



## SHORT COMMUNICATION

COMPARATIVE STUDY OF THE VENOMS OF THREE  
SUBSPECIES OF *LACHESIS MUTA* (BUSHMASTER)  
FROM BRAZIL, COLOMBIA AND COSTA RICA

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Rafael Otero, María de Fátima Furtado, Luis R. C. Gonçalves, Vitelbina Núñez, Martha E. García, Raúl G. Osorio, Marjorie Romero and José María Gutiérrez. Comparative study of the venoms of three subspecies of *Lachesis muta* (bushmaster) from Brazil, Colombia and Costa Rica. *Toxicon* 36, 2021–2027, 1998.—A comparative study was performed on the pharmacology and biochemistry of venoms from three subspecies of *Lachesis muta* (*L. m. stenophrys*, *L. m. muta* and *L. m. rhombeata*) from Brazil, Colombia and Costa Rica. All venoms induced lethal, hemorrhagic, edema-forming, myotoxic, coagulant and defibrinating effects, showing also proteolytic and indirect hemolytic activities. The venoms of *L. m. stenophrys* from Costa Rica and *L. m. muta* from Cascalheira, Brazil, had the highest lethal and hemorrhagic activities and the venom of *L. m. rhombeata* showed the highest coagulant activity, whereas no significant differences were observed in myotoxic and edema-forming activities at most of the time intervals studied. In addition, venoms showed similar electrophoretic patterns on SDS–polyacrylamide gel electrophoresis. In conclusion, despite quantitative differences in toxic and enzymatic activities, together with subtle variations in electrophoretic patterns, our results indicate that experimental envenomation by these venoms induce a qualitatively similar pathophysiological profile. © 1998 Elsevier Science Ltd. All rights reserved

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The genus *Lachesis* includes a single species, *L. muta* (Linnaeus), distributed in Central and South America from Nicaragua to Brazil (Campbell and Lamar, 1989). Four subspecies are currently recognized: *L. m. stenophrys*, distributed in the Atlantic versant of Nicaragua, Costa Rica and Panama, and in some areas of the Pacific region of Colombia; *L. m. melanocephala*, restricted to the south Pacific region of Costa Rica; *L. m. muta*, distributed in tropical rain forests of Colombia, Venezuela, Guyanas, Surinam, Peru, Ecuador and Brazil; and *L. m. rhombeata*, confined to some forest areas of the Atlantic region of Brazil (Campbell and Lamar, 1989). In a recent molecular study based on mitochondrial DNA sequences, Zamudio and Greene (1997) proposed that populations of *L. muta* should be classified in three different species: *L. stenophrys* and *L. melanocephala* in Central America, and *L. muta* in South America.

Human envenomations by *L. muta*, although infrequent, are usually serious, characterized by conspicuous local tissue damage (edema, hemorrhage and necrosis), nausea, coagulopathies, hypotension, shock and renal disturbances (Silva-Haad, 1980/81; Bolaños *et al.*, 1982; Otero *et al.*, 1992, 1993; Jorge *et al.*, 1997). In addition, bradycardia, diarrhea and vomiting have been described in some cases in Brazil, Venezuela and in the Amazonian region of Colombia (Silva-Haad, 1980/81; Torres *et al.*, 1995; Jorge *et al.*, 1997), although no such effects were described in cases from other regions of Colombia and Costa Rica (Bolaños *et al.*, 1982; Otero *et al.*, 1992, 1993). It is therefore important to perform comparative studies on the pharmacological, biochemical, and enzymatic characteristics of *L. muta* venoms from different subspecies and populations in order to substantiate differences in the effects induced by these venoms. A comparative study on various activities of the venoms of three subspecies of *L. muta* is presented in this communication.

Venoms of the following subspecies were used: (a) *L. m. stenophrys* (30 specimens; Atlantic region of Costa Rica); (b) *L. m. muta* (11 specimens; regions of Antioquia and Chocó, Colombia); (c) *L. m. muta* (4 specimens; Cascalheiras, Mato Grosso, Brazil); (d) *L. m. muta* (40 specimens; Tucuquí, Pará, Brazil) and *L. m. rhombeata* (4 specimens; Recife, Pernambuco, Brazil). Pools of venoms were obtained from adult specimens kept in captivity at Instituto Butantan, Universidad de Antioquia and Instituto Clodomiro Picado. Venoms were centrifuged at  $3000 \times g$  for 15 min and supernatants were lyophilized and stored at  $-20^{\circ}\text{C}$ . For *in vivo* experiments, Swiss-Webster mice were used.

Lethality was tested by the intraperitoneal route in 18–20 g mice. Venom was dissolved in phosphate-buffered saline solution, pH 7.2 (PBS) and injection volumes of 0.5 mL were used. Deaths were recorded during 48 h and the median lethal dose ( $\text{LD}_{50}$ ) was calculated by the Spearman-Kärber method (WHO, 1981). Hemorrhagic activity was determined according to Kondo *et al.* (1960), as modified by Gutiérrez *et al.* (1985). Minimum hemorrhagic dose (MHD) was the amount of venom inducing a hemorrhagic area of 10 mm diameter 2 h after injection. Edema-forming activity was determined in mice injected subcutaneously (s.c.) in the right foot pad with 1  $\mu\text{g}$  venom, dissolved in 50  $\mu\text{L}$  of PBS. The contralateral foot pad received 50  $\mu\text{L}$  of PBS under otherwise identical conditions. Edema was evaluated plethysmographically at 1, 2, 4, 6 and 24 h and expressed as the percentage of increase in the volume of the right foot pad as compared to the contralateral foot pad. Myotoxic activity was tested by injecting mice (18–20 g;  $n = 4$ ) intramuscularly (i.m.) into the right gastrocnemius with 50  $\mu\text{g}$  venom, dissolved in 0.1 mL PBS (Gutiérrez *et al.*, 1980). Control mice received 0.1 mL of PBS. Blood samples were obtained 3 h after injection and plasma creatine kinase (CK) activity was determined by using the Sigma kit 520 (Sigma Chemical, Missouri). Then, 24 h after injection, mice were sacrificed and tissue samples from the injected gas-

trocnemius muscle were obtained, placed in Karnovsky's fixative, and processed routinely for embedding in Spurr resin (Moreira *et al.*, 1992). Thick (1  $\mu\text{m}$ ) sections were obtained, stained with toluidine blue and examined microscopically.

Defibrinating activity was assessed by the method of Theakston and Reid (1983), as modified by Gené *et al.* (1989). Minimum defibrinating dose (MDD) corresponded to the minimum amount of venom that induced incoagulability in all mice tested. Coagulant activity was determined in human citrated plasma according to Theakston and Reid (1983). Minimum coagulant dose (MCD) was the amount of venom that induced plasma coagulation in 60 s. Proteolytic activity was studied according to Lomonte and Gutiérrez (1983), using casein as substrate. Activity was expressed in units/mg venom, determined as follows: change in absorbance at 280 nm  $\times$  100/mg venom. Indirect hemolytic activity was determined in agarose-sheep erythrocyte-egg yolk gels, according to Gutiérrez *et al.* (1988), using 0.8% agarose dissolved in PBS, pH 7.2. Minimum hemolytic dose (MHeD) was the amount of venom that induced a 20 mm diameter hemolytic halo. The significance of the differences observed was determined by analysis of variance (ANOVA). When mean values were significantly different by ANOVA ( $p < 0.05$ ), the significance of the differences between pairs of means was studied by the Newman-Keuls method and by the Tukey-Kramer multiple comparison test. Venoms were also compared by SDS-polyacrylamide gel electrophoresis, according to Laemmli (1970), using a 6-16% acrylamide gradient. Venoms were separated under reducing conditions and gels were stained with Coomassie Brilliant Blue G-250. Molecular weight markers were run in parallel.

Table 1 compares the pharmacological and enzymatic activities of the various venoms. LD<sub>50</sub>s ranged from 72.7  $\mu\text{g}/\text{mouse}$  (*L. m. muta*, Cascalheira) to 122.8  $\mu\text{g}/\text{mouse}$  (*L. m. rhombeata*). Statistical analysis indicated that venoms can be placed in two groups: *L. m. muta* (Cascalheira) and *L. m. stenophrys* venoms were more lethal than those of *L. m. muta* (Tucuruí), *L. m. muta* (Colombia) and *L. m. rhombeata* (Table 1). All venoms induced hemorrhagic, edema-forming, myotoxic, defibrinating, coagulant, proteolytic and indirect hemolytic activities, with relatively minor quantitative variations between populations and subspecies (Table 1). In the case of edema-forming activity, the five venoms induced an edema of rapid onset that peaked 1 h after injection (Fig. 1). No hemorrhage was observed macroscopically in mouse foot pads with this dose. No significant differences in the intensity of edema were observed between the venoms at 1, 2 and 6 h. A similar pattern of histological alterations was observed for all venoms in skeletal muscle 24 h after i.m. injection of 50  $\mu\text{g}$ . Hemorrhage was conspicuous, and a moderate myonecrosis was evident, as judged by a relatively low proportion of necrotic muscle fibers. An inflammatory infiltrate composed of polymorphonuclear leucocytes and macrophages was present both inside necrotic cells and in the interstitial space. The patterns of separation of venom proteins on SDS-PAGE run under reducing conditions are shown in Fig. 2. All venoms had common bands with approximate mol. wt. of 87, 80, 70, 47, 40, 35, 25, 21 and 16 kDa. In addition, a 60 kDa band was observed in the venoms of *L. m. muta* from Cascalheira and Tucuruí, but were absent in the others, and the venoms from *L. m. muta* (Colombia) and *L. m. stenophrys* had an additional band of 54 kDa. Moreover, the venom of *L. m. stenophrys* presented a band of 90 kDa which was not observed in the others, whereas the 25 kDa band was very faint in this venom (Fig. 2).

Intraspecific variation in venom composition is a common finding in a number of species, particularly in those having wide geographical distribution. Such variability has evident implications in the pathophysiology and treatment of snakebite envenomations

Table 1. Pharmacological and enzymatic activities of venoms of *L. muta* from different subspecies and populations

Venom	Lethality (LD <sub>50</sub> , µg)*	Hemorrhage (MHD, µg)	Myotoxicity (CK, U/mL)	Defibrinating (MDD, µg)	Coagulant (MCD, µg)	Proteolytic (units/mg)	Indirect hemolytic (MHeD, µg)
<i>L. m. stenophrys</i>	88.6 (69.8–112.3) <sup>a**</sup>	0.35 ± 0.12 <sup>a</sup>	251 ± 28 <sup>a</sup>	2.00 <sup>d</sup>	6.0 ± 0.9 <sup>b</sup>	235 ± 19 <sup>a</sup>	6.3 ± 1.8 <sup>a</sup>
<i>L. m. rhombeata</i>	122.8 (94.3–160) <sup>b</sup>	0.95 ± 0.20 <sup>b</sup>	233 ± 46 <sup>a</sup>	0.50 <sup>b</sup>	2.5 ± 0.6 <sup>a</sup>	242 ± 36 <sup>a</sup>	5.9 ± 0.7 <sup>a</sup>
<i>L. m. muta</i> (Colombia)	121.6 (98.7–164.6) <sup>b</sup>	0.66 ± 0.13 <sup>b</sup>	231 ± 28 <sup>a</sup>	1.00 <sup>c</sup>	8.1 ± 1.6 <sup>b</sup>	209 ± 17 <sup>a</sup>	26.0 ± 1.8 <sup>c</sup>
<i>L. m. muta</i> (Cascalheiras Brazil)	72.7 (56.4–93.9) <sup>a</sup>	0.23 ± 0.03 <sup>a</sup>	244 ± 14 <sup>a</sup>	0.25 <sup>a</sup>	5.2 ± 0.3 <sup>b</sup>	228 ± 39 <sup>a</sup>	12.7 ± 0.8 <sup>b</sup>
<i>L. m. muta</i> (Tucuruí, Brazil)	107.2 (82.0–140.3) <sup>b</sup>	0.77 ± 0.20 <sup>b</sup>	267 ± 39 <sup>a</sup>	1.00 <sup>c</sup>	8.7 ± 2.4 <sup>b</sup>	240 ± 33 <sup>a</sup>	10.5 ± 0.4 <sup>b</sup>

\*LD<sub>50</sub>: median lethal dose; MHD: Minimum hemorrhagic dose; MDD: Minimum defibrinating dose; MCD: Minimum coagulant dose; MHeD: Minimum indirect hemolytic dose (see definitions in text).

\*\*Values with different superscripts are significantly different ( $P < 0.05$ ).

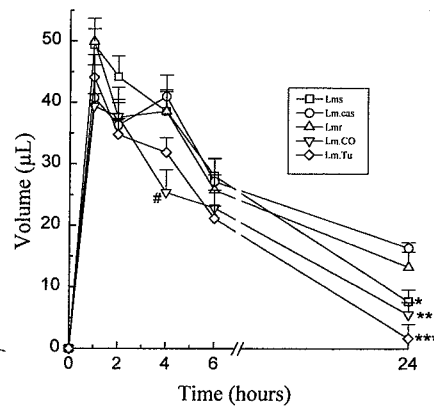


Fig. 1. Time-course of edema induced in mice by s.c. injection of *L. muta* venoms. Animals were injected with 1 µg venom, dissolved in 50 µL PBS, in the right foot pad, whereas the left foot pad was injected with 50 µL of PBS. Edema was evaluated plethysmographically at various time intervals and expressed as increment in the volume of the right foot pad as compared to the contralateral foot pad. Lms: *L. m. stenophrys*; L.M. cas: *L. m. muta* (Cascalheira); Lmr: *L. m. rhombeata*; Lm CO: *L. m. muta* (Colombia); Lm Tu: *L. m. muta* (Tucuruí). Significant differences were observed at 4 and 24 h: #  $P < 0.05$  *L. m. muta* (Colombia) vs *L. m. muta* (Cascalheira). \* $P < 0.05$  *L. m. stenophrys* vs *L. m. muta* (Cascalheira). \*\* $P < 0.01$  *L. m. muta* (Colombia) vs *L. m. muta* (Cascalheira). \*\*\* $P < 0.01$  *L. m. muta* (Tucuruí) vs *L. m. rhombeata* and  $P < 0.001$  *L. m. muta* (Tucuruí) vs *L. m. muta* (Cascalheira).

(Warrell, 1997). Intraspecific variations in some activities of the venoms of *L. m. muta* from Brazil and Peru have been described (Sánchez *et al.*, 1992), as well as in the immunoelectrophoretic patterns of venoms of *L. m. stenophrys*, *L. m. melanocephala* and *L. m. muta* from Colombia and Costa Rica (Bolaños *et al.*, 1978). In addition, a conspicuous ontogenetic variation was observed in the venom of *L. m. stenophrys* (Gutiérrez *et al.*, 1990). Three out of the four currently described subspecies were investigated in the present study, and three different populations of *L. m. muta* were included. Venom pools were prepared from adult individuals only, in order to eliminate ontogenetic vari-

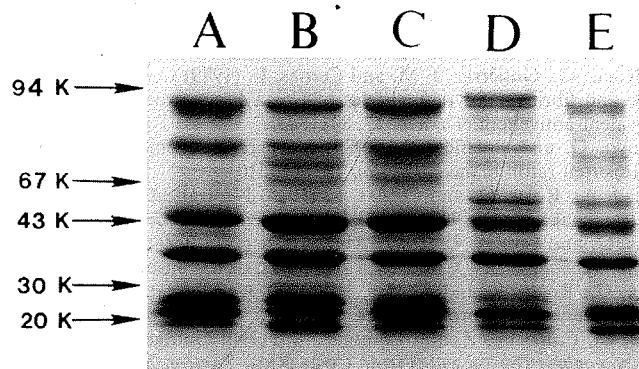


Fig. 2. SDS-polyacrylamide gel electrophoresis of *L. muta* venoms under reducing conditions in gradient acrylamide gels (6–16%) and stained with Coomassie Brilliant Blue. (A) *L. m. rhombeata*; (B) *L. m. muta* (Cascalheira); (C) *L. m. muta* (Tucuruí); (D) *L. m. muta* (Colombia); and (E) *L. m. stenophrys*. Arrows depict electrophoretic migration of mol. wt. standards.

ation. Although a recent investigation proposed the splitting of *L. muta* in three different species (Zamudio and Greene, 1997), we have maintained the traditional taxonomic arrangement in this study.

Our results show a very similar pharmacological profile between the venoms studied. All of them induced lethality, hemorrhage, edema, myonecrosis and defibrination, and had coagulant, proteolytic and indirect hemolytic activities. Both local manifestations and coagulopathies have been consistently observed in human envenomations (Bolaños *et al.*, 1982; Amaral *et al.*, 1991; Otero *et al.*, 1992, 1993), and similar effects were described in an experimental study on the venom of *L. m. muta* from Peru (Zavaleta *et al.*, 1989). In our work, despite the fact that significant differences were observed in specific pharmacological activities between some of the venoms studied, there was not a consistent pattern of variation that would suggest a divergence of one venom from the others. Moreover, a similar extent of variation was observed between the three populations of *L. m. muta* and between these populations and the other subspecies. Electrophoresis also showed a similar pattern of venom proteins, although slight differences were detected. Furthermore, despite the fact that *L. m. melanocephala* venom was not included in this study, an earlier work performed with the same methodologies indicate a similar pattern of venom activities (Gutiérrez *et al.*, 1987). Thus, although experimental studies should not be simplistically extrapolated to human envenomations, our results do not provide evidence substantiating differences in the experimental pathophysiology induced by venoms of different subspecies of *L. muta*.

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