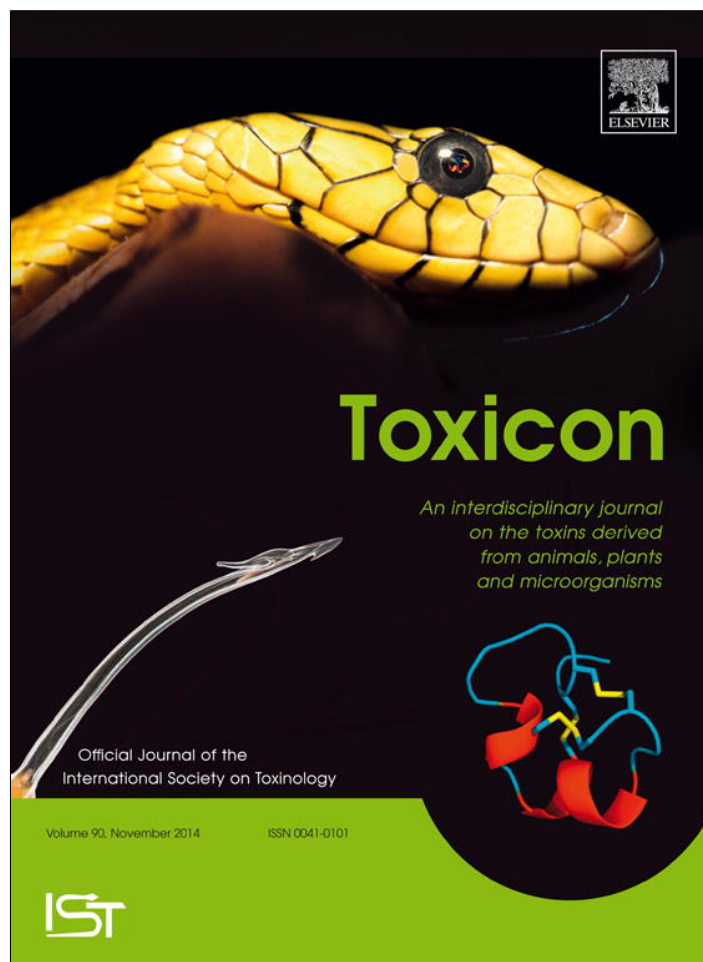


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## An alternative micromethod to access the procoagulant activity of *Bothrops jararaca* venom and the efficacy of antivenom

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### 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<sub>50</sub>). 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<sub>50</sub>) 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<sub>50</sub> 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<sub>50</sub> is displaced to the maximum threshold (1800 s). Five batches of the ABS, previously assayed for their lethality neutralizing activity (ED<sub>50</sub>) were assayed. The correlation coefficient (*r*) between both *in vitro* (ED) and *in vivo* (ED<sub>50</sub>) 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.

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### 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

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constituted by either whole IgG molecules or the immunoglobulin fragments F(ab')<sub>2</sub>; 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<sub>50</sub>), 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<sub>50</sub> survived after 48 h is defined as mean effective dose (ED<sub>50</sub>), 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 antithrombotic 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 ( $m \pm$  S.E.M) were measured according to Markwell et al. (1978).

### 2.3. Antithrombotic serum (ABS) batches

Purification protocols of F(ab')<sub>2</sub> fragments from plasma of immunized animals at the Butantan Institute include the following steps: precipitation of plasma proteins by ammonium sulfate, enzymatic fractionation and thermo-coagulation (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<sub>50</sub> = 6.1 mg of venom/mL); (2) ABS ampoule (final product) lote number 1102042 (with ED<sub>50</sub> = 7.0 mg of venom/mL); (3) Intermediary (in bulk) batch of ABS lote number IB-B 20-10-68 (with ED<sub>50</sub> = 17.5 mg of venom/mL); (4) Intermediary (in bulk) batch of ABS lote number IB-B 25-10-82 (with ED<sub>50</sub> = 17.8 mg of venom/mL) and (5) Intermediary (in bulk) batch of ABS lote number IB-B 24-10-81 (with ED<sub>50</sub> = 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<sub>50</sub> 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  $4000 \times g$  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  $\mu$ L 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  $2998 \pm 249$  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  $\mu$ g) which reduces the CT parameter to 900 s. In summary, before recalcification (with 20  $\mu$ L of 0.2 M  $CaCl_2$ ), 275  $\mu$ L of chicken plasma samples were preloaded with 45  $\mu$ L containing: (Group 1) – 0.15 M NaCl (control,  $n = 8$ ); (Group 2) – semi-logarithmic doses [0.1; 0.3; 1 and 3  $\mu$ g 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  $\mu$ L,  $n = 4$ ). The final volume of 340  $\mu$ L 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  $\pm$  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  $\mu$ g/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,  $\alpha$  angle and maximal amplitude, are particularly dependent of fibrin polymerization and platelet count (Orlikowski et al., 1996; Apelseh 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  $4000 \times g$ ). 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  $2998 \pm 249$  s,  $n = 8$ ), in contrast with rat recalcified plasma samples (range of the CT parameter values vary from 200 to

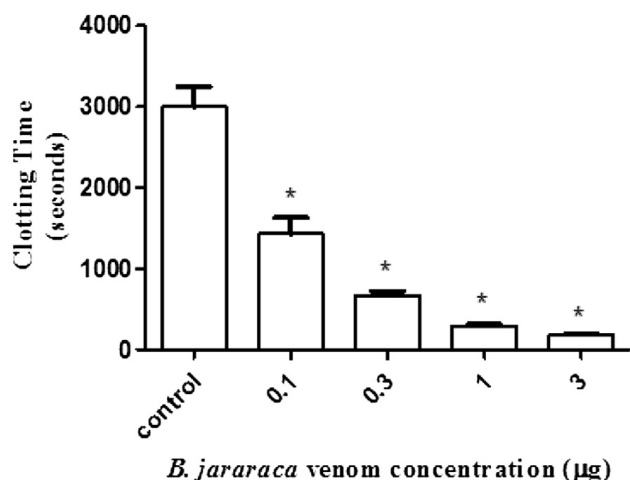
**Table 1**

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	$LD_{50}$ ( $\mu$ g/animal) with inferior and superior limits of confidence
<i>B. jararaca</i>	0.085	1.025	49.4 (32.0–74.6)
<i>B. moojeni</i>	0.057	0.952	125.1 (80.3–178.9)
<i>B. jararacussu</i>	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  $\mu\text{g}$ ) and the highest (3  $\mu\text{g}$ ) doses of *B. jararaca* venom correspond to the baseline ( $1229 \pm 295$  s) and the maximum responses ( $186 \pm 11$  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<sub>50</sub> survived after 48 h is defined as ED<sub>50</sub>. In this *in vitro* coagulant test, the mean coagulant dose 50% (CD<sub>50</sub>) was defined as the quantity of venom (in  $\mu\text{g}$ ) which reduces the CT parameter of the ROTEM profile to 900 s, between minimum and maximum responses (Fig. 2). The CT induced by 5CD<sub>50</sub> 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<sub>50</sub> is displaced to the maximum threshold (1800 s). Five batches of the ABS, previously assayed for their lethality neutralizing activity (ED<sub>50</sub>) by the *in vivo* assay using mice, were assayed (Table 2). The *in vivo* neutralizing activities, ED<sub>50</sub>, 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<sub>50</sub>) 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  $\mu\text{L}$  of this ellagic acid-based reagent led to one significant decrease ( $1287 \pm 168$  s,  $p < 0.05$ ,  $n = 4$ ) of the CT parameter when compared with control vehicle-treated samples ( $2879 \pm 212$  s). Addition of 100 nL of ABS (sufficient to significantly neutralize the procoagulant effect of one amount of venom corresponding to 5CD<sub>50</sub> 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  $\mu\text{L}$ ). \*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 anti-coagulant 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 pro-coagulant 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

**Table 2**

The *in vitro* and *in vivo* neutralization activities of five batches of anti-bothropic serum (ABS) as expressed as potency, *in vitro* effective dose (ED) and *in vivo* effective dose (ED<sub>50</sub>).

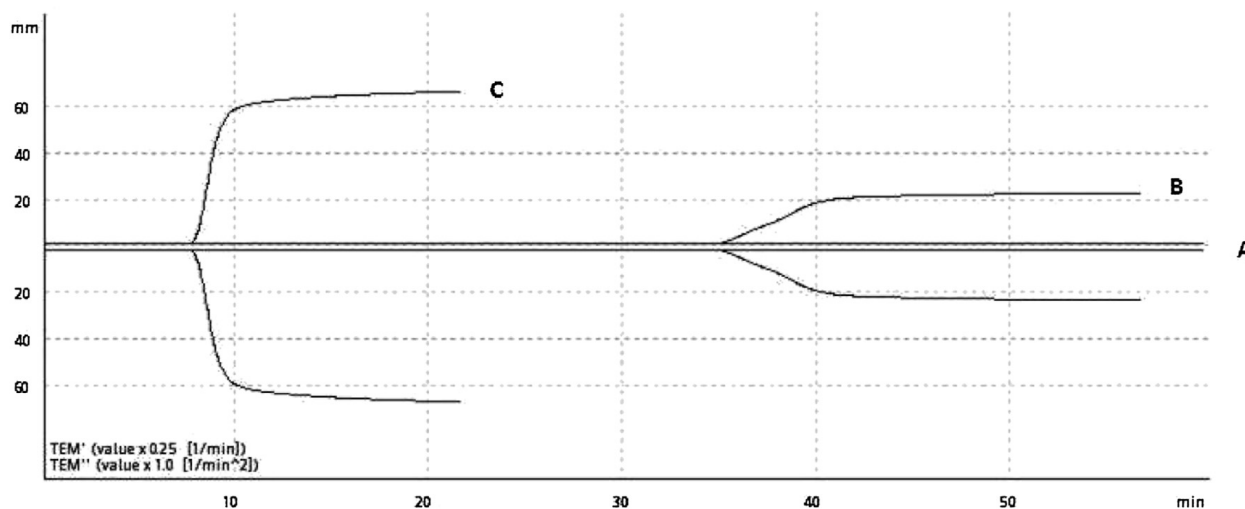
ABS batch number	<i>In vitro</i> (ED) (nL ABS/5ED <sub>50</sub> venom, <i>m</i> ± S.E.M.)	Potency (95% confidence intervals)	<i>In vivo</i> (ED <sub>50</sub> ) (μL of ABS plus 95% confidence intervals)
1102042	122.66 ± 16.40	7.0 (6.1–7.9)	27.45 (24.19–31.38)
1103068	101.32 ± 22.50	6.1 (5.4–7.0)	31.34 (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<sub>50</sub>, mean effective dose; ABS, anti-bothropic 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<sub>50</sub> 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).

(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.



**Fig. 2.** Thromboelastometric profile of chicken plasma samples activated with *B. jararaca* venom in presence and absence of antithrotophic serum (ABS). 20 μL of 0.2 M CaCl<sub>2</sub> 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<sub>50</sub>) of *B. jararaca* venom nor the half maximal inhibitory concentration (IC<sub>50</sub>) of ABS. However, it was possible to evaluate the CT parameter induced by doses of *B. jararaca* venom corresponding to both CD<sub>50</sub> and 5CD<sub>50</sub>, 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<sub>50</sub>, 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<sub>50</sub>) 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 pre-operative 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.

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## 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>.

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