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Development of a double sandwich fluorescent ELISA to detect rattlesnake venom in biological samples from horses with a clinical diagnosis of rattlesnake bite

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ABSTRACT

Rattlesnake bites in horses are not uncommon and the clinical outcomes are widely variable. Treatment of horses with anti-venom is often cost prohibitive and could have negative consequences; therefore, the development of a quantitative test to determine if anti-venom therapy is indicated would be valuable. The objective of this study was to develop an ELISA to detect rattlesnake venom in biological samples from clinically bitten horses. Nineteen horses were enrolled in the study. Urine was available from 19 horses and bite site samples were available from 9 horses. A double sandwich fluorescent ELISA was developed and venom was detected in 5 of 9 bite site samples and 12 of 19 urine samples. In order to determine if this assay is useful as a guide for treatment, a correlation between venom concentration and clinical outcome needs to be established. For this, first peak venom concentration needs to be determined. More frequent, consistent sample collection will be required to define a venom elimination pattern in horses and determine the ideal sample collection time to best estimate the maximum venom dose. This report describes development of an assay with the ability to detect rattlesnake venom in the urine and at the bite site of horses with a clinical diagnosis of rattlesnake bite.

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1. Introduction

In certain geographical areas it is common for horses to be bitten by rattlesnakes. The clinical response of horses to rattlesnake venom appears to be highly variable (Dickinson et al., 1996; Fielding et al., 2011; Gilliam et al., 2012; Lawler et al., 2008; Rashmir-Raven and Brashier, 2000). Horses bitten by rattlesnakes can experience cardiac damage evidenced by increased cardiac troponin I or abnormal electrocardiograms (Gilliam et al., 2012; Lawler et al., 2008; Rashmir-Raven and Brashier, 2000). These cardiac changes can lead to decreased performance, loss of use, or death

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(Dickinson et al., 1996; Fielding et al., 2011; Gilliam et al., 2012; Lawler et al., 2008; Rashmir-Raven and Brashier, 2000). The reported mortality rates in horses are relatively high, varying from 9 to 25% compared to <1% in people (Dickinson et al., 1996; Fielding et al., 2011; Weinstein et al., 2009). The use of anti-venom is known to significantly reduce morbidity and mortality in people (Gold et al., 2004); however, treating an adult horse with anti-venom is often cost prohibitive. Additionally, the only anti-venom products available on the veterinary market are equine origin products. Serum sickness has been reported in horses secondary to the administration of equine origin biologics (Aleman et al., 2005; Guglick et al., 1995; Smith, 1996). The severity of clinical signs seen with rattlesnake envenomation could be directly related to the quantity of venom received. If this is true, knowing the amount of venom a horse received would be beneficial in determining whether or not anti-venom is necessary and could provide helpful information when giving horse owners a prognosis.

ELISAs have been widely used in venom detection (Selvanayagam and Gopalakrishnakone, 1999). Cross reactivity amongst rattlesnake species has been documented (Li and Ownby, 1992; Minton, 1987; Ownby and Colberg, 1990) and is likely due to similar toxins in the venoms. This cross reactivity, while detrimental when trying to determine an exact species, may prove beneficial when testing patients in areas with multiple rattlesnake species where the goal is venom quantification rather than species identification. The venom used in ELISA development is primarily focused on the most likely venom to be detected in a given sample population. In a sample population where multiple rattlesnakes are present and the goal is venom quantification rather than species identification, a venom's cross reactivity should be considered. *Crotalus viridis viridis* contains at least one toxin, a small myotoxin a, that has also been found in many other rattlesnake venoms making it a venom with high cross reactivity (Bober et al., 1988; Ownby and Colberg, 1990). The type of antibodies used in ELISA development for venom detection have a significant effect on specificity (Selvanayagam and Gopalakrishnakone, 1999). Using polyclonal antibodies, is less labor intensive and costly and increases the occurrence of cross reactivity (Selvanayagam and Gopalakrishnakone, 1999).

Detection of rattlesnake venom has not been reported in equine biological samples; however, an ELISA that identifies Australian snake venom (Forbes and Church, 2010) is widely used for venom detection in horse urine as well as other species because of its low cost, high sensitivity, and rapidity (Hung et al., 2003). We developed a double sandwich fluorescent ELISA to detect venom at the bite site as well as in the urine of rattlesnake bitten horses residing in the Texas panhandle and Oklahoma. The goal of the assay was to detect venom from any of the rattlesnakes endemic to these areas including *C. viridis viridis*, *Crotalus atrox*, *Crotalus horridus horridus*, and *Sistrurus miliarius streckeri*.

2. Materials and methods

2.1. Materials

C. viridis viridis lyophilized venom generously donated from the venom laboratory at Oklahoma State University was used in the development of this assay. Anti-venoms used were equine polyvalent crotalidae anti-venom[2] and ovine polyvalent crotalidae anti-venom.[3] Ninety-six well flat bottom microtiter ELISA plates[4] were purchased from Thermo Scientific. Lyophilized donkey serum (60 mg/ml)[5] and alkaline phosphatase labeled donkey anti-sheep IgG (0.6 mg/ml)[6] were purchased from Jackson Immunolabs. 4-Methylumbelliferyl phosphate (0.6 mM)[7] was purchased from Sigma.

2.2. ELISA for rattlesnake venom

Checkerboard plates were designed and multiple concentrations of anti-venoms, blocking agent, urine/venom dilutions, conjugate, and substrate were used to define the ideal concentration for each component of the assay in order to detect the smallest amount of venom with the least amount of background. These plates were performed with known venom dilutions in incubation buffer as well as urine. The final assay recipe was as follows. Ninety-six well, flat bottom polystyrene microtiter plates were coated using 50 μl per well of sodium bicarbonate coating buffer (pH 9.6) containing 0.1 μg/ml horse polyvalent crotalidae anti-venom[2] and incubated at room temperature overnight. Plates were washed three times using PBS (pH 7.4), then blocked using 200 μl 5% donkey serum per well and incubated at room temperature overnight. Plates were washed three times using PBS, then donkey serum was added again (200 μl 5% donkey serum per well) and plates were incubated for 1 h at room temperature. The plates were washed three more times using PBS and donkey serum was added once more (for a total of 3 times including the overnight incubation) and the plate was incubated for 1 h at room temperature. Samples were centrifuged at 134.2 g for 15 min and then urine and bite site samples were diluted 1:5 and 1:100 respectively with incubation buffer (PBS/0.05% Tween-20). Control urine and wound samples were handled the same as clinical samples. The plate was washed three times using PBS and then all samples were added in triplicate (50 μl/well) and incubated for 1 h at room temperature. Plates were washed three times using PBS. Incubation buffer containing 10 μg/ml sheep polyvalent crotalidae anti-venom was added (100 μl/well) and the plate was incubated at room temperature for 1 h. Plates were washed five times using PBS. Tris Buffered Saline (pH 7.4)[8] containing alkaline phosphatase labeled donkey anti-sheep immunoglobulin was added (50 μl/well) and the plate was incubated at room temperature for 1 h. A conjugate dilution of 1:200 was used for bite site samples, while a 1:50 dilution was used for urine samples. The plate was washed using PBS-Tween six times with one minute soaks in between each wash. A 1:5 dilution of 4-methylumbelliferyl

[2] Antivenin (Crotalidae) Polyvalent, Fort Dodge, Overland Park, KS.

[3] CroFab generously donated by Dr. Steve Mackessy.

[4] Immulon 4-HBX, Thermo Scientific, Pittsburgh, PA.

[5] Donkey serum, Jackson Immunolabs, West Grove, PA.

[6] Alkaline Phosphatase-conjugated AffiniPure donkey anti-sheep IgG, Jackson Immunolabs, West Grove, PA.

[7] 4-Methylumbelliferyl phosphate liquid substrate system, Sigma, St. Louis, MO.

[8] Tris Buffered Saline, Fisher Scientific, Waltham, MA.

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