Published by Associazione Teriologica Italiana

Hystrix, the Italian Journal of Mammalogy

Volume 29 (1): 95–99, 2018

OPEN ACCESS

Available online at:

http://www.italian-journal-of-mammalogy.it

doi:10.4404/hystrix-00055-2018

Research Article

VKORC1 mutation in European populations of *Rattus norvegicus* with first data for Italy and the report of a new amino acid substitution

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Keywords:
Norway rat
anticoagulant rodencides
resistance
Vitamin K epoxide reductase 1
exon 3 mutations

Article history:
Received: 1 February 2018
Accepted: 20 April 2018

Acknowledgements
This work has been partially funded by A.N.I.D. (Associazione Nazionale delle Imprese di Disinfestazione). We are grateful to Renaldo Renzi, Fulvio Fraticelli, Giulia Sozio, Fabio Dartora, Valentina Candian and Marco Panza for the Norway rat samples and to David Halfmaerten for its help in the laboratory procedures problems solving. Thanks to Luca Santini for his help with the figures in R. Thanks are extended to two anonymous reviewers that greatly improved the earlier version of the manuscript with constructive criticisms. Finally, and most importantly, we want to dedicate this work to Mauro Cristaldi, recently passed away, who devoted much of his researches to the rodent pest control.

Abstract

In the Norway rat, *Rattus norvegicus*, anticoagulant rodenticide resistance is mainly associated with mutations in the third exon of the *Vitamin K epoxide reductase complex subunit 1* (*VKORC1*). Identification of the resistant wild populations is very important to improve the control practices and to limit the damages due to inadequate use of the anticoagulant rodenticide. In this study, we determined the distribution of the third exon mutations in poorly investigated areas of Africa, Europe and the Middle East. In particular, we investigated the phenomenon for the first time in the Italian peninsula. We obtained sequences of the third exon for 133 Norway rats from 37 localities in Africa, Europe and the Middle East. For additional analysis, we retrieved information in literature on amino acid substitution in 1136 third exon sequences of Norway rats from Europe, the Far East, North America and South America. However, we found third exon mutations only in Europe and the Far East with the Y139F mutation shared between the two areas. Europe has the higher number of mutant individuals and Y139C mutation prevails. In Italy, we found a single missense mutation (I123S) in a Venetian locality. This homozygote mutation, is not know in literature to be associated with resistance, but it is very similar to a mutation that confers resistance in humans (I123N). This similarity and its high local frequency makes it a good candidate for future testing. Our results provide useful data to better understand the resistance phenomenon and to plan targeted control actions.

Introduction

Rodents are responsible of a wide range of impacts, from agriculture to urban areas, from farming to ecosystems, from forestry to public health (Singleton et al., 1999). Among them, synanthropic species (i.e. *Rattus rattus*, *R. norvegicus* and *Mus musculus*) are recognized to be the most impacting species (Capizzi et al., 2014). For this reason, over the centuries, different strategies have been used to mitigate the impacts and the economic damage. Control actions were carried out using several type of poisons (Buckle, 1994) until the introduction of anticoagulant rodenticides (derivatives of coumarin) in the 1950s. Compounds had already been used for the treatment of thrombophilia in humans (e.g. warfarin) and they were adapted to control wild rodent populations (Seidmann et al. 1950). Coumarins target blood coagulation by inhibiting the vitamin K epoxide reductase multiprotein complex (*VKORC*; Rost et al., 2004), thus blocking the production cycle of the Vitamin K, an essential cofactor for the synthesis of various blood coagulation factors (Oldenburg et al., 2008). The inhibition of vitamin K recycling causes internal hemorrhages and death of the individual. The introduction of anticoagulant compounds made a significant change in rodent control practices. However, the first anticoagulant resistance events were observed in wild rodent populations in the United Kingdom in the 1960s (Boyle, 1960; Buckle and Prescott, 2012). Between the 1970s and the 1980s, "second generation anticoagulants", (brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen) were introduced to overcome resistance. They are more toxic and have longer half-life periods in individuals than the first generation anticoagulants (warfarin, chlorophacinon and coumatetralyl) (Breckenridge et al., 1985). Nevertheless, some of them are also affected by resistance (Rowe et al., 1981; Greaves et al., 1982; Lund, 1984; MacNicoll and Gill, 1987; Johnson, 1988).

Several studies have been performed to relate resistance to inherited characteristics of wild rodent populations (Pool et al., 1968; Hermodson et al., 1969). One important mechanism providing resistance to anticoagulant rodenticides was defined by Rost et al. (2004) who showed that mutations in the *VKORC1* gene (vitamin K epoxide reductase complex subunit 1) render the rodenticides ineffective (Rost et al., 2004; Pelz et al., 2005). These mutations have been identified in humans, mice and rats (e.g. Rost et al., 2004; Pelz et al., 2012; Gryseels et al., 2015). For humans, house mouse and Norway rat resistance the effects

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are known especially for mutation in the third exon of the *VKORC1* gene and with a high-frequency in amino acid position 128 and 139 (Pelz et al., 2005). Exon 3 mutations conferring resistance to anticoagulant compounds have been found in wild rat populations in several European countries (e.g., Denmark: Lodal, 2001, Germany: Pelz et al., 2005, Belgium: Baert et al., 2012), but data are lacking for Italy. However, there are various reports about ineffective anticoagulant applications in the Italian peninsula (*A.N.I.D. - National Association of Pest Control Companies personal communication 2015*). This suggests that also the Italian populations could show the exon 3 mutations conferring some kind of resistance.

The Norway rat (*R. norvegicus*) is a synanthropic, cosmopolite invasive species. It lives close to a large variety of human derived environments as settlement wineries and sewers, although it is also possible to find it in stalls, warehouses, holdings and seaports (Santini, 1983). It has an omnivorous diet, but it shows a neophobic behaviour for food in new or disturbed places. *R. norvegicus* lives in small groups composed of related individuals, which are led by a dominant male and some females (Amori et al., 2008). Archaeo-zoological and molecular phylogenetic data indicate that the species originated in Southwestern China around 1.3 Mya (Song et al., 2014; Puckett et al., 2016) and spread from the native area in more recent times (Song et al., 2014; Puckett et al., 2016). Specifically, the species arrived in West Europe and Britain probably between 16^{th} and the early 18^{th} century (Atkinson, 1985; Pascal et al., 2006) and then, it is believed it colonized Africa by the middle of the 19^{th} century (Long, 2003). Moreover, evidences of the presence of the species in North America were by the middle of the 18^{th} century (Armitage, 1993). In its invasion range, it is considered a serious pest on account of its damages to native animal and plant species (Atkinson, 1985), to agriculture and food pantries (Lambert et al., 2008) and its role as vector of zoonotic diseases (Meerburg et al., 2014). Its impact is mainly evident on the islands, where it is considered as one of the main causes of native species extinction (Thorsen et al., 2000; Towns et al., 2006; Howald et al., 2007; Jones et al., 2008). For the Norway rat, the most common mutations identified as conferring resistance to anticoagulant rodenticides are L120Q (amino acid change from L–leucine to Q–glutamine in the position 120), L128Q, Y139F, Y139C and Y139S, with Y139C and Y139F being the most frequent ones (Buckle and Prescott, 2012). The *VKORC1* gene mutations could not be the only explanation of the resistance to anticoagulant products. In fact, other compensatory mechanisms to avoid the internal bleeding or the action of other genes can be involved (Pelz et al., 2005). Nevertheless, the identification of these mutations in the *VKORC1* gene is still a quick and effective method to detect resistant rodent populations in the wild. Such an identification is very important to improve the control practices and to limit the risk of an inadequate use of anticoagulant rodenticides. In fact, these rodenticides tend to bioaccumulate and can be passed along the trophic chain, threatening other species (Grandemange et al., 2009; Buckle and Prescott, 2012; Eason et al., 2001; Laakso et al., 2010). Moreover, the intensive and uncontrolled use of anticoagulant rodenticides can increase the selection of resistance individuals in the wild rat populations (Greaves and Ayres, 1977). Additionally, some resistant strains are very difficult to eradicate and can enhance the spread of zoonotic diseases (i.e., hantaviruses, echinococcosis, leptospirosis, helminth zoonoses and *Salmonella*) (Davis et al., 2005; Easterbrook et al., 2007; Costa et al., 2014; Meerburg et al., 2014; Himsworth et al., 2015; Ribas et al., 2016). The aim of this study is to identify molecular signatures of anticoagulant resistance in the third exon of the *VKORC1* gene in Norway rat populations sampled in poorly investigated areas of Africa, Europe and the Middle East. We have placed emphasis on to the Italian peninsula for which data were completely unavailable. Indeed, Italy is one of the few European countries that has not been studied for the presence of wild populations of rodent resistant to anticoagulant poisons. There are many reports of the non-efficacy of anticoagulant products by the Italian deratization companies, but the lack of data on resistant wild populations does not allow to plan efficient control strategies. Hence, the identification of possible Norway rat resistant wild populations is very important both to control this invasive species and to limit damage to the environment and to other species, due to an incorrect use of the anticoagulant products (Laakso et al., 2010; Vein et al., 2013).

Materials and methods

Specimens sampling

The *Rattus norvegicus* samples, mostly consist of parts of ears, tails and paws, were fixed in 80% ethanol alcohol and stored at a temperature of 4 °C. In total 133 samples were obtained and sequenced (Tab. S1). In adding , unpublished information of the sequence of amino acidic were obtained from 32 rats from Belgium. For Italy, we collected 64 individuals from 19 localities. For the rest of Europe, 62 individuals where obtained from 24 localities of Belgium, Denmark, France, Hungary, Netherlands, Portugal (Azores Islands) and Spain. The other samples were: 21 individuals from 8 localities in Africa (Mozambique, Tanzania, Tunisia and South Africa); 9 individuals from 4 localities in the Middle East (Pakistan, Yemen, Iraq and Lebanon) and 9 individuals from 1 locality in Southeast Asia (Thailand) (Tab. S1). Samples from Flanders (Belgium) were caught and processed under permission Directive 2010/63/EU, Annex IV; KB 14/08/86 art.15 and KB 29/05/13. Samples from the La Palmyre Zoo (France) were caught and processed according to the French zoo legislation. All other analyzed samples were sourced from museum collections or derived from road killed specimens. All the national and international ethical guidelines for the animal tissues use were followed. The samples collection made for this study was opportunistic for Italy, trying to cover the entire country. Despite numerous studies done in recent years, there are no sequences of *VKORC1* from wild Norway rats in GenBank. This is because most of the screening, was done through mutant specific primers and no sequences were produced. This strongly limits any in-depth analysis of genetic variability of our datasets (see also Results and Discussion). To compare our results to other studies, we retrieved information in literature on amino acid substitution in 1136 third exons of Norway rats from Argentina (n=15; Rost et al., 2009), Belgium (n=14; Pelz et al., 2005), Denmark (n=44; Pelz et al., 2005; Song et al., 2014), France (n=281; Pelz et al., 2005; Grandemange et al., 2009; Song et al., 2014), Germany (n=439; Pelz et al., 2005; Song et al., 2014), Hungary (n=12; Rost et al., 2009), Indonesia (n=16; Rost et al., 2009), Japan (n=6; Rost et al., 2009), the Netherlands (n=220; Meerburg et al., 2014), Portugal (Azores Islands, n=16; Rost et al., 2009), South Korea (n=8; Rost et al., 2009), Thailand (n=2; Rost et al., 2009), the United Kingdom (n=42; Pelz et al., 2005; Rost et al., 2009) and the USA (n=21; Rost et al., 2009).

Laboratory procedures

A standard protocol using salts (Aljanabi and Martinez, 1997) was used to extract the genomic DNA from well preserved tissues, whereas we used DNAeasy Tissue Kit (Qiagen, Hilden, Germany) for old samples or those stored under sub-optimal conditions (n=30). For all the 165 individuals (Tab. S1), we amplified the third exon (203 bp) of the *VKORC1* gene using the following primers: forward primer 5'-CATTGGGGAGGTGTTACAGAG-3' and reverse primer 5'-GATACACTTGGGCAAGGCTC-3'. The amplification protocol included an initial denaturation at 94 °C for 3 min followed by 30 cycles of 94, 58 and 72 °C for 30 seconds each; these steps were followed by extension at 72 °C for 3 min. For the PCR purification the Sureclean protocol (Bioline) was used and the PCR products were sent to Macrogen Europe for sequencing (www.macrogeneurope.com).

Genetic polymorphism and mutations distribution pattern analysis

The 133 sequences were controlled, aligned and converted in protein using FinchTV (Geospiza, Inc.) and MEGA 6.06 (Tamura et al., 2013) software. Haplotype and nucleotide diversity were calculated with DnaSP v.5 (Librado and Rozas, 2009). A median joining network connecting haplotypes was built with popART (Leigh and Bryant, 2015). The frequencies of the third exon amino acid substitu-

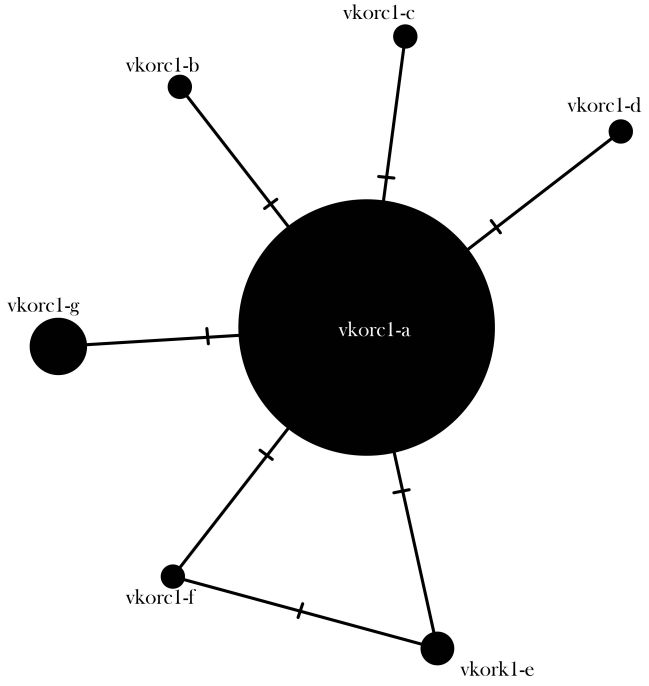

Figure 1 – Median joining network connecting haplotypes of *VKORC1* third exon. The haplotype *VKORC1*-g corresponds to the one carrying the new amino acid substitution I123S.

tion and wild type individuals of the entire dataset (including newly sequenced individuals and information retrieved from literature) were plotted on a map to evaluate their distribution in the areas using `Pegas` (Paradis, 2010) and `Maps` (Becker et al., 2015) packages of R v.3.2.1 software (https://www.r-project.org/foundation/). For some geographic area (Belgium, Denmark), the frequencies of wild individuals cannot be considered as representatives of their real occurrence in the territory. This bias is caused by the excess of sequenced individuals in areas where resistance phenomenon has been observed. However, this representation has the advantage of highlighting the areas where the mutations are completely absent.

Results

Molecular genetic diversity

Haplotype and nucleotide diversity are very low in the sample (Hd=0.166 and Pi=0.00084 respectively) in spite of the considerable geographic distance of analyzed populations spanning from South Africa to Denmark. The number of polymorphic sites is five (one silent substitution) with three singleton. No heterozygote sites has been detected. This polymorphism identified seven different haplotypes (*VKORC1*-a/f, GenBank accession numbers MH430820-6). One haplotype is the most common, present in 92% of the individuals and in all the sampled areas. The other six haplotypes are unique or very rare and were found in six different populations. Network among haplotypes revealed a star like pattern with the common haplotype connected by a single substitution to the rare ones (Fig. 1).

Global and European distribution of the *VKORC1* gene third exon amino acidic mutations

The global distribution of the VKORC1 gene third exon mutations in the wild Norway rats showed 13 aminoacidid substitutions (inset of Fig. 2).

Mutations were only found in Europe and in the Far East, instead only wild type individuals were found in Africa, Argentina, the Middle East and the USA. The Y139C and Y139F mutations prevail in Europe, being Y139F shared with the Far East where there are also high frequency of I141V and A143V. The distribution of Y139F and Y139C in Europe, show different frequencies in each country considered for this study. In fact, Y139F and Y139C have comparable frequencies in the United Kingdom, while Y139F prevails in France and Belgium and Y139C in Germany, the Netherlands, Hungary and Denmark. Hence the two mutations have a different distribution throughout the European continent: Y139C prevails in the north-east and Y139F in the west. In Italy, Spain and the Azores, there are not mutations known to provide resistance to anticoagulant rodenticides. For Italy (Fig. 2), we identified a new mutation, the I123S, in homozygous condition in six of the eight analysed individuals in a population from Veneto. Only wild type individuals were found at the other Italian localities.

Discussion

Global anticoagulant resistance

We provided an updated map of the global distribution of aminoacid mutations in the third exon of the *VKORC1* gene in wild populations of the Norway rat with new data from several new localities not studied so far (European country, Africa, Italy and Middle East, Fig. 2). These mutations represent the most rapid and reliable method to identify resistant populations since there are specific mutations that provide a resistance to the anticoagulant compounds. As explained above, the lack of *VKORC1* sequences from wild rats deposited in GenBank, limits the interpretation on the observed pattern of distribution. In fact silent substitutions, of potentially phylogenetic significance, are not available. The new samples here analyzed, representing the only available sequences, show a very low molecular diversity and, alone, are not indicative of any geographic pattern. Considering aminoacid substitutions, the most common mutations in the third exon are: Y139F, Y139C, Y139S, L120Q and L128Q. Their distribution is rather heterogeneous in the different areas being present only in Europe and in the Far East while none of the samples from Africa, Argentina, the Middle East and the USA showed any mutation known to confer resistance. This pattern can be the result of sample bias since these latter areas are also the ones with low number of samples analyzed (Tab. S1). Europe and the Far East share a single mutation (Y139F) conferring resistance. This may represent a convergent mutation or alternatively, it may be derived from a common ancestor. It is difficult to distinguish between the two hypotheses but a certain level of genetic divergence has been observed between Far East and European populations pointing out the possibility of independent evolution of such mutations (Song et al., 2014; Puckett et al., 2016). The European distribution of the two most common mutations shows a geographic pattern. Y139C prevails in the West (Denmark, Germany, Hungary and the Netherlands) while Y139F prevails in more central European areas since it's widespread in all France and Belgium (Fig. 2; Tab. S1). This pattern mirrors the one observed by genotyping of 32K SNPs that allowed the identification of two European clusters, one containing rats from Norway, Sweden, Finland Germany and Netherlands and another including those from France, Austria and Hungary (Puckett et al., 2016). All the European *VKORC1* mutations are present in the UK, but it is interesting to note the presence of the Y139S mutation that evolved locally at the Anglo-Welsh border (Buckle and Prescott, 2012). The high number of different mutations conferring resistance in Europe, and the relatively overall high frequencies, may be associated with different factors. For example, Song et al. (2014) hypothesized that the third exon mutations were already present in high frequencies in Europe before the intensive use of anticoagulant rodenticides, being characteristic of some genetic lines coming from the native areas. Rost et al. (2009) explained the presence of mutations in some areas rather than in others by independent "founder effect" events, linked to different colonization events of the areas. Moreover, a substantial increase in resistant rats has been observed in areas where rodenticides were used intensively for years (Brooks and Bowerman, 1973). Therefore, the presence of a high number of mutant individuals in Europe could be associated both with independent colonization events and with an intensive and not well-managed use of the anticoagulant rodenticides. The first hypothesis is supported by molecular genetic analyses indicating that multiple colonization occurred in Europe (Puckett et al., 2016; Iacucci et

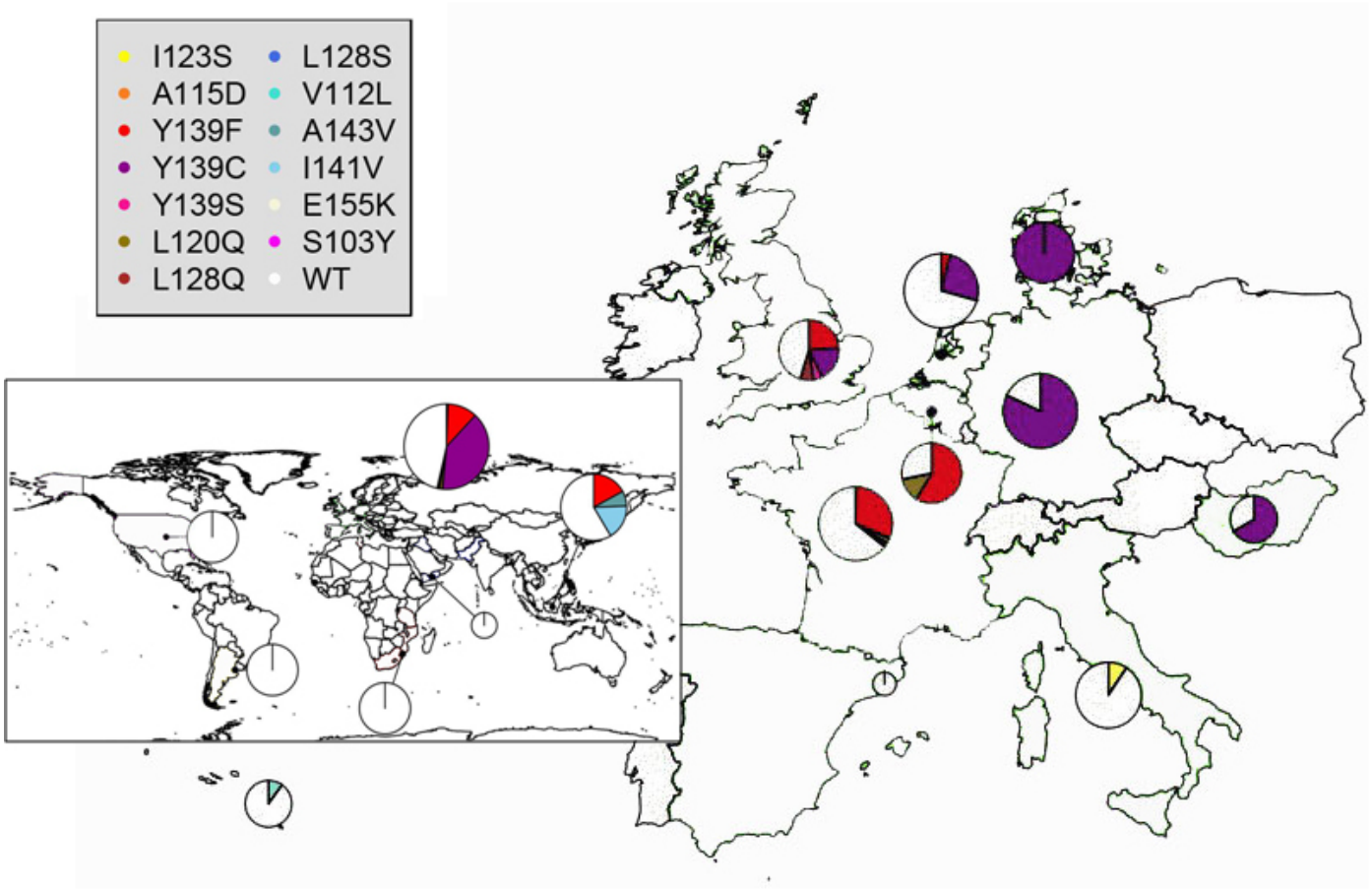

Figure 2 – Global (inset) and European distribution of the *VKORC1* gene third exon amino acid mutations. The pie charts sizes are proportionate to the sample of the countries and the slices are the frequencies of the single mutations (Italy=64, Germany=440, France=294, Belgium=47, Azores Islands=20, Spain=1, Netherlands=225, Hungary=15, Denmark=46, United Kingdom=42, USA=21, Europe=1194, Middle East=9, Far East=41, Africa=21, Argentina=15) WT: wild type individuals.

al., 2016), whereas the second one is difficult to test since the rodenticide programs have been carried out with different intensities across the European distribution area of *R. norvegicus* and no detailed data are available.

Anticoagulant resistance in Italy and the report of a new amino acidic substitution candidate for resistance

This is the first study to assess the presence of potential resistance to anticoagulant rodenticides in wild Norway rat in Italy. This total lack of information is regrettable since a better knowledge on the presence of resistance in wild rat populations is of fundamental importance from an ecological and public health point of view, because the presence of resistant wild rodent populations could facilitate the spread of zoonoses (i.e. hantavirus infection or leptospirosis) (Davis et al., 2005; Meerburg et al., 2014).

Albeit preliminary, we didn't find mutations known to confer anticoagulant resistance in Italy. However, the 75% of the individuals belonging to a rural Venetian population (n=8) carried a new mutation in the third exon of the *VKORC1* gene, namely I123S. It is not known if this mutation confers anticoagulant resistance in Norway rats, but it is located in the same position as mutation I123N which is responsible for anticoagulant resistance in humans (Oldenburg et al. 2014), allowing us to suggest its involvement in resistance. The similarity is found not only in the amino acid position, but also in the type of mutated amino acid. In fact, serine (S) and asparagine (N), unlike isoleucine (I), are polar and hydrophilic amino acids which usually participate in hydrogen bonds as proton donors or acceptors. This mutation could have evolved locally, like Y139S which is known only in the UK (Buckle and Prescott 2012). The lack of other genetic data concerning the resistance phenomenon and of the information about the rodenticides used in Italy do not allow us to confirm this hypothesis, although it would be interesting to continue this study locally in the future. We do not have direct information about the use of rodenticides in the sampled Italian localities, with one exception. In the Zoological Garden of Rome ("Bioparco") a strong suspicion of resistance to anticoagulant rodenticides was observed in the years 2013–2016 related to the use of different types of anticoagulant rodenticides (*F. Fraticelli personal communication 2015*). Surprisingly, the samples found for this area were all wild type, but we can not exclude that this result could be attributable to the small number of the sample (n=6). Moreover, the total absence in the Italian samples of known mutation associated with resistance may be attributable to the different pattern of use of anticoagulant rodenticides compared to that of other countries (e.g. UK), where a much more strict regulation does not allow to use the more potent second generation anticoagulants (i.e. brodifacoum and flocoumafen) in outdoor areas. In Italy, there is no restriction, and indeed there is often a routine use of the most powerful anticoagulants. For these last generation compounds are not known forms of resistance. Therefore paradoxically, this could have prevented the spreading of mutation conferring resistance to first generation anticoagulants, to a greater extent than in countries with stricter regulations. The problem is that, as above discussed, second and third generation rodenticides, are highly bioaccumulative and potentially harmful to predators of rodent species (Eason et al., 2001; Grandemange et al., 2009; Laakso et al., 2010; Buckle and Prescott, 2012; Vein et al., 2013).

Conclusion

This study provides updated information about the distribution of *VKORC1* polymorphisms in the Norway rat throughout the world using both new samples and literature data. The high frequencies of exon 3 mutations highlight the importance of resistance management strategies to ensure future pest control of resistant wild population in different areas. Our data also fill some gaps in knowledge of resistance

to anticoagulant rodenticides in Italy where it is very important to plan future studies to understand the role of the new mutation (I123S) in resistance and the absence of the commonest mutations in the country in order to plan targeted control actions. Indeed, if there is a good knowledge about the precence/absence of the mutation in the wild populations and about the anticoagulant compounds used, it will be possible to evaluate where and how to use the anticoagulant rodenticides.

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Associate Editor: R. Caniglia

Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 Exon 3 mutant and not-mutant rats obtained in this work.