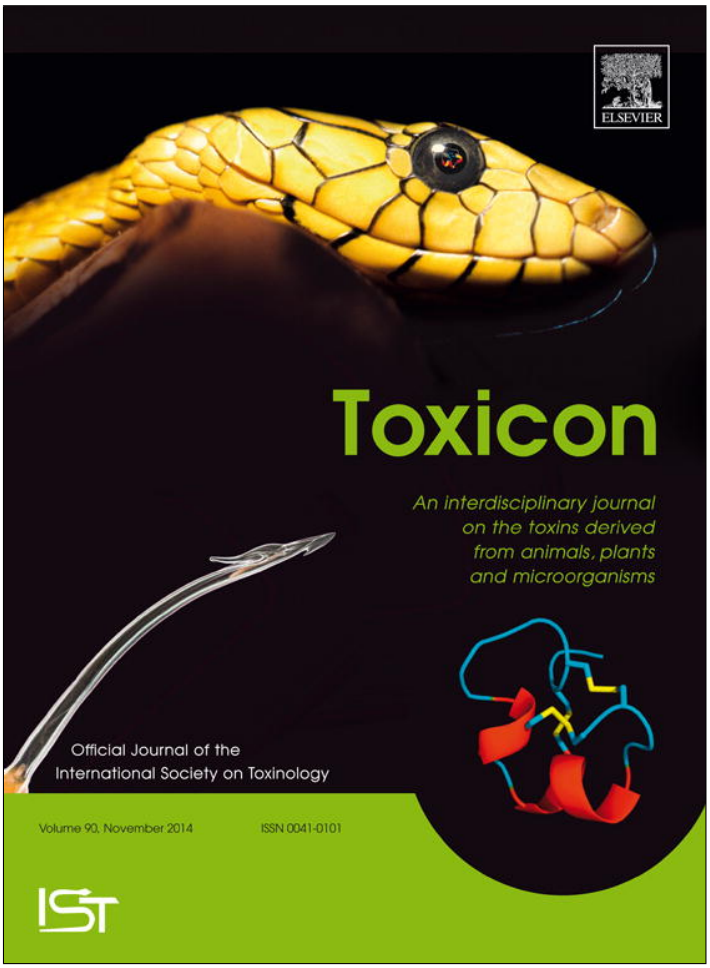

Toxicon 90 (2014) 148–154

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

An alternative micromethod to access the procoagulant activity of *Bothrops jararaca* venom and the efficacy of antivenom

N. Oguiura [a], J. Kapronezai [a], T. Ribeiro [a], M.M.T. Rocha [b], C.R. Medeiros [c], J.R. Marcelino [d], B.C. Prezoto [e, *]

[a] *Ecology and Evolution Laboratory, Butantan Institute, Av. Dr. Vital Brazil 1500, CEP 05503-900 São Paulo, SP, Brazil*
[b] *Laboratory of Herpetology, Butantan Institute, Av. Dr. Vital Brazil 1500, CEP 05503-900 São Paulo, SP, Brazil*
[c] *Vital Brasil Hospital, Butantan Institute, Av. Dr. Vital Brazil 1500, CEP 05503-900 São Paulo, SP, Brazil*
[d] *Immunology Service, Butantan Institute, Av. Dr. Vital Brazil 1500, CEP 05503-900 São Paulo, SP, Brazil*
[e] *Laboratory of Pharmacology, Butantan Institute, Av. Dr. Vital Brazil 1500, CEP 05503-900 São Paulo, SP, Brazil*

ARTICLE INFO

Article history:
Received 3 April 2014
Received in revised form 23 July 2014
Accepted 6 August 2014
Available online 13 August 2014

Keywords:
Antivenom neutralization
Bothrops jararaca venom
Coagulation
Chicken plasma
Rotational thromboelastometry

ABSTRACT

The assessment of the capacity of antivenoms to neutralize the lethal activity of snake venoms still relies largely on traditional rodent lethality assay (LD_{50}). However, adequately validated *in vitro* tests should be introduced for assessing antivenom neutralizing capacity in plasma of immunized horses as well as for in-process quality control. The dynamic of fibrin formation in recalcified avian plasma samples is extremely slow, when compared to that presented by mammalian plasmas. In this study, we present one new coagulant assay, by performing dose–response curve after plotting the clotting time (CT) parameter of the ROTEM profile of recalcified chicken plasma samples (target) against semi-logarithmic doses of *Bothrops jararaca* venom (agonist), either in absence or in presence of the semi-logarithmic doses of anti-bothropic serum (ABS) (antagonist).
The mean coagulant dose 50% (CD_{50}) was defined as the quantity of venom (in μg) which reduces CT to 900 s, between minimum and maximum responses. The CT induced by $5CD_{50}$ of the venom was used as the control for calculating the effective dose (ED) of each batch of ABS. ED was defined as the ABS dose (nanoliters, nL) at which CT induced by one amount of venom corresponding to $5CD_{50}$ is displaced to the maximum threshold (1800 s). Five batches of the ABS, previously assayed for their lethality neutralizing activity (ED_{50}) were assayed. The correlation coefficient (r) between both *in vitro* (ED) and *in vivo* (ED_{50}) values was 0.87 (p value < 0.05). We propose this micro method as highly sensitive for characterization and quantification of possible procoagulant activity of small doses of snake venoms (nanograms) and for detecting small doses (nanoliters) of specific antibodies against this effect in little volume samples of biological fluids.

1. Introduction

The parenteral administration of animal-derived antivenoms constitutes the cornerstone of snakebite envenoming therapy. Snake antivenoms are manufactured by immunizing animals, usually horses, with venoms from a single or several medically-relevant snake species and are

* Corresponding author.
E-mail addresses: nancy.oguiura@butantan.gov.br (N. Oguiura), josana.kapronezai@butantan.gov.br (J. Kapronezai), thayanep4@hotmail.com (T. Ribeiro), marisa.rocha@butantan.gov.br (M.M.T. Rocha), carlos.medeiros@butantan.gov.br (C.R. Medeiros), jose.marcelino@butantan.gov.br (J.R. Marcelino), benedito.prezoto@butantan.gov.br (B.C. Prezoto).

http://dx.doi.org/10.1016/j.toxicon.2014.08.004
0041-0101/

constituted by either whole IgG molecules or the immunoglobulin fragments F(ab')$_2$; and Fab, obtained by digestion with pepsin and papain, respectively (Theakston et al., 2003). The assessment of the capacity of antivenoms to neutralize the lethal activity of snake venoms is routinely performed by manufacturers and quality control laboratories and still relies largely on traditional rodent lethality assay (LD_{50}), defined as the amount (μg) of venom which causes the death of 50% of a group of mice within 48 h after injection. Similarly, the amount (mL) of antivenin at which half of the injected animals at doses corresponding to $5LD_{50}$ survived after 48 h is defined as mean effective dose (ED_{50}), expressing the neutralizing capacity of antivenin (Farmacopeia Brasileira, 2010). This rodent lethality assay usually is time-consuming, expensive, requires large quantities of venom and animals, and beside leads to animal suffering due to the toxic effects (in particular pain) induced by venoms (Warrell et al., 1986; Laing et al., 1992). Although it is likely that this assay will continue to be the gold standard for the final quality control of the potency of antivenoms, adequately validated *in vitro* tests should be introduced for assessing antivenom antibody titre in plasma of immunized horses as well as for in-process quality control (Gutiérrez et al., 2011).

Circulating blood cells, coagulation factors and vascular wall components of mammalian species are considered as targets of snake toxins (Du et al., 2006) and the study of snake venom procoagulant toxins has been traditionally assessed directly by simple clotting studies (Marshall and Herrmann, 1983; Masci et al., 1988; Sprivulis et al., 1996). For example, the least amount (mg) of venom that clot a solution of citrated plasma from different animal species or fibrinogen solution in 60 s at 37 °C is defined as minimum coagulant dose (MCD) (Theakston and Reid, 1983). The MCD gives a single parameter (formation of the first fibrin strands) for a complex process (Isbister, 2009). Attempts to overcome this limitation of classical coagulation tests have been tried with the use of different strategies, such as, for example, monitoring of viscoelastic changes of plasma or whole blood (WB) samples with thromboelastography and more recently, rotational thromboelastometry (ROTEM). These assays refer to the graphic display of the viscoelastic properties of fibrin clots, evaluating clot initiation, propagation and termination generated in plasma or WB samples under conditions of low shear (Zuckerman et al., 1981; Rugeri et al., 2007; Wiinberg and Kristensen, 2010; Brooks et al., 2011; Van Geffen and van Heerde, 2012).

Although being hemorrhagic *in vivo*, *Bothrops jararaca* (*B. jararaca*) venom presents procoagulant activity in *in vitro* assays with mammalian plasmas, mainly due to presence of prothrombin and factor X activators (Antunes et al., 2010). Since the venom of *Bothrops* genus is toxic for avian organisms (Zelanis et al., 2008; Arbuckle, 2010) and the dynamic of spontaneous fibrin formation in recalcified plasma samples of avian species is significantly slow (Spurling, 1981), we therefore asked whether the coagulation process can be triggered (in a dose-dependent fashion) in chicken plasma samples after preincubation with *B. jararaca* venom, both in presence and absence of the antibothropic serum (ABS), by using the ROTEM assay.

2. Material and methods

2.1. Reagents

Activated partial thromboplastin time reagent (aPTT clot, containing ellagic acid and synthetic phospholipids) from BIOS Diagnóstica (SP, Brazil); sodium citrate from Ecibra (Brazil) and calcium chloride from E. Merck (German). All chemicals were of analytical reagent grade. Citrated normal human plasma from Sigma–Aldrich (Brazil).

2.2. Venom pools

(1) *B. jararaca venom pool (lote Bj 01/08-4)* – Venoms from 925 specimens of *B. jararaca*, with protein content of 1005.7 ± 76.6 μg/mg; (2) *Bothrops jararacussu (B. jararacussu) venom pool (lote Bju Lote 01/08-1)* – From 109 specimens of *B. jararacussu*, with protein content 1259.8 ± 95.8 μg/mg and (3) *Bothrops moojeni (B. moojeni) venom pool (lote Bm Lote 01/08-1)* – From 57 specimens of *B. moojeni* with protein content of $m = 1180.1 \pm 195.7$ μg/mg. These pools were produced in the Herpetology Laboratory of the Butantan Institute and after lyophilization, maintained at −20 °C until use. Protein contents (m ± S.E.M) were measured according to Markwell et al. (1978).

2.3. Antibothropic serum (ABS) batches

Purification protocols of F(ab')$_2$ fragments from plasma of immunized animals at the Butantan Institute include the following steps: precipitation of plasma proteins by ammonium sulfate, enzymatic fractionation and thermocoagulation (Raw et al., 1995). Two ABS ampoules (final product) and three intermediary (in bulk) batches of ABS were tested: (1) ABS ampoule (final product) lote number 1103068 (with ED_{50} = 6.1 mg of venom/mL); (2) ABS ampoule (final product) lote number 1102042 (with ED_{50} = 7.0 mg of venom/mL); (3) Intermediary (in bulk) batch of ABS lote number IB-B 20-10-68 (with ED_{50} = 17.5 mg of venom/mL); (4) Intermediary (in bulk) batch of ABS lote number IB-B 25-10-82 (with ED_{50} = 17,8 mg of venom/mL) and (5) Intermediary (in bulk) batch of ABS lote number IB-B 24-10-81 (with ED_{50} = 20,7 mg of venom/mL). These batches were provided by the Technological Development and Production Division of the Butantan Institute (São Paulo, Brazil) and the ED_{50} test, assayed according to WHO (1981).

2.4. Animals

Adult female or male *white leghorn* chickens (1.0–1.7 kg) were used. All birds (10–15 weeks) were purchased from commercial breeding and kept on at 22 °C with free access to water and a commercial feed. All procedures involving animals were carried out in accordance with the Brazilian College of Animal Experimentation (COBEA). The experimental protocol was approved by the Ethical Committee for the Use of Animals of Butantan Institute.

2.5. Obtention of citrated chicken whole blood and plasma samples

The birds were restrained on their backs with wings spread. Feathers were removed and, after using xylocaine spray as a local anesthetic agent, small incisions were made around the brachial wing vein and blood samples carefully collected into syringes containing 1:10 (v/v) 3.8% trisodium citrate. Chicken plasma was obtained after centrifugation at 4000 × *g* for 20 min at 4 °C.

2.6. Determination of minimum coagulant dose (MCD)

The MCD was determined in citrated human and chicken plasmas according to the method described by Theakston and Reid (1983). One hundred microliters of plasma, previously incubated at 37 °C, were mixed with 25 μL of several dilutions of venoms of *B. jararaca*, *B. moojeni* and *B. jararacussu* snakes. The clotting time (CT) in seconds (s) was determined in a semi-automatic coagulation analyzer (1 channel) Start 4 (Stago). The venom concentration was plotted against the CT to determine the clotting activity. The MCD is defined as the concentration of venom (in mg/ml) which induces coagulation of plasma in 60 s at 37 °C under the described conditions.

2.7. Thromboelastometric assays

2.7.1. Determination of the mean coagulant dose (CD_{50})

For experimental procedures, *B. jararaca* venom and ABS were diluted in 0.15 M NaCl. Thromboelastometric (ROTEM) assays using the ROTEM® four-channel system (Pentapharm, Munich, Germany) were performed according to the manufacturer's instructions. In our conditions, control (vehicle-treated) recalcified chicken plasma samples are stable (no spontaneous clot formation) for at least 30 min (CT values from 1937 to 3600 s, mean of 2998 ± 249 s, $n = 8$). CT value of 1800 s was then considered as the maximum threshold of the dose–response curve (no procoagulant effect) and the mean coagulant dose 50% (CD_{50}) was defined as the dose of venom (in μg) which reduces the CT parameter to 900 s. In summary, before recalcification (with 20 μL of 0.2 M $CaCl_2$), 275 μL of chicken plasma samples were preloaded with 45 μL containing: (Group 1) – 0.15 M NaCl (control, $n = 8$); (Group 2) – semi-logarithmic doses [0.1; 0.3; 1 and 3 μg of *B. jararaca* venom], for determination of CD_{50} ($n = 8$) and (3) and (Group 3) – TTPa clot (ellagic acid-based reagent) both in absence ($n = 4$) or in presence of 100 nL of ABS ($n = 4$).

2.7.2. Determination of the neutralization of coagulant activity by antivenom

Neutralization was expressed as effective dose (ED). ED value is defined as the ABS dose (nanoliters, nL) at which CT parameter induced by one amount of venom corresponding to $5CD_{50}$ is displaced to the maximum threshold (1800 s). In this experimental group, the procoagulant activity of this amount of venom was tested in presence of 0 (control), 30, 60 and 120 nL of ABS (in 40 μL, $n = 4$). The final volume of 340 μL were placed into the ROTEM cups and lasted at least 60 min for evaluating the clotting time (CT, in seconds). *B. jararaca* venom doses and TTPa clot reagent were preincubated with ABS for 15 min at 37 °C.

2.8. Statistical analysis

Values of the CT parameter of the dose–response curve related to the procoagulant effect of *B. jararaca* venom were monitored in seconds and expressed as mean ± S.E.M. of 5–8 independent experiments. It was used the ANOVA, Newman–Keuls post-test for analyzing the difference in relation to the control values and we considered $p < 0.05$ as statistically significant. Correlation between the ED_{50} and ED values, obtained from the *in vivo* and the *in vitro* assays, respectively, was determined by means of linear regression analysis The linear regression plots were determined using the computer program GraphPad Prism and the correlation coefficient (r) calculated (Sokal and Rohlf, 1981).

3. Results

The coagulant activity (MCD) of pools of *B. jararaca*, *B. moojeni* and *B. jararacussu* venoms upon citrated human and chicken plasmas is shown in Table 1. This test ($n = 1$) was run in triplicate. Concentration of these bothropic venoms (in μg/ml) which induces coagulation of citrated chicken plasma in 60 s are significantly higher, when compared to that presented when assayed upon human plasma, under the described conditions. Some parameters of the ROTEM profile, such as CT, clot formation time, α angle and maximal amplitude, are particularly dependent of fibrin polymerization and platelet count (Orlikowski et al., 1996; Apelseth et al., 2010; Rumph et al., 2010). Recalcified chicken plasma samples obtained after centrifugation at greater rates render no typical ROTEM profile, probably as a consequence of low concentrations of co-factors such as, for example, platelet microparticles and phospholipids (Owens and Mackman, 2011; Wu et al., 2013). For obtaining one typical ROTEM profile after incubation with *B. jararaca* venom, *White leghorn* WB samples must be centrifugated at lower rates (up to 4000 × *g*). The control (vehicle-treated) recalcified chicken plasma samples are stable (no spontaneous clot formation) for at least 30 min (CT values from 1937 to 3600 s, mean of 2998 ± 249 s, $n = 8$), in contrast with rat recalcified plasma samples (range of the CT parameter values vary from 200 to

Table 1
Minimum coagulant dose (MCD) and Mean lethal dose (LD_{50}) of *B. jararaca*, *B. jararacussu* and *B. moojeni* venoms. MCD was determined in citrated human and chicken plasmas according to the method described in Theakston and Reid (1983) ($n = 1$) and LD_{50} was assayed with mice, according to Villarroel et al. (1978/79).

Venom pool	MCD (mg/mL) upon citrated human plasma	MCD (mg/mL) upon citrated chicken plasma	LD_{50} (μg/animal) with inferior and superior limits of confidence
B. jararaca	0.085	1.025	49.4 (32.0–74.6)
B. moojeni	0.057	0.952	125.1 (80.3–178.9)
B. jararacussu	0.106	0.543	74.5 (54.0–100.9)

450 s, data not shown) (Fig. 1). In our conditions, CT values of the lowest (0.1 μg) and the highest (3 μg) doses of *B. jararaca* venom correspond to the baseline (1229 ± 295 s) and the maximum responses (186 ± 11 s), respectively, when compared with that of vehicle-treated control samples (Fig. 1).

In the *in vivo* assay, the amount (mL) of antivenin at which half of the injected animals at doses corresponding to $5LD_{50}$ survived after 48 h is defined as ED_{50}. In this *in vitro* coagulant test, the mean coagulant dose 50% (CD_{50}) was defined as the quantity of venom (in μg) which reduces the CT parameter of the ROTEM profile to 900 s, between minimum and maximum responses (Fig. 2). The CT induced by $5CD_{50}$ of the venom was used as the control for calculating the effective dose (ED) of each batch of ABS. ED was defined as the ABS dose (nanoliters, nL) at which CT induced by one amount of venom corresponding to $5CD_{50}$ is displaced to the maximum threshold (1800 s). Five batches of the ABS, previously assayed for their lethality neutralizing activity (ED_{50}) by the *in vivo* assay using mice, were assayed (Table 2). The *in vivo* neutralizing activities, ED_{50}, potency and *in vitro* neutralizing activities with the 95% confidence intervals of five batches of ABS were compared by means of linear regression analysis. The correlation coefficient (r) between both *in vitro* (ED) and *in vivo* (ED_{50}) values was 0.87 (p value < 0.05). As a control assay, in order to verify the specificity of ABS, activation of the chicken coagulation process was induced by adding one classical activator of the intrinsic pathway of coagulation (aPTT clot), one ellagic acid-based reagent (Abildgaard and Harrison, 1974) to recalcified chicken plasma samples. In summary, addition of 45 μL of this ellagic acid-based reagent led to one significant decrease (1287 ± 168 s, $p < 0.05$, $n = 4$) of the CT parameter when compared with control vehicle-treated samples (2879 ± 212 s). Addition of 100 nL of ABS (sufficient to significantly neutralize the procoagulant effect of one amount of venom corresponding to $5CD_{50}$ of *B. jararaca* venom) presented no influence in procoagulant effect of aPTT clot reagent (data not shown).

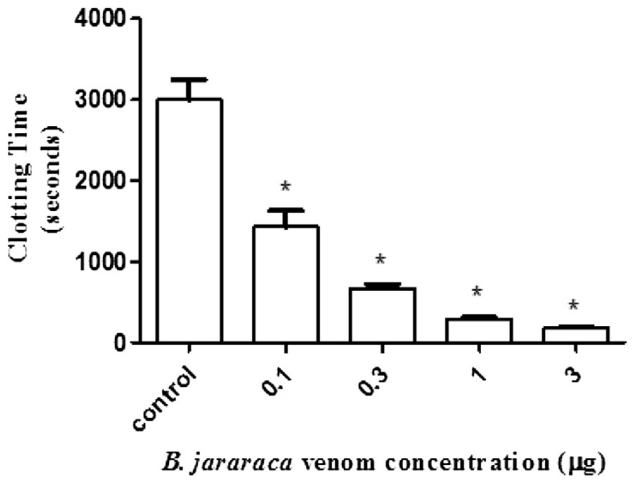

Fig. 1. Semi-log plot of *B. jararaca* venom against the clotting time parameter of recalcified chicken thrombocyte-poor plasma samples (320 μL). *Indicates a statistically significant difference from control ($n = 8$, $p < 0.05$, ANOVA, Newman–Keuls post-test).

4. Discussion

The development of *in vitro* assays to substitute for animal models is a highly relevant task for future development of antivenom quality control as a way to achieve the 'three R' goals of animal experimentation, i.e. Reduce, Refine and Replace animal tests (Sells, 2003; Van Geffen and van Heerde, 2012). A number of *in vitro* studies relatives to alternatives assays for testing several snake venoms activities in general and giving a good correlation with the *in vivo* lethality assay have been proposed (Theakston and Reid, 1979; Gutiérrez et al., 1988; Maria et al., 2001; Sells et al., 2001; Rial et al., 2006; Rafael et al., 2008; Isbister et al., 2010). Recently, Pornmuttakun and Ratanabanangkoon (2014) demonstrated a high correlation ($r = 0.957$, $p < 0.001$) between neutralization of *in vitro* coagulant activity and neutralization of lethality in the case of the venom of the viperid *Calloselasma rhodostoma*.

The imbalance of hemostatic systems of victims is an important event of snakebite envenomation. This severe toxicity is caused by the collective effect of the dozens of toxic proteins that constitute the venom mixture. The complexity of snake venom composition and toxicological profile demands that, for many venoms such as those of viperid snakes and some elapids, the neutralization of lethality be complemented with the analysis of the neutralization of other relevant toxic activities, such as hemorrhagic, myotoxic, necrotizing, procoagulant and defibrinogenating effects (Gutiérrez et al., 2013). The development of these alternative *in vitro* tests for evaluating toxic activities of animal venoms should ideally be based on a careful analysis of the predominant toxic proteins present in a particular venom, and on the understanding of the mechanisms by which these toxins exert their deleterious effects. Analysis of gene expression of the venom gland from *B. jararaca* snake showed that more than 50% of transcribed genes belong to snake venom metalloproteases (Cidade et al., 2006). These proteases, grouped in three main classes according to these structures, have been shown to participate in the hemorrhagic process by proteolytic degradation of endothelial cell surface proteins and extracellular matrix components involved in the maintenance of capillary structure and integrity, leading to disruption of capillary networks, edema and hemorrhage (Escalante et al., 2006; Moura-da-Silva et al., 2007). Additionally, class P-III bothropic metalloproteinase such as a thrombin-like, prothrombin and factor X activators are certainly responsible by significant part of these clinical manifestations, being responsible for complete consumption of fibrinogen, factor V, and factor VIII *in vivo* due to the downstream effects of the thrombin that is formed (Nahas et al., 1979; Furtado et al., 1991; Maruyama et al., 1992; Bjarnason and Fox, 1994; Sano-Martins et al., 1997; Kamiguti et al., 1998; Matsui et al., 2000; Santoro and Sano-Martins, 2004; Gutiérrez et al., 2005; Rucavado et al., 2005; Cidade et al., 2006; Berger et al., 2008; Terra et al., 2009; Moura-da-Silva and Baldo, 2012).

Several toxic activities (proteolytic, edematogenic, nucleotidasic, hyaluronidasic phospholipasic, myotoxic and procoagulant) presented by *B. jararaca* venom have been

detected by a variety of *in vitro* and *in vivo* assays (Antunes et al., 2010).

In contrast to conventional laboratory tests [e.g. MCD, prothrombin time and activated partial thromboplastin time (aPTT)], ROTEM assay can measure early variables of the clotting process, such as CT, clot formation time, alpha angle and clot amplitude. In summary, presence of anticoagulant drugs or abnormal levels of coagulant factors, platelets or fibrinolysis are detected in plasma or WB samples by the ROTEM assay. Additionally, this assay can be used as a routine screening procedure for testing a variety of activators and/or inhibitors of the coagulation process, by analyzing its influence on ROTEM parameters after incubation with recalcified plasma or WB samples.

The dynamics of fibrin formation in recalcified samples of reptilian or avian plasmas are significantly slow, when compared with mammalian plasmas (Spurling, 1981). This unusual characteristic of the avian clotting process seems to be due to the absence of important constituents of the intrinsic pathway of coagulation (Ponczek et al., 2008). There is a poor correlation between estimation of *in vitro* clotting activity (MCD) of venoms of *B. jararaca*, *B. moojeni* and *B. jararacussu* in human and chicken citrated plasma samples. One convincing explanation for this different responsiveness between these two plasmas to bothropic venoms is not available at this moment. However, when assayed upon recalcified chicken plasma samples in the ROTEM assay, *B. jararaca* venom present significant procoagulant activity, being possible to perform one typical dose–response curve. Moreover, in contrast with that happen with the hemostatic system of some reptilian species (Nahas et al., 1983; Fortes-Dias et al., 1991; de Oliveira and Tanizaki, 1992; Thurn et al., 1993; Smith et al., 2000; Tanaka-Azevedo et al., 2010), avian species do not present significant amounts of natural inhibitors against snake venom proteins. After analyzing the influence of several doses of *B. jararaca venom* on ROTEM parameters of recalcified chicken plasma samples, the following data were established: (a) The CT values of control (vehicle-treated) recalcified chicken plasma samples are significantly prolonged no spontaneous clot formation for at least 30 min (2998 ± 249 s, $n = 8$), when compared to that presented by mammalian (rat) plasma samples in similar conditions (from 200 to 450 s, $n = 5$); (b) there is an inverse relation between *B. jararaca* venom doses and CT values; (c) the lowest (0.1 μg) and the highest (3 μg) thresholds related to *B. jararaca* venom doses correspond to the minimum and maximal responses, respectively, when compared with vehicle-treated control samples and (d) the straight line portion between minimum and maximum responses occurs over two orders of magnitude of concentration of the agonist (*B. jararaca* venom, from 0.3 to 10 μg/mL of chicken plasma). These characteristics define one typical dose–response curve, with *B. jararaca* venom as agonist, coagulation zymogens of chicken plasma samples as targets and the ABS as the competitive antagonist.

Table 2
The *in vitro* and *in vivo* neutralization activities of five batches of antibothropic serum (ABS) as expressed as potency, *in vitro* effective dose (ED) and *in vivo* effective dose (ED_{50}).

ABS batch number	*In vitro* (ED) (nL ABS/5ED_{50} venom, $m \pm$ S.E.M.)	Potency (95% confidence intervals)	*In vivo* (ED_{50}) (μL of ABS plus 95% confidence intervals)
1102042	122.66 ± 16.40	7.0 (6.1–7.9)	27.45 (24.19–31.38)
1103068	101.32 ± 22.50	6.1 (5.4–7.0)	31.34 (27.46–35.57
20-10-68	28.13 ± 5.01	17.5 (15.4–20.1)	10.91 (9.5–12.4)
25-10-82	39.68 ± 5.45	17.8 (15.6–20.7)	10.72 (9.26–12.27)
24-10-81	40.92 ± 5.83	20.7 (18.0–23.8)	9.23 (8.03–10.6)

Abbreviations: ED, effective dose; ED_{50}, mean effective dose; ABS, antibothropic serum; *m*, mean; S.E.M., standard error of mean.
Potency is defined as the amount of *B. jararaca* venom neutralized by 1 mL of ABS.
Correlation between the ED_{50} and ED values was determined by means of linear regression analysis and the correlation coefficient (r) of these two assays is 0.87 ($p < 0.05$), according to Sokal and Rohlf (1981).

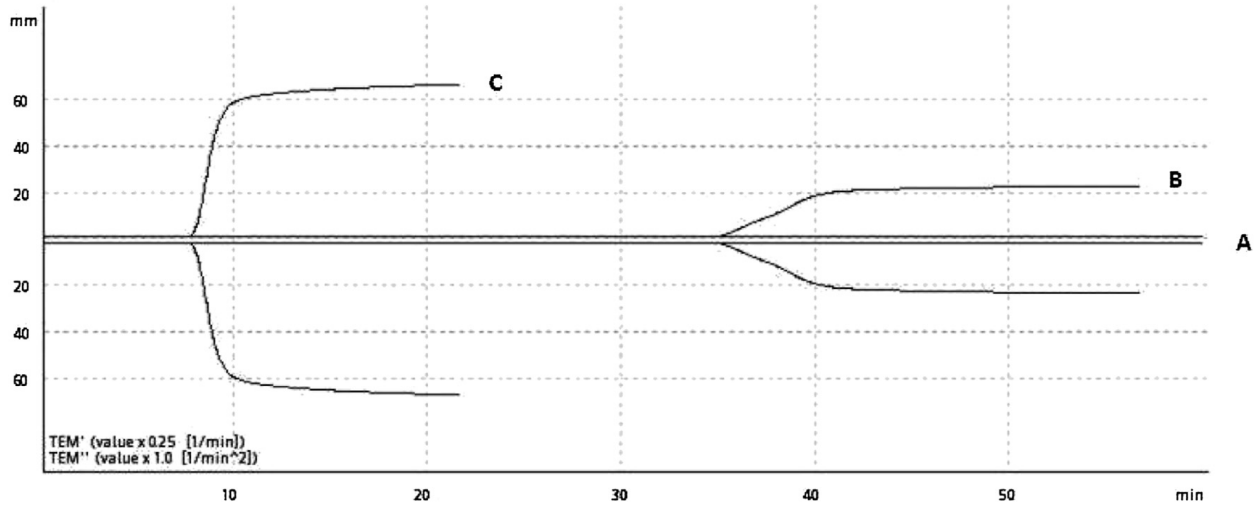

Fig. 2. Thromboelastometric profile of chicken plasma samples activated with *B. jararaca* venom in presence and absence of antibothropic serum (ABS). 20 μL of 0.2 M $CaCl_2$ were added to plasma samples (275 μL) before incubating with 45 μL containing: 0.15 M NaCl (A); 300 ng bothropic venom + 30 nL of ABS (B) and 300 ng *B. jararaca* venom (C).

Since the molar concentration of the ABS is not available, we were not able to calculate neither the half maximal effective concentration (EC_{50}) of *B. jararaca* venom nor the half maximal inhibitory concentration (IC_{50}) of ABS. However, it was possible to evaluate the CT parameter induced by doses of *B. jararaca* venom corresponding to both CD_{50} and $5CD_{50}$, similarly with doses used in the *in vivo* rodent lethality assay. Five batches of the ABS, previously assayed for their lethality neutralizing activity by the *in vivo* assay using mice, were assayed (Table 2) (Fig. 2). The *in vivo* neutralizing activities, ED_{50}, potency and *in vitro* neutralizing activities of five batches of ABS were compared by means of linear regression analysis. The correlation coefficient (r) between both *in vitro* (ED) and *in vivo* (ED_{50}) values was 0.87 (p value < 0.05). In this study, satisfactory correlation between the *in vitro* activity and the *in vivo* activity was obtained; the correlation coefficient (r) was 0.87 ($p < 0.05$). However, as mentioned by Gutiérrez et al. (1988), even when an *in vitro* test showed high correlation, it did not necessarily mean that the enzyme was responsible for the lethal toxic effect of the venom. Rather, it suggested that the antibody production against the enzyme paralleled those against the predominant lethal toxins of the venom.

The aPTT clot reagent have been used as a routine preoperative screening procedure, to detect qualitative deficiencies of constituents of the intrinsic pathway of coagulation (factors VIII, IX, XI, XII and plasma prekallikrein) (Abildgaard and Harrison, 1974). Since the mechanism of activation of the clotting process elicited by this ellagic acid-based reagent is not related to that induced by *B. jararaca* venom, the absence of neutralizing activity of ABS against this reagent is not surprising, indicating its specificity.

5. Conclusion

Our findings are very similar to the *in vitro* potency assay of antiserum against the *C. rhodostoma* venom reported by Pornmuttakun and Ratanabanangkoon (2014). We propose this functional assay with chicken plasma samples as a highly sensitive method for: (a) quantification of the activity of small doses (nanograms) of snake venoms that present procoagulant effect *in vitro*; (b) detection of very low levels of antibodies against this procoagulant effect in little volume samples (nanoliters) of biological fluids (such as plasma, cerebrospinal fluid, urine, tears, saliva …); and (c) assessing antivenom relative potency in some intermediary steps of antivenom production, to quality control test. Moreover, it is easy, reproductive, fast and inexpensive, and many samples can be studied simultaneously. The total blood volume of one determined organism is very difficult to determine (McGuill and Rowan, 1989) and depends on species, sex, age and health as well as nutritional condition. Total circulating blood volume is in the range of 55–70 ml/kg body weight and adult chickens allow the collection of at least 8 ml of WB samples from each wing vein (allowing at least 20 ROTEM assays), without significant animal distress or necessity of euthanasia.

Acknowledgments

We thank Dr Paulo Lee Ho, Technological Development and Production Division Director for providing the antibothropic serum and to Venom Commission of the Butantan Institute for providing the *B. jararaca* venom pool.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2014.08.004.

Funding

The study was supported partially by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) 2010/08580-8 and Programa Institucional de Bolsas de Iniciação CientíficaCnpq/Instituto Butantan (PIBIC/CNPq).

References

Abildgaard, C.F., Harrison, J., 1974. Fletcher factor deficiency: family study and detection. Blood 43, 641–644.
Antunes, T.C., Yamashita, K.M., Barbaro, K.C., Saiki, M., Santoro, M.L., 2010. Comparative analysis of newborn and adult *Bothrops jararaca* snake venoms. Toxicon 56, 1443–1458.
Apelseth, T.O., Bruserud, O., Wentzel-Larsen, T., Hervig, T., 2010. Therapeutic efficacy of platelet transfusion in patients with acute leukemia: an evaluation of methods. Transfusion 50, 766–775.
Arbuckle, K., 2010. Suitability of day-old chicks as food for captive snakes. J. Anim. Physiol. Anim. Nutr. 94, 296–307.
Berger, M., Pinto, A.F., Guimaraes, J.A., 2008. Purification and functional characterization of Bothrojaractivase, a prothrombin-activating metalloproteinase isolated from *Bothrops jararaca* snake venom. Toxicon 51, 488–501.
Bjarnason, J.B., Fox, J.W., 1994. Hemorrhagic metalloproteinases from snake venoms. Pharmacol. Ther. 62, 325–372.
Brooks, M.B., Stokol, T., Catalfamo, J.L., 2011. Comparative hemostasis: animal models and new hemostasis tests. Clin. Lab. Med. 31, 139–159.
Cidade, D.A., Wermelinger, L.S., Lôbo-Hajdu, G., Dávila, A.M., Bon, C., Zingali, R.B., Albano, R.M., 2006. Molecular diversity of disintegrin-like domains within metalloproteinase precursors of *Bothrops jararaca*. Toxicon 48, 590–599.
de Oliveira, E.P., Tanizaki, M.M., 1992. Effect of a proteinase inhibitor from the plasma of *Bothrops jararaca* on coagulant and myotoxic activities of *Bothrops* venoms. Toxicon 30, 123–128.
Du, X.Y., Sim, D.S., Lee, W.H., Zhang, Y., 2006. Blood cells as targets of snake toxins. Blood Cells Mol. Dis. 36, 414–421.
Escalante, T., Shannon, J., Moura-da-Silva, A.M., Gutierrez, J.M., Fox, J.M., 2006. Novel insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases: a biochemical and immunohistochemical study. Arch. Biochem. Biophys. 455, 144–153.
Farmacopeia Brasileira, 2010, 904pp., 2v/il. Soro Antibotrópico Pentavalente – Immunoserum Bothropicum, vol. 2. Agência Nacional de Vigilância Sanitária, Brasília, pp. 1289–1290. Brasil: Anvisa.
Fortes-Dias, C.L., Fonseca, B.C., Kochva, E., Diniz, C.R., 1991. Purification and properties of an antivenom factor from the plasma of the South American rattlesnake (*Crotalus durissus terrificus*). Toxicon 29, 997–1008.
Furtado, M.F.D., Maruyama, M., Kamiguti, A.S., Antonio, L.C., 1991. Comparative study of nine *Bothrops* snake venoms from adult female snakes and their offspring. Toxicon 29, 219–226.

Gutiérrez, J.M., Avila, C., Rojas, E., Cerdas, L., 1988. An alternative in vitro method for testing the potency of the polyvalent antivenom produced in Costa Rica. Toxicon 26, 411–413.
Gutiérrez, J.M., Rucavado, A., Escalante, T., Díaz, C., 2005. Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. Toxicon 45, 997–1011.
Gutiérrez, J.M., León, G., Burnouf, T., 2011. Antivenoms for the treatment of snakebite envenomings: the road ahead. Biologicals 39, 129–142.
Gutiérrez, J.M., Solano, G., Pla, D., Herrera, M., Segura, Á., Villalta, M., Vargas, M., Sanz, L., Lomonte, B., Calvete, J.J., León, G., 2013. Assessing the preclinical efficacy of antivenoms: from the lethality neutralization assay to antivenomics. Toxicon 69, 168–179.
Isbister, G.K., 2009. Procoagulant snake toxins: laboratory studies, diagnosis, and understanding snakebite coagulopathy. Semin. Thromb. Hemost. 35, 93–103.
Isbister, G.K., Woods, D., Alley, S., O'Leary, M.A., Seldon, M., Lincz, L.F., 2010. Endogenous thrombin potential as a novel method for the characterization of procoagulant snake venoms and the efficacy of antivenom. Toxicon 56, 75–85.
Kamiguti, A.S., Zuzel, M., Theakston, R.D., 1998. Snake venom metalloproteinases and disintegrins: interactions with cells. Braz. J. Med. Biol. Res. 31, 853–862.
Laing, G.D., Theakston, R.D., Leite, R.P., da Silva, W.D., Warrell, D.A., 1992. Comparison of the potency of three Brazilian *Bothrops* antivenoms using *in vivo* rodent and in vitro assays. BIASG (Butantan Institute Antivenom Study Group). Toxicon 30, 1219–1225.
Maria, W.S., Pacheco, B.G., Barbosa, C.F., Velarde, D.T., Chavez-Olortegui, C., 2001. Determination of the neutralizing potency of horse antibothropic and anticrotalic antivenoms in blood samples collected on filter paper. Toxicon 39, 1607–1609.
Markwell, M.A., Haas, S.M., Bieber, L.L., Tolbert, N.E., 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87, 206–210.
Marshall, L.R., Herrmann, R.P., 1983. Coagulant and anticoagulant actions of Australian snake venoms. Thromb. Haemost. 50, 707–711.
Maruyama, M., Sugiki, M., Yoshida, E., Shimaya, K., Mihara, H., 1992. Broad substrate specificity of snake venom fibrinolytic enzymes: possible role in haemorrhage. Toxicon 30, 1387–1397.
Masci, P.P., Whitaker, A.N., de Jersey, J., 1988. Purification and characterization of a prothrombin activator from the venom of the Australian brown snake, *Pseudonaja textilis textilis*. Biochem. Int. 17, 825–835.
Matsui, T., Fujimura, Y., Titani, K., 2000. Snake venom proteases affecting hemostasis and thrombosis. Biochim. Biophys. Acta 1477, 146–156.
McGuill, M.W., Rowan, A.N., 1989. Biological effects of blood loss: implications for sampling volumes and techniques. ILAR. News 31, 5–18.
Moura-da-Silva, A.M., Baldo, C., 2012. Jararhagin, a hemorrhagic snake venom metalloproteinase from *Bothrops jararaca*. Toxicon 60, 280–289.
Moura-da-Silva, A.M., Butera, D., Tanjoni, I., 2007. Importance of snake venom metalloproteinases in cell biology: effects on platelets, inflammatory and endothelial cells. Curr. Pharm. Des. 13, 2893–2905.
Nahas, L., Kamiguti, A.S., Barros, M.A.R., 1979. Thrombin-like and factor X-activator components of *Bothrops* snake venoms. Thromb. Haemost. 41, 314–328.
Nahas, L., Kamiguti, A.S., Sousa e Silva, M.C., Ribeiro de Barros, M.A., Morena, P., 1983. The inactivating effect of *Bothrops jararaca* and *Waglerophis merremii* snake plasma on the coagulant activity of various snake venoms. Toxicon 21, 239–246.
Orlikowski, C.E., Rocke, D.A., Murray, W.B., Gouws, E., Moodley, J., Kenoyer, D.G., Byrne, S., 1996. Thromboelastography changes in preeclampsia and eclampsia. Br. J. Anaesth. 77, 157–161.
Owens, A.P., Mackman, N., 2011. Microparticles in hemostasis and thrombosis. Circ. Res. 108, 1284–1297.
Ponczek, M.B., Gailani, D., Doolittle, R.F., 2008. Evolution of the contact phase of vertebrate blood coagulation. J. Thromb. Haemost. 6, 1876–1883.
Pornmuttakun, D., Ratanabanangkoon, K., 2014. Development of an in vitro potency assay for antivenom against Malayan pit viper (*Calloselasma rhodostoma*). Toxicon 77, 1–5.
Rafael, A., Tanjoni, I., Fernandes, I., Moura-da-Silva, A.M., Furtado, M.F., 2008. An alternative method to access in vitro the hemorrhagic activity of snake venoms. Toxicon 51, 479–487.
Raw, I., Higashi, H.G., Kelen, E.M., 1995. Antivenom production and organization of the public health system for the treatment of envenoming in Brazil. In: Bon, C., Goyffon, M. (Eds.), Envenomings and Their Treatments. Editions Fondation Maecel, Lyon, p. 173.
Rial, A., Morais, V., Rossi, S., Massaldi, H., 2006. A new ELISA for determination of potency in snake antivenoms. Toxicon 48, 462–466.
Rucavado, A., Soto, M., Escalante, T., Loría, G.D., Arni, R., Gutiérrez, J.M., 2005. Thrombocytopenia and platelet hypoaggregation induced by *Bothrops asper* snake venom. Toxins involved and their contribution to metalloproteinase-induced pulmonary hemorrhage. Thromb. Haemost. 94, 123–131.
Rugeri, L., Levrat, A., David, J.S., Delecroix, E., Floccard, B., Gros, A., Allaouchiche, B., Negrier, C., 2007. Diagnosis of early coagulation abnormalities in trauma patients by rotation thromboelastography. J. Thromb. Haemost. 5, 289–295.
Rumph, B., Bolliger, D., Narang, N., Molinaro, R.J., Levy, J.H., Szlam, F., Tanaka, K.A., 2010. *In vitro* comparative study of hemostatic components in warfarin-treated and fibrinogen-deficient plasma. J. Cardiothorac. Vasc. Anesth. 24, 408–412.
Sano-Martins, I.S., Santoro, M.L., Castro, S.C.B., Fan, H.W., Cardoso, J.L., Theakston, R.D.G., 1997. Platelet aggregation in patients bitten by the Brazilian snake *Bothrops jararaca*. Thromb. Res. 87, 183–195.
Santoro, M.L., Sano-Martins, I.S., 2004. Platelet dysfunction during *Bothrops jararaca* snake envenomation in rabbits. Thromb. Haemost. 92, 369–383.
Sells, P.G., 2003. Animal experimentation in snake venom research and *in vitro* alternatives. Toxicon 42, 115–133.
Sells, P.G., Laing, G.D., Theakston, R.D., 2001. An *in vivo* but insensate model for the evaluation of antivenoms (ED_{50}) using fertile hens' eggs. Toxicon 39, 665–668.
Smith, A., Marshall, L.R., Mirtschin, P.J., Jelinek, G.A., 2000. Neutralisation of the clotting activity of Australian snake venoms by snake plasma. Toxicon 38, 1855–1858.
Sokal, R.R., Rohlf, F.J., 1981. Biometry. In: Wilson, J., Cotter, S. (Eds.), The Principles and Practice of Statistics in Biological Research, second ed. W. H. Freeman and company, New York, pp. 561–601.
Sprivulis, P., Jelinek, G.A., Marshall, L., 1996. Efficacy and potency of antivenoms in neutralizing the procoagulant effects of Australian snake venoms in dog and human plasma. Anaesth. Intensive Care 24, 379–381.
Spurling, N.W., 1981. Comparative physiology of blood clotting. Comp. Biochem. Physiol. A. Physiol. 68, 541–548.
Tanaka-Azevedo, A.M., Morais-Zani, K., Torquato, R.J., Tanaka, A.S., 2010. Thrombin inhibitors from different animals. J. Biomed. Biotechnol., 1–9.
Terra, R.M., Pinto, A.F., Guimarães, J.A., Fox, J.W., 2009. Proteomic profiling of snake venom metalloproteinases (SVMPs): insights into venom induced pathology. Toxicon 54, 836–844.
Theakston, R.D., Reid, H.A., 1979. Enzyme-linked immunosorbent assay (ELISA) in assessing antivenom potency. Toxicon 17, 511–515.
Theakston, R.D.G., Reid, H.A., 1983. Development of simple assay standard procedures for the characterization of snake venoms. Bull. World Health Organ. 61, 949–956.
Theakston, R.D., Warrell, D.A., Griffiths, E., 2003. Report of a WHO workshop on the standardization and control of antivenoms. Toxicon 41, 541–557.
Thurn, M.J., Broady, K., Mirtschin, P.J., 1993. Neutralisation of tiger snake (*Notechis scutatus*) venom by serum from other Australian elapids. Toxicon 31, 909–912.
Van Geffen, M., van Heerde, W.L., 2012. Global haemostasis assays, from bench to bedside. Thromb. Res. 129, 681–687.
Villarroel, M.S., Zelante, F., Rolim Rosa, R., Furlanetto, R.S., 1978/79. Padronização da titulação da atividade tóxica de venenos botrópicos, em camundongos. Mem. Inst. Butantan 42/43, 311–323.
Warrell, D.A., Looareesuwan, S., Theakston, R.D., Phillips, R.E., Chathavanich, P., Viravan, C., Supanaranond, W., Karbwang, J., Ho, M., Hutton, R.A., Vejcho, S., 1986. Randomized comparative trial of three monospecific antivenoms for bites by the Malayan pit viper (*Calloselasma rhodostoma*) in southern Thailand: clinical and laboratory correlations. Am. J. Trop. Med. Hyg. 35, 1235–1247.
WHO, 1981. Progress in the Characterisation of Venoms and Standardisation of Antivenoms. Offset Publication no. 58. World Health Organization, Geneva.
Wiinberg, B., Kristensen, A.T., 2010. Thromboelastography in veterinary medicine. Semin. Thromb. Hemost. 36, 747–756.
Wu, Z.H., Ji, C.L., Li, H., Qiu, G.X., Gao, C.J., Weng, X.S., 2013. Membrane microparticles and diseases. Eur. Rev. Med. Pharmacol. Sci. 17, 2420–2427.
Zelanis, A., Travaglia-Cardoso, S.R., Furtado, M.F.D., 2008. Ontogenetic changes in the venom of *Bothrops insularis* (Serpentes: Viperidae) and its biological implication. South Am. J. Herpetol. 3, 43–50.
Zuckerman, L., Cohen, E., Vagher, J.P., Woodward, E., Caprini, J.A., 1981. Comparison of thromboelastography with common coagulation tests. Thromb. Haemost. 46, 752–756.