Evaluation of a point-of-care anticoagulant rodenticide test for dogs

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Abstract

Objective – To evaluate a point-of-care anticoagulant rodenticide lateral flow analyzer for the detection of various rodenticide compounds.
Design – Prospective, laboratory study.
Setting – University teaching hospital.
Animals – The study utilized a serum sample from one healthy canine donor. Samples were centrifuged and serum samples were aliquoted and either used within 4 hours or frozen at −70°C for further quantitative analysis.
Interventions – Samples were spiked with clinically relevant concentrations of 1 of 6 rodenticide compounds (warfarin, pindone, chlorphacinone, brodifacoum, bromethalin, and its metabolite desmethylbromethalin). Seventy-five microliters of spiked serum (or unaltered serum) was introduced into the lateral flow test.
Measurements and Main Results – Three readers who were blinded to the sample preparation interpreted the lateral flow test as either positive or negative for the presence of anticoagulant rodenticide. All readers were in agreement for the results of each serum sample. The point-of-care test kit was able to detect a single anticoagulant rodenticide (warfarin) at concentrations below the manufacturer's recommended limit of detection, but was unable to detect any other anticoagulant rodenticide.
Conclusions – The results of this test and therapeutic interventions must be considered in light of history, physical examination, and other clinical data. Based on results from this study, the test kit only detects warfarin and not other more common second-generation anticoagulant rodenticides.

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Introduction

Anticoagulant rodenticide toxicosis is one of the most common intoxications in small animals[1] and is a major cause of morbidity and mortality.[2–4] Anticoagulant rodenticides are widely used in agriculture and rodent control and can result in uncontrolled hemorrhage and death.[5–7] These compounds inhibit hepatic vitamin K_1 epoxide reductase thereby reducing synthesis of functional vitamin K_1-dependent clotting factors (factors II,

Abbreviations

aPTT	activated partial thromboplastin time
PT	prothrombin time

VII, IX, X) by preventing carboxylation of glutamate and formation of gamma-carboxyglutamate (Gla) residues.[8] Formation of Gla domains is essential for functional vitamin K_1-dependent clotting factors. Anticoagulant rodenticides are categorized as either first-generation anticoagulants such as chlorphacinone and warfarin or second-generation anticoagulants such as brodifacoum, bromadiolone, and difenacoum. The second-generation agents are more toxic due to their extended half-life resulting in prolonged effects on hemostasis.[9]

While anticoagulant rodenticide toxicosis is a potentially fatal condition, patients may be treated successfully if the diagnosis is made quickly and appropriate therapy is instituted. Diagnosis of anticoagulant

rodenticide toxicosis is often made based on a history of exposure, clinical signs, and coagulation testing. In the emergency setting, screening tests of coagulation involving in vitro activation of parts of the clotting cascade such as one step prothrombin time (PT) and activated partial thromboplastin time (aPTT) are performed to aid diagnosis in animals with coagulopathy. PT and aPTT are not specific tests and may be affected by other disease processes such as liver failure,[10,11] disseminated intravascular coagulopathy,[12] clotting factor deficiencies,[13] and inflammatory processes.[14,15] The qualitative or quantitative chemical measurement of an anticoagulant rodenticide concentration in blood, liver, or other tissues remains the gold standard for confirming rodenticide exposure.[16] However, chemical assay availability and the time required to complete and report the analysis limits their immediate usefulness in the emergency setting.[16–20]

Simple standardized point-of-care tests for anticoagulant rodenticides are desirable to improve the ability of clinicians to rapidly diagnose anticoagulant rodenticide poisoning. Recently, a semiquantitative lateral flow assay for anticoagulant rodenticides[a] has been developed for the detection of "super-rodenticides, warfarin, coumadin, and their derivatives at concentrations as low as 75 ng/mL limit of detection (LOD) in serum or plasma."[b]

Lateral flow assays have been used extensively as diagnostic tools for monitoring toxins,[21] pathogens,[22–24] and biomarkers[25] in veterinary medicine. These assays are simple to use and require minimal training. The basis of this test is utilization of a target compound-antibody reaction on a membrane that results in the formation of a color band by a colorimetric reaction. The flow test is scored by visual inspection for staining of the antigen line, and therefore interpretation is subjective. The purpose of the study reported here was to evaluate the ability of this point-of-care rodenticide toxicity kit to detect several common anticoagulant and nonanticoagulant rodenticide compounds. Our hypothesis is that this point-of-care anticoagulant rodenticide lateral flow analyzer will be able to detect various anticoagulant rodenticides.

Materials and Methods

Sample preparation

A single dog was used for sampling and deemed healthy based on physical examination, no history of bleeding, or other medical problems and a normal PT. A single 60 mL blood sample, not exceeding 5% of the dog's blood volume, was obtained without difficulty by jugular venipuncture using a 20-Ga needle and vacutainer. The sample was allowed to clot for 15 minutes then immediately separated. Nonhemolyzed serum was placed in a plastic tube for further testing. Except for the

quantitative analyses, all samples were processed within 4 hours of serum collection. The serum sample was maintained at room temperature during stock solution preparation. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

Analytical standards

Analytical standards of each of the anticoagulant rodenticide groups: warfarin,[c] pindone,[d] chlorphacinone,[e] and brodifacoum[f] were evaluated (Figure 1). The nonanticoagulant rodenticide bromethalin[g] and its metabolite desmethylbromethalin[h] were also evaluated for specificity. Analytical grade methanol[i] (MeOH) was used to prepare all rodenticide stock solutions.

All rodenticides were weighed[j] separately, transferred to volumetric flasks, and dissolved in MeOH to prepare 0.5 mg/mL stock solutions.[26] Each rodenticide stock solution was thoroughly mixed and stored at room temperature until use. Additional volumes of MeOH were added to dilute each of the stock solutions to a working concentration of 5 ng/μL. An appropriate volume of the second-stock solution was added to 100 μL serum to obtain desired final concentrations (10–500 ng/mL) of each test compound. The spiked serum sample was inverted 10 times to ensure sample mixing in the final dilution of test samples.

Experimental protocol: All assays were performed in accordance with the manufacturer's instructions.[b] Spiked serum samples with the following concentrations were tested for each group: warfarin (10, 25, 75, 250 ng/mL), pindone (75, 250, 500 ng/mL), chlorphacinone (75, 250, 500 ng/mL), bromethalin (500 ng/mL), and desmethylbromethalin (500 ng/mL). Negative controls using unaltered serum and serum/MeOH were also tested. The assay test apparatus has a small sample well that receives 75 μL of serum.

In this test kit, which is designed differently than the majority of lateral flow assays, the absence of a color band in the test line indicates a positive test. The manufacturer's product insert[b] states that the test portion of the membrane is coated with warfarin-like particles, and an antibody to warfarin and its derivatives is conjugated to a detector particle binding to either the anticoagulant on the test line (if the animal serum is void of warfarin) resulting in a colorimetric reaction or to the anticoagulant in the animal serum (if the sample is positive) resulting in the failure of a colorimetric reaction to develop at the test line. A single red line in the control area on the membrane is visible to indicate if the sample migrated across the membrane as intended.

At exactly 5 minutes, 3 blinded readers who recorded their answers as positive for the presence of rodenticide
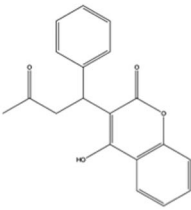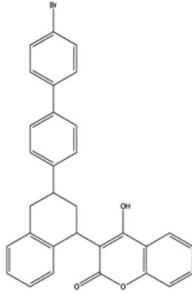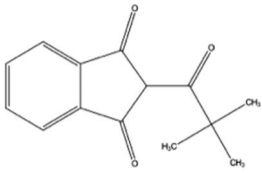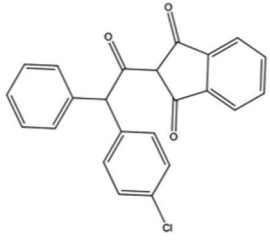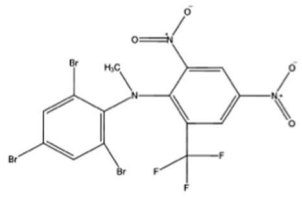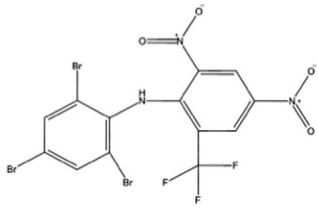
Rodenticide	Structure
Warfarin	
Brodifacoum	
Pindone	
Chlorophacinone	
Bromethalin	
Desmethylbromethalin	

Figure 1: Chemical structures of rodenticides.

or negative for the presence of rodenticide evaluated the test kit. Only the preparer of the samples had understanding of the sample concentration and compound identity. As per the manufacturer's instructions, the test result was only considered valid when the control line was clearly visible (Figure 2). The appearance of a red line in the control area and a second red line in the test area was considered negative for the presence of
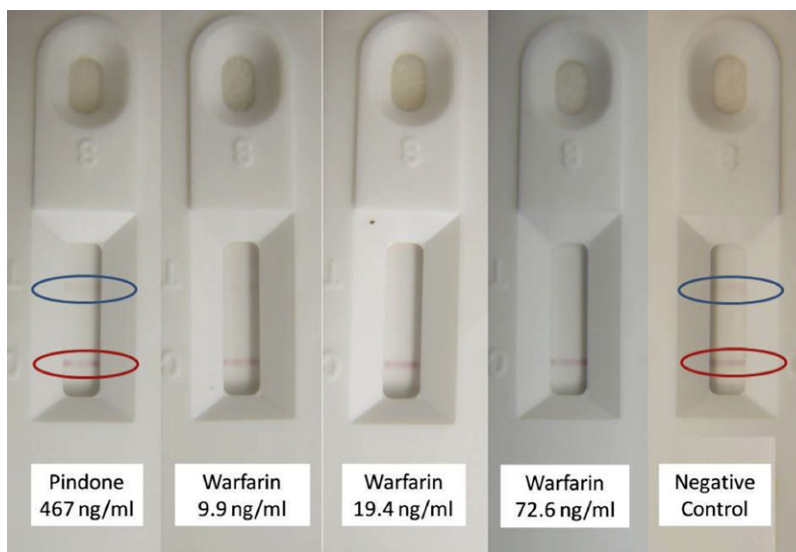
Figure 2: Negative control area (red). Test area (blue). A negative control line present in each test indicated the testing was performed correctly. The additional faint line in the test area of the 1st and 5th tests indicates the test was negative for anticoagulant rodenticide, while the absence of this additional line in the test area of the 2nd, 3rd, and 4th tests indicates the tests were positive for anticoagulant rodenticide.

rodenticide. The absence of a red line in the test area with the presence of a red line in the control area was considered positive for the presence of rodenticide.

Chemical confirmation

A 500 µL aliquot of each spiked serum sample was immediately stored at −70°C for future chemical analysis. Spiked serum samples were sent to a diagnostic laboratory for analysis. All spiked serum samples were analyzed using high-performance liquid chromatography to quantitate sample concentrations. All sample analysis was completed within 2 months.

Results

The results for each compound, nominal concentration, and quantitative chemical assay results are reported in Table 1. The lateral flow assay produced a consistently positive result with concentrations below the reported assay LOD for warfarin. No other anticoagulant rodenticide compound tested was recorded to be positive. The negative controls yielded a negative result, indicating no anticoagulant was present in the samples. All 3 readers examined each test kit and reported identical answers.

Discussion

The goal of this study was to determine whether a commercially available test kit was effective for the detec-tion of warfarin, brodifacoum, pindone, and chlorphacinone. As with any colorimetric reaction, the subtle difference between positive and negative for the presence of anticoagulant rodenticide could be misinterpreted. We therefore used 3 individuals to independently interpret the test results using the manufacturer's instructions. We also found the test kit to be rapid (sample preparation and time to complete the assay was <30 min). As expected, the test kit was effective at detecting serum samples spiked with warfarin even at concentrations below the stated assay LOD. In addition, the test was not associated with false-positive results resulting from either control or bromethalin- or desmethylbromethalin-spiked serum sample. However, the ability of this test to detect brodifacoum, pindone, and chlorphacinone in blood was not supported by our study results. We observed false-negative test results with serum samples spiked with brodifacoum, pindone, and chlorphacinone at >6 times higher than the manufacturer's stated LOD. This inability to detect second-generation anticoagulants is clinically important since the majority of anticoagulant rodenticide poisonings result from the ingestion of brodifacoum and other second-generation rodenticides.[20,27–29] A negative test result may lead to withholding administration of vitamin K_1 or other life-saving treatments. The anticoagulant serum concentrations used in our study (75–500 ng/mL) are similar to those found in animals with clinical rodenticide intoxication.[30]

One possible explanation for the inability of this test to detect "super-rodenticides" is the specificity of the

Table 1: Actual serum rodenticide concentrations (in ng/mL) and assay results

	Actual serum concentrations HPLC (ng/mL)/lateral flow results									
	10 ng/mL		20 ng/mL		75 ng/mL		250 ng/mL		500 ng/mL	
Rodenticide	HPLC	Lateral flow	HPLC	Lateral flow	HPLC	Lateral flow	HPLC	Lateral flow	HPLC	Lateral flow
Warfarin	9.87	+	19.4	+	72.6	+	191	+	ND	NT
Brodifacoum	NT	NT	NT	NT	72	−	288	−	548	−
Pindone	NT	NT	NT	NT	59	−	199	−	467	−
Chlorophacinone	NT	NT	NT	NT	35	−	175	−	375	−
Bromethalin	NT	NT	NT	NT	NT	NT	NT	NT	NT	−
Desmethyl-bromethalin	NT	NT	NT	NT	NT	NT	NT	NT	NT	−

+, assay was considered positive for the presence of the rodenticide; −, assay was considered negative for the presence of the rodenticide; HPLC, high-performance liquid chromatography; ND, not detected; NT, not tested

antibody used in production of the test kit, or direction toward a functional group not present in most other anticoagulant rodenticides. Attempts made to contact the manufacturer regarding direction of the antibody at specific functional groups were unsuccessful.

There are a number of limitations in this report. First, the in vitro nature of the study and use of MeOH to prepare stock solutions may have led to inaccurate results. Given this possibility, MeOH and serum were evaluated and produced a negative result on the lateral flow analyzer. A second limitation of this bedside test is the potential misinterpretation of the colorimetric reactions given the subtle difference between the presence and absence of a colorimetric reaction in the test area. However, the readers in this study did not differ in their visual interpretations of the results.

A third limitation in this study is the use of one canine serum donor. It is plausible that the results could be altered and possibly strengthened had the study included multiple canine and feline serum donors. However, it is unlikely that the use of a single donor resulted in spurious false-negative assay results since positive results were seen with serum samples spiked with warfarin. Finally, the variability in measured spiked serum sample concentrations versus the expected sample concentrations is also considered a limitation. The samples were prepared by a single operator and mixed manually. It is unknown whether the decrease in measured concentration may reflect instability in the stock solution. Although the measured concentration was often lower than expected, these concentrations still reflect clinically relevant concentrations in toxicosis.

There remains a need for the development of point-of-care test kits for the rapid diagnosis of anticoagulant rodenticide exposure. In our study, the point-of-care anticoagulant rodenticide kit demonstrated excellent results for the detection of warfarin. However, the test was unable to detect brodifacoum and several other more commonly encountered second-generation anticoagulant rodenticides. Therefore, this test kit must be used and interpreted in an emergency setting with extreme caution and in combination with history, standard coagulation testing, quantitative send-out testing, and clinical signs. This test kit is best suited for the detection of exposures arising from the ingestion of warfarin-based rodenticides.

Acknowledgment

Footnotes

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