Single Nucleotide Polymorphisms (SNPs) Within Vkorc1 in Rodent Populations in a Tropical City-state: Implications for Anticoagulant Rodenticide Use for Rodent Control

Cliff Chua (Cliff.CHUA@nea.gov.sg)
Environmental Health Institute, National Environment Agency

Mahathir Humaidi
Environmental Health Institute, National Environment Agency

Lee Ching Ng
Environmental Health Institute, National Environment Agency

Joel Aik
Environmental Health Institute, National Environment Agency

Research Article

Keywords: Anticoagulant rodenticides, Single Nucleotide Polymorphisms (SNPs), Vkorc1, rodent populations, tropical city-state, rodent control

DOI: https://doi.org/10.21203/rs.3.rs-691977/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Anticoagulant rodenticides are commonly used in rodent control because they are economical and have great deployment versatility. However, rodents with Single Nucleotide Polymorphism (SNP) mutations within the Vkorc1 gene are resistant to the effects of anticoagulant rodenticide use and this influences the effectiveness of control strategies that rely on such rodenticides. This study examined the prevalence of rat SNP mutations in Singapore to inform the effectiveness of anticoagulant rodenticide use. A total of 130 rat tail samples, comprising 83 Rattus norvegicus (63.8%) and 47 Rattus rattus spp. (36.2%) were conveniently sampled from November 2016 to December 2019 from urban settings and sequenced at exon 3 of Vkorc1. Sequencing analysis revealed 4 synonymous and 1 non-synonymous mutations in Rattus rattus spp. samples. A novel synonymous mutation of L108L was identified and not previously reported in other studies. Non-synonymous SNPs were not detected in the notable codons of 120, 128 and 139 in Norway rats, where these regions are internationally recognised to be associated with resistance from prior studies. Our findings suggest that the prevalence of anticoagulant rodenticide resistance in Singapore is low. Continued monitoring of rodenticide resistance is important for informing rodent control strategies aimed at reducing rodent-borne disease transmission.

Introduction

Rodents are identified globally to be pests and vectors for a variety of diseases transmissible to humans. Leptospirosis is a common zoonotic disease worldwide, communicable to humans through direct excreta contact from infected mammals, but rodents are well-known reservoirs for it. Approximately 1.03 million cases are reported with 58,900 deaths annually mainly in tropical regions, resulting it to be a prominent zoonotic pathogen of concern in poorer regions of South and Southeast Asia [1,2]. Hantavirus haemorrhagic fever with renal syndrome (HFRS) or Hantavirus pulmonary syndrome (HPS) is another rodent-borne pathogen which can be contracted via inhalation of excreta aerosols. HFRS is widespread in Asia and Europe with mortality rates of about 40% [3,4]. Rats also serve as amplifying hosts to diseases transmitted by arthropod vectors that are parasitic to rats. The Oriental Rat Flea (Xenopsylla cheopis) can harbour Rickettsia typhi, which is responsible for causing murine typhus. Based on international seroepidemiological studies, murine typhus has a morbidity rate of 3–36% and is endemic in coastal areas of temperate climates in America, Europe and Asia [5]. Apart from the disease burden they carry, rodents increase economic costs to society by causing damage to structures, consumer goods and food. It was estimated that United States incurred losses of about $19 billion dollars per year due to the destruction caused by rats [6].

In order to curtail the spread of rodent-borne diseases, reducing the vector population is a key approach implemented across many countries. Physical methods such as cage traps, snap traps and glue boards are frequently used. Physical traps do not result in any chemicals that could leach into the environment, pose no risk of secondary poisoning to other animals, and unintended trapped wildlife can be subsequently released. The use of chemical control in food production premises could be risky due to its potential to contaminate food. Therefore, the use of physical methods can be useful in such environments to avoid compromising food safety. Moreover, carcasses of rats can also be easily located and disposed to avoid the smell of decay. However, these methods are labour intensive and not as cost-effective, especially in countries where labour costs are higher than rodenticides [7]. Due to the neophobic nature of rodents, trap success is usually low and declines significantly after one to two days of trapping [8].

Alternatively, the use of rodenticides offers a much cheaper option with lesser man hours required for placement and maintenance [9]. These baits can come in various forms such as bait blocks or in powdered form and can be mixed with food-based materials to make it highly attractive to rats for consumption. It is commonly placed directly into burrows or suspended as hanging bait blocks in the sewer lines, where it may be more physically challenging to deploy traps due to space constrains. The use of bait stations helps to reduce non-target animal ingestion. In addition to rodenticides effectiveness in reducing rat population [10], long-term preventive baiting can also help in keeping rodent populations low.

Over the years, inappropriate or excessive use of these rodenticides has resulted in resistance, which has been a prevailing issue in many countries for the past few decades [11–13]. It has been postulated that the emergence of rodenticide resistance is associated with the selection pressure exerted from rigorous use of anticoagulants [11,12,14]. Another possible reason for increasing prevalence of rodenticide resistance is that it is transferrable from parent to offspring [15,16]. This poses a challenge for vector control, as physical control may be the only alternative for reducing rodent populations. Apart from the high labour costs associated with recurrent visits to check traps, only a fraction of rodents is removed from the site of infestation, allowing rapid population recovery in weeks to months. As such, the main objective of reducing the vector for rodent-borne diseases may only be temporary. Ironically, culling may even increase the prevalence of certain zoonotic pathogens in rats, as physical intervention disrupts the social hierarchy of rats [17].

Anticoagulant resistance in rodents occurs when rodents develop resistance to the detrimental effects of anticoagulant, thus resulting in its efficacy loss despite correct application [18]. The mechanism for anticoagulants is such that these agents bind specifically to the Vitamin K epoxide reductase complex subunit 1 (VKORC1) enzyme and inhibits its activity. This enzyme is encoded by Vkorc1 located in chromosome 1.
for rats, chromosome 7 for mice and chromosome 16 for humans \cite{19, 20}. VKORC1 plays a crucial role in the vitamin K cycle, where its function is to reduce vitamin K 2,3-epoxide to its reduced form, vitamin K hydroquinone. It is an essential precursor for another enzyme γ-glutamyl carboxylase, which is involved in catalysing post-translational modification of vitamin K dependent proteins required for the cessation of bleeding \cite{21, 22, 23, 24}. Resistance to rodenticide happens when mutations known as single nucleotide polymorphisms (SNPs) occurs in \textit{Vkorc1}. This could cause non-synonymous mutations that results in a different amino acid being incorporated into the VKORC1 enzyme during translation. Consequently, anticoagulants are unable to bind effectively to VKORC1 to exert its effect \cite{22, 23, 24}. In-vivo studies done previously have identified that SNPs in codons 139, 128 and 120 of exon 3 confers resistance to rats \cite{20, 25, 26}.

Extensive studies on the distribution and prevalence of \textit{Vkorc1} mutations have been carried out mainly in the European regions \cite{16, 26, 27, 28, 29, 30}. The growth and geographical expansion of mutant rat populations may compel an increased reliance on physical population control methods, thus increasing the cost burden to health and pest control authorities. The use of anticoagulant rodenticides is common in Southeast Asia but few studies have examined if rodenticide resistance has reduced the effectiveness of anticoagulant rodenticide use. One particular study in Indonesia has demonstrated that the rats displayed resistant phenotypes in oil palm plantations that have been subjected to intensive anticoagulant baiting \cite{21}.

This study aimed to: (a) identify molecular indicators or novel mutations of anticoagulant resistance in exon 3 of \textit{Vkorc1} for both the Norway (\textit{Rattus norvegicus}) and Roof rats (\textit{Rattus rattus} spp.), and (b) the prevalence and distribution of these SNPs, in order to inform the development and review of rodent control strategies.

**Materials And Methods**

**Study site**

Singapore is located in Southeast Asia, at the southernmost region of Peninsular Malaysia. There are approximately 5.7 million residents living on this island with a size of 728.3 km\textsuperscript{2} \cite{32, 33}, a country with very high population density. Large majority of the island is comprised of high-rise buildings, whereby about 80% of the citizens reside in public housing known as Housing & Development Board (HDB) flats. General wastes from household buildings are consolidated into bin chutes before transportation by external waste management companies to incineration plants for treatment. There were a total of 37,527 licensed food establishments in Singapore as at 2019 \cite{34}, an average of about 51.5 per square kilometer.

**Ethics approval**

This study was part of the National Environment Agency's existing integrated national programme of rodent surveillance and control. Our study did not involve any human subjects. The Environmental Health Institute of the National Environment Agency, Singapore (NEA) reviewed the protocols and gave study approval (TS217). All applicable national guidelines for the care and use of animals were followed. Rodents trapped in the study did not belong to endangered or protected species.

**Rat tissue sampling**

\textit{R. norvegicus} and \textit{R. rattus} spp. samples were selected for this study as they were the two most common species of rats in Singapore. This study employed a cross-sectional study with Norway and Roof rat samples collected via opportunistic sampling from shopping malls, common residential areas, back alleys of food establishments and parks located across Singapore. Samples comprised tails and carcasses that were provided by pest control companies directly after their population control operations. For whole carcasses received, the tails were separated from the body and used for DNA extraction. Samples delivered or collected were labelled with their address location along with the species through morphological identification. A total of 83 \textit{R. norvegicus} and 47 \textit{R. rattus} spp. were tested from November 2016 to December 2020. A small portion of flesh and muscle of each tail sample (1 cm from proximal end) were carefully excised, while avoiding the outer most layer of skin. This was done is to avoid any cross-contamination from the sampling carried out by the pest control, as well as any residual soil and dirt that could hamper downstream extraction procedures. DNA for these tail samples were extracted with the DNeasy Blood & Tissue Kit (QIAGEN\textsuperscript{®}), following their standard protocol provided.

**PCR amplification of \textit{Vkorc1} and sequencing procedures**

Polymerase Chain Reaction was carried out in 20µL reactions to amplify exon 3 of \textit{Vkorc1}. Specific primers that were designed in accordance to Grandemange et al. (2009) were used to flank exon 3. Primer sequence are as follows: Exon 3 Forward 5'-TTTACCAGAAGCACCTGCTGCC-3' and Exon 3 Reverse 5’-ACACTTGGGCAAGGCTCATGTG-3', with an expected amplified fragment size of 354 base pairs \cite{28}. The amplification was carried out using the Thermo Scientific\textsuperscript{™} Phusion\textsuperscript{™} Flash High-Fidelity PCR Master Mix with the following parameter: Initial denaturation at 98°C for 10 seconds, 35 cycles of denaturation at 98°C for 5 seconds, annealing at 69°C for 5 seconds and extension at 72°C for 10 seconds,
following with the final extension of 72°C for 1 minute. Amplified fragments were subsequently confirmed by gel electrophoresis on 1.5% agarose gel with GelRed® Nucleic Acid Gel Stain. Amplified fragments were then purified by FavorPrep™ GEL/PCR Purification Kit (FAVORGEN®) before sending to 1st Base Asia for DNA sequencing.

Species confirmation

For tail samples that are ambiguous, 2µL of extracted DNA was amplified using primers that are specific towards cytochrome b, which is located within the mitochondrial DNA. Primer sequences for mcytb forward and mcytbHb reverse were 5’-CCATCGTTGTAATTCAACTATAG-3’ and 5’GAATGGGAGAATGAAGTGGAATGCG-3’ respectively, designed with reference to Aplin et al. (2011) [35]. PCR amplification was carried out using the Thermo Scientific™ Phusion™ Flash High-Fidelity PCR Master Mix with the following parameter: Initial denaturation at 98°C for 10 seconds, 35 cycles of denaturation at 98°C for 5 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 10 seconds, followed by the final extension of 72°C for 1 minute. The PCR product was then purified and sequenced before performing Basic Local Alignment Search Tool (BLAST) analysis and subsequently the construction of phylogenetic tree using the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software.

Screening of single nucleotide polymorphisms

Sequencing files were visually analysed using DNASTAR Lasergene SeqMan Pro and any low-quality ends were edited. BioEdit Sequence Alignment Editor was then used for ClustalW multiple alignment to compare the sequenced R. norvegicus and R. rattus spp. samples to a published wild-type reference sequence from GenBank [25, 36, 37] (Accession No. AY423047). SNPs identified while using the alignment editor were traced back to each individual sample in SeqMan Pro to determine if the mutation was heterozygous or homozygous.

Results

Rodent population

R. norvegicus samples were collected from outdoor areas such as burrows and bin chutes around public residential estates. R. rattus spp. samples were obtained from indoor premises within false ceilings of shopping malls, industrial buildings, private residential buildings and suburban regions like parks or nature reserves. The sampling locations are portrayed in Fig. 1.

In total, 63.8% (83/130) of R. norvegicus and 36.2% (47/130) of R. rattus spp. tail samples were received and tested for SNPs. None of the R. norvegicus samples had mutations in codons 139, 128 or 120. No novel mutations were detected in the whole of exon 3, when compared with the GenBank AY423047 sequence.

SNP mutations in R. rattus spp. samples were not detected in the key codons of 139, 128 or 120. However, we detected the presence of heterozygous and homozygous mutations in other codons of exon 3. The electropherogram profiles in Figure 2 shows the comparison of three different R. rattus spp. samples at the same codon. Samples that have heterozygous haplotypes will have double-coloured peaks at a particular nucleotide location as seen in Figure 2b. SNPs that were detected in exon 3 of R. rattus spp. samples are denoted by the wild type amino acid, followed by the codon position and then the mutant amino acid. These SNPs and their codon location can be found in Table 1.

| Codon and Mutation | Codon Position | Codon WT | Codon Mutant | Amino Acid WT | Amino Acid Mutant |
|--------------------|----------------|----------|--------------|---------------|------------------|
| S103S<sup>a</sup>  | 103            | TCT      | TCC          | Serine        | Serine           |
| I107I<sup>a</sup>  | 107            | ATC      | ATA          | Isoleucine    | Isoleucine       |
| L108L<sup>a</sup>  | 108            | CTG      | TTG          | Leucine       | Leucine          |
| T137T<sup>a</sup>  | 137            | ACC      | ACT          | Threonine     | Threonine        |
| A143V<sup>b</sup>  | 143            | GCG      | GTG          | Alanine       | Valine           |

<sup>a</sup> SNP mutation that is silent

<sup>b</sup> SNP mutation that brings about a substitution in amino acid

WT: Wild-type sequence/amino acid
Results for SNP in exon 3 of *R. rattus spp.*

Five SNPs were detected at exon 3 for the *R. rattus spp.*, of which one caused a substitutional mutation of GCG to GTG at codon 143 resulting in the amino acid valine replacing alanine (see Fig. 2). The other four mutations at codons 103, 107, 108 and 137 were silent. Allelic frequencies for both species of rats in exon 3 are summarised in Table 2.

Table 2
Summary of SNPs found in exon 3 of *Vkorc1* with the respective substitution of amino acids of *R. norvegicus* (*n* = 83) and *R. rattus spp.* (*n* = 47) when compared to the GenBank AY423047 wild type sequence.

| Codon Position | Codon WT | Codon Mutant | Substitution mutation & Amino acid change | Codon WT | Codon Mutant | Substitution mutation & Amino acid change | Frequency (%) | Homozygous Genotype | Heterozygous Genotype | Frequency (%) | Homozygous Genotype | Heterozygous Genotype |
|----------------|----------|--------------|------------------------------------------|----------|--------------|------------------------------------------|-------------|---------------------|---------------------|-------------|---------------------|---------------------|
| 103            | TCT      | TCC          | Ser103Ser                                | 100      | 83           | 0                                        | 0           | 0                   | 0                   | 0           | 0                   | 0                   |
| 107            | ATC      | ATA          | Ile107Ile                                | 0        | 0            | 0                                        | 0           | 0                   | 0                   | 0           | 0                   | 0                   |
| 108            | CTG      | TTG          | Leu108Leu                                | 0        | 0            | 0                                        | 0           | 0                   | 0                   | 0           | 0                   | 0                   |
| 137            | ACC      | ACT          | Thr137Thr                                | 0        | 0            | 0                                        | 0           | 0                   | 0                   | 0           | 0                   | 0                   |
| 143            | GCG      | GTG          | Ala143Val                                | 0        | 0            | 0                                        | 17.0        | 3                   | 5                   | 17.0        | 3                   | 5                   |

All of the *R. rattus spp.* samples carried silent homozygous SNP mutations in codons 107 and 137. In addition, 27.7% (13 out of 47, 3 homozygous and 10 heterozygous) of the samples had the silent SNP mutation in codon 108 (Leu108Leu). 17.0% of the samples (8 out of 47, 3 homozygous and 5 heterozygous) had SNP mutations in both codons 103 (Ser103Ser) and 143 (Ala143Val). These samples were located approximately 6km or more apart from each other and were obtained from settings such as shopping malls and parks (Fig. 1).

Discussion

In this study, we sought to determine the prevalence of anticoagulant rodenticide resistance in Singapore to inform rodent management strategies. We did not find any evidence of anticoagulant rodenticide resistance in the *R. norvegicus* populations. Moreover, SNPS were absent for the whole of exon 3 for all Norway rat samples, identical to that of the wild-type. This finding was surprising, given that *R. norvegicus* was the most common species of rodents found in urban environments of Singapore [38], and that anticoagulant rodenticide has been widely used by pest control operators (PCOs) to treat heavily rodent infested places in the last 20 years. The findings in our study for *R. norvegicus* differed from others undertaken in Netherlands, France, England, Germany, Hungary, Azores, USA and Argentina [12, 14, 15, 28, 30]. The majority of the missense SNPs proven to cause resistance in Norway rats that were detected in the European regions were Tyr139Cys, Tyr139Phe, Leu128Gln, Leu128Ser and Leu120Gln. Interestingly, *R. norvegicus* samples in Indonesia and Thailand (which are located in Southeast Asia) were reported to contain both missense and silent mutations [12]. A possible explanation for the absence of the above-mentioned SNPs in Singapore is that the urban conditions could be relatively harsh for Norway rats to thrive. In Singapore, there are well established refuse management and public cleaning programmes that reduces access to food waste and keeps the environment clean. Strict laws and regulations are in place to minimize littering, poor waste management and food hygiene practices in food retail establishments that can generate food sources to facilitate rodent population growth. This reduces the accessibility to food sources, leading to tougher competition amongst rat colonies. Even if spontaneous resistance mutations occurred within a few individuals of the rat population, they might not be fit enough to survive until they produce the next generation of pups. Similarly, a good and comprehensive rodent control programme that comprises of pulsed-baiting techniques [39] and alternating between types of rodenticide usage and physical trapping strategy may also result in low resistance frequency.

In contrast, the Roof rats contained multiple SNPs within exon 3 itself comprising of a variety of SNPs that are either silent or missense mutations. Although SNPs were not detected in the widely studied codons of 139, 128 or 120 that have been proven to confer resistance [20, 25, 26], it was interesting to find other mutations and this suggests at the possibility of local rat populations in acquiring rodenticide resistance phenotypes. In our study, roof rats carried multiple silent mutations within exon 3 of *Vkorc1*, namely at codons Ser103Ser, Ile107Ile, Leu108Leu and Thr137Thr. This essentially means the nucleotide variants do not result in a substitution of amino acid, allowing the VKORC1 enzyme to retain its protein structure and function. The two most common silent mutations observed were Ile107Ile and Thr137Thr and this was found in all of the Roof rats carrying both SNPs in homozygous form. In fact, all of the *R. rattus* samples tested in New Zealand and India demonstrated the exact same pattern of silent mutation at both of these codons [36, 40]. One reason for this finding could be the outcome of genetic bottleneck
events or founder effect that occurred in the past. As SNPs in the \textit{Vkorc1} gene is heritable from parents\cite{15,16}, all that is required is for a single founder individual having these two SNPs to pass down the mutations to modern day rat populations. Due to Singapore's strategic location as a trading hub since the 19th century, ship vessels frequent this island and it is likely that stowaway rats from neighbouring countries are imported many years ago. Nevertheless, it is interesting to note that \textit{R. norvegicus} from Indonesia and Thailand have also been reported to exhibit similar SNP mutation patterns at codons 107 and 137\cite{12}. Hence, the silent mutations in these two codons are not only exclusive to \textit{R. rattus}, but also able to occur in \textit{R. norvegicus} as well.

A new silent SNP mutation that has not been described before was identified in this study. It was located in codon 108 with the substitution of a single cytosine nucleotide to tyrosine. About a quarter of the Roof rat population possess the Leu108Leu variant, which is quite substantial given that there is no substitution of amino acid involved. The final silent mutation detected in this study was the Ser103Ser variant. What makes this SNP interesting is that all the Roof rats with it simultaneously also carried the Ala143Val SNP, the only amino acid substitution observed in this study. Rats that are heterozygous for the SNP at codon 103 were observed to be heterozygous for the Ala143Val variant and likewise if it was homozygous. It is quite likely that the Ala143Val genotype has a strong association with the 103Ser103 silent mutation in exon 3. Although there were only eight samples exhibiting this genotype amongst the roof rats, they were obtained from the four corner regions of Singapore with distances of 6km or more apart. Therefore, it is quite unlikely that these groups of roof rats were related in any manner. As this genotype is known to be seen in the wild type VKORC1 protein of other species such as humans and mice, it is considered to be a neutral mutation\cite{12}. However, a slightly more recent study in Indonesia in 2013 tested the Asian House Rats, \textit{R. tanezumi}, sampled from areas with intensive coumatetralyl usage had comparable genotypic patterns. It was reported that the Ala143Val mutation was found in 9 rats and 7 of them displayed resistant phenotypes\cite{21}. Further research is required to elucidate the mechanism by which this amino acid substitution truly confers resistance.

Molecular analysis of mitochondrial DNA from fossils found in present day traced that Norway rats historically originated from Southwestern China about 1.3 million years ago\cite{41}. Also, for the Roof rats, a strong phylogeographic pattern indicates that they were native to South Asia and Indochina regions\cite{35}. Despite studies depicting these two species having Asian origins, rodenticide resistance was first described in Europe and then United States about one to two decades after the introduction of rodenticide in the early 1950s\cite{16,42,43}. This strongly suggests that the evolution of anticoagulant resistance could be the result of massive or improper use of warfarin in the western regions of the world where it was developed and used. As such, the exon 3 SNP profile of rats in Singapore was unexpected, since rodenticides have been used for the past 20 years. Likewise for China, anticoagulant rodenticides have been widely used for over 30 years and yet the frequency of resistance has been reported to be low\cite{44}.

\textit{Vkorc1} has been extensively studied and alterations to the protein structure is known to affect the blood clotting mechanism. Future studies can include investigating SNP mutation profiles of exon 1 and 2 for other non-synonymous mutations, coupled along with blood clotting response (BCR) test. It will also be interesting to investigate rodents’ ability in metabolising the ingested rodenticide by measuring gene expression of cytochrome P450 gene. The P450 enzyme breaks down a wide range of drugs and an enhanced expression of certain P450 genes can increase drug metabolism rate\cite{45}. This could result in anticoagulants being cleared from their cardiovascular system quickly, causing rodenticides to be ineffective.

Our results indicate that the prevalence of SNP mutations in the rat population in Singapore was low and it is reassuring that there is no widespread resistance. Nonetheless, further studies to provide a better geographical representation and spatial resolution of assessed rodenticide resistance would benefit the review of site-specific intervention strategies. Regular monitoring of the extent and evolution of mutations in rodent populations would facilitate the review of resistance management strategies.

Given that this was the first study carried out in Singapore since the registration and use of rodenticides, we did not have historical information on the status of anticoagulant resistance or types of SNP mutations for comparison over time. We obtained our samples through convenience sampling and our results may not be generalizable to the entire population of rodents in Singapore. However, we have no reason to believe that rodenticide resistance in the areas from which the rodent samples were obtained had lower resistance compared to other areas that had no samples since population activity was higher and control activities more extensive in the former. While screening for SNPs in \textit{Vkorc1} does provide some information on the mutations associated with resistance, it is a proxy of the actual level of resistance towards rodenticides.

**Conclusion**

A new \textit{Vkorc1} silent SNP mutation of Leu108Leu was discovered in a few \textit{R. rattus} populations of this study, though it was not associated with anticoagulant resistance. Our study findings suggest that rodenticide resistance in Singapore was either low or an absent, even after nearly two decades of rodenticide use. This provides support for the continued use of anticoagulants as a means for effective rodent population control. Continued rodenticide resistance monitoring will inform rodent control strategies for reducing rodent-borne disease transmission.
Declarations

Data availability

The data used in this study are owned by a third party. Requests for the data can be made to the Environmental Health Institute of the National Environment Agency at Contact_NEA@nea.gov.sg.

Acknowledgements

We would like to thank the various pest control operators who helped to collect rat tail samples for this study.

Author contributions

J.A., C.C, and M.H. conceptualised the study. C.C. curated the data. J.A. and C.C. were responsible for the methodology. J.A. and C.C. were responsible for the project administration. C.C. and M.H. facilitated in the collection of samples for the study. J.A. supervised this study. C.C. generated the map presented. C.C. wrote the first draft of the paper. J.A., C.C. and N.L.C reviewed and edited the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no conflict or competing interests.

References

1. Costa, F. et al. Global morbidity and mortality of leptospirosis: A systematic review. *PLoS Negl. Trop. Dis.* 9, e0003898, doi:10.1371/journal.pntd.0003898 (2015).
2. Cosson, J.-F. et al. Epidemiology of Leptospira transmitted by rodents in southeast asia. *PLoS Negl. Trop. Dis.* 8, e2902, doi:10.1371/journal.pntd.0002902 (2014).
3. Jonsson, C. B., Figueiredo, L. T. M. & Vapalahti, O. A global perspective on Hantavirus ecology, epidemiology, and disease. *Clin. Microbiol. Rev.* 23, 412-441, doi:10.1128/CMR.00062-09 (2010).
4. Vaheri, A. et al. Uncovering the mysteries of Hantavirus infections. *Nat. Rev. Microbiol.* 11, 539-550, doi:10.1038/nrmicro3066 (2013).
5. Peniche Lara, G., Dzul-Rosado, K. R., Zavala Velázquez, J. E. & Zavala-Castro, J. Murine typhus: Clinical and epidemiological aspects. *Colomb. Med. (Cali).* 43, 175-180 (2012).
6. Pimentel, D., Lach, L., Zuniga, R. & Morrison, D. Environmental and economic costs of nonindigenous species in the United States. *Bioscience.* 50, 53-65, doi:10.1641/0006-3568(2000)050[0053:EAECON]2.3.CO;2 (2000).
7. Smith, R. & Meyer, A. Rodent Control Methods: Non-chemical and Non-lethal Chemical, with Special Reference to Food Stores in Buckle, A.P. (eds) *Rodent Pests and their Control* (2nd edn) 101-122 (CAB International, 2015).
8. Himsworth, C. G., Jardine, C. M., Parsons, K. L., Feng, A. Y. T. & Patrick, D. M. The characteristics of wild rat (*Rattus spp*.) populations from an inner-city neighborhood with a focus on factors critical to the understanding of rat-associated zoonoses. *PLoS ONE.* 9, e91654, doi:10.1371/journal.pone.0091654 (2014).
9. Mari Saez, A. et al. Rodent control to fight Lassa fever: Evaluation and lessons learned from a 4-year study in Upper Guinea. *PLoS Negl. Trop. Dis.* 12, e0006829-e0006829, doi:10.1371/journal.pntd.0006829 (2018).
10. Baldwin, R., Quinn, N., Davis, D. & Engeman, R. Effectiveness of rodenticides for managing invasive roof rats and native deer mice in orchards. *Environ. Sci. Pollut. Res.* 21, 5795-5802, doi:10.1007/s11356-014-2525-4 (2014).
11. Hadler, M. R. & Buckle, A. P. Forty five years of anticoagulant rodenticides - past, present and future trends. *Proc. 15th Vertebr. Pest Conf.* 149-155 (1992).
12. Rost, S. et al. Novel mutations in the VKORC1 gene of wild rats and mice – a response to 50 years of selection pressure by warfarin? *BMC Genet.* 10, 4, doi:10.1186/1471-2156-10-4 (2009).
13. Buckle, A., Prescott, C. & Ward, K. J. Resistance to the first and second generation anticoagulant rodenticides - A new perspective. *Proc. 16th Vertebr. Pest Conf.* 138-144 (1994).
14. Goulois, J., Lambert, V., Legros, L., Benoit, E. & Lattard, V. Adaptive evolution of the Vkorc1 gene in *Mus musculus domesticus* is influenced by the selective pressure of anticoagulant rodenticides. *Ecol. Evol.* 7, 2767-2776, doi:10.1002/eece.3.2829 (2017).
15. Meerburg, B. G., van Gent-Pelzer, M. P. E., Schoelitzs, B. & van der Lee, T. A. J. Distribution of anticoagulant rodenticide resistance in *Rattus norvegicus* in the Netherlands according to Vkorc1 mutations. *Pest Manag. Sci.* 70, 1761-1766, doi:10.1002/ps.3809 (2014).
16. Lund, M. Rodent resistance to the anticoagulant rodenticides, with particular reference to Denmark. *Bull. World Health Organ.* **47**, 611-618 (1972).
17. Lee, M. J. *et al.* Effects of culling on *Leptospira interrogans* carriage by rats. *Emerg. Infect. Dis.* **24**, 356-360, doi:10.3201/eid2402.171371 (2018).
18. Greaves, J. H. Resistance to anticoagulant rodenticides in *Buckle, A. P. and Smith, R. (eds) Rodent Pests and their Control* (2nd edn) 187-208 (CAB International, 2015).
19. Lefebvre, S. B., Benoit, E. & Lattard, V. Comparative biology of the resistance to vitamin K antagonists: An overview of the resistance mechanisms in *Anticoagulation Therapy* (Basaran O, Biteker M eds) 20-45 (Intech Open, 2016).
20. Grandemange, A. *et al.* Consequences of the Y139F Vkorc1 mutation on resistance to AVKs: in-vivo investigation in a 7th generation of congenic Y139F strain of rats. *Pharmacogenet. Genomics.* **19**, 742-750, doi:10.1097/FPC.0b013e32832ee55b (2009).
21. Sadowski, J. A., Esmon, C. T. & Suttie, J. W. Vitamin K-dependent carboxylase. Requirements of the rat liver microsomal enzyme system. *J. Biol. Chem.* **251**, 2770-2776 (1976).
22. Mooney, J. *et al.* VKORC1 sequence variants associated with resistance to anticoagulant rodenticides in Irish populations of *Rattus norvegicus* and *Mus musculus domesticus*. *Sci. Rep.* **8**, 4535, doi:10.1038/s41598-018-22815-7 (2018).
23. Thijsen, H. H. W. Warfarin-based rodenticides: Mode of action and mechanism of resistance. *Pestic. Sci.* **43**, 73-78, doi:https://doi.org/10.1002/ps.2780430112 (1995).
24. Bell, R. G. & Caldwell, P. T. Mechanism of warfarin resistance. Warfarin and the metabolism of vitamin K1. *Biochemistry.* **12**, 1759-1762, doi:10.1021/bi00733a015 (1973).
25. Pelz, H.-J. *et al.* The genetic basis of resistance to anticoagulants in rodents. *Genetics.* **170**, 1839-1847, doi:10.1534/genetics.104.040360 (2005).
26. Baert, K., Stuyck, J., Breyne, P., Maes, D. & Casaer, J. Distribution of anticoagulant resistance in the brown rat in Belgium. *Belg. J. Zool.* **142**, 39-48 (2012).
27. Prescott, C. V., Buckle, A. P., Gibbons, J. G., Allan, E. N. W. & Stuart, A. M. Anticoagulant resistance in Norway rats (*Rattus norvegicus* Berk.) in Kent – a VKORC1 single nucleotide polymorphism, tyrosine139phenylalanine, new to the UK. *Int. J. Pest Manag.* **57**, 61-65, doi:10.1080/09670874.2010.523124 (2010).
28. Grandemange, A., Lasseur, R., Longin-Sauvageon, C., Benoit, E. & Berny, P. Distribution of VKORC1 single nucleotide polymorphism in wild *Rattus norvegicus* in France. *Pest Manag. Sci.* **66**, 270-276, doi:10.1002/ps.1869 (2009).
29. Goulois, J. *et al.* Evidence of a target resistance to antivitamin K rodenticides in the roof rat *Rattus rattus*: identification and characterisation of a novel Y25F mutation in the Vkorc1 gene. *Pest Manag. Sci.* **72**, 544-550, doi:10.1002/ps.4020 (2015).
30. Endepols, S., Klemann, N., Jacob, J. & Buckle, A. P. Resistance tests and field trials with bromadiolone for the control of Norway rats (*Rattus norvegicus*) on farms in Westphalia, Germany. *Pest Manag. Sci.* **68**, 348-354, doi:10.1002/ps.2268 (2011).
31. Andru, J., Cosson, J.-F., Caliman, J.-P. & Benoit, E. Coumatetralyl resistance of *Rattus tanezumi* infesting oil palm plantations in Indonesia. *Ecotoxicology.* **22**, 377-386, https://doi.org/10.1007/s10646-012-1032-y (2012).
32. Department of Statistics Singapore. *Population and Population Structure* https://www.singstat.gov.sg/find-data/search-by-theme/population-and-population-structure/latest-data (2020).
33. Department of Statistics Singapore. *Environment* https://www.singstat.gov.sg/find-data/search-by-theme/society/environment/latest-data (2020).
34. Department of Statistics Singapore. *M890531 - Licensed Food Establishments (End Of Period), Annual* https://www.tablebuilder.singstat.gov.sg/publicfacing/createDataTable.action?refId=14624 (2021).
35. Aplin, K. P. *et al.* Multiple geographic origins of commensalism and complex dispersal history of black rats. *PLoS ONE.* **6**, e26357, doi:10.1371/journal.pone.0026357 (2011).
36. Cowan, P. E. *et al.* Vkorc1 sequencing suggests anticoagulant resistance in rats in New Zealand. *Pest Manag. Sci.* **73**, 262-266, doi:10.1002/ps.4304 (2016).
37. Rost, S. *et al.* Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature.* **427**, 537-541, doi:10.1038/nature02214 (2004).
38. Wong, T. W. *et al.* Hantavirus infections in humans and commensal rodents in Singapore. *Trans. R. Soc. Trop. Med. Hyg.* **83**, 248-251, doi:https://doi.org//10.1016/0035-9203(89)90666-4 (1989).
39. Dubock, A. Pulsed baiting - A new technique for high potency, slow acting rodenticides. *Proc. 10th Vertebr. Pest Conf.* **10**, 123-136 (1982).
40. Garg, N., Singla, N., Jindal, V & Babbar, B. Studies on bromadiolone resistance in *Rattus rattus* populations from Punjab, India. *Pestic. Biochem. Physiol.* **139**, 24-31 (2017).
41. Song, Y., Lan, Z. & Kohn, M. H. Mitochondrial DNA phylogeography of the Norway rat. *PLoS ONE*. 9, e88425, doi:10.1371/journal.pone.0088425 (2014).

42. Boyle, C. M. Case of apparent resistance of *Rattus norvegicus* Berkenhout to anticoagulant poisons. *Nature*. 188, 517, doi:10.1038/188517a0 (1960).

43. Jackson, W. B. & Kaukeinen, D. Resistance of wild Norway rats in North Carolina to warfarin rodenticide. *Science*. 176, 1343 (1972).

44. Ma, X. *et al.* Low warfarin resistance frequency in Norway rats in two cities in China after 30 years of usage of anticoagulant rodenticides. *Pest Manag. Sci.* 74, 2555-2560, doi:10.1002/ps.5040 (2018).

45. Markussen, M. D. K., Heiberg, A.-C., Fredholm, M. & Kristensen, M. Differential expression of cytochrome P450 genes between bromadiolone-resistant and anticoagulant-susceptible Norway rats: a possible role for pharmacokinetics in bromadiolone resistance. *Pest Manag. Sci.* 64, 239-248, doi:10.1002/ps.1506 (2008).

**Figures**

**Figure 1**

Map of Singapore depicting the locations of rats being sampled. Rats with the various SNPs are represented with different symbols and colours.
Figure 2

DNA nucleotide bases are each highlighted with a different colour. Electropherogram profiles of three different Rattus rattus spp. samples depicting wild type (a), heterozygous (b) and homozygous (c) SNP mutations at codon 143 of exon 3.