Detection of Snake Venom in Horse Urine and Plasma

— Validation of the snake venom detection kit in horses —

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by Sally Church and Grace Forbes

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Foreword

The veterinary profession and horse owners in Australia are sometimes confronted with horses that have clinical signs suggestive of snake bite, but the diagnosis is rarely confirmed. A snake venom detection kit (CSL Ltd Parkville Australia) produced for use in people, has found increasing application in the diagnosis of snake bite in dogs and cats, but has often returned negative results in horses demonstrating clinical signs suggestive of snake bite. These negative results can influence the decision as to whether or not antivenene is administered.

There is no information on the clinical effectiveness of the snake venom detection kit test in horses. Clinical impressions have been that horses with signs suggestive of snake bite rarely test positive, even when subsequent treatment with multivalent snake antivenene is accompanied by clinical improvement. This suggests that either elements in horse body fluids interfere with the test or the levels of venom present in horses after a snake bite may be too low for the test to detect. There is a need to determine the clinical usefulness of the snake venom detection kit test in horses. Firstly its ability to detect snake venom in horse body fluids, and the limits of the concentrations it can detect. Subsequently whether these concentrations are likely to occur in horses that have been bitten by a snake, and whether those concentrations are likely to still be present when horse start showing the signs suggestive of envenomation.

The project determined that the commercially available CSL produced snake venom detection kit is very effective at detecting the presence of each of the five major genera of snake venoms (tiger, brown, black, death adder and taipan) in horse urine and plasma. The lower limit of detection appears to be 5-10 nanograms per millilitre. Whether this concentration is reached in horses bitten by snakes, and whether it is still present when horses start to show clinical signs of snake bite, remains to be determined. Both pieces of information are important in establishing the usefulness of the snake venom detection kit test in horses and should be the aims of further research in this area.

This project was funded by RIRDC, the University of Melbourne and the first author (Sally Church).

This report is an addition to RIRDC’s diverse range of over 2000 research publications and it forms part of our Horse R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

Most of RIRDC’s publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
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Acknowledgments

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Abbreviations

SVDK  Snake venom detection kit
ug/ml  micrograms per millilitre
ng/ml  nanograms per millilitre
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Executive Summary

What the report is about

Assessing the ability of the CSL produced Snake Venom Detection Kit (SVDK) to detect Australian snake venoms in horse urine and plasma.

Who is the report targeted at?

Equine veterinarians and horse owners who need to diagnose and treat potential cases of snake bite in horses.

Background

There is no detailed clinical or laboratory data published on venomous Australian snake bites in horses and confirming envenomation is difficult. The Australian snake venom detection test kit (SVDK) produced by CSL, is often used to detect and identify venom in people and its use in dogs and cats is frequently successful. Unfortunately our clinical experience suggests horses, in which snake-bite is suspected, rarely test positive for venom with this kit, even when subsequent treatment with multivalent snake antivenene is accompanied by clinical improvement. This suggests that either something in horse urine or plasma interferes with the test or that the concentration of venom in horses with clinical signs is too low for the test to detect. As false negative test results can cause appropriate treatment to be delayed or withheld there is a need to establish the value of using this SVDK in horses.

Aims/objectives

To determine the sensitivity and specificity of the SVDK in detecting known quantities of the five major snake venoms in horse plasma and urine. This represents the first step in determining the value of this test in horses and assisting veterinarians and horse owners in establishing a diagnosis of snake bite in horses.

Methods used

Five venoms were each tested in 10 different horse urine samples at two different concentrations previously indicated to be either the optimal concentration or the lower limit of detection for the SVDK in non-biological fluids. The five venoms were Notechis scutatus (Tiger snake), Pseudonaja textilis (Common Brown snake), Pseudonaja australis (Black snake), Oxyuranus scutellatus (Taipan) and Acanthophis antarcticus (Death Adder). These five venoms were also tested in 10 different horse plasma samples. Control samples (with no venom added) of urine and plasma from 10 different horses were also assessed.

Results/key findings

The SVDK reliably detected all five genera of snake venoms in both horse urine and heparinised plasma. When venom concentrations were optimal for detection by the kit (1 to 10 ug/ml) the test sensitivity and specificity was 100% (with a 95% confidence interval of 69.2-100%). This 100% sensitivity and specificity was also seen at concentrations of 100 ng/ml (death adder, tiger snake) 10 ng/ml (taipan and black snake) and 5 ng/ml (common brown snake). Results were less accurate when tiger snake venom was tested at 10 ng/ml and were essentially useless when testing common brown snake venom at 2 ng/ml or tiger snake venom at 1 ng/ml.

The intensity of the colour reaction demonstrated by the SVDK test varies according to the concentration of venom in the sample. At low venom concentrations the colour change is difficult to
see when test wells are viewed from above, and viewing the test wells from in front, against a white background, is strongly recommended to detect low concentrations of venom.

**Implications for relevant stakeholders**

The SVDK can reliably and accurately detect Australian snake venoms from five different snake genera when it is present in horse urine and plasma, provided venom is present at a concentration within the SVDK’s limits of detection.

The lower limit of detection for the different venoms in equine biological fluids appears to be close to:

- 10 ng/ml for tiger, black and taipan venoms
- 5 ng/ml for brown snake venom
- less than 100 ng/ml for death adder venom (the lowest concentration tested)

Just how long venom remains at detectable concentrations in horses bitten by snakes is not known, however this knowledge is fundamental to determining whether the SVDK has any clinically useful role to play in diagnosing Australian snake bite in horses. The authors believe this information should be determined by further experimental investigation.

**Recommendations**

Further experimental work needs to be done to determine how long detectable concentrations of Australian snake venom remain in horse body fluids after envenomation and whether horses are likely to be showing any clinical signs of envenomation during the period that venom is detectable.
Introduction

Little is published about the effect of Australian snake bites in horses, possibly because snake bite is rarely confirmed in this species. Owners rarely, if ever, witness a horse being bitten and the presence of venom is rarely confirmed in a horse. There are no detailed accounts of clinical signs or laboratory parameters in envenomated horses to assist veterinarians diagnose snake bite in horses. Kellaway has provided very brief clinical descriptions and autopsy reports of two or three horses injected with differing amounts of five venoms (Kellaway 1929a, 1929b, 1929c, 1930, 1931).

The commercially available snake venom detection kit (SVDK) (CSL Ltd, Parkville, Australia) is a rapid in vitro sandwich enzyme immunoassay. It is able to detect the venom for the five major genera of snakes within Australia (Tiger, Brown, Black, Death Adder and Taipan). It is designed for use in humans, but has found increasing application in detecting and identifying Australian snake venoms in dogs and cats (Moisidis et al. 1996, Heller et al. 2007). Unfortunately clinical experience suggests that horses in which snake-bite is suspected rarely test positive for venom with this kit, even when subsequent treatment with multivalent snake antivenene is accompanied by clinical improvement. The inference is there may be something in horse urine or serum that interferes with the test or alternatively the levels of venom present in horse urine or plasma after a snake bite may be too low for the test to detect. Either way, the presumed, false negative test results can cause appropriate treatment with antivenene to be delayed or withheld. There is a need to determine the clinical usefulness of the SVDK test in horses.

In contrast to urine from other species, equine urine has a unique composition, it is alkaline and contains a lot of mucus and calcium carbonate crystals. It is possible that something in horse urine or plasma could interfere with venom detection by the SVDK. If however the SVDK can detect venom in horse urine and plasma, then the limits of concentrations detectable by the SVDK and the concentration of venom that occurs in envenomated horses become very relevant to the clinical usefulness of this test in horses.
Objectives

To determine the effectiveness (sensitivity and specificity) of the commercially available snake venom detection kit (SVDK), produced by CSL in detecting snake venom in horse urine and plasma using known concentrations of five venoms (Brown, Tiger, Black, Death Adder and Taipan).
Methodology

Biological fluids and reagents

Individual urine samples were collected from 10 different horses into clean, sterile containers either by free catch or passing a lubricated, sterile, urethral catheter. The same 10 horses were not necessarily used for each of the venoms assessed. Urine appearance ranged from clear, pale yellow to viscous, turbid and amber, consistent with the range seen in normal horses. Urine was used fresh the day it was collected or after storage overnight in a refrigerator at 4°C.

Individual blood samples were collected from the jugular vein of 10 different horses into lithium heparin anticoagulant. Plasma was then separated from the cells by centrifugation. Plasma was used either on the day of collection or after storage overnight in a refrigerator at 4°C.

Freeze dried venom of *Notechis scutatus* (Tiger snake), *Pseudonaja textilis* (Common Brown snake), *Pseudonaja australis* (Black snake or King Brown snake), *Acanthophis antarcticus* (Death Adder) and *Oxyuranus scutellatus* (Taipan) was purchased from Venom Supplies Pty Ltd (Tanunda South Australia) and stored in a refrigerator at 4°C until used.

Snake venom detection kits (SVDK) were purchased from CSL laboratories (Parkville, Melbourne Victoria) and stored at 4°C until used according to the manufacturer’s directions.

Venom concentrations

Five venoms were each tested in 10 different horse urine samples at two different concentrations previously suggested to be either the optimal concentration or the lower limit of detection for the SVDK in non-biological fluids (Steuten et al 2007). For each venom these concentrations were respectively:

- *Notechis scutatus* (Tiger snake) 1 microgram/ml (µg/ml) and 1 nanogram/ml (ng/ml)
- *Pseudonaja textilis* (Common Brown snake) 1 µg/ml and 2 ng/ml
- *Pseudonaja australis* (Black snake) 1 µg/ml and 10 ng/ml
- *Oxyuranus scutellatus* (Taipan) 1 µg/ml and 10 ng/ml
- *Acanthophis antarcticus* (Death Adder) 10 µg/ml and 100 ng/ml.

When the test failed to reliably detect the lower limit tested for Tiger and Brown snake venom, these venoms were re tested at a slightly higher concentration (10 and 5 ng/ml respectively) in an attempt to identify more clearly the lower limit of detection for these venoms.

The five venoms were also each tested in 10 different horse plasma samples. The concentration tested was either the optimal concentration for detection by the SVDK (Black and Brown snake venom 1µg/ml), a 10 fold dilution of the optimal concentration (Tiger snake venom 100 ng/ml) or the lower limit of detection anticipated for the venom (Taipan venom 10 ng/ml and Death adder venom 100 ng/ml).

Venom concentrations were prepared by dissolving 20 milligrams of freeze dried venom in 10 ml of sterile distilled water, then serially diluting 1 ml of venom solution into 9 ml of distilled water until a concentration 10 or 20 times that required was attained. One millilitre of these solutions was then diluted in either 4 ml or 9 ml respectively of urine or plasma.
Solutions tested by the SVDK are diluted 1:1 with a test diluent, which effectively halves the concentration of venom in the tested sample. Our dilutions were calculated so that the concentration tested represented the concentration of venom in the test sample after it was diluted with the test diluent. A clinical sample would have to contain twice the concentration tested to provide equivalent results.

**Sample testing and controls**

Each sample was tested with the snake venom detection kit (SVDK) according to the manufacturer’s instructions. Two drops of test sample were added to each well of an eight well enzyme immunoassay test strip (five test wells, a positive and negative control and a blank well). Following ten minutes incubation at room temperature, the wells were washed, (seven times for urine and 14 times for plasma) under running tap water before adding one drop each of a chromogen solution and a peroxide solution to every well. Wells were observed continuously, against a white background, for the development of blue colour over 10 minutes. The result was determined visually by the first well to show colour development, after the positive control and before 10 minutes had elapsed.

To validate the test for clinical use in horses it was most important to assess the test visually, as would be done in clinical practice. We had initially anticipated obtaining quantitative results by reading the colour change with spectrophotometry. However, it is not the colour intensity at a predictable time interval that determines the test outcome but rather the test well that first develops colour after the positive control. The development of colour is not completed at a predictable time interval, it may take anywhere between a few seconds and 10 minutes depending on the venom concentrations. Australian snake venoms are known to be immunologically cross-reactive (Steuten et al 2007) so more than one test well may show a colour change within the 10 minute observation period. It is not colour intensity that is diagnostic but rather the timing of the colour change.

**Statistical Analysis**

The 95% confidence interval for the results were determined by the binomial exact method using STATA version 10.1 software (Stata Corp, College Station Texas)
Results

The results of the venom assays in horse urine and plasma using the snake venom detection kit (SVDK) are shown in Table 1 and Table 2 respectively. The SVDK reliably detected and correctly identified each of the five snake venoms in horse urine when it was present at the optimal concentration for detection. Optimal concentration has been estimated to be 10 ug/ml for Death adder venom and 1 ug/ml for Tiger, Brown, Black and Taipan venoms (Steuten et al 2007). At these concentrations the SVDK demonstrated positive results for each of the 10 samples tested (100% sensitivity with a 95% confidence interval of 69.2 – 100%). At venom concentrations estimated to be at the lower limit of detection for the SVDK, results varied. Sensitivity of 100% (95% confidence interval 69.2-100%) was obtained for Death adder venom at 100 ng/ml as well as Taipan and Black snake venom at 10 ng/ml. Brown snake venom was not detected at all at 2 ng/ml (0% sensitivity with 95% confidence interval 0-30.8%). Tiger snake venom was correctly detected in only two of 10 samples at 1 ng/ml (20% sensitivity, 95% confidence interval 2.5-55.6%), in three samples at this concentration it was incorrectly identified as brown snake venom, in the other five samples no venom was detected. We believe these results reflect true cross reactivity between the venoms. There was no possibility that any of the tiger snake venom samples had been contaminated by brown snake venom because, at the time of tiger snake venom testing, no brown snake venom had ever been in the laboratory or handled by either of the authors (who performed all the testing).

The poor SVDK test results obtained for the lowest levels of tiger and brown snake venom tested prompted us to test them at slightly higher concentrations in an attempt to better approximate the lowest limit at which the SVDK could reliably detect and identify these venoms in horse urine. Brown snake venom at 5 ng/ml was correctly identified in 10 out of 10 samples (100% sensitivity 95% confidence interval 69.2-100%). Tiger snake venom at 10 ng/ml was correctly identified in eight out of 10 samples (80% sensitivity 95% confidence interval 44-97.5%).

Testing brown and black snake venom in horse plasma at optimal concentrations for the SVDK gave the same results as when testing in urine (100% sensitivity with a 95% confidence interval of 69.2-100%), suggesting nothing in horse plasma appeared to interfere with the test. Lower than optimal concentrations in plasma were subsequently tested for the remaining three venoms (10 or 100 ng/ml). All returned positive test results (100% sensitivity with 95% confidence interval of 69.2-100%).

The lower limit of reliable venom detection in horse body fluids appears to be close to 10 ng/ml (tiger, black and taipan venoms) 5 ng/ml (brown snake venom) and less than 100 ng/ml for Death adder venom (lowest concentration tested for this venom).

No positive result for venom was obtained when testing the 10 urine control or 10 plasma control samples, indicating a specificity of 100% (95% confidence interval 69.2-100%).

The intensity of the colour reaction demonstrated by the SVDK test varied according to the concentration of venom in the sample. At low venom concentrations the colour change was difficult to see when test wells were viewed from above, and viewing the test wells from in front against a white background is strongly recommended to detect low concentrations of venom.
### Table 1  
Snake venom detection kit test results detecting venom in horse urine.

<table>
<thead>
<tr>
<th>Venom</th>
<th>Optimal concentrations</th>
<th>Concentrations near lower limits of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ug/ml</td>
<td>1 ug/ml</td>
</tr>
<tr>
<td>Tiger</td>
<td>na</td>
<td>10/10</td>
</tr>
<tr>
<td>Brown</td>
<td>na</td>
<td>10/10</td>
</tr>
<tr>
<td>Black</td>
<td>na</td>
<td>10/10</td>
</tr>
<tr>
<td>Death adder</td>
<td>10/10</td>
<td>na</td>
</tr>
<tr>
<td>Taipan</td>
<td>na</td>
<td>10/10</td>
</tr>
</tbody>
</table>

Positive test result/Number of samples tested; na = not assessed; ug/ml = microgram per millilitre; ng/ml = nanogram/millilitre

### Table 2  
Snake venom detection kit test results detecting venom in horse plasma.

<table>
<thead>
<tr>
<th>Venom</th>
<th>Concentration</th>
<th>Positive results/Samples tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiger</td>
<td>100 ng/ml</td>
<td>10/10</td>
</tr>
<tr>
<td>Brown</td>
<td>1 ug/ml</td>
<td>10/10</td>
</tr>
<tr>
<td>Black</td>
<td>1 ug/ml</td>
<td>10/10</td>
</tr>
<tr>
<td>Death adder</td>
<td>10 ng/ml</td>
<td>10/10</td>
</tr>
<tr>
<td>Taipan</td>
<td>100 ng/ml</td>
<td>10/10</td>
</tr>
</tbody>
</table>

ug/ml = microgram per millilitre; ng/ml = nanogram/millilitre
Implications

There is no substance in horse urine or plasma that interferes with the detection of Australian snake venoms by the SVDK. Provided venom is present at a concentration within the SVDK’s limits of detection, it should return a positive result.

Just how long venom remains at detectable concentrations in horses bitten by snakes is not known, however this knowledge is fundamental to determining whether the SVDK has any clinically useful role to play in diagnosing Australian snake bite in horses. The authors believe this information should be determined by further experimental investigation.

Recommendations

Further experimental work needs to be done to determine how long detectable concentrations of Australian snake venom remain in horse body fluids after envenomation and whether horses are likely to be showing any clinical signs of envenomation during the period that venom is detectable.
References


Note – from 1914 to 1983 the Medical Journal of Australia published two volumes each year, but the volume numbering began each year with volume 1.
Veterinarians and horse owners in Australia are sometimes confronted with horses that have clinical signs suggestive of snake bite, but the diagnosis is rarely confirmed. A snake venom detection kit (CSL Ltd Parkville Australia) produced for use in people, has found increasing application in the diagnosis of snake bite in dogs and cats, but has often returned negative results in horses demonstrating clinical signs suggestive of snake bite. These negative results can influence the decision as to whether or not antivenene is administered.

This study assesses the ability of the CSL produced Snake Venom Detection Kit (SVDK) to detect Australian snake venoms in horse urine and plasma.

The report is targeted at equine veterinarians and horse owners who need to diagnose and treat potential cases of snake bite in horses.

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