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Anticoagulant Rodenticides: Resistance and Residues in Norway Rats in France

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ABSTRACT: In the European Union (EU), anticoagulant rodenticides (AR) represent more than 90% of the commercially available products for use against commensal rodents. The only other active ingredients (CO₂, chloralose, corn cob) represent minor alternatives. A major issue in the EU is the resistance level of rat and mice populations, as well as potential non-target species exposure. This study presents results of surveys of anticoagulant resistance in Norway rats based on the sequencing of the VKORC1 gene, the major gene involved in AR and an investigation of the presence of AR residues detected in rodents trapped alive in urban and rural areas in order to investigate the potential risk of secondary poisoning of predators and scavengers. For resistance monitoring, rats were either trapped alive in the city of Lyon or its surroundings, or alternatively rat tails were obtained from pest control operators from France. Specific DNA primers were used for DNA sequencing and mutation identifications. AR residues were monitored by LC-MS-MS (for the 8 ARs marketed in Europe), with a limit of quantification of 1.0 µg/kg in liver samples. AR resistance appears to be extremely common (45-70% of all rats tested, depending on the part of France), with the notable exception of downtown Lyon where all rats are susceptible to AR. AR residues are detected in almost 100% of the rats trapped and tested (>200 individuals in/around Lyon). These results show that resistance is common in France, and evidence from neighboring countries suggests that this is an EU-wide problem. More surprising is the fact that all rodents tested contain detectable residues of AR, which could potentially result in secondary poisoning.

KEY WORDS: anticoagulants, France, genetic resistance, Norway rat, *Rattus norvegicus*, residues, resistance, rodent control, rodenticides

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INTRODUCTION

There is published evidence showing that resistant rodents will have a prolonged survival time in the presence of anticoagulant rodenticides (AR) as compared with susceptible (wild-type) rodents (Vein et al. 2013). The development of molecular tools has been a cornerstone for the monitoring of AR resistance detection in Norway rats (*Rattus norvegicus*). It is possible to identify the most common mutations in rat strains suspected of being of resistance with specifically designed primers to perform Q-PCR (Grandemange et al. 2009b). This technique allows for rapid and cost-effective determination of the resistance status, provided the effects of each identified mutation on VKOR activity have been confirmed *in vivo* or *in vitro* (Hodroge et al. 2011). Using these techniques, we were able to demonstrate the reality of resistance among Norway rat populations in France (Grandemange et al. 2010b) and similar work has been conducted in other European countries (Buckle 2012, Tanaka et al. 2013, Meerburg et al. 2014). Several mutations in the VKORC1 gene confer resistance to first generation AR (Pelz et al. 2005). Resistance is a co-dominant trait and susceptible individuals are homozygous wild-type individuals.

The purpose of the present study was:

- to determine the nature and frequency of resistance in Norway rat populations in rural and urban settings;
- to determine the nature and amount of AR residues detected in rats; and

- to determine the existence of a relationship between the resistance status and the presence of AR residues.

MATERIALS AND METHODS

Trapping

Trapping was conducted in 20 different farms around Lyon, France. The farms were selected in two different parts of the western suburbs of Lyon, 20 km apart: St Romain (ROM), and Givors (GIV). In the downtown area of Lyon (LYO), 7 different trapping sites were eventually selected, based on accessibility, safety, and evidence of rodent activity. Municipal areas were included (waste incinerator, waste water treatment plant, municipal parks, and gardens) as well as private buildings. Both live traps and snap traps were used. All traps were left unarmed with baits for several days. Trapping was conducted for 4-5 consecutive nights and all traps were left unarmed during weekends. Because of personal constraints, trapping was conducted in different sessions between October 2010 and July 2013. For each geographical area (LYO, GIV, ROM), 1- or 2-month trapping sessions were conducted. Trapping was concentrated in late winter and spring 2012 for the LYO area. Rodents trapped alive were transferred to the rodent unit at the veterinary college and euthanized with CO₂ according to our local procedures and AVMA recommendations. Rats were weighed and measured (from the nose to the tail) on that occasion. Gender, approximate age (based on body weight, hair coat,

gonadal development, or evidence of sexual activity or prior gestation) was recorded at the same time.

Resistance Monitoring

The resistance status was determined by RT-PCR on liver or tail samples with specific primers for each mutation tested (L120Q, L128Q, Y139F, Y139C, Y139S). Complete sequencing of the VKORC1 gene was done on all samples for which the abovementioned mutations were not identified (Grandemange et al. 2009b). Only the Y139F mutation was identified in all 3 areas.

AR Residue Determination

For each animal, the liver was removed immediately after death and kept frozen (-20°C) until analysis. A small piece (0.5g) of this liver was cut and used for AR residue determination. Prior testing conducted in the lab showed that AR distribution in the liver is fairly homogeneous (unpubl.). AR residues were determined by LC-MS-MS after adaptation of a former method (Fourel et al. 2010). All solvents and standards used were of the highest purity available (Cluzeau Info Labo, Ste Foy la Grande, France).

Liver samples were spiked with internal standard solution (dicoumarol, final concentration 50µg/L), then ground (Ultrathurax®), and extracted twice with acetone (final volume 20 ml). The liquid extract was centrifuged and the supernatant evaporated to dryness under gentle nitrogen stream at 40°C. Dry pellets were resuspended in 200 µL acetonitrile and sonicated for 30 s. Each sample was placed in the freezer for ca 10 min to allow for separation of the lipid upper phase. This procedure was repeated twice. All supernatants were combined and filtered (0.2 µm) to fill a vial for analysis.

In order to avoid cross contamination, all material was single-use whenever possible or thoroughly cleaned, rinsed, and solvent-rinsed between each individual sample. For each day of analysis, blank liver samples were extracted and treated with other field samples, known positive samples were added to the series, and confirmatory calibration points were used. An Agilent triple quad HPLC-MS-MS (Agilent Technologies, Santa Clara, CA, USA) was used (MS6410 with a 1200 series HPLC).

The method allows for the simultaneous determination of all ARs marketed in Europe. The limit of quantification could be determined at 1 µg.kg⁻¹ for each active substance.

Statistics

Because of the skewed distribution of AR residues as well as the differences in trapping efficiency between sites, all data were compared using non-parametric Kruskal-Wallis test with a significance level of 0.05.

Geographic Information System (GIS)

Each trap location was georeferenced with Global Positioning System (GPS) technology, and data were computed in GIS (a.k.a., Système d'Information Géographique or "SIG") using Quantum[®] 1.8.0. Lambert 93 projection.

RESULTS AND DISCUSSION

Rodent Population Description

The total number of rats trapped was 462. Gender and age distribution, as well as body weight, are described in Table 1. Overall, 40% of the individuals trapped were females; 52% were sexually mature individuals. These results are quite comparable to the population characteristics obtained in a longitudinal study in Vancouver, Canada by Himsworth et al. (2014). Rats from LYO were significantly different from the rural rats, with a higher proportion of males and of young adults. This difference may be attributed to the different period of trapping (mostly late winter and early spring in Lyon city), with young adults being more active to establish new burrows. Himsworth et al. (2014) obtained a very similar result during their 1-year study. For each age group, mean body weight and length were not significantly different between areas of trapping. As a consequence, these rodent populations appear quite similar and comparable to earlier descriptions of Norway rat populations in France (Le Louarn and St Girons 1977).

According to site accessibility and rodent population size, almost 30 different trapping sites were maintained throughout the study. Figure 1 presents the results in terms of number of individuals trapped for each location.

Resistance Status

Only the Y139F mutation was detected in the rat samples eventually analyzed. All susceptible (S) individuals were sequenced to confirm the absence of any other unexpected mutation and were all found to be actual wildtype (susceptible) rats. Overall, the allelic frequency of the mutation was 54.5%. Homozygous resistant (RR) rats accounted for 44% of the population, heterozygous (RS or I: intermediate) for 21%, and wild type (SS) 35%. As can be seen in Figure 2, there was a statistically significant difference in the distribution of the R and S genes across trapping areas, with the LYO inner city almost devoid of resistant rats (the few RR or RS individuals trapped were caught in the suburbs of the city), while the two selected rural areas were very similar and had >80% individuals with at least one R gene. This mutation has already been described as being the most prevalent in France (Grandemange et al. 2010b) but also is present in other countries in Europe (Buckle 2012, Pelz et al. 2005, Prescott et al. 2010). Very few studies, however, have investigated the frequency of resistance among urban or rural populations, apart from focal resistance areas (Buckle 2012, Endepols et al. 2012). In 2009, we showed that resistance could be a very common phenomenon at a national level (Grandemange et al. 2009b). In a recent survey in the Netherlands, Meeburg et al. (2014) identified 39% resistant individuals (no information on the homozygote status) carrying either Y139C or Y139F mutations, with some geographical link with either Germany or Belgium/France where these mutations are commonly detected. Our study is the first investigation, however, of the frequency of resistance in a large area with no specific resistance issue identified before analysis.

The most surprising phenomenon was the absence of

Table 1. Rat trapping results by county.

| County | N | F/M | Juvenile/sub-adult/adult | BW(g) |
|-----------------|-----|--------------|--------------------------|--------------------|
| LYON | 104 | 27/59* (18?) | 19/15/56*(14?) | 210.4 [23.0-520.0] |
| GIVORS (GIV) | 211 | 98/81 (32?) | 13/51/118 (29?) | 199.1 [11.0-470.0] |
| St Romain (ROM) | 147 | 63/42(42?) | 11/44/62 (30?) | 170.8 [9.8-388.0] |

? = undetermined

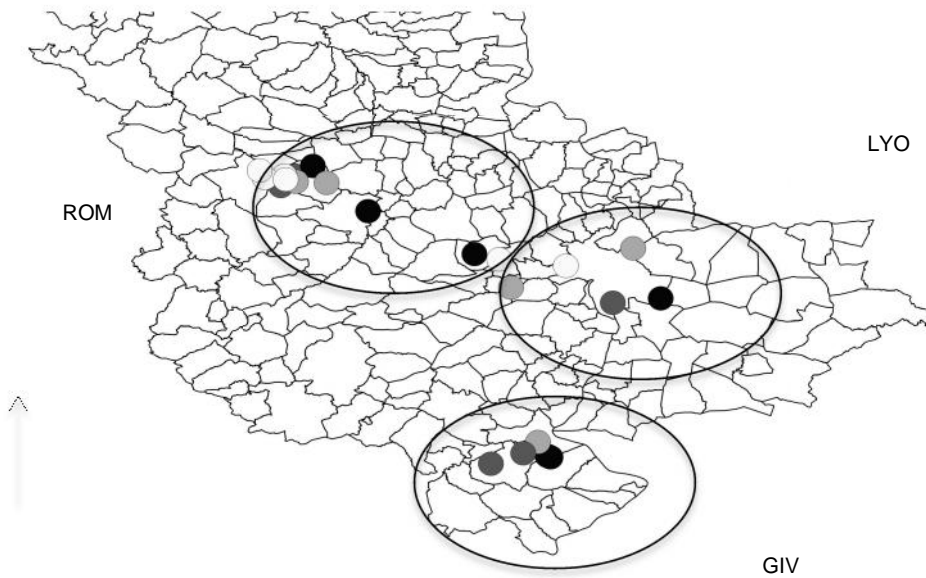


Figure 1. Sampling sites and number of rats trapped per site in the Lyon area (France). Circles represent quartiles.
 (white: Q1, 1-4 rats; light grey: Q2, 5-14 rats; dark grey: Q3, 15-28 rats; black: Q4, 29-64 rats).

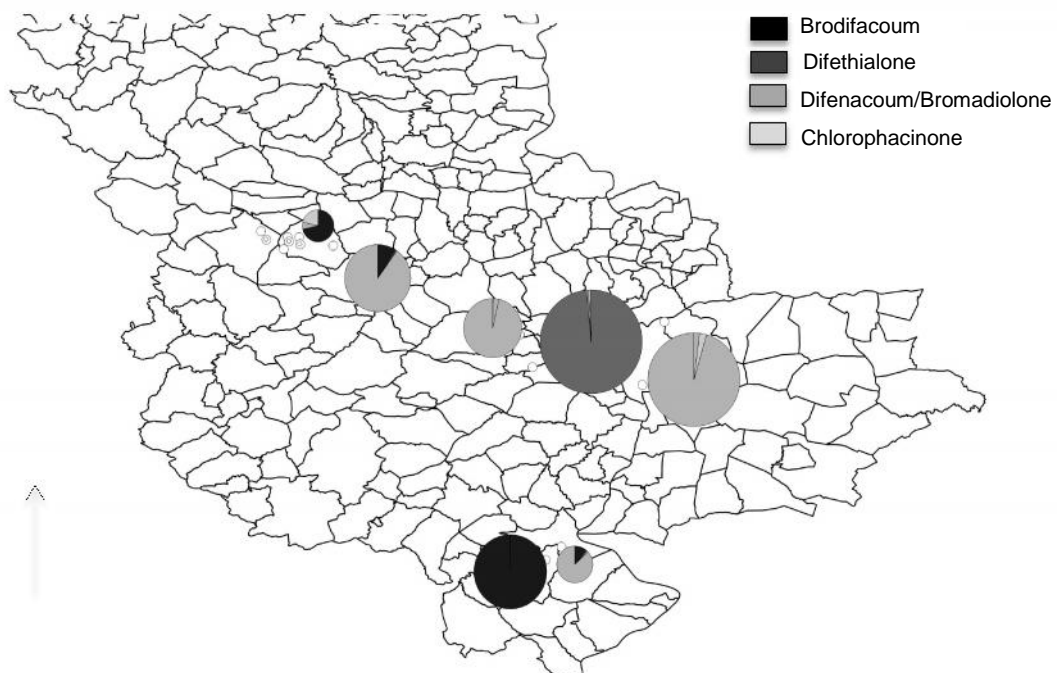


Figure 3. Median concentration of anticoagulant rodenticides and proportion of active substances detected in rat livers.
 Circles are proportional to the $\text{Log}_{10} [\text{AR}]+1$.

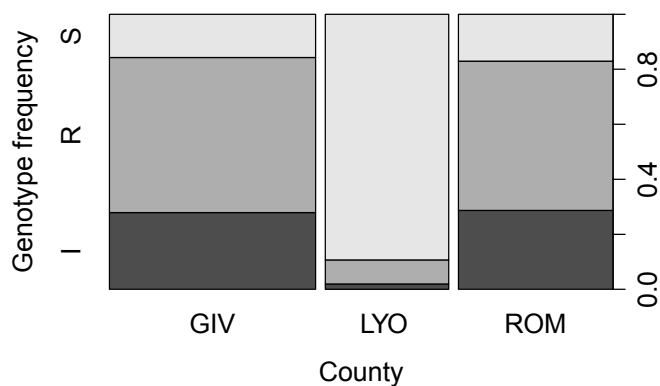


Figure 2. Distribution of R(RR), I(RS), or S(SS) individuals with respect to VKORC1 mutation Y139F by area of trapping.

any resistant genotype in the city limits of Lyon. Our ongoing studies on rat genetics indicate that the rat populations from LYO are not genetically related to the rural rats, which appear to be related [one single microsatellite associated with the mutation (J.F. Cosson, INRA-CBGP, Montpellier, France, pers. comm.)]. There is no reason to consider that this result could be biased by the sampling season, since subsequent trapping sessions consistently caught susceptible individuals (unpubl.). Interestingly, Heiberg (2009) described some level of resistance in rats from the sewer system in Copenhagen, but in the absence of any mutation in the VKORC1 gene.

AR Residues

Only 121 individuals could eventually be tested for AR residues in the liver (poor sample quality). Out of those, 119 contained at least one AR > LOQ (98.3%). The median content was 75 µg/kg, and 45% of all rats had [AR] > 100 µg/kg, a value often considered as significant of potential AR poisoning in non-target species (Thomas et al. 2011). More than 50% of all rats tested contained two active substances. The presence of residues down to a few µg/kg should be considered cautiously; there is evidence of exposure, but no evidence of effect at these low levels (this was not the purpose of this study).

There was a significant association between the status of the rat (found dead with hemorrhages or trapped alive) and the amount of AR residues detected, but also between the resistance status and the amount of AR found in the liver: the median liver concentration was 98 µg/kg in R and I rats, versus 31 µg/kg in S rats. Figure 3 presents the distribution of AR residues per sampling site. There was a great variety among sites, both in rural and urban areas. Overall, the total amount of AR residues was not different between sites, but the distribution of residues for each AR was sometimes different between sites (Table 2). As can be seen, residues detected in rats from the Lyon city were mainly bromadiolone and difenacoum, and the most potent second-generation ARs (difethialone and brodifacoum) were almost never detected. In the two rural areas, more potent AR were detected (mostly difethialone). Current regulations in France allow the use of all

ARs in and around buildings both in urban and rural settings. The frequency of resistance was much lower in LYO. VKORC1 mutations confer a strong resistance to first generation AR but also some degree of resistance towards bromadiolone and difenacoum. It is hypothesized that most PCOs do not apply difethialone or brodifacoum in LYO, since most rats are susceptible to ARs.

Table 2. Residues of AR per County
(median values in µg.kg⁻¹, * p < 0.05).

| | CHLORO | BROMA* | DIFEN* | BRODIF* | DIFET* |
|------|--------|--------|--------|---------|--------|
| GIV | 0.0 | 0.5 | 0.4 | 0.0 | 1.0 |
| LYON | 0.0 | 9.2 | 0.9 | 0.0 | 0.0 |
| ROM | 0.0 | 2.1 | 1.0 | 0.7 | 29.4 |

It was anticipated to find more AR in the liver of rats found dead with hemorrhages. The higher concentration of AR residues in resistant individuals was more surprising. One limitation of this finding, however, is that there was also a significant site-dependent distribution of resistance with most S individuals found in the city of Lyon. It may be considered that rodenticide baiting strategies may be quite different between rural and urban areas. It is known that baiting may be extensive in relation to the sewer system in large cities (Heiberg 2009) and is usually conducted by trained professionals, while baiting in rural areas may usually be done by farmers or untrained professionals, who barely follow the general recommendations and guidelines (Stuart et al. 2010).

However, all studies conducted so far have pointed out that resistant rats usually do not carry more residues than susceptible ones, neither with first-generation products such as chlorophacinone (Vein et al. 2013), nor second-generation products such as bromadiolone and difenacoum (Atterby et al. 2005, Berny et al. 2012). All authors conclude on the prolonged survival time, but do not describe any significant accumulation of AR in resistant rats. With chlorophacinone, evidence of liver metabolism and rapid turnover may play a role in this lack of accumulation. For the other ARs, all studies have been conducted with *ad libitum* feeding for several days (5-14), but this may potentially overcome the accumulation potential of some AR. Indeed, there is evidence that AR uptake is governed by two different mechanisms (passive uptake and active transport; Ockner et al. 1983). This probably is responsible for the increased toxicity of AR administered over several days at low doses. Saturation of transporters is more likely to occur when rats are fed *ad libitum* with rodenticides. The values detected in rats exposed experimentally are usually much higher than the ones detected in this field survey (median liver concentration 3,000-4,000 µg/kg with chlorophacinone, 600-1,000 µg/kg for whole body residues with difenacoum, for instance). Therefore, we can estimate that the rats trapped/killed in our study accumulated ARs to a more significant extent when they were resistant, probably for lack of saturation of their active transport system.

The median level detected (98 µg/kg) in the resistant rats also raises questions with respect to susceptibility of rats to anticoagulants. The Y139F mutation is known to confer resistance to first-generation ARs and to a certain extent to bromadiolone, and poorly so to difenacoum, but there is no evidence that this mutation can confer resistance to the most potent rodenticides (brodifacoum, difethialone, flocoumafen), which were detected in a large proportion of rats (Grandemange et al. 2009a). It is suggested, however, that rats survived mostly because the concentrations of AR detected in the liver were low enough.

CONCLUSIONS

The conclusions of this study are that resistance is usually common in European countries, mostly in rural areas, reaching levels close to 80% allelic frequency. There is also evidence of a high prevalence of presence of AR residue in Norway rats, with higher concentrations in resistant individuals, suggesting that AR residues accumulate to a higher extent in resistant animals exposed to low levels. The high proportion of individuals with AR residues >100 µg/kg also questions the diagnostic value of this threshold.

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