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Short Note

First record of *VKORC1* sequence mutation associated with resistance to anticoagulant rodenticides in Italian individuals of *Mus musculus domesticus*

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Abstract

Non-synonymous mutations in the gene encoding vitamin K epoxide reductase (VKORC1) are responsible for resistance to anticoagulant rodenticides in rodents. Detection of resistant individuals is crucial to implement effective management plans and guarantee the success of eradication campaigns. Resistant individuals of the house mouse *Mus musculus* have been reported in several European countries and in North America. However, to date no data is available for *M. musculus domesticus* in Italy. In this study, we sequenced the three exons of the *VKORC1* gene in 30 mice from the Pontine Archipelago, currently subject to an eradication campaign of invasive rodents. We recorded the presence of a mutation known to confer resistance to rodenticides (Tyr139Cys) in one individual (3.3% of the total samples set). Our data represent the first record of a resistant *M. musculus domesticus* in Italy.

The impact of invasive rodents on public health, human activities and ecosystems is commonly tackled by using a range of rodenticides, mainly anticoagulants which inhibit the production of Vitamin K by blocking the activity of the vitamin K epoxide reductase (Rost et al., 2009; Goulois et al., 2017). This enzyme, encoded by the *VKORC1* gene, is involved in the recycling of vitamin K, essential for the synthesis of various blood coagulation factors. Inhibition of vitamin K recycling hinders blood clotting and causes internal hemorrhages eventually leading to the death of an individual (Li et al., 2004; Rost et al., 2004; Oldenburg et al., 2008). Since 1950s, the use of anticoagulant rodenticides as chemical pest control has proved to be very efficient. However, their massive use resulted in the development of resistant rodent populations (Buckle, 2013; Goulois et al., 2017).

The house mouse *Mus musculus* is listed as one of the 100 most invasive species in the world by the IUCN, affecting public health, agriculture and other human activities such as the food industry, animal husbandry and residential premises (e.g. Pocock et al., 2004; Witmer and Jojola, 2006; Capizzi et al., 2014). It also poses serious threats for the conservation of many animal and vegetal autochthonous species worldwide. Critical impacts on plants, invertebrates, land and seabirds have been well documented, particularly in insular ecosystems (Cuthbert and Hilton, 2004; Angel et al., 2009; Harper and Cabrera, 2010).

House mice, together with rats, are primary targets of anticoagulant rodenticides (Capizzi et al., 2014). Resistance to anticoagulants in these species, reported for European and North American populations (Siddiq and Blaine, 1982; Pelz et al., 2005; Mooney et al., 2018 and references therein), is mainly linked to single nucleotide polymorphisms (SNPs), found in the three exons of the *VKORC1* gene, (Pelz et al., 2005) that generate structural changes in the VKORC1 protein, causing a reduction in the activity of the enzyme (Rost et al., 2004; Pelz et al., 2005). The most frequent SNPs in the *VKORC1* gene known to confer anticoagulant resistance in rodents map to exon 3, in the amino acid position 128 and 139 (Leu128Ser and Tyr139Cys, respectively), while additional mutations have also been described for exon 1 and 2 (Pelz et al., 2012; Goulois et al., 2017 and references therein).

The occurrence of anticoagulant resistance may compromise the control of rodent pests and the success of eradication campaigns, resulting in an overabundant and ineffective use of toxic baits (Pelz and Prescott, 2015). The identification of resistant rodent individuals is therefore crucial for developing efficient management strategies in natural and urban areas (Buckle, 2013). Moreover, in case of *M. musculus* populations resistant to anticoagulants, the effectiveness of eradication measures against other sympatric invasive rodents, such as rats, can be compromised due to consumption of baits, originally placed for rat eradication, by house mouse (e.g. Howald et al., 2007).

A comprehensive program for the conservation of threatened endemic species and their habitat is currently undergoing in the Pontine Archipelago (Central Italy) under the financial support of the European Union (Celesti-Grapow et al. 2017; http://www.ponderat.eu/en-home). The project foresees the management of rodent invasive species (namely the black rat and the house mouse) to preserve the ecosystem of the Archipelago. As part of this project, we sequenced the *VKORC1*

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gene of 30 house mice collected from two Pontine islands in order to investigate the presence of resistance to anticoagulants in this species and, if found, to measure its percentage of occurrence. Anticoagulants are commonly used in the Archipelago to control rodents, both in natural and urbanized (i.e harbors and small towns) areas. This is the first investigation on resistance to anticoagulant rodenticides in house mouse in Italy and it will be of significant help to set up efficient management plans of *M. musculus domesticus* and co-occurring rat populations in the Pontine Islands.

The Pontine Archipelago is located in the Tyrrhenian Sea, off the coast of Central Italy, and includes five volcanic islands (Ponza, Palmarola, Ventotene, Santo Stefano and Zannone). The Archipelago is a popular touristic destination in summer and represents a hotspot for Mediterranean biodiversity. It harbours numerous faunistic and floristic endemisms and is a site of ornithological importance. Thirty individuals of *M. musculus domesticus* were captured from the islands of Ventotene (n=19) and Santo Stefano (n=11) using snap traps (Model T-Rex, Bell Laboratories INC), in accordance with Italian regulation for sampling of non protected species (L. 157/92, art. 2).

For each captured individual, 10 mg–50 mg of tail muscle tissue was collected and preserved in absolute ethanol. DNA was extracted by overnight digestion at 55 °C in a lysis buffer containing 0.1 м Tris buffer, 0.005 м EDTA, 0.2 м NaCl and 0.4% SDS, pH 8.0, and 0.1 mg proteinase K followed by isopropanol-ethanol precipitation (Sambrook and Russel, 2001). Samples were resuspended in DNAase-free water and preserved at −80 °C.

Exons 1, 2 and 3 of the *VKORC1* gene were amplified by polymerase chain reaction (PCR) using three primer pairs designed to anneal to the adjacent intronic regions (see Table 1). Primer set 1 was designed by the authors in order to obtain amplification products in both the house mouse and black rat. Primer set 2 was specifically designed for this study, while primer set 3 was modified from Grandemange et al. (2010). Primers were designed using Primer3 4.1.0 (http://primer3.ut.ee/).

The PCR amplifications were performed using 1X reaction buffer, 300 µм dNTPs, 1.5 мм MgCl2, 0.5 µм of each primer and 0.5 U Taq polymerase. Thermal profiles consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 45 s at 94 °C, annealing for 45 s at locus-specific temperature (Table 1) and extension for 90 s at 72 °C, with a final extension step of 10 min at 72 °C.

Amplicons were first visualized in a 1% agarose gel, then purified by precipitation with Sure Clean (Bioline), resuspended in water, and cycle-sequenced using BigDye Terminator v3.1 chemistry (Life Technologies). Sequencing products were isopropanol-precipitated and resolved by capillary electrophoresis in an Applied Biosystems 3130xl genetic analyzer.

The *VKORC1* sequences were corrected and aligned using Geneious 8.0.5 (Kearse et al., 2012). The SNPs known to be responsible for resistant phenotypes were identified by comparison with annotated reference sequences of *M. musculus*, *Rattus norvegicus* and *R. rattus* downloaded from GenBank (NCBI reference numbers: NM178600, GQ905715, NM203335 and LC164812).

For each of the 30 individuals analysed, we sequenced three fragments (253 bp, 801 bp and 308 bp long) of the *VKORC1* gene . These fragments included the three exons and their combination allowed analysis of the entire coding region of the gene (486 bp long). Exon 1, 2 and 3 are 174 bp, 110 bp and 202 bp long and encode in total 161 amino acids. The sequences were deposited in GenBank (accession numbers MN244946 and MN244947).

Table 1 – Primer sequences for the PCR amplification of the three exons of the *VKORC1* gene and relative annealing temperature.

Primer	Sequence 5'–3'	Annealing Temperature
VKORC1_ex1_F	TCTTCCCTCCTGTSYCTGGG	52 °C
VKORC1_ex1_R	AAATYATCTGGYAACCTGGC	
VKORC1_ex2_F	CTGTGCTGAGGGGACAAAGT	50 °C
VKORC1_ex2_R	TTGCCATAAAACTGAGATTGTGA	
VKORC1_ex3_F	TTTCACCAGAAGCACCTGCTGYC	54 °C
VKORC1_ex3_R	ACACTTGGGCAAGGSTCATGTG	

When compared to the reference sequences, the obtained sequences showed no variability except for a sample from Ventotene Island. This individual presented a non-synonymous SNP mapping to exon 3 and corresponding to aminoacid position 139 (Tyr139Cys). This mutation was recorded in heterozygosity and consists in a nucleotide change from TAT to TGT. The Tyr139Cys is one of the most frequent amino acid substitutions occurring in the *VKORC1* gene in resistant house mice and rats in Europe (Pelz et al., 2012), and it is known to confer resistance to anticoagulants also in heterozygosity condition (Rost et al., 2004).

Our finding is the first record of the presence of a resistant *M. musculus domesticus* individual in Italy. Two studies investigated the occurrence of mutations that might be involved in the development of resistant phenotypes of rodents in Italy (Iacucci et al., 2018, Fratini et al. unpublished data). Iacucci et al. (2018) found a single missense mutation (Iso123Ser) in exon 3 of the *VKORC1* gene in eight individuals of the brown rat *R. norvegicus* in a Venetian locality. However, Iso123Ser has never been reported as associated with resistance in rodents (Pelz et al., 2012; Goulois et al., 2017). In the Pontine Archipelago, Fratini et al. (unpublished data) analysed sequence variations in 119 individuals of black rat (*Rattus rattus*) at the three exons of the *VKORC1* gene and found no missense mutations. Goulois and collaborators (2017) reported that in other European countries anticoagulant resistance is much more common in mice than in black and brown rats, probably because of a greater selective pressure exerted by anticoagulant rodenticides on *M. musculus* (Goulois et al., 2017). Control of house mice is, in fact, carried out mostly by non-professionals not aware of the consequences that a massive use of these molecules can have on the spread of resistance. Rat control is instead usually performed by informed specialists, well aware of the risks associated with the use of biocides (Goulois et al., 2017).

Detection of rodent individuals resistant to anticoagulant is crucial to set up efficient management strategies. Once resistant phenotypes are detected, alternative pest control methods should be employed (Buckle, 2013). Among these, the use of more effective second-generation anticoagulants such as brodifacoum and flocoumafen has not so far resulted in the development of resistant individuals. However, such compounds are highly bioaccumulative and may lead to secondary poisoning in rodent predators (Eason et al., 2001; Laakso et al., 2010; Vein et al., 2013). Physical removal of invasive rodents is another alternative to anticoagulant rodenticides. While the use of traps may not be very effective for eradicating large populations of invasive rats (e.g. Howald et al., 2007), live trapping can work relatively well for controlling small rodent infestations, such as those of the house mouse (Clapperton, 2006; Buckle, 2013). Nevertheless, trapping is both labour intensive (traps must be visited daily) and expensive (Corrigan, 1997).

The detection of a resistant individual of *M. musculus domesticus* in the Pontine Archipelago suggests that more effective anticoagulant rodenticides (such as brodifacoum) should be used to manage invasive rodent populations in general, keeping the use of less toxic compounds (such as bromadiolone) only in areas where the target species is not present, as suggested in Pelz and Prescott (2015). Increasing the efficiency of eradications is particularly important in insular contexts: a large percentage of the world's threatened biodiversity, in fact, resides on islands where invasive species are the major threat (Howald et al., 2007). In case of sympatry between rats and mice (as in the Pontine Archipelago), the two species compete for food and the control of only one species can result in a dramatic increase of the competitor (Caut et al., 2007). Thus, despite no resistant individuals of black rat have been detected so far in the Archipelago (Fratini et al., unpublished data), the use of the most effective second-generation anticoagulants would be necessary in order to reduce competition for baits between rats and mice. In fact, the use of first-generation and less effective second-generation anticoagulants in the Archipelago would bring to a failure of rat eradication because resistant mice could outcompete rats for baits. However, considering the potential threat of powerful second-generation antico-

agulants to non-target species (e.g. pets, predators), it is necessary to carefully evaluate when and how to use such rodenticides in natural habitats.

This study reports, for the first time, the occurrence of *M. musculus domesticus* resistance to anticoagulants in Italy. Our results therefore support the importance of conducting more comprehensive investigations at a national scale in order to assess the extent of mice resistance to pesticides. This will help identifying appropriate management strategies for potential use of more effective but noxious rodenticides in areas where resistance is recorded.

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