ORIGINAL PAPER

The Journal of Venomous Animals and Toxins including Tropical Diseases
ISSN 1678-9199 | 2012 | volume 18 | issue 2 | pages 173-179

Development of a sensitive enzyme immunoassay (ELISA) for specific identification of *Lachesis acrochorda* venom

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Abstract: The snake genus *Lachesis* provokes 2 to 3% of snakebites in Colombia every year. Two *Lachesis* species, *L. acrochorda* and *L. muta*, share habitats with snakes from another genus, namely *Bothrops asper* and *B. atrox*. *Lachesis* venom causes systemic and local effects such as swelling, hemorrhaging, myonecrosis, hemostatic disorders and nephrotoxic symptoms similar to those induced by *Bothrops*, *Portidium* and *Bothriechis* bites. *Bothrops* antivenoms neutralize a variety of *Lachesis* venom toxins. However, these products are unable to avoid coagulation problems provoked by *Lachesis* snakebites. Thus, it is important to ascertain whether the envenomation was caused by a *Bothrops* or *Lachesis* snake. The present study found enzyme linked immunosorbent assay (ELISA) efficient for detecting *Lachesis acrochorda* venom in a concentration range of 3.9 to 1000 ng/mL, which did not show a cross-reaction with *Bothrops*, *Portidium*, *Botriechis* and *Crotalus* venoms. Furthermore, one fraction of *L. acrochorda* venom that did not show cross-reactivity with *B. asper* venom was isolated using the same ELISA antibodies; some of its proteins were identified including one Gal-specific lectin and one metalloproteinase. This test may be useful to physicians, since it could be applicable for tracking the kinetic distribution of antigens in patients or experimentally envenomed animals.

Key words: snake venom, *Lachesis, Lachesis acrochorda*, enzyme-linked immunosorbent assay.

INTRODUCTION

Snakebite is a common and frequently devastating environmental and occupational pathology, especially in rural areas of tropical developing countries (1). According to the National Health Institute of Colombia, 3405 snakebite cases occurred in the country during 2009, of which 3.2 % were induced by Verrugoso (*Lachesis* spp.) (2).

The genus *Lachesis* is unique among New World vipers in having a high number of ventral, dorsal, and cephalic scales, and in laying eggs (3). *Lachesis* spp. are found preferentially in primary forests and comprise four species: *L. stenophrys*, distributed in lower Central America in the Atlantic lowlands of central and possibly southern Nicaragua through northern Costa Rica to about central Panama; *L. melanocephala*, located in southern Costa Rica and possibly extreme western Panama, where it is restricted to the Pacific versant; *L. acrochorda*, distributed in both the Atlantic and Pacific versants of western Panama and into northwestern Colombia on the Atlantic coast where it extends southward into the Cauca and Magdalena river Valleys, and along the Pacific versant of Colombia into northwestern Ecuador; and *L. muta*, found in South America east of the Andes (3).

Envenomations by *Lachesis* spp. are characterized by severe coagulopathy with fibrinogen depletion, edema, hemorrhaging, pain and necrosis which may result in permanent sequelae or even death (4). These symptoms

are very similar to those of *Bothrops asper and B. atrox*, whereas such distinctive symptoms as profuse sweating, nausea, vomiting, abdominal cramps, diarrhea and hypotension may not be manifested by all victims of *Lachesis* bites (4-7).

Geographical variation in the venom composition of the different *Lachesis* species may explain differences in the frequency and type of clinical features observed (8). *Lachesis* genera are frequently distributed in the same regions inhabited by *Bothrops* genus (3). The lack of treatment for *Lachesis* bites is still a health problem in some regions, since some clinical observations suggest that *Bothrops* antivenoms are not efficacious at neutralizing *Lachesis* toxins (9). Thus, as reported by Jorge *et al.* (4), patients bitten by *Lachesis muta muta* snakes may die or have persistently incoagulable blood after being treated with bothropic/crotalic antivenoms.

Distinguishing between *Bothrops* and *Lachesis* bites depends on the identification of the snake or on enzyme immunoassay diagnosis. Enzyme linked immunosorbent assay (ELISA) is a method that has been demonstrated to be efficient for the detection of snake venom (10, 11).Thus, the aim of the present study was to develop an enzymatic immunoassay as a basis for immunodiagnostic envenomations caused by *Lachesis.*

MATERIALS AND METHODS

Venoms

Venoms of *Lachesis acrochorda* (three specimens), *Bothrops asper* (20 specimens), *Porthidium nasutum* (ten specimens), *Bothriechis schlegelii* (ten specimens), and *Crotalus durissus cumanensis* (five specimens), all from Colombia, were obtained by manual extraction of specimens maintained in captivity at the animal house of the Universidad de Antioquia (Medellin, Colombia). Venoms were centrifuged at 800 g for 15 minutes, and the supernatants were lyophilized and stored at –20°C until use.

Antibody Production

One rabbit (female 1.8 to 2 kg body mass) was subcutaneously (SC) immunized with 1 mL containing 0.5 mg/mL of the *L. acrochorda* venom emulsified in complete Freund's adjuvant. After 20 days, the animal was SC injected with 1.0 mg/mL of venom in incomplete Freund's adjuvant. Two venom boosters of 2.0 and 3.0 mg, each diluted in incomplete Freund's adjuvant, were injected at 20-day intervals. Blood was collected from the rabbit one day before immunization (preimmune sera) and ten days after the last booster dose. The serum was separated and stored at –20°C until use.

Antibody Purification

Affinity purification of rabbit IgG

Hyperimmune sera produced against *L. acrochorda* venom were used for the purification of rabbit IgG by means of a protein A Sepharose column (Amersham Biosciences AB, Sweden). Three milliliters of rabbit hyperimmunized sera was loaded into the column. The column was washed with 0.12 M NaCl, 0.04 M sodium phosphate and pH 7.2 buffer (PBS). The bound protein was eluted with 0.1 M glycine-HCl buffer, pH 3.0. The fraction containing total IgG was collected into tubes containing 0.5 M Tris, pH 8.8 (0.5 mL Tris /4 mL solution). The fraction was dialyzed against PBS, and conserved at 4°C until use.

Affinity purification of rabbit IgG anti-*L. acrochorda* without anti-*B.asper* cross-reactivity

B. asper venom was coupled to CNBr activated Sepharose 4B. For this, CNBr-activated Sepharose 4B (Amersham Biosciences AB, Sweden) was incubated overnight at 4°C with *B. asper* venom (5 mg/mL gel) dissolved in 1 mL 0.1 M $NaHCO_3$, and 0.5 M NaCl, pH 8.3 (binding buffer). The gel was later treated with 0.2 M glycine and incubated at room temperature for two hours. The gel was washed sequentially with binding buffer and 0.1 M acetate buffer, pH 4.0. Ten milliliters of the prepared affinity matrix was suspended in PBS, pH 7.2, and later packed in a chromatography column (12 x 1.5 cm). The fraction of total IgG was loaded into the column and washed with PBS. Unbound protein was collected with PBS, pH 7.2. The fraction was dialyzed against PBS, and conserved at 4°C until use.

Affinity purification of specific IgG anti-*Lachesis* venom

CNBr-activated Sepharose 4B was incubated overnight at 4°C with *L. acrochorda* venom (5 mg/mL gel) dissolved in 1 mL 0.1 M $NaHCO_3$, and 0.5 M NaCl, pH 8.3 (binding buffer). The gel

was later treated with 0.2 M glycine and incubated at room temperature for two hours. The gel was washed sequentially with binding buffer and 0.1 M acetate buffer, pH 4.0. Ten milliliters of the prepared affinity matrix was suspended in PBS, pH 7.2, and later packed in a chromatography column (12 x 1.5 cm). Previously obtained unbound protein solution was loaded into the column and washed with PBS. The proteins bound to the column were eluted with 0.1 M glycine-HCl buffer, pH 3.0. The bound fraction was collected into tubes containing 0.5 M Tris, pH 8.8 (0.5 mL Tris/4 mL solution). The fraction was dialyzed against distilled water, lyophilized, and conserved at –20°C until use. This fraction was denominated "IgG anti-*L. acrochorda* – not *B. asper*".

Biotinylation of IgG anti-*L. acrochorda* – not *B. asper*

Purified IgG anti-*L. acrochorda* – not *B. asper* was labeled with biotin according to the instructions of the manufacturer (Sigma-Aldrich, USA). Briefly, 2 mg of IgG was diluted in 1 mL of carbonate buffer, pH 9.6, and mixed with 100 µL of biotin (2.2 mg/1 mL DMSO). The mixture was incubated at room temperature for four hours. Biotin-IgG conjugate was separated from free biotin by dialysis against PBS.

ELISA

Plates of 96 wells (Nunc Inc., USA) were coated overnight at 4°C with 100 µL of IgG per well at 100 µg/mL. The anti-*L. acrochorda* – not *B. asper* was diluted in 50 mM carbonate/bicarbonate buffer, pH 9.6. The plates were then washed five times with washing buffer (PBS pH 7.2: 0.12 M NaCl, 0.04 M sodium phosphate and 0.05% tween 20). The remaining binding sites were blocked with PBS pH 7.2, containing bovine serum albumin 1% for two hours at 37°C (100 µL/well). Afterwards, the plates were washed again five times with washing buffer. Next, different concentrations (2 up to 1000 ng/mL) of *L. acrochorda*, *B. asper*, B. *schlegelii* and *P. nasutum* venoms in sample buffer (PBS and bovine serum albumin 1%) were added to the plates (100 µL/well) and incubated for one hour at 37°C.

Plates were washed five times with washing buffer, and then 100 µL/well of biotinylated Ig G anti-*L. acrochorda* – not *B. asper* (diluted in sample buffer 1:100) was added and incubated for one hour at 37°C. After washing, streptavidine (Sigma, USA) diluted in sample buffer (1:1000) was added and incubated for one hour at 37°C. Immediately after washing, 100 µL/well of ABTS (Sigma, USA) diluted in citrate 0.1 M, pH 5.0, containing 30% hydrogen peroxide was added and incubated for 30 minutes at 37°C while protected from light. Subsequently, the absorbance was obtained at 405 nm in an ELISA plate reader (Awareness Technology, USA). Serum samples from people not bitten were included as controls. The assay was repeated six times in duplicate.

Affinity Purification of the Specific Fraction from *L. acrochorda* Venom

CNBr-activated Sepharose 4B was incubated overnight at 4°C with IgG anti-*L. acrochorda* – not *B. asper* (5 mg/mL gel) dissolved in 1 mL 0.1 M $NaHCO_3$, and 0.5 M NaCl, pH 8.3 (binding buffer). The gel was later treated with 0.2 M glycine and incubated at room temperature for two hours. The gel was washed sequentially with binding buffer and 0.1 M acetate buffer, pH 4.0. Ten milliliters of the prepared affinity matrix was suspended in phosphate buffer (0.02 M PBS pH 7.2) and later packed in a chromatography column (12 x 1.5 cm). The venom of *L. acrochorda* (20 mg/2 mL) was loaded in the column and washed with PBS. Proteins bound to column were eluted with of 0.1 M glycine-HCl, buffer pH 3.0. The bound fraction was collected into tubes containing 0.5 M Tris pH 8.8 (0.5 mL Tris/4 mL solution). The fraction was dialyzed against distillated water, lyophilized, and conserved at –20°C until use. This fraction was denominated "*L. achrochorda* specific fraction" (*La*SF).

Electrophoresis – SDS PAGE

The whole venoms or the fraction *La*SF was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide gels (12). Respective samples of 40 µg were separated under non-reducing conditions and the gels were stained with Coomassie brilliant blue R-250. Molecular weight markers were run in parallel.

RP-HPLC Fractionation and Characterization of the Fraction *La*-SF

Two milligrams of the fraction *La*-SF was dissolved in 200 µL of 0.1% trifluoroacetic acid (TFA), centrifuged for five minutes at 13,000

rpm, and loaded into a C_{18} column (250 x 4.6 mm, 5 µm particle; Teknokroma, Spain) using an Agilent 1200 chromatograph (USA). Elution was performed at 1 mL/minute by applying a gradient towards solution B (acetonitrile, containing 0.1% TFA) as follows: 5% B for five minutes, 5-15% B for ten minutes, 15-45% for 60 minutes, and 45-70% B for 12 minutes. Absorbance was monitored at 215 nm, and fractions were manually collected and dried in a vacuum centrifuge (Savant, USA) for subsequent characterization.

The major fractions obtained were separated by SDS-PAGE under reducing conditions, using 12% gels. Protein bands were excised from Coomassie blue R-250-stained gels and subjected to reduction with dithiothreitol and alkylation with iodoacetamide. This was followed by in-gel digestion with sequencing grade bovine trypsin on an automated processor (ProGest Digilab, USA), according to the manufacturer's instructions. The resulting peptide mixtures were analyzed by MALDI-TOF-TOF mass spectrometry on an Applied Biosystems 4800-Plus instrument (USA). The resulting spectra were analyzed using ProteinPilot v.4 (ABSciex, USA) to identify proteins using the UniProt/SwissProt database (20100622) and the Paragon® algorithm method, at a confidence level of 99%.

Statistical Analysis

The data were subject to non-parametric statistical analysis. Results are presented as mean ± standard error. Significant differences between observed absorbance values of *L. acrochorda* and other venoms were determined by the Kruskal-Wallis test. A Mann-Whitney U test was used to determine the detection limit by comparing absorbances at each concentration of all venoms. Differences were considered significant at $p < 0.05$.

RESULTS

The anti-*L. acrochorda* IgG purified by several affinity chromatography steps was highly specific. The sandwich ELISA reactivities for known concentrations of *L. acrochorda*, *B. asper, B. schlegelii* and *P. nasutum* whole venoms are shown in Figure 1. Concentrations of 2.0 ng/mL of *L. acrochorda* venom gave measurable absorbance signals. No cross-reactivity was observed with other venoms evaluated in the concentration range of 3.9 to 1000 ng/mL. ($p \leq 0.01$). Similar results were observed using *B. atrox* and *C. d. cumanensis* venoms (data not shown). Higher quantities of *L. acrochorda* venoms produced dose-dependent absorbance values. Similar results were obtained when known concentrations of *Lachesis* venom were put in pooled human serum from healthy individuals (data not shown).

Additionally, the specific IgG used in the ELISA was employed in affinity chromatography to isolate specific components of *L. acrochorda* venom that displayed no cross-reactivity with *B. asper* venom. SDS-PAGE under non-reducing conditions of the obtained fraction denominated *La*SF showed three bands, one major at ~29 kDa and two minor bands at ~28 kDa and 14 kDa (Figure 2). Nevertheless, under reducing conditions the major band disappeared and the intensity of the 14 kDa band increased, indicating that the

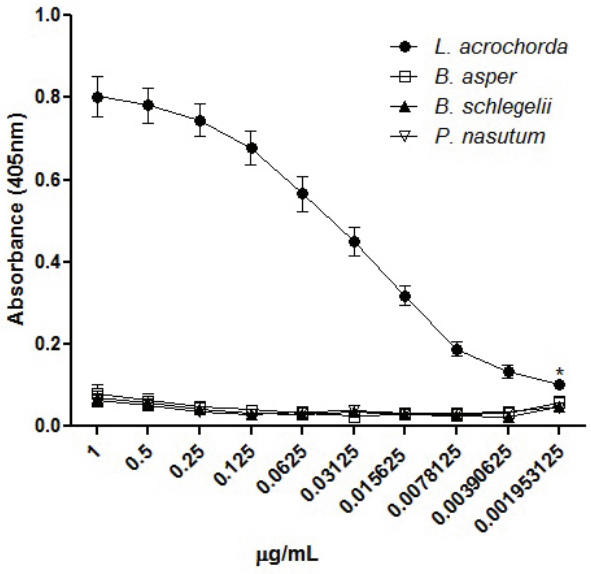

Figure 1. Sandwich-ELISA titration of *L. acrochorda*, *B. asper*, *B. schlegelii* and *P. nasutum* Colombian snake venoms: 96-well plates were coated with IgG specific anti-*L. acrochorda* previously obtained by several procedures of affinity chromatography with hyperimmune rabbit serum. The plates were incubated with varying amounts of *L. acrochorda* (●), *B. asper* (□), *B. schlegelii* (▲) and *P. nasutum* (▽) venom. Biotinylated anti-*L. acrochorda* IgG was added and incubated. These values are the average of six experiments, performed in duplicate, ± SE. Assay details are described in the Materials and Methods section.

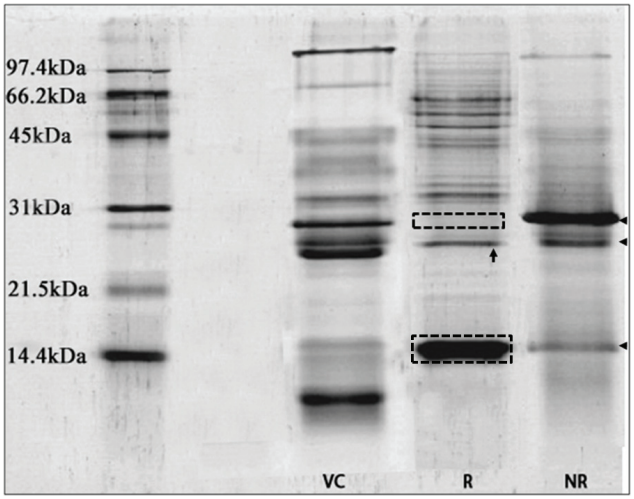

Figure 2. SDS-polyacrylamide gel electrophoresis. VC: whole venom. NR: specific fraction of *L. acrochorda* (*La*SF) isolated using IgG anti-*L. acrochorda* – not *B. asper* in non-reducing conditions. R: *La*SF in reducing conditions. Arrows show the main proteins observed in the fraction (29, 28 and 14 kDa). The rectangles show changes in 29 kDa protein, which disappears while augmenting the 14 kDa band in reducing conditions. Molecular mass markers (Biorad, USA) are shown on the left.

protein is a dimer. Similarly, the separation of *La*-SF by RP-HPLC showed two major peaks (Figure 3). Four peptides – YGESLEIAEYISDYHK, EFCVELVSLTGYR, DFSWEWTDR and YGESLEIAEYISDYHKGQAEVWIGLWDK – obtained from the major peak corresponded to Gal-specific lectin, while the minor peak was identified as metalloproteinases (VHEIVNFINGFYR, YIELVVVADHGM$_{ox}$FTK, NSVGIVQDHSPK and YNGNLNTIR).

DISCUSSION

Antibodies against specific components are important tools for establishing venom detection immunoassays useful in snakebite diagnosis (13). The identification of the snake involved in the accident allows selection of the correct specific antivenom. In the present work, the anti-*L. acrochorda* IgG purified by several affinity chromatography steps was highly specific and may be important in the development of diagnostic kits to differentiate among envenomations caused by *L. acrochorda* and B. *asper/atrox*, *P. nasutum*, and *B. schlegelii*, the species responsible for most snakebites in Colombia. In addition, this highly specific IgG may contribute to epidemiological

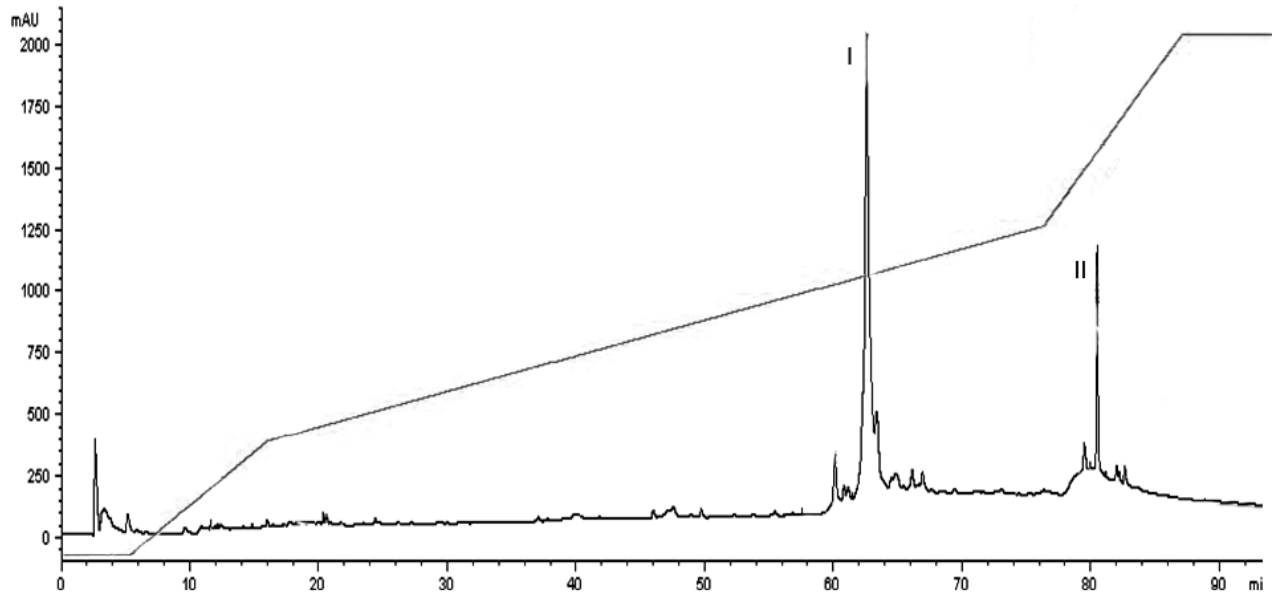

Figure 3. Reverse HPLC of *La*SF (*Lachesis acrochorda* specific fraction). Fraction *La*SF was loaded into a C_{18} column using an Agilent 1200 chromatograph (USA). Elution was performed at 1 mL/minute by applying acetonitrile gradient containing 0.1% TFA. Absorbance was monitored at 215 nm; fractions were manually collected and dried in a vacuum centrifuge (Savant, USA). Peak I was identified as a Gal-specific lectin and peak II as a metalloproteinase.

statistics by assessing the number and geographical distribution of ophidian accidents.

Colombini *et al.* (14) showed considerable antigenic cross-reactivity between *B. asper*/*atrox* and *Lachesi*s venoms. However, they showed by using species-specific monoclonal antibodies that some molecules were particular to *L. m. muta* venom in some regions. Several proteins had been reported from *Lachesis* species such as a lectin-like dimer protein, with molecular mass of 28 kDa, isolated from *L. muta* venom (15). An acidic kininogenin from *Lachesis muta* venom was purified and shown to be a highly stable serine protease with a molecular mass of 27.9 kDa, and capable of releasing bradykinin from bovine kininogen (16). Giovanni-de-Simone *et al.* (17) isolated a kalikrein-like protein from *Lachesis muta rhombeata* from Brazil with a molecular mass of 32 kDa. Several reports about phospholipases A_2 have been published (18-20). Sanchez *et al.* (21) identified a serine proteinase of 33 kDa, denoted LV-PA, from *Lachesis muta muta* venom; this toxin selectively converts plasminogen into plasmin *in vitro*. LV-PA, detected at the rate of 1.5 ng of venom per assay, was used to develop a specific ELISA to detect *Lachesis muta muta* venom (13).

In the present work the specific IgG used in the ELISA was employed in affinity chromatography to isolate a specific fraction of *Lachesis acrochorda* venom that did not cross-react with *B. asper* venom (i.e. *La*SF). The main components present in this fraction were identified as a lectin and a metalloproteinase.

One study on *Lachesis* species from Costa Rica, Brazil and Colombia found some significant differences in the pharmacological activities induced by their venoms (22). However, there was not a consistent variation pattern in those activities. Furthermore, subtle variations in the electrophoretic patterns of the venoms were observed. A recent study to develop a proteomic characterization of venoms of *L. muta* from Brazil and *L. stenophrys* from Costa Rica revealed that these venoms share (or contain highly similar) proteins, in particular bradykinin-potentiating/C-natriuretic peptide (BPPs), serine proteinases, a galactose-specific C-type lectin, and L-amino acid oxidase (LAO). However, they dramatically differ in their respective PLA_2 complement (23).

Various immunological methods have been reported for detecting *Lachesis* venom, but *Lachesis acrochorda* has not been included (11, 13, 24). And as previously mentioned, some differences among *Lachesis* species had been observed (23). Furthermore, geographical variations in the venom components demand the development of regional immunodiagnostic tests specific to snakes that inhabit a given region (25). Thus, this ELISA assay may contribute to improving the diagnosis and treatment of snakebites in Colombia. Finally, the *La*SF fraction could be used in the immunization mixture for the production of polyvalent antivenom in Colombia.

ACKNOWLEDGEMENTS

The authors thank Prof. Bruno Lomonte (Instituto Clodomiro Picado, Costa Rica) for identification of peaks of *Lachesis acrochorda* venom (*La*SF).

SUBMISSION STATUS

Received: September 8, 2011.
Accepted: December 20, 2011.
Abstract published online: January 25, 2012.
Full paper published online: May 31, 2012.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

FINANCIAL SOURCE

Universidad de Antioquia (CODI project), and COLCIENCIAS (project 1115-459-21441 and Programa de Jóvenes Investigadores) provided the financial grants for this project.

ETHICS COMMITTEE APPROVAL

The present study was approved by the Ethics Committee of Antioquia University, Medellin, Colombia.

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