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EFFICACY OF ANTICOAGULANT DRUGS AS RODENTICIDES AND GENETIC VARIATION ON *Vkorc1* OF *Mus musculus* FROM BUENOS AIRES PROVINCE (ARGENTINA)

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ABSTRACT

The relationship between anticoagulant rodenticides and the gene for vitamin K epoxide reductase has been extensively studied The significance of mutations in *Vkorc1* on the degree of response to anticoagulants is controversial. The implication of the VKORC1 function in pest control drives the attention to a wide range of factors that may be influencing the resistance to rodenticides, an issue that will be discussed in this article. Anticoagulants are used as rodenticides as well as to improve human health. The interactions between *VKORC1* and *CYP4F18* (cytochrome P450) are complex. It is likely that the coagulation response may be due to a diverse expression of the *Vkorc1* gene and its interaction with the expression of *Cyp4f18*, and to the polymorphisms present in both genes. We analyzed the presence of polymorphisms (especially the presence of mutations that produce substitutions of Tyr139Cys and Leu128Ser) in *Vkorc1* of the genome of *Mus musculus* belonging to local wild populations living in farms. The animals studied were from areas in which rodenticide anticoagulants (ej. bromadiolone) are commonly used as pest control. None of the studied mice showed any signs of anticoagulant resistance-related mutations in the *Vkorc1* gene. This study will enable a proper selection of the rodenticide method for pest control in local areas.

Key words: Vitamin K epoxide reductase, rodenticides, rodents.

RESUMEN

La relación entre los rodenticidas anticoagulantes y el gen de la vitamina K epóxido reductasa se ha estudiado ampliamente. La influencia e importancia de las mutaciones en *Vkorc1* sobre la respuesta a los anticoagulantes es un tema polémico. La importancia de la función para el control de plagas de *Vkorc1* conduce a centrar la atención en una amplia gama de factores que pueden influir en la resistencia a los rodenticidas. En este trabajo discutiremos este tema. Los anticoagulantes se utilizan tanto como rodenticidas como para mejorar la salud en humanos. Las interacciones entre *VKORC1* y *CYP4F18* (citocromo P450) son complejas. Es probable que la respuesta de coagulación se deba a una expresión diversa del gen *Vkorc1* y su interacción con la expresión de *Cyp4f18* y a los polimorfismos presentes en ambos genes: En este trabajo, se analizó la presencia de polimorfismos (especialmente la presencia de mutaciones que producen sustituciones de Tyr139Cys y Leu128Ser) en *Vkorc1* del genoma de *Mus musculus* de animales de poblaciones silvestres locales que habitan en granjas. Los animales estudiados provinieron de áreas en las que se utilizan comúnmente rodenticidas anticoagulantes (ej. bromadiolona) como control de plagas. Ninguno de los ratones estudiados presentó las mutaciones descriptas en el gen *Vkorc1* relacionadas con la resistencia a los anticoagulantes. Este estudio permitirá la elección adecuada del método rodenticida para el control de plagas en áreas locales.

Palabras clave: Vitamina K epóxido reductasa, rodenticidas, roedores.

INTRODUCTION

Vitamin K epoxide reductase complex subunit 1 (*VKORC1*) is a gene that codifies a transmembrane protein located in the endoplasmic reticulum, VKORC1. This protein was described in mammals (human, rat, mouse, dog and cow), fruit fly and zebra fish. It has three exons and, in the previously mentioned *taxa*, a similar amino acid sequence. Moreover, *VKORC1* is homologous -and conserved- in *Mus musculus*, humans and other *taxa*.

The *Vkorc1* gene is located in *Mus musculus* chromosome 7 (7F3; 7 61.0 cM). Research on this gene is continuous and active because VKORC is a key protein for the metabolic pathway of vitamin K, for which there are several features that remain unknown.

The amino acids related to resistance to warfarin are Tyr139 and Leu128, because the changes observed in animals with resistance to this anticoagulant are the replacement of tyrosine by cysteine, serine or phenylalanine and substitution of leucine by glutamic acid or serine (Pelz *et al.*, 2005).

The key enzyme responsible for vitamin K metabolism, vitamin K epoxide reductase complex subunit 1 (VKORC1), was identified as the target of anticoagulants used as rodenticides in pest control (warfarin, bromadiolone, and other related compounds) by Li *et al.* (2004) and Rost *et al.* (2004). Inhibition of blood coagulation is the basic principle used as pest control around the world. It was first observed in 1958 that rodents were able to develop resistance to anticoagulant rodenticides (Buckle *et al.*, 1994). After identification of the *Vkorc1* gene and mutations related to warfarin resistance (Rost *et al.*, 2004), it was confirmed that this is a genetic heritable condition and the *Vkorc1* gene is the subject of mutations that confer resistance to anticoagulants (Rost *et al.*, 2009). In rodents, three genomic sequence variants associated with loss of rodenticide efficacy are known. It is important to study the distribution and frequency of *Vkorc1* sequence variants to achieve a better understanding of the mechanism of action of anticoagulants and to improve performance on pest control. The first data about frequency and distribution of resistance-conferring sequence variants of *Vkorc1* in house mice were found in 30 populations in Germany, Switzerland and the Azores (Pelz *et al.*, 2012).

In this study, we analyzed the presence of mutations in *Vkorc1* in wild populations of *Mus musculus*. We performed sequence analysis and mutation screening in genomic DNA by PCR amplification of the three *Vkorc1* gene exons. The three exons of 20 mice revealed high homology with the *Vkorc1* gene. We did not find any significant polymorphism or mutations in any of the three exons.

MATERIALS AND METHODS

The animals studied were collected in the field from wild populations (Buenos Aires province) and they were treated following international rules for animal welfare. We used the recommendations from the Canadian Council of Animal Care Guide (resources on line). The locations of capture were Diego Gaynor (34°16' S; 59°13' W) and Capilla del Señor (34°17' S; 59°06' W), Buenos Aires Province. Animals were trapped with Sherman traps during summer (January and February) and spring (September and October) 2011, using an experimental design similar to the reported by Guidobono *et al.* (2010). Traps were conditioned with a bait of oats and peanut butter and were distributed in the field near poultry farms. Mice falling in traps were mostly adults (20-35 g of body weight). They were classified as *Mus musculus* on the basis of morphological phenotypes and geographic distribution. DNA was extracted from 20 specimens that were taken at random to analyze the presence of mutations in the *Vkorc1* gene by PCR and sequencing. Genomic DNA was obtained from small pieces of hepatic tissue according to standard procedures. Briefly, the tissue was transferred into a polypropylene microfuge tube containing 0.5 ml of DNA buffer and was mechanically disaggregated to obtain a cell suspension. After removal of the supernatant, the cell suspension was incubated overnight at 50-55 °C with gentle shaking in DNA digestion buffer (with proteinase K, 0.5 mg/ml final concentration). DNA extraction was carried out with neutralized phenol/chloroform/isoamyl alcohol (25:24:1). DNA was transferred to a new tube and precipitated with 100 % ethanol at room temperature. A range of 20 to 50 µg DNA was obtained per sample; dried DNA was resuspended in 100-200 µl TE buffer.

All PCR reactions were performed at the following cycling conditions: 94°C for 3', 25 cycles of 94 °C for 30", 58 °C for 30" and 72 °C for 1'30", followed by an extension time of 72 °C for 10'. After that, the tubes were stored at 4 °C until processing. Each PCR mixture contained PCR buffer minus Mg [1X], dNTP mixture [0.2 mM], $MgCl_2$ [1.5mM]; primers F and R [0.5 µM each]; DNA

template 1µl; Taq DNA polymerase [1.5 units] (Taq DNA Polymerase, Recombinant - Invitrogen Cat Nº 11615-010, as well as PCR buffer and $MgCl_2$, were purchased from the local Invitrogen representative). The final volume was 20 µl completed with sterile deionized water. PCR products were analyzed by electrophoresis in agarose gels with a 100 bp DNA ladder (Invitrogen Cat Nº 15628-019). After electrophoresis, the desired DNA bands were excised using a clean and sterile scalpel blade. Bands were visualized under UV transilluminator light and cut quickly (Figure 1). They were isolated in approximately 300 mg of agarose slices, that were transferred into 1.5 ml microcentrifuge tubes and incubated at 4 °C until derivation to the sequencing service.

Sequencing of PCR products was performed in an automatic sequencer, by the Sanger method, at the *Unidad de Genómica* of *Instituto de Biotecnología del INTA* (Argentina). Sense and antisense primers were purchased from Invitrogen. Nucleotide sequences of primers were (a) exon 1: 5' GACCAATCTTCCGGTAGGAG (forward primer), 5' CGACCCCAGACTCCAAAAT (reverse primer); (b) exon 2: 5' TGGAGCTTCTTGCTAATCACT (forward primer), 5' GGTGTCAATTGTCTGGGTCA (reverse primer); (c) exon 3: 5' GAAGCACCTGCTGTCTGTCA (forward primer), 5' GCCTTCTAGGAACCCACACA (reverse primer).

Sequences analysis of selected PCR products was performed with the Nucleotide Database (NCBI) using the Basic Local Alignment Search Tool (Mega BLAST). The nomenclature followed to designate genes and proteins was from the International Committee on Standardized Genetic Nomenclature for Mice (Guidelines for Nomenclature of Genes, Genetic Markers, Alleles, and Mutations in Mouse and Rat; http://www.informatics.jax.org/mgi-home/nomen/gene.shtml)

RESULTS

The PCR products (Figure 1) were sequenced. The *Vkorc1* sequences for the three exons for wild type mice (n= 20) were compared with the NCBI Reference Sequence: NT_039433.8 corresponding to *Mus musculus* strain C57BL/6J chromosome 7 (Figure 2). Length of sequences ranged from 36 to 315 base pairs. A maximum of 6 gaps was found in 3 % of the sequences. Identities between the sequences for vitamin K epoxide reductase complex from *Mus musculus* strain C57BL/6J and the wild type ranged from 87 to 100 %. There was no mutation for the *Vkorc1* sequence in any of 20 wild rodents analyzed. These analyses show that the *Vkorc1* exons sequence is well conserved in wild mice from the Buenos Aires populations used in this study. Although the sequences obtained partially cover the *Vkorc1* region, we consider them to be low-frequency single nucleotide polymorphisms (SNPs).

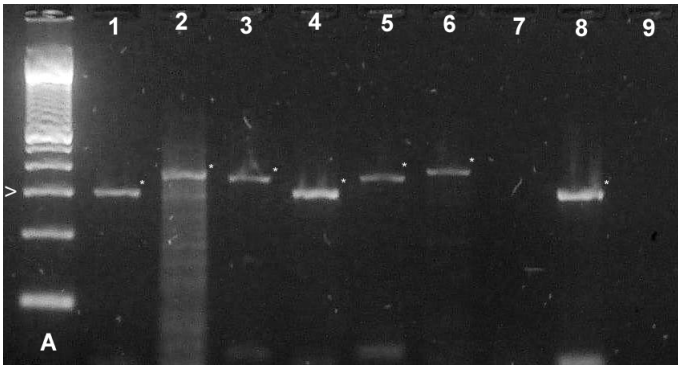

Figure 1. PCR products obtained from three representatives samples. Lines 1, 2 and 3 correspond to specimen 118 (exons 1, 2 and 3 respectively). Lines 4, 5 and 6 belong to the three exons of specimen 119, and lines 7, 8 and 9 match the three exons of specimen 120. Each DNA band (*) was sliced out of the gel and then purified and sequenced. Exons 1 and 3 (DNA template from sample 120) were not amplified in this PCR experiment. In cases like these, in which PCR was negative, amplification was repeated in order to obtain the corresponding amplification fragments.
In the first line (A), molecular weight marker (Invitrogen; Cat. Nº 15628-019) was placed; the indicated band (>) corresponds to 300 base pairs.

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Query  1         GCANGTTAGGNTAGTCTGGCATGAGGTGGGAACTCAGTGCTTTTTGGTCTNNNNNNNNNG  60
                 ||| |||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  45641933  GCAAGTTAGGTTAGTCTGGCATGAGGTGGGAACTCAGTGCTTTTTGGTCTTGTGTTCTGG  45641992

Query  61        TACCTTCTGGAAGCTAAGCAACATCAGACCCACATTGATGGNNTAGGTGGTAATGCACAC  120
                 ||||||||||||||||||||||||||||||||||||||||||  ||||||||||||||||
Sbjct  45641993  TACCTTCTGGAAGCTAAGCAACATCAGACCCACATTGATGGCATAGGTGGTAATGCACAC  45642052

Query  121       AATGCAGAAATCATATAACACAAAGAACAGGATCCAGGCCANGTACACGGAACCANCGAC  180
                 |||||||||||||||||||||||||||||||||||||||||| |||||||||||| ||||
Sbjct  45642053  AATGCAGAAATCATATAACACAAAGAACAGGATCCAGGCCAGGTACACGGAACCAGCGAC  45642112

Query  181       GGACACCANGGAACTCAGCACCAGTAGGATAGAGGCCCAACGTCCCCTCAAGCAACCTGT  240
                 |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  45642113  GGACACCAGGGAACTCAGCACCAGTAGGATAGAGGCCCAACGTCCCCTCAAGCAACCTGT  45642172

Query  241       AAAACAAGAGACGGTCAGTAATGGTATATCNAGAACTCTGTCATGCCTCCTCAATGACAG  300
                 |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  45642173  AAAACAAGAGACGGTCAGTAATGGTATATCTAGAACTCTGTCATGCCTCCTCAATGACAG  45642232

Query  301       ACAGCAGGTGC  311
                 ||||||||||
Sbjct  45642233  ACAGCAGGTGC  45642243
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Figure 2. Results from sequence alignment of Mus musculus, exon 3, reverse primer. BLAST alignment shows that the sequence corresponds to Mus musculus strain C57BL/6J chromosome 7 genomic contig, MGSCv37, C57BL/6J MMCHR7_CTG11. Sequence ID reference: NT_039433.8. Number of matches: 1. Range covered in the genomic context (Map Viewer): 45641933 to 45642243. Alignment parameters for segment: identities 294/311 (95%) and gaps 0/311 (0 %). Features: the sequence corresponds to vitamin K epoxide reductase complex subunit 1 precursor.

DISCUSSION

Anticoagulant drugs have been used as pesticides against rats and mice from city and farm populations for more than 60 years. Rodents die due to internal bleeding. The alleged cause is that the activated form of vitamin K cannot be produced because the enzyme that catalyzes the reduction has mutated in resistant individuals.

Tyr139Cys and Leu128Ser were the mutations commonly seen in populations of mice from Germany, Switzerland and the Azores. It was found that less than 10 % of the individuals studied (n= 434) carried the wild-type genotype whereas more than 90 % of them presented mutations. However, in some geographical areas, particularly in the Azores and Switzerland, the frequency of mutations was very low or non-existent (Pelz *et al.*, 2012).

Recently it has been shown that resistance in sewer rats from Danish populations would not be associated with any mutation in the *Vkorc1* gene (Heiberg, 2009). In humans, it is known that not only the *VKORC1* gene is related to the response to anticoagulants; certain interactions between alleles of *CYP2C9* and *CYP4F2* with *VKORC1* affect the response to anticoagulant treatment in patients with thromboembolic risk (Rettie *et al.*, 2006; Kringen *et al.*, 2011). The potential polymorphisms in the cytochrome P450 family related to resistance to anticoagulants have not been studied in mice, but it is possible that not only genetic variants of *Vkorc1* may be acting as factors that influence resistance. Warfarin, bromadiolone and 4-hydroxycoumarin are the active substances found in rodenticides and these drugs are inhibitors in the synthesis of vitamin K-dependent clotting factors and coagulation. The poisoning treatment used by rodenticides is the delivery of intravenous vitamin K, which acts as "antidote". Given that vitamin K (forms K_1 and K_2) is found in vegetables, dairy products, meat and eggs, and considering that mice are omnivores, they could be eating the antidote at the same time as the poison. Even if farmers are applying rodenticides, rodents could be avoiding the ingestion of the poison. If house mice have a good source of food, they may avoid taking the poison. We think that the wrong management of feeding in poultry farms may be causing an overestimation of resistance to rodenticides. The basis of the low efficacy of anticoagulants used for rodent pest control may be a combination of the *Mus* sp. genotype and an inappropriate management during the application of anticoagulants in areas where rodents are plague.

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