

## Duration of *Loxosceles reclusa* Venom Detection by ELISA from Swabs

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**BACKGROUND:** Diagnosis of *Loxosceles reclusa* envenomations is currently based upon clinical presentation. An enzyme-linked immunosorbent assay (ELISA) can detect surface *Loxosceles* venom at the envenomation site, allowing diagnostic confirmation. The length of time that venom on the skin is recoverable non-invasively is unknown.

**MATERIALS AND METHODS:** To investigate duration of recoverable venom antigen, whole venom and fractionated sphingomyelinase D venom aliquots were injected subcutaneously in New Zealand White rabbits. Cotton and Dacron swabs were compared for venom recovery over a 21-day period using a surface swab technique.

**RESULTS:** Significant amounts of *Loxosceles reclusa* antigen were found on the surface of rabbit skin after experimental injection of whole venom and sphingomyelinase D. The duration of recoverable antigen using this experimental model appears to be at least two weeks and as long as 21 days in some cases.

**CONCLUSIONS:** Because the duration of the recoverable antigen is seen to be at least two weeks,

the ELISA venom test appears capable of detecting venom on most patients presenting with *Loxosceles* envenomations. This detection system will allow the physician more accurate determination of whether the lesion is from a brown recluse spider or some other agent that can cause this type of necrotic ulcer.

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*injection of venoms and swab collection was instrumental in the accomplishment of this protocol.*

## INTRODUCTION

*Loxosceles reclusa* (common name: Brown Recluse Spider) and related arachnids are indigenous American spiders that possess a venom capable of causing painful necrotic ulcers with surrounding inflammation and possibly severe systemic effects<sup>1,2,3,4</sup>. The diagnosis of a brown recluse spider bite is made clinically, based on the appearance of the lesion<sup>1,2,3,4</sup>. Definitive diagnosis is usually not possible because few patients bring the offending spider to the clinician for identification. Even then, misidentification of the spider is a distinct possibility<sup>5</sup>. The appearance of significant envenomation with cutaneous necrosis is the usual basis for diagnosis but is not specific for *Loxosceles* species envenomation<sup>1,3,6</sup>. Indeed, a variety of treatable illnesses can also give rise to cutaneous necrotic ulcers, including staphylococcal, and streptococcal infections such as impetigo and cellulitis, herpes simplex infection, factitial injury, squamous cell carcinoma, toxicodendron dermatitis (poison ivy), diabetic ulcer, fungal infection, localized vasculitis, erythema nodosum, Stevens-Johnson syndrome, toxic epidermal necrolysis, and other arthropod bites including bites from ticks, triatomid bugs, hymenoptera, blister beetles, crickets, and grasshoppers<sup>5</sup>, sporotrichosis<sup>7</sup>, and even Lyme disease<sup>8</sup>. A test for *Loxosceles* envenomation is desirable for cases with significant systemic findings, for often misdiagnosed cases in nonendemic areas, and for cases of nonhealing ulcers and other wounds. All of these are often falsely attributed to loxoscelism<sup>5,7,9,10</sup>.

A polyclonal ELISA, derived from New Zealand White rabbits, was reported by Gomez et al<sup>11</sup>. In that study, 17 North American arthropod venoms elicited no cross-reactivity when assayed at relevant venom amounts<sup>12</sup>. The present study was designed to test the hypothesis that venom could be detected by using a swab assay and to determine how long, up to three weeks, venom could be detected after subcutaneous venom injection. The present study included controls

for both injection (a saline-only injection was performed) and for the type of swab (both Dacron and cotton swabs were used).

## MATERIALS AND METHODS

### Venom necrotic fraction purification

Purification of the fraction of the venom that causes necrosis in rabbits proceeded as previously described<sup>13</sup>. Polyacrylamide gel electrophoresis<sup>14</sup> was conducted using seven percent 5mm diameter acrylamide gels at pH 9.5. All electrophoresis reagents were purchased from Bio-Rad Laboratories, Richmond, CA. Stacking gels were 1.0 cm long and separating gels 4.1 cm long. Electrophoresis with ice water cooling was initiated at 2 mA/gel and continued at 4 mA/gel after samples entered the separating gel. Protein collection was accomplished by using dialysis tubing, with samples marked by bromphenol blue tracking dye. Proteins were concentrated using CF25 Centriflo ultra-filtration membrane cones.

Purification of an enriched venom necrotic fraction was accomplished by sequential fractionation over diethylaminoethyl cellulose (DE 52), carboxymethyl cellulose (CM 52) (Whatman, Clifton NJ) and Bio-Gel P-100 gel resin (Bio-Rad, Richmond CA). All chromatography steps were performed at 4°C. All dialysis tubing (Fisher, St. Louis MO) was boiled for 0.5 hr in a 0.3 M ethylenediamine tetraacetate (EDTA) solution followed by washing with deionized distilled water to avoid a major loss of lethal activity as measured in mice<sup>13</sup>. The protein purified by the method of Babcock et al. is a single subunit protein of 33 kD. This purified protein is the major fraction in the venom, comprising about 40% by weight<sup>15</sup>. The fraction has the lethal effect on mice of whole venom, is dermonecrotic to rabbits<sup>13</sup> and has sphingomyelinase D activity<sup>16</sup>. Accordingly, we have labeled this fraction the sphingomyelinase D fraction.

### Rabbit Inoculation

Animal testing and euthanization following the procedure was approved by the animal care committee (IACUC) of Lackland Air Force Base. All of the test animals were shaved in the area of the mid-

dorsal spine prior to injection. Three New Zealand White rabbits (*Oryctolagus cuniculus*) were injected with a 4.0 or 5.0 µg aliquot of whole venom of *Loxosceles reclusa* (SpiderPharm, Yarnell, AZ) in 0.2 mL saline, in the mid-dorsal back area subcutaneously. In addition, three rabbits were injected with 5.0 µg of sphingomyelinase D fraction extract, purified from whole venom as noted above. Three control rabbits were injected with 0.2 mL normal saline. Swab specimens were collected daily for 21 days. Each type of individual swab (Dacron and cotton) was dipped in normal saline and the inoculation site was then swabbed for 30 seconds. Biopsies were obtained at 24 and 72 hours at 1 cm from the injection site. Biopsies and swabs were frozen in liquid nitrogen and kept frozen at (-70°C) and transported under dry ice to the laboratory for ELISA testing.

The swab was thawed and the absorbent end was removed from the swab stick and was placed in a 1.5 mL microcentrifuge tube. The swab was centrifuged at 10,000g for 10 minutes to recover the saline from the absorbent material. The presence of venom proteins in the solution was detected with an ELISA designed to detect *Loxosceles* venom. The assay was originally described by Gomez et al<sup>11</sup>; the assay employed for these experiments was modified slightly from the original format. Polyclonal antibodies for recognition of whole venom were raised in New Zealand white rabbits with unfractionated *Loxosceles reclusa* venom. Antibodies were purified from serum by means of protein A column liquid chromatography<sup>17</sup>. The concentration of blocking agents as noted in Gomez et al,<sup>11</sup> were increased and nonfat milk solids were added to the blocking buffer. The detection agent was changed from horseradish peroxidase to alkaline phosphatase after standard curves showed slightly greater sensitivity with the alkaline phosphatase in the current assay design. Product generation was monitored at 405 nm on a model ELx808, BIO-TEK, Inc. microplate reader.

Sensitivity and specificity were determined by the following methods:

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}} \times 100\%$$

$$\text{Specificity} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}} \times 100\%$$

## RESULTS

The lesions in the rabbits caused by the experimental whole venom and sphingomyelinase injections were characterized by more hemorrhage and less cutaneous necrosis than is seen in humans. A typical 24-hour post inoculation lesion in rabbits is shown in Figure 1.



**Figure 1.** 24 hours post envenomation with brown recluse spider venom in rabbit model.

The assay results showed that the cotton swabs allowed for more detectable venom recovery than were obtained from the Dacron swabs (Figures 2 and 3). The average signal detected from the animals treated with venom was significantly above the background signal detected from the saline treatment for up to 21 days after the initial treatment (Figure 2, 3, and 4). The sphingomyelinase D injections showed greater separation of amounts of recoverable antigen, when compared to saline injections, than did the whole venom injections (Figures 3 and 4).

The average amounts of venom detected by ELISA are shown in figures 2-5. The averages were determined from the amount detected in each of six rabbits for figures 2 and 3 and for each of three

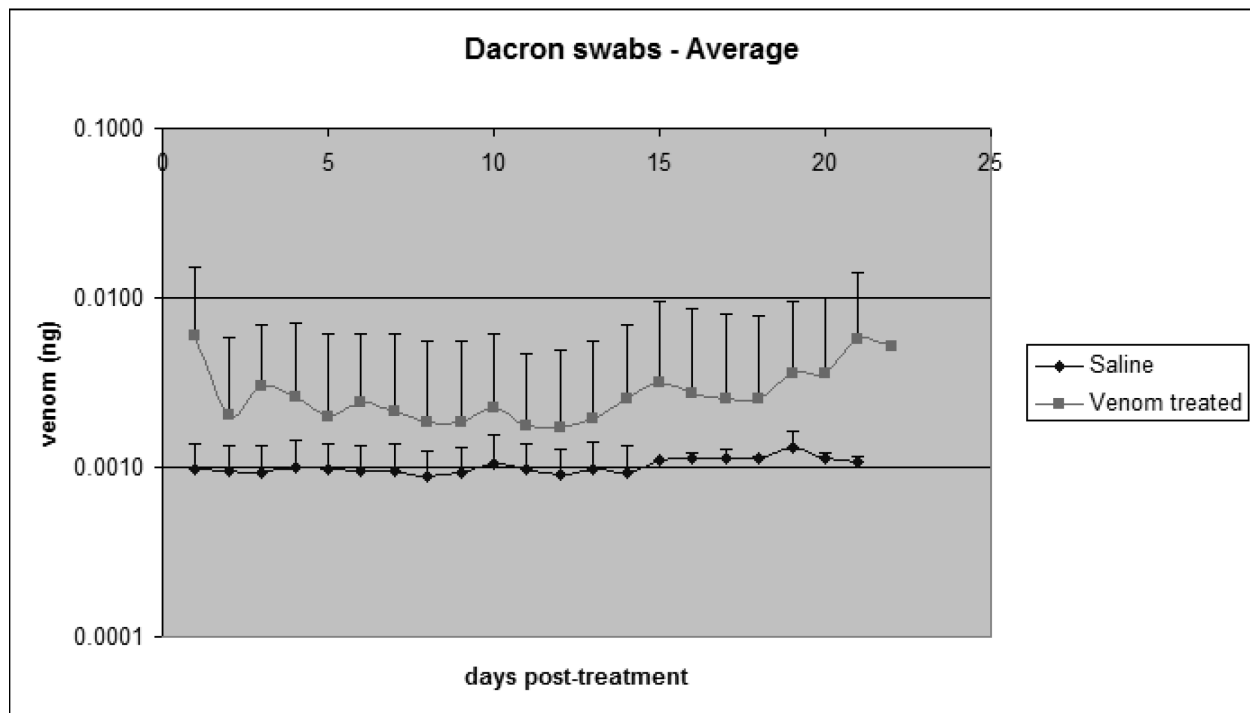


Figure 2. Venom recovery using Dacron swabs.

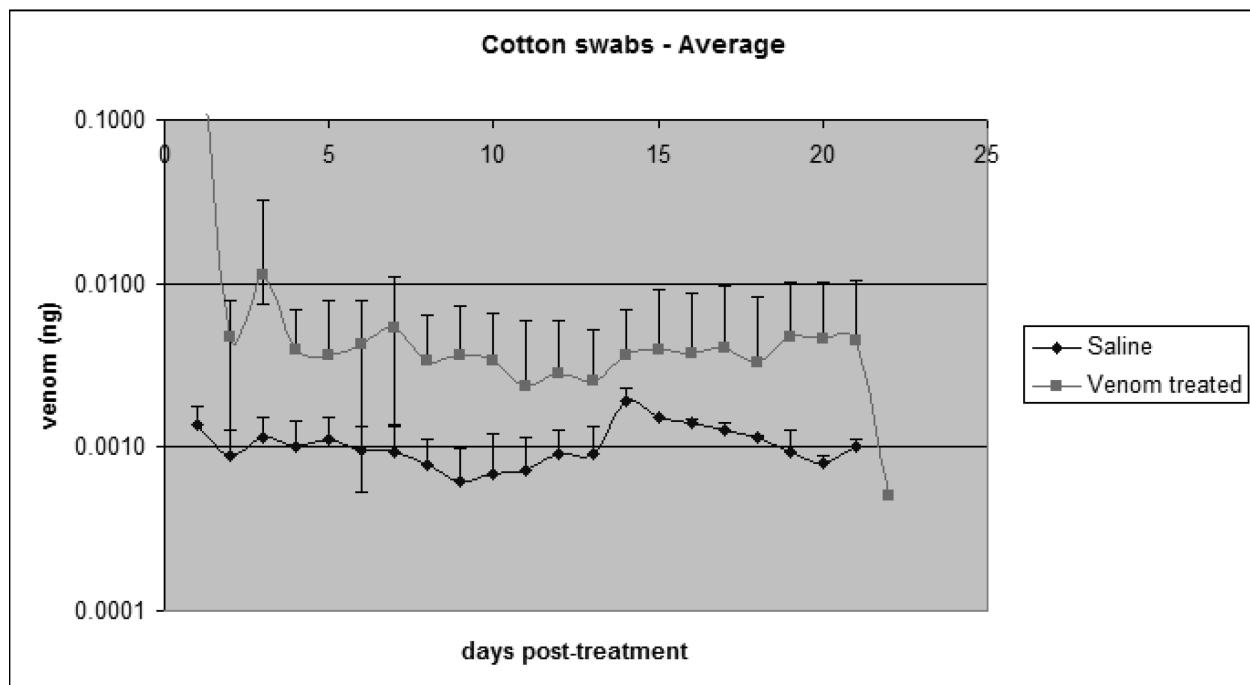
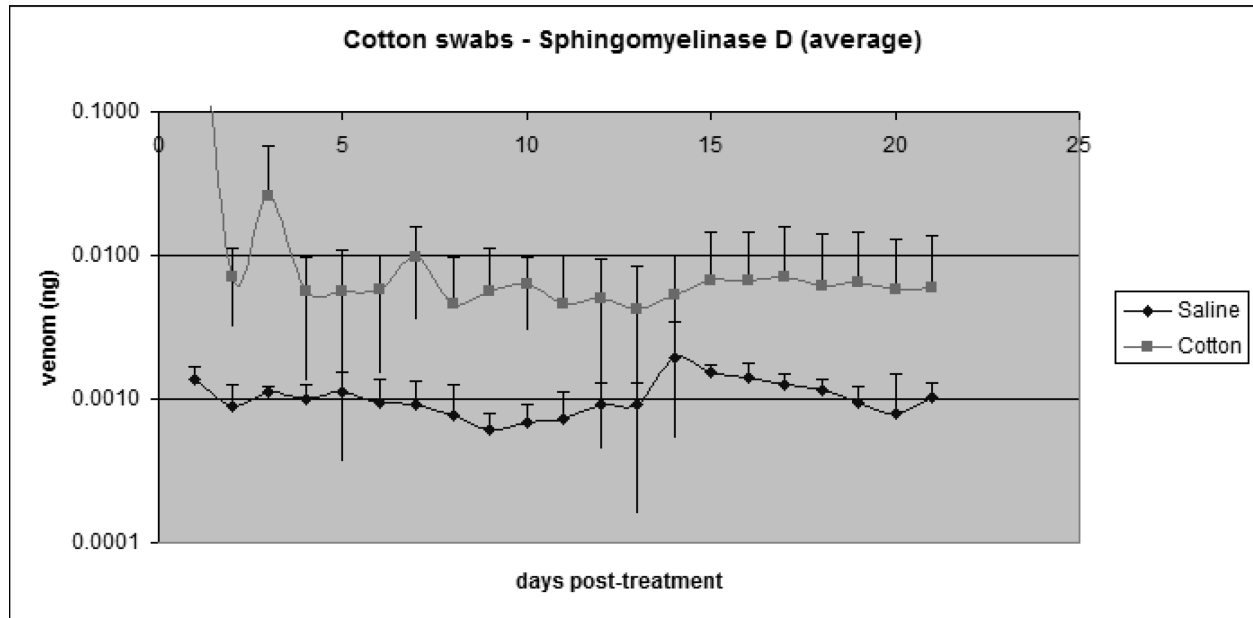
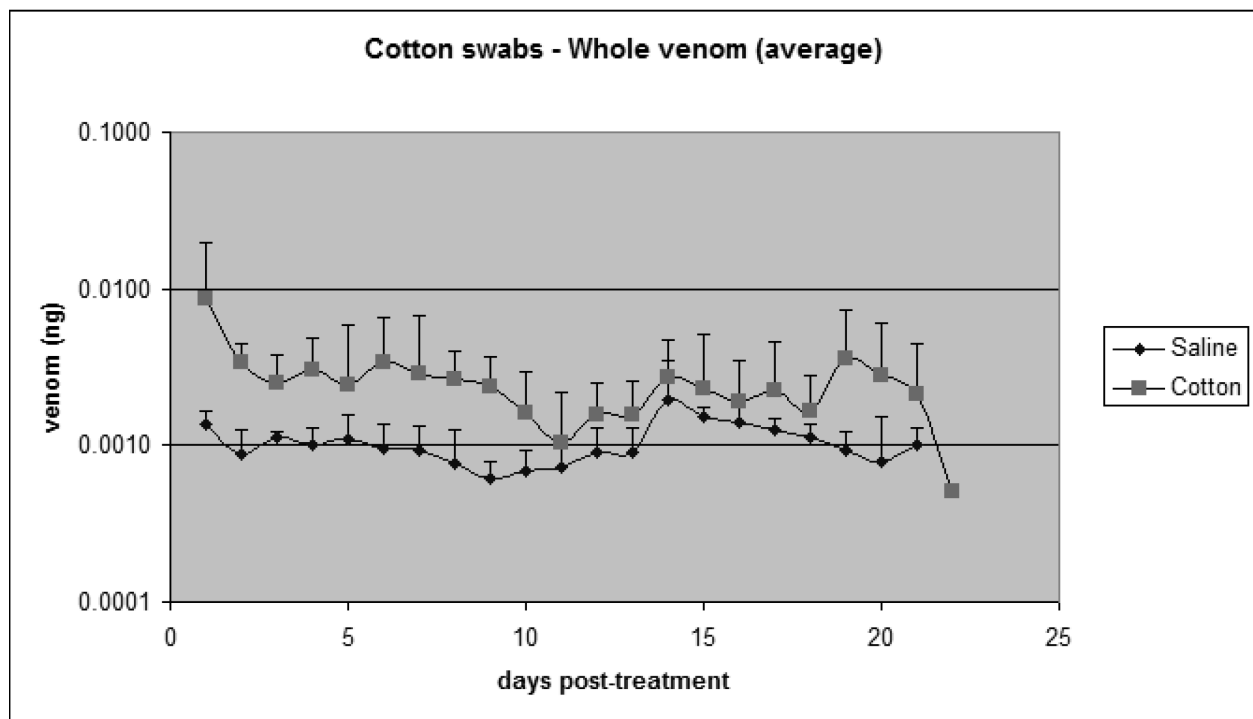


Figure 3. Venom recovery using cotton swabs.



**Figure 4.** Comparisons of recoverable antigen with sphingomyelinase D vs saline injections.



**Figure 5.** Comparisons of recoverable antigen with venom vs saline injections.

rabbits for figures 4 and 5. ELISA performed on control biopsy extracts exhibited no detectable venom immuno-reactivity.

The sensitivities of the ELISA for the whole venom with the cotton swabs up to and including 7, 10, 14, and 21 days were 67%, 65%, 62%, and 60% respectively. For the sphingomyelinase D, the sensitivity of the ELISA test was 95%, 90%, 83%, and 77%. The overall specificity remained high throughout the tests, at 95%, 96%, 93%, and 92% on days 7, 10, 14, and 21, respectively. The values were above three times the standard deviation of the background signal. The small number of test animals did not allow establishment of a confidence interval between venom amounts and background.

## DISCUSSION

Patients with suspected spider bites bring in the culpable spider in only a minority of cases. In one series, 19 of 274 (7%) of patients diagnosed with brown recluse spider bites between 1987 and 1993 brought in the spider<sup>18</sup>. The spider may be found after a significant delay, leading to uncertainty that the arachnid presented is the offending agent. Therefore, the diagnosis of most spider bites is generally dependent upon bite morphology. Many bites lack the moderately specific 'red, white and blue' sign<sup>3</sup> and the atrophic, bluish patch<sup>1</sup>. Additional confusion is created by the diagnosis of "spider bite," often for nonspecific necrotic wounds, in areas where *L. reclusa* and similar species have never been verified<sup>5</sup>.

A sensitive and specific *Loxosceles* species venom assay is clinically needed. With so many alternative diagnoses, diagnostic error in spider bites remains high. These alternative diagnoses in our clinic have included staphylococcal or streptococcal infection, herpes simplex, herpes zoster, pyoderma gangrenosum, granulomatous rosacea, and squamous cell carcinoma. A sensitive and specific assay would provide the "more strict diagnostic criteria" called for by Vetter and Bush<sup>10</sup>. Misdiagnosing loxoscelism

"may lead to unnecessary, expensive, or even harmful therapy"<sup>10</sup>. Additionally, it may lead to delays in appropriate care that may lead to adverse, and possibly fatal, consequences. This, in turn, can lead to increased medical-legal risk if there is a treatment for the actual diagnosis<sup>5</sup>. It is our experience that patients with suspected spider bites report the bite most commonly 2-8 days after the appearance of the possible bite. Therefore, to correctly evaluate those who delay reporting the bite, it is essential to have a clinical assay that is able to detect the venom after at least one week. This study demonstrates that the *Loxosceles* venom antigens that are detectable by ELISA persist for up to two weeks.

The sensitivity for the whole venom was between 60 and 70% over the test interval. If we exclude the results for a single rabbit, the sensitivity of both the whole venom and the sphingomyelinase D tests would have been above 85%. The remaining rabbits had no significant difference in the venom sensitivity.

In summary, our research has established an ELISA for the detection of brown recluse venom present in swab samples. The assay is effective in identifying venom up to two weeks after exposure. Further refinement of the polyclonal ELISA may make the assay even more sensitive. Studies are currently underway to determine if it is possible to obtain an increase in the sensitivity and specificity of the assay if venom-affinity purified antibodies are employed in the ELISA.

## CONFLICTS OF INTEREST

Dr. Stoecker owns a controlling interest in Spider-Tech, which develops tests for spider bites and spider traps. Drs. Stoecker, Green, and McGlasson have filed a provisional patent for a diagnostic test for *Loxosceles* envenomations.

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## RESEARCH AND REPORTS

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