Anticoagulant rodenticide poisoning in animals of Apulia and Basilicata, Italy

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Summary
This study evaluates the presence of anticoagulant rodenticides in animals with a diagnosis of suspected poisoning and in bait samples. The survey was carried out from 2010 to 2012, in 2 regions of South Italy (Puglia and Basilicata) on 300 organs of animals and 90 suspected bait samples. The qualitative and quantitative analyses were conducted using an analytical method based on high-performance liquid chromatography (HPLC) with fluorimetric detection (FLD) for the simultaneous determination of 8 anticoagulant rodenticides (bromadiolone, brodifacoum, coumachlor, coumafuryl, coumatetralyl, difenacoum, flocoumafen, and warfarin). The presence of anticoagulant rodenticides was detected in 33 organs of animals (11% of the total) and 6 bait samples (7% of the total). The most commonly detected compound was coumachlor (47% of 39 positive samples) followed by bromadiolone (24%), and brodifacoum (11%). The species mostly involved in anticoagulant rodenticide poisoning were dogs and cats. This study emphasizes the relevance of the determinations of anticoagulant rodenticides in cases of suspected poisoning in veterinary practice.

Indagine sull'avvelenamento da rodenticidi anticoagulanti in Puglia e Basilicata (Italia)

Riassunto
Questo studio valuta la presenza di sospetti avvelenamenti da rodenticidi anticoagulanti in animali deceduti in due regioni italiane (Puglia e Basilicata) e inviati presso il Reparto di Diagnostica dell'Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata con una diagnosi di sospetto avvelenamento. L' indagine è stata condotta su 300 organi di animali e su 90 campioni di presunte esche inviate alla struttura dal 2010 al 2012. Le determinazioni quali-quantitative sono state effettuate mediante metodica analitica che impiega la cromatografia liquida ad elevate prestazioni abbinata alla rilevazione fluorimetrica. La metodica consente la determinazione simultanea di 8 rodenticidi anticoagulanti (bromadiolone, brodifacoum, coumachlor, coumafuryl, coumatetralyl, difenacoum, flocoumafen e warfarin) in organi di animali ed esche. In 33 organi di animali (11% del totale) e in 6 esche (7% del totale) è stata verificata la presenza di rodenticidi anticoagulanti. Il composto più frequentemente rilevato è stato il coumachlor (47% dei 39 campioni positivi) seguito dal bromadiolone (24%) e dal brodifacoum (11%). Le specie animali maggiormente coinvolte in casi di avvelenamento da rodenticidi anticoagulanti sono i cani ed i gatti. Questo studio sottolinea l'importanza della determinazione di rodenticidi anticoagulanti nei casi di sospetto avvelenamento nella pratica veterinaria.

Introduction

Due to the frequent use of baits prepared for criminal intents to kill companion and wildlife animals, in 2008 an Italian Ministerial Decree[1] banned the preparation, possession, and deployment of poisoned baits and, at the same time, to place more responsibility onto owners and veterinarians involved in animal poisoning cases. In particular, the legislation establishes the specific rules and duties of the veterinarians and official laboratories, such as the Istituti Zooprofilattici Sperimentali (IIZZSS). By law, veterinarians have to send the organs of suspected poisoned animals or suspected baits to the competent IZS in order to detect the substances responsible of the poisonings. Each sample should be accompanied by ananamnestic report, which might address the analytical investigation. Specialized departments of IIZZSS perform the autopsies on the suspected poisoning animals and search for the poisoning substances. The analysis results have to be communicated to the mayor of the city, the official veterinary service, and to the judicial authorities in case of positive samples.

The absence of a National reference Veterinary Poison Control Centre which organises a system to collect data and prepares official reports contribute to underestimate the problem. Consequently, clinicians and veterinarians find difficult to obtain information on poisoning episodes (Amorena *et al.* 2004, Calzetta *et al.* 2002, Giorgi *et al.* 2011, Albo *et al.* 2004). The causes of poisoning in animals are manifold and it is not always possible to make a precise diagnosis due to the presence of non-specific symptoms such as vomiting, diarrhoea, and tremors. The veterinarian has the task of diagnosing the state and the cause of intoxication on the basis of semeiotic and laboratory analysis. In most cases, clinic investigation alone allows the veterinarian to find the most appropriate remedy. However, additional knowledge on poisoning cases in the area is essential to perform the diagnosis and to give an adequate therapy.

Among the various substances used to prepare baits, anticoagulant rodenticides are quite common in European countries, such as Italy, Portugal, Spain, and France (Belas *et al.* 2010, Berny *et al.* 2010, Caloni *et al.* 2003, Albo *et al.* 2004, Sánchez-Barbudo *et al.* 2012).

All anticoagulants have the basic coumarin or indanedione nucleus. Within the hydroxycoumarin rodenticides, first and second-generation compounds clinically differ in some relevant aspects; in particular, the first-generation appears to have a much shorter half-life, coumatetralyl, a first-generation product, showed a plasma elimination half-life of 0.52 days. Whereas, brodifacoum, a second-generation product, showed a plasma elimination half-life of 91.7 days. The elimination half-lives in liver varied from 15.8 days for coumatetralyl to 307.4 days for brodifacoum (Vandenbrouche *et al.* 2008, Valchev *et al.* 2008). These differences obviously lead to a more prolonged therapy necessity for second-generation rodenticide intoxications. Additionally, unlike first-generation compounds, a single dose of second-generation anticoagulant rodenticides is sufficient to cause haemorrhages (Petterino *et al.* 2001). Finally, second-generation anticoagulants such as brodifacoum and bromadiolone have higher toxicity to non-target species than the first-generation anticoagulants such as warfarin and coumachlor (Langford *et al.* 2013).

Both hydroxycoumarin and indanedione anticoagulant rodenticides antagonize vitamin K, interfering with the normal synthesis of coagulation proteins (factors I, II, VII, IX, and X) in the liver (Ishizuma *et al.* 2008). For both first and second-generation anticoagulants intoxications, clinical signs generally reflect manifestations of haemorrhages such as haematomas, melena, and haemothorax. Signs depending on haemorrhage, such as weakness, ataxia, colic, and polypnea, may be seen. Depression and anorexia are noted in all species even before bleeding occurs (Hirsh 1991).

The aim of this study was to evaluate the occurrence of coumarin anticoagulants in suspected poisoning cases in the Italian regions of Apulia and Basilicata, to produce useful data for preliminary surveillance, to contribute to stop the public concern and highlight the problems related to animal poisoning. Moreover, considering therapeutic differences between first and second-generation coumarin anticoagulants, which are not accompanied by any difference of detectable clinical signs, our objective was to study how often the different classes of compounds (first- and second-generation coumarin anticoagulants) were found in the suspected cases of poisoned animals reported in this assay. Finally, the increasing demand of official analyses requires sensitive and validated analytical methods. Analyses reported in this paper were executed employing a method based on high performance liquid chromatography (HPLC) with fluorimetric detection (FLD) (Armentano *et al.* 2012). A comparison with previous data obtained in the period 2007-2009 using thin layer chromatography (TLC) was also conducted.

[1] Ministerial Decree of 18/12/2008. Norme sul divieto di utilizzo e di detenzione di esche o di bocconi avvelenati. Official Journal n. 13 of 17/1/2009.

Materials and methods

Sample collection

In the years 2010-2012, anticoagulant rodenticides analyses were performed on 300 organs of animals, for which death by poisoning had been ascertained by necropsy examination conducted at the Veterinary Diagnostic Department of IZS of Apulia and Basilicata. Ninety suspicious baits were also analysed. In 14 cases, the organs of suspected poisoned animals with the related suspicious baits were addressed to the IZS. Among the total samples (n = 390), the request for the research of anticoagulant rodenticides was specified in only 29 anamnestic reports, where the most common animal species were represented by dogs (174) and cats (49). Other species included ruminants (15 between goats and sheep) and birds (36 among magpies, seagulls, pigeons, turtledoves, waterfowls, and birds of prey). The geographical area covered in this study corresponds to the Apulia region including the provinces of Foggia (153 samples), Bari (15 samples), Brindisi (28 samples), Lecce (46 samples), Taranto (52 samples) and the Basilicata region including the provinces of Matera (24 samples), and Potenza (72 samples).

Sample treatment, equipment, analytical conditions and validation parameters

Sample treatment, equipment, and analytical conditions of the validated analytical method based on HPLC/FLD used for the simultaneous determination of 8 anticoagulant rodenticides (bromadiolone, brodifacoum, coumachlor, coumafuryl, coumatetralyl, difenacoum, flocoumafen, and warfarin) have been largely described elsewhere (Armentano *et al.* 2012). Briefly, 4 g of sample, homogenised just before extraction, were treated with 40 ml of a mixture dichloromethane/acetone, (70:30 v/v) and shaken by vortex mixer (VWR Int. PBI, Milano, Italy) for 15 minutes. The mixture was filtered and purified by an alumina-based column (500 mg to 6 ml column). The eluate was evaporated to dryness at 60 °C using a nitrogen flow. The residue was solubilized in 1.0 ml of methanol, filtered, and injected for HPLC analysis.

Chromatographic separations were performed on a HPLC system (Agilent Technologies SL 1200 Series; Waldbronn, Germany). The HPLC system was equipped with a silica-based column (250 × 4.6 mm internal diameter; particle size: 5 μm) set at 30°C. Mobile phases A and B were water with 0.25% glacial acetic acid and methanol with 0.25% glacial acetic acid, respectively. The optimised gradient consisted of an initial mobile phase composition of 75% A, followed by an elution gradient (t = 5 min, A = 5%, B = 95%; t = 20 min, A = 0%, B = 100%, t = 35 min, A = 0%, B = 100%; t = 38 min, A = 75%, B = 25%, t = 45 min, A = 75%, B = 25%). The flow rate was 0.5 ml min^{-1}, and the injection volume was 20 μl. Fluorescence detection was performed at the emission and excitation wavelengths of 310 nm and 390 nm, respectively.

The most important validation performances of this method were evaluated. A good linearity for each analyte was observed with a determination coefficient higher than 0.992 The limits of detection (LOD) and limits of quantification (LOQ) are reported in Table I. The LODs and LOQs are slightly higher than those obtained with analytical methods based on the use of liquid chromatography coupled to mass spectrometry (LC/MS) (Sánchez-Barbudo *et al.* 2012), which are widely used but require more expensive experimental setup. The method is also characterised by recovery percentage in the range between 70-100%. The intra-laboratory reproducibility, expressed as coefficient of variation (CV%) for all analytes, was not greater than 14%. Shewhart control charts on analytes concentration using spiked samples were periodically performed.

Results

Over the total of 300 animal organs and 90 suspected baits, the presence of anticoagulant rodenticides was detected in 10% of the samples. The positive samples include 33 poisoned animals (11%). Among the animals poisoned with anticoagulants, dogs (21 samples) and cats (7) represent the most common species. The remaining positive samples include pigeons (1), waterfowls (2), foxes (1), and rabbits (1). Regarding suspected bait samples, 7% of them turned to be positive to anticoagulant rodenticides (6 positive baits). The spectrum of individual anticoagulant rodenticides and their percentages detected in this 3-year study are

Table I. *Limit of detection (LOD) and limit of quantification (LOQ) values for each anticoagulant molecule of the HPLC/FLD method employed in the survey.*

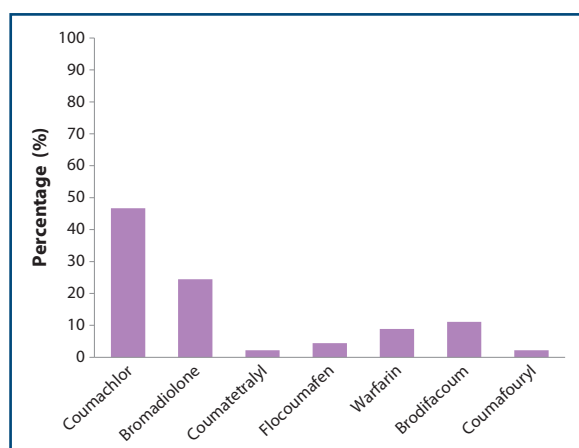| Analyte	LOD μg kg^{-1}	LOQ μg kg^{-1}
Brodifacoum	13.9	42.1
Bromadiolone	15.7	47.6
Coumachlor	14.5	43.9
Coumafouryl	99.5	301.5
Coumatetralyl	5.2	15.7
Difenacoum	14.7	44.5
Flocoumafen	7.1	21.5
Warfarin	31.4	95.2

Figure 1. *Percentage distribution of the cumarinic anticoagulant rodenticides detected in the positive samples of animal organs and baits collected in the Italian regions of Apulia and Basilicata.*

given in Figure 1. The most commonly detected compound was coumachlor (47% of total positive samples) followed by bromadiolone (24%), and brodifacoum (11%).

Samples containing more than 1 compound were also found. Presence of multiple anticoagulants in the same specimen was detected in 7 cases. Most of these samples (6 samples) were characterised by the

Table II. *Survey results and geographic distribution of samples collected in the Italian regions of Apulia and Basilicata.* The numbers between brackets indicate the number of times that each anticoagulant molecule was detected.

Province	Number of samples	Number of positive samples	Molecules detected
Foggia	153	4 (3%)	Coumachlor (4)
			Bromadiolone (1)
Bari	15	2 (13%)	Coumachlor (1)
			Bromadiolone (1)
Brindisi	28	0 (0%)	-
Lecce	46	15(33%)	Coumachlor (6)
			Warfarin (2)
			Coumatetralyl (1)
			Brodifacum (3)
			Bromadiolone (4)
			Flocoumafen (1)
Matera	24	1(4%)	Coumachlor (1)
Taranto	52	6(12%)	Coumachlor (3)
			Warfarin (2)
			Coumafouryl (1)
			Bromadiolone (1)
Potenza	72	11(15%)	Coumachlor (6)
			Brodifacum (2)
			Bromadiolone (4)

presence of bromadiolone associated with 1 of the other molecules. The most frequent combination was bromadiolone/coumachlor.

In 2 cases, the presence of bromadiolone was detected in both animal organs and the related suspicious baits.

As reported in Table II and in Figure 2, the 2 Italian regions (Apulia and Basilicata) show a wide spatial distribution of the anticoagulant rodenticides in almost all provinces with the only exception of Foggia, where a significant prevalence of coumachlor is observed. The province of Lecce has the highest ratio of positive sample/sample analysed (0.4), while the province of Foggia shows a value 10 times lower (0.04).

The higher average level of anticoagulant rodenticides has been found in animal tissues containing coumachlor and bromadiolone (see Table III). The concentration distribution for baits and animal organs is reported in Figure 3. As
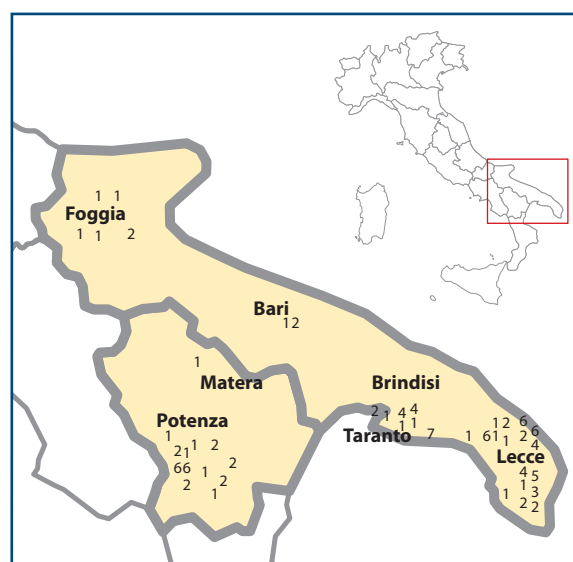

Figure 2. *Geographic distribution of the anticoagulant molecules detected in the positive samples of animal organs and baits collected in the Italian regions of Apulia and Basilicata.* 1 = coumachlor; 2 = bromadiolone; 3 = flocoumafen; 4 = warfarin; 5 = coumatetralyl; 6 = brodifacoum; 7 = coumafuryl.

Table III. *Average detected concentrations for each anticoagulant molecule in animal organs and baits analysed in this study employing the HPLC/FLD method.*

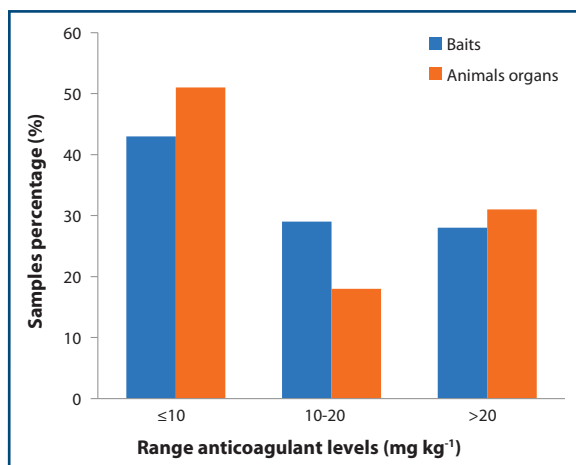| Average concentration (mg kg^{-1})		
Molecule	**Animal organs**	**Baits**
Coumachlor	35	115
Bromadiolone	28	2,900
Brodifacoum	2	3
Warfarin	3	5

Figure 3. *Percentage distribution of cumarinic anticoagulant rodenticide concentrations in positive samples of animals and baits collected in the Italian regions of Apulia and Basilicata.*

Figure 4. *Correlation between the numbers of tissue samples and coumachlor concentration (mg kg^{-1}) in positive organ samples collected in the Italian regions of Apulia and Basilicata.*

shown in Figure 3, the animal organ samples were mostly found with a concentration of anticoagulant rodenticides lower than 10 mg kg^{-1} (51% of total number of rodenticide molecules found in poisoned animals). The graphical distribution of organs positive for coumachlor is shown in Figure 4. The data were log-distributed. Of the 29 samples for which the research of anticoagulant rodenticides was specifically requested in the anamnestic reports, the presence of anticoagulant rodenticides was confirmed in 10 samples.

Discussion

The present study has permitted to investigate the animal poisoning cases of anticoagulant rodenticides in 2 Southern Italian regions. From our assay, dogs and cats represent the most common species victims of anticoagulant rodenticide poisoning. By analysing the detected concentrations, it was not possible to absolutely establish if each animal poisoning was due to a casual exposure to these substances or to ingestion of baits made for criminal intents. Although the first-generation anticoagulant rodenticides were most prominent (coumachlor was detected in 47% of positive samples), second-generation rodenticides were also largely represented (bromadiolone and brodifacoum were detected in 35% of positive samples). The high percentage of samples with the presence of coumachlor can be explained taking into account that the first-generation rodenticides are still largely used and available in the South of Italy. On the contrary, a comparison with the data available shows that the second generation anticoagulants were usually found in North Italian regions and other European countries (Antonoiou *et al.* 2005, Berny *et al.* 2008, Caloni *et al.* 2003, Fournier-Chambrillon
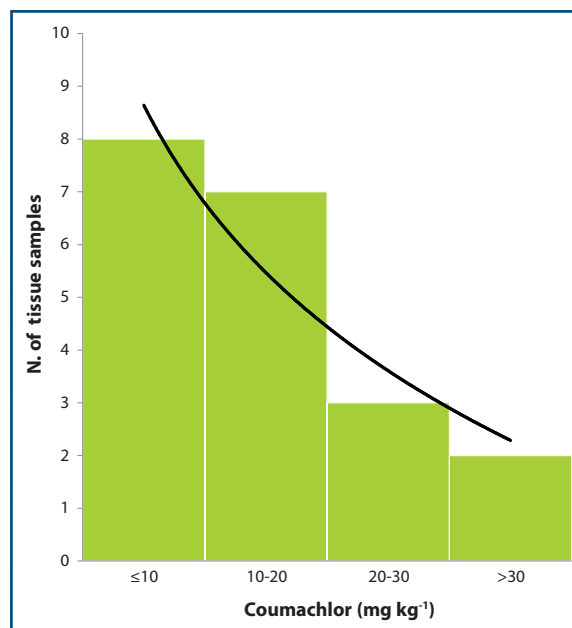
et al. 2004, Giuliano Albo *et al.* 2004, Grobosh *et al.* 2006, Sánchez -Barbudo *et al.* 2012).

Moreover, the present study revealed that over 300 cases of animal suspected of poisoning, but only 33 samples (11%) were confirmed. A similar trend was already found in France, where, over 1,074 samples of dogs and cats suspected of poisoning, laboratory tests confirmed only 79 cases (equal to 7.3%) (Berny *et al.* 2010).

Rare cases of ruminants poisoned by anticoagulant rodenticides were reported in Europe (Antonoiu *et al.* 2005, Berny *et al.* 2010, Guitart *et al.* 2010). In this study, no traces of anticoagulants were found in 15 ruminant samples (sheep and goats) suspected of poisoning.

In some European countries, anticoagulant secondary poisoning is reported in birds, especially predators and scavengers (mainly birds of prey). It is reported less frequently in pigeons, partridges, and waterbirds (Berny *et al.* 2010) and even in granivorous birds (Sánchez Barbudo *et al.* 2012). In our study anticoagulant rodenticides were detected in 3 birds (1 pigeon and 2 waterfowls).

The use of bromadiolone to control water voles in some areas of France was associated also to toxicity in many cases of wild animals [wild boars (*Sus scrofa*)], red foxes, hares, and small mustelids, including the European mink (Fournier-Chambrillon *et al.* 2004). Regarding wild mammals, few cases were analysed in this survey (4 foxes and 1 wolf) and 1 (a fox) was found to be positive for coumachlor.

Despite the relatively high complexity of the analysed matrices and low concentrations of rodenticides in analysed samples, the good selectivity and sensitivity of the employed HPLC method allowed for detecting the presence of anticoagulant rodenticides that may remain undetected by other less sensitive techniques (Palermo *et al.* 1995). This is demonstrated by comparing this monitoring assay with previous studies related to the detection of anticoagulants conducted in our laboratory in the period 2007-2009. The analyses were performed on a total of 340 samples (117 samples in 2007, 110 in 2008 and 113 in 2009). The samples consisted of 39 baits and 301 organs of suspected cases of poisoned animals. Also in this case, poisoned animals were mainly dogs and cats. The liver of each animal was used for the analysis, employing TLC as analytical technique. Only 7 samples (equal to 2%) were found positives (6 poisoned baits and 1 organ). The percentage of positives resulted 5 times lower than the one found in the period between 2010-2012 (10%).

These findings corroborate the experimental results carried out by Rengel and colleagues (Rengel *et al.* 1993). In this study, samples from animals suspected to be poisoned (5 livers, 1 gastric content, 1 serum) and suspected baits were examined using both TLC and HPLC/FLD. No positive sample was detected by TLC in the 5 liver extracts, whereas the presence of anticoagulant rodenticides was detected by HPLC/FLD. Also the blood serum was found positive only by the HPLC/FLD method. Thin layer chromatography method was able to establish the positivity only in the 3 baits and the gastric content sample.

The occurrence of anticoagulant rodenticide poisoning in domestic animals and livestock in 2 regions of Italy (Apulia and Basilicata) in 2010-2012 was investigated by using an accurate HPLC method with fluorescence detection for the simultaneous determination of 8 anticoagulant of first and second generation. The presence of anticoagulant rodenticides was detected in 10% of total analysed samples. Among the anticoagulant rodenticides found, the first generation substances were most prominent. Dogs and cats represent the most common species victims of anticoagulant rodenticide poisoning. The more sensitive and selective HPLC method allowed for detecting a higher number of positive samples compared to TLC method. The high sensitivity (limit of quantification ranging from 16 $\mu g\ kg^{-1}$ to 302 $\mu g\ kg^{-1}$) and selectivity of the method might allow its analytical use for the detection of the toxic substances for the poisoning diagnosis caused by anticoagulant rodenticides to possibly prevent the animal death. As observed in this monitoring assay, an incomplete anamnestic report causes the absence of a guide for the analytical investigation with a consequent economic and time loss. A collaborative attitude is highly needed by all the groups' involved (general public, environmental police, veterinarians, and animals' owner).

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