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A functional and thromboelastometric-based micromethod for assessing crotoxin anticoagulant activity and antiserum relative potency against *Crotalus durissus terrificus* venom

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ABSTRACT

The assessment of the capacity of antivenoms to neutralize the lethal activity of snake venoms still relies on traditional rodent *in vivo* lethality assay. ED_{50} and LD_{50} assays require large quantities of venoms and antivenoms, and besides leading to animal suffering. Therefore, *in vitro* tests should be introduced for assessing antivenom neutralizing capacity in intermediary steps of antivenom production. This task is facilitated when one key lethal toxin is identified. A good example is crotoxin, a β-neurotoxin phospholipase A_2-like toxin that presents anticoagulant activity *in vitro* and is responsible for the lethality of venoms of *Crotalus durissus* snakes. By using rotational thromboelastometry, we reported recently one sensitive coagulation assay for assessing relative potency of the anti-bothropic serum in neutralizing procoagulant activity of *Bothrops jararaca* venom upon recalcified factor-XII-deficient chicken plasma samples (CPS). In this study, we stablished conditions for determining relative potency of four batches of the anti-crotalic serum (ACS) (antagonist) in inactivating crotoxin anticoagulant activity in CPS (target) simultaneously treated with one classical activator of coagulation (agonists). The correlation coefficient (r) between values related the ACS potency in inactivating both *in vitro* crotoxin anticoagulant activity and the *in vivo* lethality of whole venom (ED_{50}) was 0.94 (p value < 0.05). In conclusion, slowness in spontaneous thrombin/fibrin generation even after recalcification elicit time lapse sufficient for elaboration of one dose-response curve to pro- or anti-coagulant agonists in CPS. We propose this methodology as an alternative and sensitive assay for assessing antivenom neutralizing ability in plasma of immunized horses as well as for in-process quality control.

1. Introduction

The assessment of the capacity of antivenoms to neutralize the lethal activity of snake venoms still relies on traditional rodent *in vivo* lethality assay (ED_{50}). ED_{50}, defined as mean effective dose, corresponds to the amount (μL) of antivenin necessary to protect half of animals injected with $5LD_{50}$ of snake venom (Farmacopeia Brasileira, 2010). These *in vivo* assays usually are time-consuming, requiring relatively large quantities of venoms and antivenoms, besides leading to animal suffering. Therefore, 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. However, this task is not so simple, since the biochemical/toxicological complexity of snake venoms demands careful analysis of most medically-relevant toxins, to design the most appropriated assay. Imbalance of the hemostatic system of victims as well as coagulation derangements are commonly present and are sometimes fatal in accidental envenomation by Viperidae snake venoms. Circulating blood cells, coagulation factors and

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Abbreviations

ACS	Anti-crotalic serum
CPS	Chicken plasma samples
ED_{50}	Mean effective dose
LD_{50}	Mean lethal dose
MAD	Minimum anticoagulant dose
MCD	Minimum coagulant dose
PLA_2	Phospholipases A_2

vascular wall components of most of vertebrate species may be considered as targets for snake venom toxins (Du et al., 2006). Although promoting venom-induced consumption coagulopathy with hemorrhagic episodes during accidental envenomation, many Viperid snake toxins present procoagulant activity when added to normal blood or plasma samples *in vitro* (Isbister, 2009). The effects of snake venoms on coagulation process in mammalian plasma samples have been traditionally assessed by simple clotting studies (Isbister, 2009). Minimum coagulant dose (MCD) is defined as the least amount of venom that clots a solution of citrated mammalian plasma in 60 s at 37° C and minimum anticoagulant dose (MAD) prolongs its recalcification time to more than 30 min at 37° C (Theakston and Reid, 1983; Pla et al., 2017).

Most of *in vitro* techniques designed for assaying procoagulant activity of snake venoms or toxins on mammalian plasma samples give a single parameter for one complex enzymatic process (Isbister, 2009). Moreover, they present additional limitations, such as: (a) Use of non-human plasma as substrate for assessing antivenom efficacy is polemic, since venoms that cause relevant coagulopathy in humans may have differential effects in non-human plasmas (Maduwage et al., 2016); (b) Non-recalcified plasma samples (such as used in the MCD assay) require significantly higher amounts of venoms (μg or mg), since only prothrombin or fibrinogen activators can be detected in optimal conditions. Important factors of coagulation cascade such as factors XII, XI, IX and X trigger a series of Ca^{2+} and phospholipid-dependent events that lead to thrombin/fibrin formation (Davie et al., 1991). On the other hand, if recalcification increases sensitivity of mammalian plasma samples to procoagulant toxins (Isbister et al., 2010; O'Leary and Isbister, 2010; Nielsen et al., 2017), it triggers simultaneously spontaneous thrombin/fibrin formation process.

New technologies that show good correlation coefficient between antivenom's ability in inactivating venom pro- or anticoagulant effects of venoms *in vitro* and lethality *in vivo* have been reported (Gené et al., 1989; Laing et al., 1992; Clemens et al., 1995; Sprivulis et al., 1996; Sells, 2003; Isbister et al., 2007, 2010; Segura et al., 2010; Pla et al., 2014, 2017; Pornmuttakun and Ratanabanangkoon, 2014; Sánchez et al., 2015; Calvete et al., 2016).

Rotational thromboelastometry (ROTEM) provides an evaluation of the kinetics of all stages of clot initiation, formation, stability, strength, and dissolution in whole blood (Whiting and DiNardo, 2014). The ROTEM assay has been used in several studies reporting pro- or anticoagulant activities of several snake venoms on citrated human whole blood or plasma samples of rats and dogs (Dambisya et al., 1994, 1995; Nagel et al., 2014; Hiremath et al., 2016; Nielsen et al., 2018).

By using rotational thromboelastometry, we have shown earlier that recalcified factor XII-deficient chicken plasma samples (CPS) seems to be an appropriated target for elaboration of one typical dose-response curve to the procoagulant effect of *Bothrops jararaca* (*B. jararaca*) venom, in virtue of the extreme slowness in dynamics of its spontaneous thrombin/fibrin formation as well as to its high sensitivity to procoagulant snake venom toxins. We reported satisfactory correlation coefficient ($r = 0.87$) after comparing relative potencies of five batches of the anti-bothropic serum in neutralizing *in vitro B. jararaca* venom procoagulant effect and their respective ED_{50} values previously determined by the *in vivo* rodent lethality assay (Oguiura et al., 2014).

Crotalus durissus terrificus (*C. d. terrificus*) is a snake of public health importance in South America because it is responsible for the most lethal snakebite events in Brazil (Azevedo-Marques et al., 1985; Cupo et al., 1988; Fan and Cardoso, 1995). *C. d. terrificus* venom is a complex mixture of biologically active substances including the toxins crotamine, crotoxin, giroxin and convulxin (Santoro et al., 1999; de Sousa-e-Silva et al., 2003). Crotoxin, a complex toxin constituted of an acidic, non-toxic and non-enzymatic subunit (crotoxin A or crotapotin) and a basic and weakly toxic phospholipase A_2 (PLA_2) subunit (crotoxin B), is considered as the most lethal component of this venom (Fan and Cardoso, 1995). It displays myotoxic, neurotoxic, nephrotoxic, cardiotoxic, edema-inducing, liposomal-disrupting and anticoagulant activities (Hendon and Fraenkel-Conrat, 1971; Gutiérrez et al., 2008; Sampaio et al., 2010).

We hypothesized that this strategy could be used to determine relative potency of the anti-crotalic serum (ACS) in inactivating crotoxin anticoagulant activity in CPS simultaneously treated with one classical activator of coagulation. In order to address this question, we standardized appropriated conditions for quantifying, in recalcified CPS, coagulant and anticoagulant activities of aPTT clot reagent and crotoxin, respectively, either in presence or absence of the ACS.

2. Material and methods

2.1. Reagents

Activated partial thromboplastin time reagent (aPTT) clot, containing ellagic acid and synthetic phospholipids) from BIOS Diagnostica (SP, Brazil); sodium citrate from Ecibra (Brazil); calcium chloride from E. Merck (German); Hepamax-s®, heparin (Blausiegel Ind. e Com. Ltda, Brasil); Peroxidase-conjugated second antibody (anti-horse IgG); and *o*-phenylenediamine from Sigma (USA). All chemicals were of analytical reagent grade.

2.2. Anticrotalic serum (ACS) batches

The serum batches were provided by the Technological Development and Production Division of the Butantan Institute (São Paulo, Brazil) and consists of F(ab')2 fragments from plasma of immunized horses purified using the following steps: precipitation of plasma proteins by ammonium sulfate, enzymatic fractionation and thermocoagulation (Raw et al., 1995). Four ACS batches, with ED_{50} assayed according to WHO (2010), were tested: two in bulk batches IB-C 04/09 and IB-C 05/09, and intermediary batches IB-C 02-12-05 and IB-C 01-12-04.

2.3. Animals

Adult female or male white leghorn chickens (1.0–1.7 kg) were used. All birds were a donation from commercial breeding (Granja Ino, SP, Brazil) and male Swiss mice (weighing between 18 and 22 g were bred at the Instituto Butantan. The animals had free access to water and food, and were kept under a 12-h light/dark cycle. The Ethical Committee for the Use of Animals of the Butantan Institute approved all procedures involving animals (CEUAIB nº 6587140616).

2.4. Citrated chicken plasma samples (CPS)

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 for cleaning around the brachial wing vein. The vessel was perforated for collecting blood samples into syringes containing 1:10 (v/v) 3.2% trisodium citrate and then closed with cotton-yarn. During the blood collection is necessary to avoid bubble formation into syringes. Chicken plasma was obtained after centrifugation at 4000 × g for 20 min at 4 °C.

2.5. Purification of the crotoxin

Lyophilized crude venom pool of *C. d. terrificus* snake (449 mg), produced in the Laboratory of Herpetology of the Butantan Institute was dissolved in 2.5 mL of 50 mM Tris, 0.1 M NaCl buffer (pH 7.4), centrifuged at 4500 × g for 15 min at 4 °C and was filtered using a 0.45 microfilter to remove the insoluble materials. The clear supernatant was applied on Sephacryl S200HR Hiprep 26/60 (G.E. Healthcare-Sweden), previously equilibrated with 50 mM Tris, 0.1 M NaCl; pH 7.4. Fractions of 5 mL/tube were collected at a flow rate of 1.5 mL/min.

2.6. Studies on lethality of crotoxin

Median lethal dose (LD_{50}) was estimated by injecting five increasing doses of crotoxin (diluted in 0.5 mL of 0.9% saline solution) into groups of six male Swiss mice (weighing between 18 and 22 g) by intraperitoneal route. Deaths occurring during 48 h (Villarroel et al., 1978/79) were recorded and LD_{50} was calculated by "Full probit analysis" (Finney, 1971). $5LD_{50}$ of crotoxin were then incubated with increasing doses (μL) of the ACS for 30 min at 37 °C and injected at final volume of 500 μL into 5 groups of 5 animals by intraperitoneal route. ED_{50} was considered as μL of antivenin necessary to protect half of injected animals.

2.7. Thromboelastometric assays

2.7.1. Standardization of the activator (aPTT clot) effective coagulant dose (ECD) and crotoxin effective anticoagulant dose (EAD) on recalcified chicken plasma samples

Clotting time (CT) parameter was recorded in a computerized ROTEM® four-channel system (Pentapharm, Munich, Germany), according to the manufacturer's instructions for the standard activated ROTEM method (INTEM) during 1 h (n = 5, each experimental group). In summary, before recalcification (with 20 μL of 0.2 M $CaCl_2$), 240 μL of CPS were incubated for 1 min at 37 °C with 80 μL of 0.9% saline solution containing: (Group 1) - 0.9% saline solution (control); (Group 2) - semilogarithmic crescent doses of aPTT clot, an elagic acid-based activator of coagulation, for determination of activator ECD, (volume necessary for shortening CT values to a region near of the middle of the discriminatory region of this dose-response); semilogarithmic crescent doses of purified crotoxin (Group 3) or heparin (Group 4), in presence of the activator ECD, for determining both crotoxin and heparin EAD, defined as minimum amount of these anticoagulants sufficient to displace the CT value related to activator ECD to the maximum threshold (≥1800 s in 100% of assays).

2.7.2. Neutralization of in vitro crotoxin anticoagulant activity by anti-crotalic serum (ACS)

On the basis of the initial results, the following substances were gently mixed and incubated for 1 min at 37 °C, before addition of 240 μL of CPS (n = 5, each experimental group): 20 μL of 0.2 M $CaCl_2$ plus activator ECD (60 μL of the aPTT clot) and 10 μL of crotoxin EAD (100 ng crotoxin, Group 5) or heparin EAD (0.035 UI, Group 6), plus 10 μL containing semi-logarithmic doses of the ACS.

2.8. Statistical analysis

Values of the CT parameter of the dose-response curve related to all experimental groups were monitored in seconds and expressed as mean ± S.E.M. of five independent experiments. It was used the ANOVA, Newman-Keuls post-test for analyzing the difference in relation to the control values. ACS mean *in vitro* effective dose and correlation with *in vivo* ED_{50} values were determined by means of linear regression analysis. The linear regression plots were performed with the GraphPad Prism 5.0 software and the correlation coefficient (r) calculated. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Purification and lethality of crotoxin

The venom was fractionated in four peaks denominated as S1 to S4 (Supplementary material). The protein of S3 fraction used in this work was analyzed by Liquid Chromatography Mass Spectrometry - LC-MS/MS (Supplementary material). Database search of MS/MS spectra acquired from in-gel digested peptides resulted in the identification of the basic phospholipase A_2 crotoxin (P24027) or its isoforms, composing >98% of the gel band (Table S1, Supplementary Material). LD_{50} presented by crotoxin/animal was 1.7 μg (1.1–2.5 plus 95% confidence intervals) and ED_{50} of batch lot number IB-C 05/09 against $5LD_{50}$ of crotoxin was 3.6 μL/animal (2.7–4.7 plus 95% confidence intervals).

3.2. Thromboelastometric assays: standardization and ACS relative potency

In our conditions, CT parameter of the ROTEM profile of control (0.9% saline solution -treated) recalcified CPS is significantly prolonged (from 1789 to 2489 s, mean of 2047 ± 119 s, n = 5) (Fig. 1-A) and 1800 s were standardized as the maximum threshold of the response curve (no coagulant response). 60 μL of aPTT were then defined as activator ECD (Group 2), displacing the CT parameter of control recalcified CPS to 412.4 ± 30.8 s (n = 5) (Fig. 1-B). Crotoxin presented anticoagulant activity in a dose-dependent manner (Fig. 2), and 100 ng (333 ng/mL of plasma, final concentration) was considered as its EAD (Fig. 1-C); similarly, heparin EAD was of 0.0375 IU/mL of plasma (final concentration). Addition of ACS recovered the coagulation capacity of aPTT clot reagent on crotoxin EAD-treated CPS (Fig. 1-D) (Group 5). ACS mean *in vitro* effective dose (determined by means of linear regression analysis) was defined as that dose (nanoliters, nL) necessary for re-stablishing CT values of CPS simultaneously treated with activator ECD and crotoxin EAD from incoagulability (≥1800 s) to 900 s (Table 1). Addition of 100 nL of all batches of the ACS presented no influence in anticoagulant effect induced by heparin EAD (0.0375 IU/mL of plasma, final concentration) (data not shown).

Fig. 2 shows the activity of different doses of crotoxin on the clotting time parameter of recalcified chicken plasma samples (240 μL) activated with 60 μL of aPTT clot.

4. Discussion

The development of alternative *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 '3 Rs' goals of

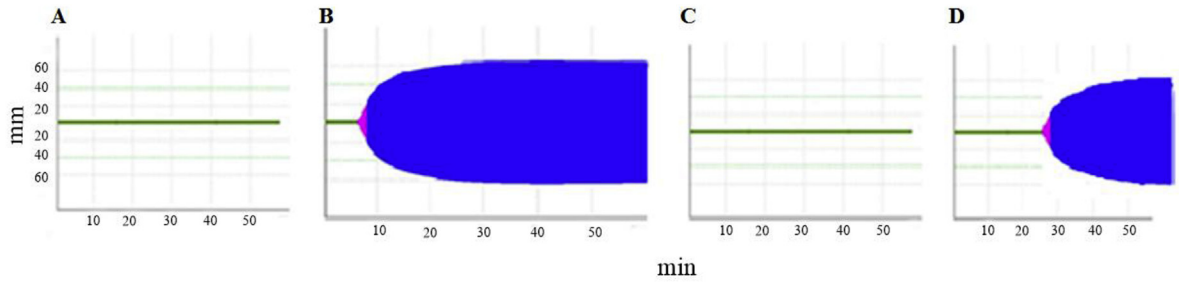

Fig. 1. Thromboelastometric profile of 240 μL of recalcified chicken plasma samples treated with 60 μL containing control-vehicle (A) or aPTT clot activator (an ellagic acid and phospholipid-based reagent) (B). In (C), (B) pre-incubated with crotoxin (100 ng) before recalcification. In (D), (C) in presence of 60 nL of the anticrotalic serum.

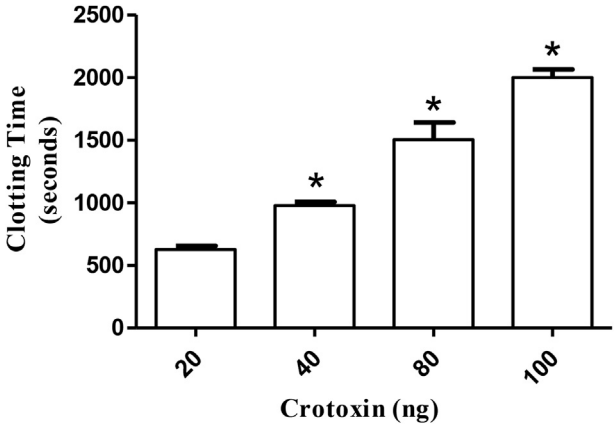

Fig. 2. Semi-log plot of crotoxin anticoagulant activity on the clotting time parameter of recalcified chicken plasma samples (240 μL) activated with 60 μL of aPTT clot. *Indicates a statistically significant difference from control (n = 5, $p < 0.05$, ANOVA, Newman-Keuls post-test).

Table 1
The *in vitro* and *in vivo* neutralization activities of four batches of anticrotalic serum (ACS) upon crotoxin and *C. d. terrificus*, respectively, as expressed as potency, *in vitro* mean effective dose (ED) and *in vivo* effective dose (ED_{50}).

ACS batch number	*In vivo* (ED_{50})	Potency	*In vitro* mean ED
IB-C04/09	3.19 (2.81–3.62)	1.80 (1.62–1.91)	75.28 ± 7.97
IB-C05/09	3.68 (3.25–3.92)	1.58 (1.42–1.78)	80.16 ± 7.41
IB-C02-12-05	2.38 (2.19–2.68)	2.20 (2.08–2.88)	74.48 ± 10.11
IB-C02-12-05	1.47 (1.31–1.62)	3.60 (3.41–3.92)	67.60 ± 6.73

Abbreviations: ACS, anticrotalic serum; *In vivo* ED_{50} (mean effective dose) means μL of ACS plus 95% confidence intervals; Potency is defined as the amount of *C. d. terrificus* venom (mg) neutralized by 1 mL of ACS (95% confidence intervals); *In vitro* mean ED (effective dose) means nanoliters of ACS/100 ng crotoxin, m ± S.E.M(-standard error of mean). Correlation between *in vivo* ED_{50} and mean *in vitro* ED values was determined by means of linear regression analysis and the correlation coefficient (r) of these two assays is 0.94 ($p < 0.05$), according to Sokal and Rohlf (1981).

animal experimentation, i.e. Reduce, Refine and Replace animal tests (Sells, 2003). In the case of snake venoms, this task should be facilitated when venom lethal activity is based on a single toxin or a group of few toxins (Gutiérrez et al., 2010). A good example is crotoxin, one dimeric PLA_2 β-neurotoxin that constitute more than 50% of the dry weight of *C. d. terrificus* venom, being considered responsible for three of the most conspicuous clinical manifestations in envenomation by the South American rattlesnake *C. d. terrificus* (Sampaio et al., 2010). PLA_2 toxins are abundant in snake venoms and are responsible for disruption of cell membrane integrity via hydrolysis of its phospholipids. In addition to their normal digestive action, a wide range of pharmacological activities such as neurotoxic, hemolytic, myotoxic, edema-inducing, hypotensive, platelet-aggregating, cardiotoxic, and anticoagulant effects have been attributed to these venom toxins. In this context, antivenom capacity in neutralizing *in vitro* one of its biological activities will probably run in parallel with neutralization of *in vivo* whole venom lethality. Another important pharmacological effect of some secreted PLA_2 toxins is an *in vitro* anticoagulant activity on human plasma samples (Kini and Evans, 1995; Kerns et al., 1999; Kini, 2005). This *in vitro* effect of crotoxin B seems to be mainly due to inhibiting formation of the prothrombinase complex via a phospholipid-independent mechanism, result of direct binding with human coagulation factor Xa (Faure et al., 2007; Oliveira et al., 2009). Classical coagulation assays using human or mammalian plasmas for assaying procoagulant activity of snake venoms or toxins and neutralizing ability of antivenoms present sensibility at the microscale level (in micrograms and microliters, respectively) (Antunes et al., 2010; Pornmuttakun and Ratanabanangkoon, 2014). Oliveira et al. (2009) reported that 2.5 μg of crotoxin B (or 10 μg of crotoxin/mL of plasma, final concentration) are necessary to cause complete anticoagulant effect on recalcification time of sheep plasma.

Our crotoxin preparation displayed: (a) dose-dependent anticoagulant activity (Fig. 2) and (b) lethality [LD_{50} value of 1.7 μg/animal (1.1–2.5)] confirming data previously reported (Sampaio et al., 2010). Moreover, according to previous studies (Freitas et al., 1990; Fan and Cardoso, 1995; Beghini et al., 2004, 2008, 2005; Rodriguez et al., 2006) and like what happened with crude *C. d. terrificus* venom, presented cross-reactivity with one of our studied antiserum batches tested (ACS batch number IB-C 05/09, included in Supplementary material). Besides, the absence of neutralizing activity of ACS batches against heparin anticoagulant activity is not surprising, indicating its specificity against crotoxin and *C. d. terrificus* venom. We determined relative potency of these four batches of ACS, since the molar concentration of substances (aPTT clot activator and the ACS) were not available, being impossible to determine their half maximal effective concentrations (EC_{50}) and their half maximal inhibitory concentrations (IC_{50}). We consider as very satisfactory the correlation coefficient 0.94 ($p < 0.05$) between ability of these four ACS batches (IB-C04/09, IB-C05/09, IB-C02-12-05 and IB-C01-12-04) in inactivating both *in vitro* crotoxin anticoagulant activity (nanoliters ACS/100 ng crotoxin) (Table 1) and *in vivo* lethal activity of *C.d. terrificus* venom. The standard protocol for antivenom potency assessment involves the incubation of venom and antivenom for 30 min at 37 °C (WHO, 2010). Since the interaction of venom toxins and antivenom antibodies *in vivo* occurs in conditions in which there is no incubation, it has been proposed that a shorter incubation time, could be a more appropriate setting (Solano et al., 2010; Debono et al., 2017; Lister et al., 2017; Rogalski et al., 2017). Below, we present some arguments justifying why, in our study, we used rotational

thromboelastometry and recalcified CPS and not classical coagulation assays and mammalian plasma samples, as usual is reported in literature: (1) Recalcification time and activated partial thromboplastin time (aPTT) in plasma samples of patients with factor XII deficiency are significantly prolonged (Ratnoff and Colopy, 1955). Dynamics of spontaneous thrombin/fibrin generation in recalcified plasma samples of avian species are significantly slow (Spurling, 1981; Oguiura et al., 2014). This slowness of the blood clotting process in recalcified CPS seems not to be due to the presence of coagulation inhibitors (Weir et al., 2004) such as occurs with reptilian plasmas (Nahas et al., 1983; Fortes-Dias et al., 1991; de Oliveira and Tanizaki, 1992; Smith et al., 2000; Joseph et al., 2002) but rather due to one interesting evolutionary adaptation of chicken hemostatic system: natural factor XII deficiency (Ponczek et al., 2008). Therefore, even recalcified, CPS provide time lapse sufficient for constructing a typical dose-response curve and for stablishing mean effective or inhibitory doses (and the relative potency) of agonists (such as pro- or anticoagulant toxins) and antagonists (such as antivenoms), respectively; (2) Total circulating blood volume in vertebrates is in the range of 55–70 mL/kg body weight and adult chickens allow the collection of at least 8 mL of whole blood samples from each wing vein, without significant animal distress or necessity of euthanasia; and (3) Finally, although presenting architectural differences in amino acid composition when compared with mammalian coagulation factors (Spurling, 1981), constituents of avian coagulation cascade are naturally sensitive to at least some snake procoagulant venom toxins (Johnson et al., 1985; Bernardoni et al., 2014; Oguiura et al., 2014), being these animal species suitable as food for snakes (Zelanis et al., 2008; Arbuckle, 2010).

In summary, the pharmacological preparation here described (aPTT/chicken plasma) present sensitivity to crotoxin *in vitro* anticoagulant activity almost hundred times greater when compared to that presented by assays with mammalian and human plasmas (Petroianu et al., 2000; Kini, 2005; Oliveira et al., 2009; Prado et al., 2010; Pla et al., 2014), being possible to determine the mean effective doses of crotoxin as well as of the antiserum at the nanoscale level (nanograms and nanoliters, respectively). We propose this methodology as an alternative and sensitive assay for assessing antivenom neutralizing ability in plasma of immunized horses as well as for in-process quality control.

5. Conclusion

Considering that crotoxin is the key lethal toxin from *C. d. terrificus* venom and that values presented by our *in vitro* assay and that correlation presented by rodent *in vivo* lethality assay is satisfactory, we consider this *in vitro* micromethod as one functional and highly sensitive assay for: (a) assessing antivenom relative potency in some intermediary steps of antivenom production against venom toxic components with both anticoagulant activity and lethality, such as occurs with PLA_2-like substances present in venoms such as that of *C. d. terrificus* snake and of *Apis mellifera* bee (Petroianu et al., 2000; Ferreira et al., 2010); (b) assaying large numbers of fractions during purification procedures of anticoagulant PLA_2-like toxins from several sources and (c) detection of very low levels of specific antibodies against this *in vitro* anticoagulant effect in little volume samples (nL) of biological fluids (such as plasma, cerebrospinal fluid, urine, tears, saliva …).

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Transparency document

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Appendix A. Supplementary data

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