations Arg12Trp, Ala26Ser, Ala48Thr and Arg61leu into the *M. musculus domesticus Vkorc1* gene on the susceptibility to rodenticides, we expressed recombinant wild type VKORC1^{dom} and VKORC1^{spr} enzymes as a c-myc fused recombinant protein in *P. pastoris* and determined for both enzymes the inhibition constants (Ki) towards various rodenticide molecules. Whatever the enzyme we analyzed, all the molecules inhibited the VKOR activity in a non-competitive manner (data not shown). K_i values obtained for VKORC1^{dom} and VKORC1^{spr} enzymes are reported in Table 1. For VKORC1^{spr}, K_i values obtained towards the first generation rodenticides (i.e., warfarin, coumatetralyl, chlorophacinone) used in this study were dramatically increased (~80 to 140-fold) compared to those obtained for VKORC1^{dom}. Towards bromadiolone and the second generation molecules (i.e., difenacoum, difethialone), K_i obtained for VKORC1^{spr} were also increased compared to VKORC1^{dom}. Nevertheless this increase was more moderated compared to the first generation molecules. Taking together, these results suggest that mice carrying *Vkorc1*^{spr} gene must be more resistant to rodenticides than mice carrying *Vkorc1*^{dom}. The use of second generation molecules must be preferred in the presence of mice populations carrying *Vkorc1*^{spr}. This is coherent with the results obtained by Song et al. (2011). Indeed, they demonstrated that 80%, 91% and 20% of *M. musculus domesticus* carrying *Vkorc1*^{spr} survived coumatetralyl, bromadiolone and difenacoum trials, respectively.

Table 1: Ki values towards various rodenticides for VKORC1^{dom} and VKORC1^{spr} and resistance factor to rodenticides of VKORC1^{spr} comparatively to VKORC1^{dom}

	K*i* (µM) VKORC1^{dom}	**K*i* (µM) VKORC1^{spr}**	**Resistance factor**
Warfarin	1.1	97	88
Coumatetralyl	0.14	20	137
Chlorophacinone	0.16	12	73
Bromadiolone	0.15	1.4	7
Difenacoum	0.17	0.8	5
Difethialone	0.05	0.2	5

Field Trial

In order to assess the efficiency of second generation molecules, especially difethialone for the control of mice populations, we performed a field trial on the mice infested bakery located in Lyon.

Reocurring problems of mice infestation were observed in this bakery for several years, with numerous unsuccessful attempts to control them. Infestation signs where important at the beginning of the treatment (droppings, stains, urine smell, dead bodies found…). Control attempts where there, in particular baiting box and old baits contained brodifacoum (blocks, wrapped soft baits, injected soft bait).

About 30 bait boxes with difethialon wheat tech (25 ppm) where positioned in the bakery and its basements. The total consumption rose until 885g / 5 day after only 5 days of baiting. Consumption started to decrease until nothing after 23 days (see Fig 2). All the dead mice found during this baiting period where collected to run analysis and sequencing of *Vkorc1*. All the mice found were homozygous

for *Vkorc1*^{spr}. These field results show that the second generation rodenticide Difethialone remains, inspite of resistance due to *Vkorc1*^{spr}, efficient to control *Vkorc1*^{spr} mice populations.

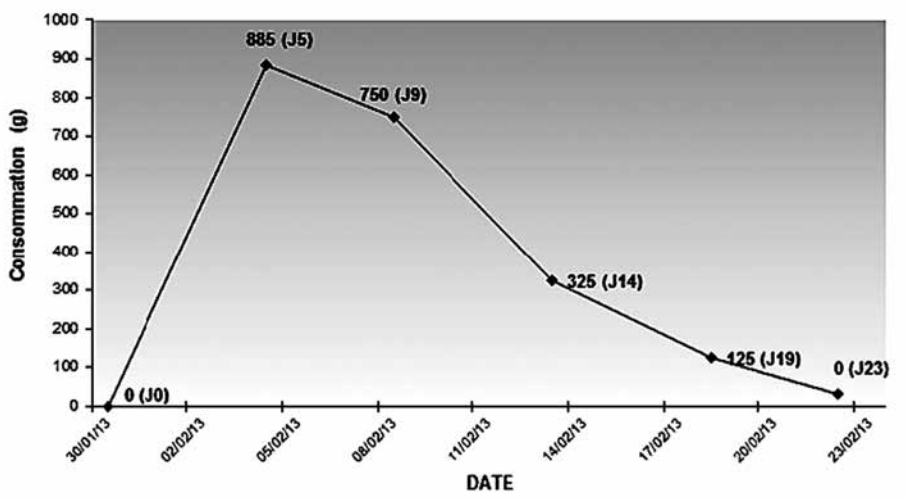

Figure 2. Daily Difethialon Wheat Tech consumption in the Lyon bakery

CONCLUSION

The present study highlighted that despite *Mus spretus* were not found in Lyon (France), evidence of natural gene introgression of *Vkorc1*^{spr} into *Mus musculus* was found, due to natural cross breeding in natural conditions. The *in vitro* analysis of VKORC1^{spr} involves a more important resistance factor to Antivitamin K anticoagulants, especially for first generation anticoagulants, but also for second generation anticoagulants. Despite this resistance factor, field trials showed that the control is possible with difethialone, when correctly applied in the right conditions. The next step will be to confirm the *in vitro* results of K*i* values with live mice from the lab and *in vivo* results. That way all the anticoagulants will be screened and better control program will be issued.

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