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A Reappraisal of Blood Clotting Response Tests for Anticoagulant Resistance and a proposal for a standardised BCR Test Methodology

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Rodenticide Resistance Action Committee

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SUMMARY

Blood clotting response (BCR) resistance tests are available for a number of anticoagulant rodenticides. However, during the development of these tests many of the test parameters have been changed, making meaningful comparisons between results difficult. It was recognised that a standard methodology was urgently required for future BCR resistance tests and, accordingly, this document presents a reappraisal of published tests, and proposes a standard protocol for future use (see Appendix).

The protocol can be used to provide information on the incidence and degree of resistance in a particular rodent population; to provide a simple comparison of resistance factors between active ingredients, thus giving clear information about cross-resistance for any given strain; and to provide comparisons of susceptibility or resistance between different populations.

The methodology has a sound statistical basis in being based on the ED_{50} response, and requires many fewer animals than the resistance tests in current use. Most importantly, tests can be used to give a clear indication of the likely practical impact of the resistance on field efficacy.

The present study was commissioned and funded by the Rodenticide Resistance Action Committee (RRAC) of CropLife International.

1 RESISTANCE TEST METHODOLOGIES

Since the 1960's many tests have been developed to identify anticoagulant-resistant Norway rats. New tests are developed for various anticoagulants and to detect different types of resistance. Very often, differences in methodology reflect the interests of the centre concerned and especially whether the individual worker is orientated towards practical aspects of rodent control or towards a particular scientific discipline.

Survival of a laboratory feeding test with commercial anticoagulant bait must always be the final proof of resistance, irrespective of the resistance mechanism, but procedures tend to be slow and labour intensive and have many other shortcomings. Direct in vitro tests for a basic biochemical or genetic mechanism would be ideal for many purposes, but would probably be too specific for general use and, in any case, are not yet a practical proposition.

In contrast, Blood Clotting Response (BCR) tests can usually be performed easily, can give meaningful results within 24 h, do not rely on consistent feeding

The first BCR methodologies were developed to distinguish one of these laboratory resistant strains from a susceptible strain, while later BCR methodologies were based on a discriminating dose that would make susceptible animals respond; failure to respond was taken as evidence of resistance.

2.1 BCR TESTS DEVELOPED USING A LABORATORY RESISTANT STRAIN

The advantage of these tests is that they are designed to detect a form of resistance that actually exists. Their disadvantage is that they may be so closely matched to the particular form of resistance that they fail to detect or differentiate it from other, possibly unknown forms. The first BCR test, based on the 24h prothrombin response to a small dose of warfarin (Greaves and Ayres, 1967) was an entirely ad hoc procedure adopted in the course of a study of Welsh-type warfarin resistance. Subsequent adaptations of the procedure were proposed based on various advances and hypotheses concerning a number of factors, including the type or origin of the resistance, resistance mechanism, route of administration, role of vitamins K, genotype of the rodents, and the nature of the tolerance distribution. This type of BCR test has been established for warfarin (Martin et al., 1979; MacNicoll and Gill, 1993) and difenacoum (Gill et al., 1993).

2.2 BCR TESTS BASED ON THE RESPONSE OF SUSCEPTIBLE RODENTS

The advantage of these tests is that they can be designed to detect the smallest possible change in susceptibility. Their disadvantage is that they do not, in themselves, indicate whether the decrease in susceptibility (or resistance), is or is likely to be of any operational significance. Therefore, it is important to specify independently the change in response that will be considered to be significant. Hitherto, these tests have employed conventional bioassay methodology to estimate a supposed 'discriminating dose' (a high response percentile such as the ED_{95}, ED_{98}, or ED_{99} and/or its upper 95% fiducial limit) from quantal dose-response data; failure to respond to this dose is presumed to indicate resistance.

The method is based upon techniques previously developed to detect insecticide resistance. Although the principle of the tests is unexceptionable, the details of the procedures are open to criticism. This type of BCR test has been established for bromadiolone (Gill et al., 1994), and for chlorophacinone and diphacinone (Prescott and Buckle, 2000).

3 TOWARDS A STANDARDISED METHODOLOGY

BCR tests for resistance in the Norway rat have been developed for five anticoagulant active ingredients, but with each development, a number of parameters of the protocol have been changed (Table 1). These will now be considered further, with the objective of standardising the procedure.

NOTE

Table 1. Some parameters used in published BCR tests for detecting resistance to anticoagulants in Norway rats.

PCA = Percentage Clotting Activity

Active Ingredient	Sex	Discriminating dose ($mg.kg^{-1}$)	Type of Vitamin K used	Dose of Vitamin K ($mg.kg^{-1}$)	Route of administration of a.i.	Time to PCA	Threshold PCA
warfarin	both	5.0	epoxide	1.0	interperitoneal	24h	17%
sodium warfarin	both	5.4	K_3	1.0	gavage	24h	17%
difenacoum	both	5.0	K_3	2.0	gavage	96h	10%
bromadiolone	male	1.0	K_3	10.0	gavage	96h	10%
bromadiolone	female	2.4	K_3	10.0	gavage	96h	10%
diphacinone	male	1.26	K_3	1.0	gavage	24h	17%
diphacinone	female	1.60	K_3	1.0	gavage	24h	17%
chlorophacinone	male	0.86	K_3	1.0	gavage	24h	17%
chlorophacinone	female	1.03	K_3	1.0	gavage	24h	17%

3.1 BCR RESISTANCE TESTS DEVELOPED FOR A LABORATORY RESISTANT STRAIN OR BASED ON THE RESPONSE OF THE SUSCEPTIBLE STRAIN

Laboratory resistant strains have been developed only for the Norway rat and house mouse, so for other species, tests must be based on the susceptible strain. For laboratory resistant strains of Norway rat, there are considerable differences in their response to anticoagulant active ingredients (Greaves and Cullen-Ayres, 1988). For example warfarin resistance in female animals is more than an order of magnitude greater in the Welsh strain than in the Scottish strain, so resistance tests established using one strain might not detect resistance in another. Furthermore, it is questionable whether laboratory homozygous resistant strains are representative of field populations that typically contain a high frequency of heterozygotes.

It is therefore recommended that future BCR tests be based on the response of the susceptible strain.

3.2 SUSCEPTIBLE ANIMALS USED TO GENERATE BASELINE DATA

Animals used to generate susceptibility baselines must be fully susceptible to all anticoagulants, and all reference strains must be demonstrably parametric in their response. Wild strains may be preferred because they are directly representative of the field population, although their provenance may be in question, and their responses are likely to have greater variance than a commercial laboratory strain.

For Norway rat and house mouse, the use of animals from a reputable commercial supplier is therefore recommended on grounds of availability and quality assurance. Where wild strains of known provenance are used, comparison of their response with that of the commercial laboratory strain is recommended. For other species the use of wild strains is unavoidable.

3.3 DATA ANALYSIS

Resistance tests based on the response of susceptible rodents rely on the statistical analysis of dose-response data generated using minimal numbers of animals. Probit and similar analyses are designed for the efficient estimation of the ED_{50}. For mathematical reasons estimates are increasingly subject to error at higher percentiles. Also, all estimates of the higher percentiles rely on extrapolation, thus violating a basic principle of scientific inference. Discriminating doses should, therefore, either be developed completely empirically or be based upon the ED_{50}. The use of a multiple of the ED_{50} has much to commend it (see Section 4.2).

3.4 METHOD OF ADMINISTRATION OF THE ANTICOAGULANT

In the early BCR resistance tests, the active ingredient was administered by intraperitoneal or subcutaneous injection, but in more recent tests, gavage has been used. Gavage is convenient for domesticated strains but can be traumatic for wild rodents unless they are anaesthetised. Subcutaneous or intraperitoneal injection while restraining the rodent in a handling bag may be easier. The route of administration is generally not considered to be significant as far as results are concerned.

3.5 CO-ADMINISTRATION OF VITAMIN K

The biochemical mechanism of certain forms of resistance imposes an enhanced dietary requirement for vitamin K. Consequently, such animals may develop symptoms of vitamin K deficiency indistinguishable from those of anticoagulant poisoning. In some BCR tests a form of vitamin K is routinely co-administered with the anticoagulant to ensure that prolonged clotting times are due to the anticoagulant, and not to vitamin K deficiency. In the Welsh-resistant strain of Norway rat, Hussain (1998) induced vitamin K deficiency with a vitamin K deficient diet, but found that the deficiency was prevented by levels of vitamin K_3 as low as 0.5 $mg.kg^{-1}$ body weight (a level that is readily available from commercial laboratory diets); he also co-administered massive doses of vitamin K_3 with potentially lethal doses of anticoagulant, and found no antidotal effect.

Co-administration of low levels of vitamin K may be applicable in special studies of certain forms of resistance, particularly where the resistance is of an unknown mechanism. There must always be some doubt whether a rodent that receives an artificially increased supply of a possible antidote can be claimed to be resistant in any true sense. Therefore this procedure is not recommended in routine tests.

3.6 ASSESSMENT OF COAGULATION – FACTOR SPECIFIC OR NON-SPECIFIC

There are four vitamin K dependent blood-clotting factors, and in a normal animal endogenous levels are maintained under physiological control. Following anticoagulant dosing, the nature of the clotting defect changes over several days due to the different half-lives of the various factors, and there may be strain differences in this respect (Kerrins and MacNicoll, 1999). Some workers advocate measuring the activity of a single factor instead of the more broadly based one-stage prothrombin time. However, blood clotting depends on complex interactions between many clotting factors, rather than on the level of any single factor. It is therefore recommended for both theoretical and practical reasons that the one-stage prothrombin time should be retained.

3.7 TIME-INTERVAL BETWEEN ANTICOAGULANT DOSING AND BLOOD SAMPLING

The interval between dosing and blood sampling must be sufficient to allow a clearly discernable response to occur. With large doses, the prothrombin time of susceptible strains of Norway rat and house mouse is typically more than eight times the resting prothrombin time after an interval of 24 hours, which is outside the tabulated range of sensitivity for the test methods. Where longer intervals are proposed (e.g. 96 hours), the dose required to achieve a comparable response tends to be greater, because of pharmacokinetic effects. With a short interval, clotting time is primarily affected by pharmacodynamically based resistance (i.e. altered enzyme biochemistry), but with a longer interval pharmacokinetically based effects (enhanced clearance) may increase (Thijssen, 1995). While there is no definite evidence that significant anticoagulant resistance due to enhanced clearance exists, it is desirable to avoid the risk that apparent resistance is induced by the test procedure. Other disadvantages of a long time delay are that prolongation of the test procedure is costly and that many animals may die from haemorrhage before blood sampling. Thus, an interval of 24 hours is recommended for the generation of quantal dose response data in BCR tests.

3.8 THE LEVEL OF COAGULATION THAT IS DEFINED AS A RESPONSE

To generate dose-response data it is necessary to specify the coagulation time that will be regarded as a response, indicating that the animals' coagulation system had been compromised. Traditionally, an animal is considered to be a responder if, a specified period after dosing, its plasma "percent coagulation activity" (PCA) is less than 17% (Martin et al., 1979; MacNicoll and Gill, 1993; Prescott and Buckle, 2000) or 10% (Gill et al., 1993; Gill et al., 1994). Coagulation times are converted to PCA using calibration curves based on serial dilutions of normal plasma in saline. When determined using the same thromboplastin reagent, calibration curve replication is poor, particularly at low dilutions. When determined using different thromboplastin reagents, the calibration curves can differ markedly. The PCA values thus arrived at therefore imprecise and should not be used.

4 DEVELOPMENTS FOR THE NEW PROTOCOL

In previous BCR resistance tests, the methods used to identify a response in terms of PCA and a discriminating dose in terms of a high ED percentile are considered flawed, and are now re-considered.

4.1 THE USE OF THE INTERNATIONAL NORMALISED RATIO (INR) TO DEFINE A BCR RESPONSE

Different thromboplastin test methods are used to assess clotting activity, but their sensitivities vary considerably. Standardisation is therefore desirable, and to accommodate this in human haematology, all methods are calibrated against selected reference material based on a W.H.O. Standard (Denson, 1998). Each thromboplastin test method is provided with an International Sensitivity Index (ISI), and a list of clotting times tabulated against the corresponding International Normalised Ratio (INR). The ISI is a measure of the sensitivity of the thromboplastin reagent, and the INR is the multiple of normal human clotting time that would have been obtained had the reference material been used. The INR thus provides a common scale of measurement for all methods.

For example, Diagen freeze dried rabbit brain thromboplastin (Diagen RBT) and Roche Hepato Quick (Roche HQ) are two thromboplastin reagents, batches of which had ISI values of 1.4 and 0.89 respectively. When clotting times of blood samples obtained from a control Norway rat and three anticoagulated rats were determined using the two test reagents, the INR values were found to correspond well (Table 2). However, when Norway rat PCA calibration curves were produced independently using the two reagents, a PCA of 17% corresponded approximately with an INR of 5 using Diagen RBT, and an INR of 2.5 using Roche HQ. The use of INR to identify a responder in BCR tests will therefore be used to control for differing sensitivities of thromboplastin reagents.

NOTE

Table 2. Clotting times with corresponding International Normalised Ratio for blood samples obtained from a control Norway rat and three anticoagulated rats, determined using Diagen Rabbit Brain Thromboplastin and Roche Hepato Quick reagent.

	Diagen RBT		Roche HQ	
	Clotting time	INR	Clotting time	INR
control rat	19 sec	1.4	33.8 sec	1.33
anticoagulated rat 1	43.8 sec	4.5	124.3 sec	4.27
anticoagulated rat 2	30.5 sec	2.7	79.5 sec	2.86
anticoagulated rat 3	39.0 sec	3.8	112.7 sec	3.91

Commercially available thromboplastin reagents were developed for use in human medicine, where the objective is to monitor therapeutic anticoagulation. Typically, INR values are tabulated from 1 to 6, with a useful and safe therapeutic range of 2 to 4.5. However, in BCR resistance tests it is desirable to produce higher levels of anticoagulation. It is therefore proposed for Norway rat and house mouse, that an INR value equal to or greater than 5 be used as the response in the BCR resistance test. The resting clotting times of **Bandicota bengalensis** have been found to be markedly longer than those of the Norway rat and house mouse (Hussain, 1998), indicating that the INR value adopted as the response should be assessed separately for each species.

4.2 ANALYSIS OF SUSCEPTIBLE BASELINE DATA AND THE 'DISCRIMINATING' TEST DOSE

In recent BCR resistance tests, the ED_{99} is used as the discriminating test dose. Norway rat BCR ED_{50} and ED_{99} data for bromadiolone (re-analysed data of Gill et al., 1994), for warfarin (Hussain, 1998) and for chlorophacinone and diphacinone (Prescott and Buckle, 2000), are presented in Table 3, together with the multiple of the ED_{99} against the ED_{50}. In these studies, using existing BCR resistance testing methodologies, the ED_{99} is between 1.25 and 1.9 times the ED_{50}. Thus, when subjected to the ED_{99} as the test dose, over 50% of a population of rodents with a resistance factor of 2 would be identified as resistant in any of the above tests. Such tests are overly sensitive, and would be of limited value as predictors of practical resistance.

NOTE

Table 3. Norway rat BCR ED_{50} and ED_{99} data for bromadiolone (re-analysed data of Gill et al.,1994), for warfarin (Hussain, 1998) and for chlorophacinone and diphacinone (Prescott and Buckle, 2000),presented with the multiple of the ED_{99} against the ED_{50}.

	Compound	ED_{50} ($mg.kg^{-1}$)	ED_{99} ($mg.kg^{-1}$)	ED_{99}/ED_{50}
male	bromadiolone	0.62	0.77	1.25
female	bromadiolone	0.70	1.33	1.9
male	warfarin	1.5	2.3	1.6
female	warfarin	2.3	3.9	1.7
male	chlorophacinone	0.56	0.86	1.6
female	chlorophacinone	0.67	1.03	1.6
male	diphacinone	0.88	1.26	1.5
female	diphacinone	1.11	1.60	1.5

For each species the ED_{50} for susceptible rodents can be determined accurately using fewer animals than current methodologies. For the checking test, animals suspected of being resistant are tested with a multiple of the ED_{50} as the discriminating dose, the particular multiple depending upon the slope of the dose-response curve, the field concentration of the anticoagulant, and the resistance factor considered to be significant. If 2x the ED_{50} were the test dose for each anticoagulant the results would give a similar level of information to that of BCR resistance tests developed following the existing guidelines (OEPP/EPPO, 1995). If a two-fold level of resistance were considered to be insignificant then a test dose corresponding to a higher multiple of the baseline ED_{50}, and thus to a more realistic level of resistance would be chosen. Extending this line of reasoning, the multiple corresponding to the ED_{50} of the resistant strain would be equal to the resistance factor.

Table 4 and Table 5 present a range of pertinent susceptible data for Norway rat and house mouse respectively. For Norway rat, data is for warfarin (re-analysis of data from Hussain, 1998), for diphacinone and chlorophacinone (re-analysis of data from Prescott and Buckle, 2000), and for coumatetralyl, bromadiolone, difenacoum, brodifacoum, difethialone and flocoumafen (the present study). For house mouse, data is for bromadiolone, difenacoum, brodifacoum, difethialone and flocoumafen (the present study).

NOTE

Table 4. BCR Resistance Tests – The ED_{50}'s of nine anticoagulants for the CD strain of Norway rats. The multiples shown indicate the range within which a test dose might be specified. The ED_{50}'s are based on the coagulation response corresponding to an INR $\geq$ 5.0.

		ED_{50}	test dose = multiple of the ED_{50}			
Anticoagulant	Sex	($mg.kg^{-1}$)	x 2	x 4	x 8	x 16
warfarin	male	1.51	3.02	6.0	12.1	24.2
warfarin	female	2.13	4.26	8.5	17.0	34.1
diphacinone	male	0.86	1.72	3.4	6.9	13.8
diphacinone	female	1.12	2.24	4.5	9.0	17.9
chlorophacinone	male	0.54	1.08	2.2	4.3	8.6
chlorophacinone	female	0.67	1.34	2.7	5.4	10.7
coumatetralyl	male	0.36	0.72	1.4	2.9	5.8
coumatetralyl	female	0.44	0.88	1.8	3.5	7.0
bromadiolone	male	0.47	0.94	1.9	3.8	7.5
bromadiolone	female	0.61	1.22	2.4	4.9	9.8
difenacoum	male	0.65	1.30	2.6	5.2	10.4
difenacoum	female	0.79	1.58	3.2	6.3	12.6
difethialone	male	0.43	0.86	1.72	3.44	6.88
difethialone	female	0.49	0.98	1.96	3.92	7.84
flocoumafen	male	0.29	0.58	1.16	2.32	4.64
flocoumafen	female	0.34	0.68	1.36	2.72	5.44
brodifacoum	male	0.22	0.44	0.9	1.8	3.5
brodifacoum	female	0.23	0.46	0.9	1.8	3.7

NOTE
Table 5. BCR Resistance Tests – The ED_{50}'s of five anticoagulants for the CD-1 strain of house mouse. The multiples shown indicate the range within which a test dose might be specified. The ED_{50}'s are based on the coagulation response corresponding to an INR $\geq$ 5.0.

		ED_{50}	test dose = multiple of the ED_{50}			
Anticoagulant	Sex	($mg.kg^{-1}$)	x 2	x 4	x 8	x 16
bromadiolone	male	1.96	3.92	7.84	15.68	31.36
bromadiolone	female	1.68	3.36	6.72	13.44	26.88
difenacoum	male	0.85	1.7	3.4	6.8	13.6
difenacoum	female	0.56	1.12	2.24	4.48	8.96
difethialone	male	0.83	1.66	3.32	6.64	13.28
difethialone	female	0.83	1.66	3.32	6.64	13.28
flocoumafen	male	0.51	1.02	2.04	4.08	8.16
flocoumafen	female	0.44	0.88	1.76	3.52	7.04
brodifacoum	male	0.39	0.78	1.56	3.12	6.24
brodifacoum	female	0.35	0.7	1.4	2.8	5.6

4.3 INTERPRETATION OF RESISTANCE TEST RESULTS

A summary of published resistance factors for the Norway rat is presented in Table 6, for guidance. Although the values were generated using LD_{50}'s rather than ED_{50}'s, the data could be used to help assess the multiple of the ED_{50} that would provide a practical discriminating dose.

NOTE
Table 6. A summary of resistance factors for the Norway rat (Greaves and Cullen-Ayres, 1988).

Resistance Focus	Warfarin (RF)	Coumatetralyl (RF)	Bromadiolone (RF)	Difenacoum (RF)	Brodifacoum (RF)
Wales	97 - 2296	33 - 168	2.7 - 6.9	1.1 - 1.3	1.0 - 1.1
Scotland	51 - 115	34 - 56	2.3 - 2.5	2.7 - 3.4	2.5 - 2.7
Hampshire			1.5 - 2.9	3.9 - 4.1	2.0 – 2.0

RF = Resistance Factor

In the present study, the ED_{50}'s of bromadiolone and difenacoum were determined for the CD-susceptible strain and the Hampshire-resistant strain of Norway rat, and resistance factors were calculated, and found to be comparable with previously published data (Table 7).

NOTE
Table 7. BCR derived ED_{50}'s of bromadiolone and difenacoum, for the CD-susceptible strain and the Hampshire-resistant strain of Norway rat. BCR resistance factors calculated from the present study are presented alongside comparable data from Greaves and Cullen-Ayres (1988).

Active Ingredient	Sex	Albino Susceptible ($mg.kg^{-1}$)	Hampshire Resistant ($mg.kg^{-1}$)	BCR Resistance factor	LD_{50} Resistance factor @
bromadiolone	male	0.47	1.40	3.0	1.5
bromadiolone	female	0.61	4.12	6.8	2.9
difenacoum	male	0.65	1.40	2.2	3.9
difenacoum	female	0.79	3.96	5.0	4.1

@ Lethal dose resistance factors from Greaves and Cullen-Ayres (1988)

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APPENDIX

Blood Clotting Response (BCR) resistance test protocol.

1. Objectives: to provide a quick, simple test to:

- Measure the baseline response of non-resistant rodents to anticoagulants
- Check suspect samples of rodents for resistance
- Estimate the resistance factor for a given anticoagulant resistant strain

2. Principle of the Method: for each rodent species and anticoagulant to:

- Specify the coagulation time (seconds) that will be considered to constitute a response.
- Determine the baseline ED_{50} for the response in the susceptible strain.
- Specify a multiple of the baseline ED_{50} as the test dose to detect resistance.
- Use the test dose in resistance monitoring, or to confirm field evidence of resistance.
- Estimate the resistance factor for each resistant strain as the multiple of the baseline ED_{50} that constitutes the ED_{50} for the resistant strain.

3.The coagulation time to be defined as a response

3.1 Permissible resting coagulation times shall be established for each species (to take account of inter-species variation).

3.2 Resting coagulation times shall be determined in each case for strains of unknown provenance, and in a representative sample of animals for attested laboratory strains of known provenance.

3.3 Where resting coagulation times are prolonged, animals shall be excluded from the study.

3.4 The response is specified as the coagulation time corresponding to a specific INR (in order to make results comparable when generated with different thromboplastin reagents).

3.5 For Norway rats and house mice, the blood clotting response is defined as that corresponding to an INR equal to or greater than 5.0.

3.6 For other species, the INR defining the response is yet to be assessed.

3.7 For each species, the INR defined as the response shall be adopted for all resistance tests.

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4 Animals

4.1 Animals shall be healthy, active and sexually mature.

4.2 Animals shall be maintained on a balanced diet appropriate to the species with an unrestricted supply of tap water.

4.3 To avoid the risk of artificially inducing an antidotal effect, the drinking water shall not be supplemented with vitamin K. Commercially formulated diets used should have a vitamin K_3 content in the range 3 - 8 $mg.kg^{-1}$ as a safeguard against primary vitamin K deficiency.

4.4 In the event of ectoparasite infestation, or on receipt of stock from the wild, animals may be treated with an approved formulation (eg. ivermectin) not less than 7 days before the start of the test.

4.5 Animals may be caged either singly or in groups, sexes separate, and shall be held in the laboratory (preferably in the test room) for not less than four days before testing.

4.6 The risks of pregnancy and lactation may be excluded for females by separating them for minimum periods of 24 days from adult males and 7 days from suckling offspring.

4.7 Repeat tests on an individual animal with the same or different anticoagulants could possibly be affected by the persistence of the previous dose, or by induction of metabolising enzymes by the previous dose. For these reasons, it is recommended that a succession of resistance tests should not be performed on the same animals.

5. Production of baseline data

5.1 Rodents shall be susceptible to all anticoagulants.

5.2 Where available, an attested, laboratory-bred strain shall be used.

5.3 For consistency in future tests, all of the susceptible ED_{50} data presented in Table 4 and Table 5 of the accompanying document have been generated using the outbred CD strain of Norway rat and the outbred CD-1 strain of house mouse supplied by Charles River UK Ltd.

5.4 All reference strains, whether wild or domesticated, shall be demonstrably parametric in their response to the anticoagulant.

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5.5 The weight of each animal shall be recorded to the nearest 1g at the beginning of the procedure. If group-caged at the time of weighing, each animal shall be identified with tail marks. Prior to gavage, food may be withheld overnight.

5.6 Animals may be lightly anaesthetised by an appropriate method during gavage and blood collection.

5.7 A blood sample may be collected prior to dosing, to ensure that resting coagulation times are normal.

5.8 The anticoagulant shall be administered by gavage. If desired, water-insoluble compounds may be dissolved in 1 volume of triethanolamine and diluted with 99 volumes of PEG 200.

5.9 Blood samples shall be collected by any convenient route 24 h after anticoagulant administration.

5.10 Coagulation times shall be determined using an established test methodology that tabulates coagulation time against INR. Animals are responders when their coagulation times are equivalent to an INR value equal to or greater than 5.

5.11 A ranging study may be used to determine appropriate dosages. For accurate estimation of the ED_{50}, a minimum of two dosage groups shall be established for each sex with high and low percentage responders respectively, and where each dosage group contains at least 15 animals. The choice of the dosage groups shall be such as to ensure that the 95% fiducial limits of the ED_{50} are within 10% of the value of the ED_{50}.

5.12 The dose response data shall be analysed by Probit analysis (or a similar analysis) in two stages.

a) The probit lines for the two sexes are compared to determine whether they differ significantly from a parallel response or a coincident response.

b) Taking account of the first analysis, the ED_{50} is estimated for each sex, and is rounded up to the nearest 0.01 $mg.kg^{-1}$.

APPENDIX

6 Specifying the test dose

6.1 For each active ingredient and for both sexes of each species, a multiple of the ED_{50} is specified as the test dose.

6.2 The test dose(s) shall be administered to at least six males and six females of the susceptible strain, to verify that they all respond.

6.3 It may be useful to specify test doses at two levels, 'alert' and 'confirmed'.

6.4 A test dose of twice the ED_{50} would be expected to generate significant numbers of false positives, but would nevertheless be more stringent than previously published BCR resistance tests based on the ED_{99} (Table 3).

7 Applying the test procedure to suspected-resistant rodents

7.1 Each animal is weighed to the nearest 1g and caged singly.

7.2 Animals may be lightly anaesthetised by an appropriate method during gavage and blood collection.

7.3 Blood samples shall be collected prior to dosing, to ensure that resting coagulation times are within the permissible range (see 3.1).

7.4 Animals with resting coagulation times outside the permissible range may have previous anticoagulant exposure and should be excluded from the test.

7.5 The test dose shall be administered by gavage. If desired, water-insoluble compounds may be dissolved in 1 volume of triethanolamine and diluted with 99 volumes of PEG 200.

7.6 Food may be withheld overnight prior to dosing.

7.7 Blood samples shall be collected by any convenient route 24 h after anticoagulant administration.

7.8 Coagulation times shall be determined using an established test methodology that tabulates coagulation time against INR.

7.9 An animal whose coagulation time corresponds to a value less than the specified INR would be provisionally identified as resistant. For Norway rat and house mouse, the specified INR value is 5.0 (see 3.4 and 3.5 above).

EDITOR

Rodenticide Resistance Action Committee of CropLife International
Following companies are represented in the RRAC:
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CropLife International
Avenue Louise 143
B - 1050 Brussels
Belgium

Tel +32 542 04 10
Fax +32 542 04 19

croplife@croplife.org
http://www.croplife.org