Establishing a protocol for thromboelastography in sea turtles

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ABSTRACT

Thromboelastography (TEG) provides a global evaluation of haemostasis. This diagnostic test is widely used in mammals but has not previously been performed in reptiles, mainly due to the limited availability of taxon-specific reagents. The objective of this pilot study was to establish a protocol to perform TEG in sea turtles. Pooled citrated plasma, stored at −80°C, from four green turtles (*Chelonia mydas*) was assayed on a TEG 5000. Several initiators were evaluated: kaolin (n=2), RapidTEG (n=2), fresh (n=2) and frozen (n=6) thromboplastin extracted from pooled brain tissue from several chelonian species, human recombinant tissue factor at 1:100 (n=1), Reptilase (n=2), and rabbit thromboplastin (n=1). Both fresh and frozen chelonian thromboplastin were superior in producing quantifiable TEG reaction time compared with all other reagents. These findings are consistent with the lack of an intrinsic pathway in turtles and confirmed a lack of coagulation in the turtle samples in response to mammalian thromboplastin. A TEG protocol was subsequently established for harvested species-specific frozen thromboplastin. The frozen thromboplastin reagent remained stable after one year of storage at −80°C. The developed protocol will be useful as a basis for future studies that aim to understand the pathophysiology of haemostatic disorders in various stranding conditions of sea turtles.

INTRODUCTION

All species of sea turtles are classed as threatened, vulnerable or endangered.[1] Improving diagnostic capabilities to include haemostasis will contribute to further understanding of the pathophysiology of some conditions known to affect sea turtles, including cold-stunning. Thromboelastography (TEG) studies are relatively limited in the veterinary literature compared with human medicine, but several recent manuscripts involving TEG in the evaluation of the haemostatic system in dogs (*Canis familiaris*), cats (*Felis catus*), horses (*Equus caballus*) and cows (*Bos taurus*) have been published.[2–5] There are also several recent publications evaluating TEG in wildlife and exotic mammalian species including manatees (*Trichechus manatus latirostris*) and various avian species.[6][7][8] However, TEG remains unexplored in reptiles, including sea turtles, despite clinical and postmortem indications that they can suffer from coagulopathies.[9] The main reason for this is the lack of taxon-specific reagents for non-mammalian species. It appears that thromboplastin needs to be sourced from the same or related species to initiate coagulation.[6]

Although studies of reptile coagulation are sparse, previous work has indicated that there is a lack of an intrinsic system and factors XI and XII.[10][11] As a result, the contact pathway (intrinsic pathway), which begins with high molecular weight kininogen, prekallikrein and factor XII, is incomplete in reptiles, resulting in reptile blood being slow to clot on foreign surfaces.[12][13] How sea turtles maintain effective haemostasis following trauma is still not fully understood; however, the fact that sea turtles have existed since the Jurassic period[14] indicates that the lack of intrinsic pathway is not detrimental to their survival. Previous studies in sea turtles confirmed the von Willebrand factor (vWF)-platelet glycoprotein (GP) Ib pathway facilitating thrombocyte adhesion to damaged epithelium as well as a collagen-induced thrombocyte aggregation pathway are intact.[11] This and possibly other not yet identified mechanisms may be sufficient alternatives to compensate for the gaps in the intrinsic pathway in sea turtle coagulation.

A delicate balance between clot formation and clot dissolution is required to prevent haemorrhage and thromboses and result in a functional haemostatic system.[15] TEG is the only diagnostic test to provide global evaluation of the haemostatic process rather than focusing on a specific pathway, such as in the more commonly used prothrombin and activated partial thromboplastin tests.[16] The thromboelastograph generates a thromboelastogram tracing representing the mechanics of the clot formation: a precoagulation phase depicted by a flat line, a coagulation phase where the line diverges into two, and a fibrinolysis phase where the lines converge.[17–19] Recently, TEG has been used to diagnose hypercoagulability,

hypocoagulability and disseminated intravascular coagulation during various disease processes including parvovirus, immune-mediated haemolytic anaemia and sepsis in mammals.[20–23] Although conventional coagulation tests can usually identify hypocoagulation in mammals, they are often insensitive in identifying hypercoagulation.[24] In contrast, thromboelastogram allows assessment via five main components (R, K, α angle, MA, LY30) that provide an overview of primary and secondary haemostasis and fibrinolysis[25] (R, reaction time; K, clot formation time; α angle, clot formation rate; MA, maximum amplitude (clot strength); LY30, percentage of clot lysed after 30 minutes).

The objective of this study was to establish a protocol for TEG in sea turtles.

MATERIALS AND METHODS
Initial protocol development
In the absence of published literature on TEG protocols in reptile species, several different protocols were tested to develop a standard method for sea turtle TEG. Frozen reptile thromboplastin was produced from pooled brain samples from one Kemp's ridley, one loggerhead and two green turtles. All brains were obtained from naturally deceased and previously frozen turtles used in postmortem examination teaching laboratories. Fresh reptile thromboplastin was extracted from one river cooter turtle (*Pseudemys concinna*) that died naturally in rehabilitation and one box turtle (*Terrapene carolina)* that was euthanased due to extensive trauma. All brains were obtained through the North Carolina State University College of Veterinary Medicine and the North Carolina State University Center for Marine Sciences and Technology (CMAST).

The turtle brain thromboplastin preparation protocol was as follows:
1. Remove turtle brain and remove the meninges if possible.
2. Rinse brain with 0.9 per cent saline.
3. Weigh brain and macerate with a mortar and pestle using a ratio of 1.25 ml of 0.9 per cent saline to 1 g of brain.
4. Centrifuge the emulsion for 25 minutes at 800 x *g*.
5. Collect the supernatant and discard the sediment.
6. Aliquot the supernatant into 50-µl aliquots and freeze at −80°C.
7. Fresh thromboplastin was maintained at room temperature and used within 90 minutes.

Both plasma and whole blood can be used for TEG testing. Due to limited availability and small sample volumes, all experimental protocols were performed with a pooled sample of frozen citrated plasma from four turtles, and one opportunistic fresh whole blood sample from a loggerhead sea turtle using kaolin only. TEG was performed using two TEG 5000 machines (TEG 5000 Hemostasis Analyzer, Haemonetics), each with two channels. Since reptiles are poikilothermic, the operating temperature of both machines was reduced from 37°C, the standard temperature used for mammalian samples, to 30°C, which is representative of sea turtle temperatures in summer in some regions, as well as in rehabilitation. Before running any samples TEG machines were E-tested (equilibrium test) for balance, and levels 1 and 2 quality controls were performed to ensure all values were within normal limits for each channel. The protocols used were randomised to channels 1–4 on the TEG machines. Data capture was performed using the manufacturer's software (version 4.1.73, Haemoscope, Niles, Illinois). Citrate inhibits clotting via calcium chelation; therefore, calcium chloride (calcium chloride 0.2 M, Haemoscope) was added to the cup to exceed chelation capacity before the addition of the sample, consistent with the manufacturer's recommendation. Samples were thawed before pooling, mixed and then allowed to rest for 30 minutes. Samples were run for 90 minutes or until the endpoint A60 (60 minutes after the MA) was achieved, whichever occurred first.

Several activators were evaluated to identify a successful protocol and are summarised in Table 1. Seven methodologies were attempted to facilitate TEG on a pooled

TABLE 1: Methods of activation evaluated to establish a protocol for sea turtle thromboelastography

Protocol	Cup contents for each method of activation
1	Kaolin (Kaolin, Haemoscope, Niles, IL.): 1-ml fresh whole blood is added to a kaolin vial. 20 µl of CaCl$_2$ and 340 µl of kaolin-activated fresh whole blood.
2	RapidTEG (RapidTEG, Haemoscope): 20 µl of CaCl$_2$, 10 µl of reagent and 340 µl of citrated plasma.
3	Fresh reptile thromboplastin (river cooter/box turtle): 20 µl of CaCl$_2$, 20 µl of reagent and 330 µl of citrated plasma.
4	Frozen reptile thromboplastin (sea turtle): 20 µl of CaCl$_2$, 20 µl of reagent and 330 µl of citrated plasma.
5	Dade Innovin Tissue Factor at 1:100 (Innovin, Siemens AG): 20 µl of CaCl$_2$, 36 µl reagent and 304 µl citrated plasma.
6	Reptilase (Pefakit Reptilase, Pentapharm, Switzerland): 20 µl of CaCl$_2$, 10 µl of reagent and 340 µl of citrated plasma.
7	Rabbit thromboplastin (Sigma-Aldrich): 20 µl of CaCl$_2$, 20 µl of reagent and 330 µl of citrated plasma.
8	Fresh reptile thromboplastin (river cooter/box turtle): 20 µl of CaCl$_2$, 10 µl of reagent and 340 µl of citrated plasma.
9	Frozen reptile thromboplastin (sea turtle): 20 µl of CaCl$_2$, 10 µl of reagent and 340 µl of citrated plasma.

turtle citrated plasma sample. Only three of the initial seven protocols produced a thromboelastogram. Two further modified protocols were then attempted to identify the most effective method for TEG in sea turtles. Two plasma TEGs were performed on each activator initially, and additional plasma TEGs were performed to optimise the final protocol.

Protocol evaluation

During September 2015, wild sea turtle captures were scheduled by the National Oceanographic and Atmospheric Administration biologists in conjunction with the CMAST in North Carolina. Turtles were captured using a pound net in Core Sound near Harkers Island, North Carolina (coordinates: ~34.6N, −076.5W), as previously described.[26] One loggerhead and one Kemp's ridley were selected for sampling for assay development. Physical examinations were performed to determine body condition to rule out the presence of trauma or disease, and to confirm each individual's apparently healthy status. Venous blood was collected from the dorsal cervical sinus by standard sea turtle blood sampling technique[27] directly into two Vacutainer tubes anticoagulated with 0.32 per cent sodium citrate (BD Vacutainer Plus Plastic, Citrate tubes, Becton Dickinson, Franklin Lakes, New Jersey, USA). Samples were kept on ice for transport to the laboratory for processing.

Two citrated whole blood samples from each turtle were available for processing at one and three hours postsampling. For each sample, protocol 9 was used in which 10 µl of frozen brain thromboplastin was added to 20 µl of $CaCl_2$ and 340 µl of whole blood. The remaining whole blood was centrifuged at 800 x g for six minutes to obtain plasma. Citrated plasma samples were refrigerated and TEGs performed at one and three hours in the loggerhead, and due to insufficient sample volume only at three hours in the Kemp's ridley (to enable a 24-hour sample). The remaining plasma was frozen and the TEG performed after thawing at 24 hours. Plasma sample TEGs were also performed with 10 µl of frozen brain thromboplastin and 20 µl of $CaCl_2$ with 340 µl of citrated plasma with the loggerhead sample. After thawing, the sample was allowed to sit at room temperature for 30 minutes before use. For both citrated whole blood and plasma samples, channel selection was randomised. Preinstalled software (TEG Analytical Software version 4.2.3, Haemonetics, Braintree, Massachusetts, USA) was used to generate and capture variables for the TEG tracing.

Thromboplastin storage

The thromboplastin that was produced in 2015 was stored at −80°C for one year to facilitate a comparison with a fresh batch of thromboplastin prepared in 2016 for the assessment of possible deterioration during storage. Twelve TEGs were performed using the frozen 2015 thromboplastin and an additional 11 TEGs performed with 2016 thromboplastin. The same methodology using protocol 9 described above was utilised. The

new 2016 batch of thromboplastin was obtained using one loggerhead brain, one Kemp's ridley brain and one green turtle brain. The four channels were randomised with two samples employing activators from the 2015 and two samples from the 2016 batch of thromboplastin. A two-way analysis of variance with pooled plasma as a fixed effect and thromboplastin as a random effect was performed to assess the variation between 2015 and 2016 thromboplastin and determine if the variance observed was within acceptable limits. Statistical software R (www.R-project.org) was used to perform statistical analysis, with statistical significance defined as a P value of <0.05.

RESULTS

Two TEGs were performed for each of the nine protocols using the same pooled plasma sample. The results from each method using the pooled plasma are presented in Table 2. RapidTEG, tissue factor, kaolin and rabbit thromboplastin produced no reaction after 85 minutes, demonstrated by a flat line on the TEG. The Reptilase produced a hypocoagulable reading compared with the sea turtle thromboplastin. The sea turtle-specific thromboplastin was deemed superior compared with all other methods, as it yielded a complete tracing with values comparable with mammalian tracings. For practical application, the frozen thromboplastin was selected as the most suitable method. This methodology was initially performed with 20 µl of fresh and frozen thromboplastin, protocols 3 and 4. However, the R time was very rapid (0.8 minutes) so the amount was subsequently reduced to 10 µl, protocols 8 and 9, to obtain a slightly slower reaction time (2.2 minutes) and provide increased precision in assessing the results.

The selected protocol was assessed on fresh whole blood within one hour of sampling in the field, fresh plasma at one hour and three hours, as well as frozen plasma thawed 24 hours later. A low sample number inhibited statistical analysis, but the results confirmed the protocol was successful in initiating clot formation and producing a thromboelastogram in loggerhead and Kemp's ridley sea turtles. These results are summarised in Table 3. Figure 1 illustrates the variation in three different TEGs produced from the study using frozen thromboplastin on fresh plasma and fresh whole blood, and the flat line thromboelastogram produced when using rabbit thromboplastin as the reagent.

The results of the thromboplastin reagent stability study are presented in Table 4. Statistical comparison of these values demonstrated that the TEG assays were reproducible with both 2015 and 2016 thromboplastin and had acceptable coefficients of variation (CV%) for routine practice, with R having the maximum CV% at 12 per cent.

DISCUSSION

This pilot study offers a successful methodology for the performance of TEG in sea turtles using whole blood,

TABLE 2: Thromboelastography results from nine different methods of activation using pooled sea turtle citrated plasma, with the exception of protocol 1 which used whole blood

Protocol		R (minutes)	K (minutes)	angle (°)	MA (mm)	LY30 (%)
1	Kaolin (whole blood)	98.3	–	–	–	–
2	RapidTEG Reptilase	96.6	–	–	–	–
3	Fresh turtle thromboplastin 20 µl	1.8	0.8	72	37.5	0
4	Frozen turtle thromboplastin 20 µl	0.8	0.8	82.7	33.7	0
5	Dade Innovin Tissue Factor 100	90.9	–	–	–	–
6	Reptilase	1.1	n/a	49.5	18.4	0
7	Rabbit thromboplastin	86.6	–	–	–	–
8	Fresh turtle thromboplastin 10 µl	8.8	6.3	22.1	29.8	0
9	Frozen turtle thromboplastin 10 µl	2.3	0.8	78.0	35.5	0.1

Reagents 1, 2, 5 and 7 did not initiate any reaction after a minimum of 85 minutes. Reagent 6 produced a suboptimal tracing. Protocols 3 and 4 and modified versions using a reduced volume of thromboplastin, protocols 8 and 9, were considered successful protocols. Method 9 was selected as the optimal protocol based on measurable results consistent with mammalian methodology. Values are reported as the mean of the two samples performed apart from method 9, where 5 TEGs were performed.

angle, clot formation rate; K, clot formation time; LY30, percentage of clot lysed after 30 minutes; MA, maximum amplitude (clot strength); n/a, not available; R, reaction time; TEG, thromboelastography.

fresh and frozen plasma, and an approach for the application of this methodology in reptiles in general. Protocol 9 was found to be the superior method from the quality of thromboelastograms produced and a quick reaction time. As TEG machines are still not readily available in veterinary medicine, the ability to freeze plasma for delayed processing provides a practical alternative to the requirement of fresh whole blood for immediate processing. Establishing this protocol for use on frozen plasma increases the feasibility of testing in diagnostic and research settings, since frozen/archived samples from other institutions can be shipped for analysis. Despite the thromboplastin reagent not being commercially available, the harvesting protocol yielded enough reagent for over 1000 TEGs when used in 10-µl aliquots. Confirming the thromboplastin was still fully functional after a period of prolonged storage (one year at −80°) also improves practicality and economics of performing this diagnostic test. The results comparing 2015 and 2016 thromboplastin (CV% −12 per cent) were similar to other studies assessing TEG reproducibility using kaolin and thromboplastin, which had CV% ranging from 3 per cent to 13 per cent.[28]
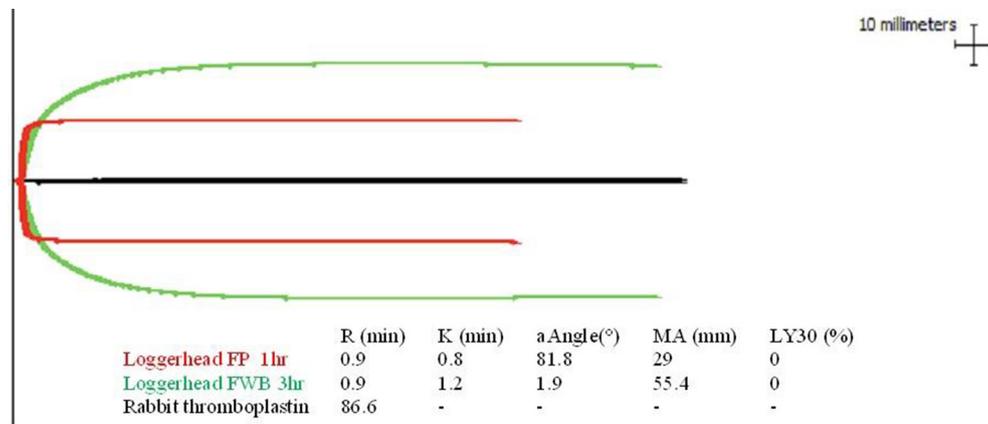
Reagents other than sea turtle thromboplastin also produced positive results. The Reptilase produced a very slow response. Reptilase is isolated from the venom of pit vipers (*Bothrops atrox*) and acts by replacing thrombin to initiate clot formation by cleaving fibrinogen.[29] Reptilase did initiate clotting and produced a TEG in the sea turtle; however, the tracing is considered suboptimal for clinical usage as it is not robust, lacks a K value and has the appearance of a hypocoagulable state.

TABLE 3: Thromboelastography results from a wild loggerhead and a wild Kemp's ridley sea turtle using protocol 9

Species	Sample	Time (hours)	R (minutes)	K (minutes)	α angle (°)	MA (mm)	LY30 (%)
Loggerhead	FWB	1	0.8	0.8	79.2	56.7	0
Loggerhead	FWB	3	1.1	0.8	80.1	55.9	0
Loggerhead	FWB	3	0.9	1.2	71.9	55.4	0
Loggerhead	Fresh plasma	1	0.9	0.8	81.8	29	0
Loggerhead	Fresh plasma	3	2.8	0.9	74.7	29.4	0
Loggerhead	Frozen plasma	24	1.2	0.8	81.5	26.2	0.7
Kemp's ridley	FWB	1	1.4	0.8	79	72.4	0
Kemp's ridley	FWB	3	1.7	0.8	77.2	72	0
Kemp's ridley	Fresh plasma	3	6.9	0.8	83.4	51.7	0
Kemp's ridley	Frozen plasma	24	7.2	2.7	42.7	51.3	0

Fresh whole blood (FWB), and fresh and frozen plasma were used from each individual to determine the superior protocol using sea turtle-specific frozen thromboplastin. Insufficient sample volume prevented a fresh plasma sample being performed at a 1-hour time interval in the Kemp's ridley.

α angle, clot formation rate; FWB, fresh whole blood; K, clot formation time; LY30, percentage of clot lysed after 30 minutes; MA, maximum amplitude (clot strength); R, reaction time.

	R (min)	K (min)	a Angle(°)	MA (mm)	LY30 (%)
Loggerhead FP 1hr	0.9	0.8	81.8	29	0
Loggerhead FWB 3hr	0.9	1.2	1.9	55.4	0
Rabbit thromboplastin	86.6	-	-	-	-

FIG 1: Three representative thromboelastographic tracings using citrated plasma or whole blood from sea turtles and various methods of activation. Red TEG: activation with frozen thromboplastin using fresh plasma obtained from a wild-caught loggerhead sea turtle. Green TEG: activation with frozen thromboplastin using fresh whole blood, three hours postsampling, from the same loggerhead. Note the larger MA in the fresh whole blood tracing compared with the plasma tracings due to the presence of thrombocytes. Black TEG: activation with rabbit thromboplastin on a pooled citrated plasma sample. Note the flat line produced when using rabbit thromboplastin as the initiator. α angle, clot formation rate; FP, fresh plasma; FWB, fresh whole blood; K, clot formation time; LY30, percentage of clot lysed after 30 minutes; MA, maximum amplitude (clot strength); R, reaction time; TEG, thromboelastography.

Both the frozen thromboplastin (pooled sea turtle species) and the fresh tissue factor (river cooter and box turtle) performed well producing thromboelastograms. Species-specific differences likely exist; therefore, for future reptile TEG, utilising thromboplastin from the brain of the species being tested will likely yield the best results to establish baselines. Frozen thromboplastin was deemed the better option due to the impracticalities of utilising fresh thromboplastin. It is also possible that the variations observed within this study are within normal ranges; however, insufficient samples and species were assessed to establish this as part of this study.

The MA is the ultimate strength of the fibrin clot and is the greatest distance between the two lines on the graph. It primarily illustrates the contribution of platelet aggregation to the clot rigidity in whole blood or platelet-rich plasma (PRP) of mammals (or thrombocytes of other reptiles and other taxa). MA can also be affected by both fibrinogen concentration and haematocrit. In platelet-poor/thrombocyte-poor plasma TEG, the functional fibrinogen is represented because the platelets or thrombocytes have already been removed.[30] Although it

would have been optimal for global evaluation to utilise whole blood, it is not practical in this setting. There are considerable differences in the MA values produced between whole blood samples and plasma (Table 2), and these can be accounted for by the absence of thrombocytes in plasma, since they are considered major contributors to the MA.[25] Differences were observed between the MA in the loggerhead and Kemp's ridley turtles; therefore, species-specific differences should be considered when establishing normal ranges. The advantage of performing a plasma TEG is that it gives a more accurate assessment of the enzymatic processes involved in coagulation not involving thrombocytes, while still evaluating the global coagulation process. Analysing the formation and breakdown of fibrin can provide an indication as to whether enzymatic inhibitors, such as warfarin and heparin, would be beneficial treatment options. If the tracing is potentially hypocoagulable, plasma transfusion may be of benefit.

Studies in human beings have elected to use PRP instead of platelet-poor plasma when performing plasma TEG.[31 32] Due to the size and weight of thrombocytes

TABLE 4: Comparison of thromboelastography results utilising protocol 9 and frozen thromboplastin from 2015 (stored at −80°C for one year; n=12) and frozen thromboplastin from 2016 (n=11)

Thromboplastin	Time	R (minutes)	K (minutes)	α angle (°)	MA (mm)	LY30 (%)
2016 (n=11)	Mean	2.23	0.8	81.49	45.02	0.02
	sd	0.27	0.00	2.037	1.48	0.06
2015 (n=12)	Mean	2.31	0.80	83.21	48.37	0.00
	sd	0.45	0.00	1.46	2.01	0.00
Analysis of variance P value		0.50	1.0	0.26	0.17	0.69

A single pooled batch of citrated plasma (from 4 green turtles) was used to perform all TEG analyses.
α angle, clot formation rate; K, clot formation time; LY30, percentage of clot lysed after 30 minutes; MA, maximum amplitude (clot strength); R, reaction time; TEG, thromboelastography.

in reptiles, the production of a PRP equivalent is less practical.

Reports investigating different methods for TEG activation in mammals include native samples (no activator), commercially available thromboplastin and kaolin.[2] Commercially available agents such as RapidTEG, Dade Innovin, kaolin and rabbit thromboplastin activate both the intrinsic and extrinsic systems in mammals; however, it appears that reptiles cannot recognise mammalian tissue factor, and therefore no reaction is produced and an alternative methodology was required.

The main limitation of this study was the low number of available TEG tracings for statistical analysis. Ideally each protocol would have been repetitively performed instead of only twice to ensure consistency. Due to the complexities of the reptilian coagulation system, the authors did anticipate that several of the commercially available reagents would not initiate clotting. It was, however, clearly apparent in the tracings the difference when the protocol was successful and a thromboelastogram and more importantly a clot were produced. Due to the complete lack of reaction in protocols 1, 2, 5 and 7, altering the concentration of these reagents would have been of little value.

The accommodation of the lower reptilian temperature is important to achieve representative results for these species. Previous non-mammalian studies such as in the Amazon parrot did not adjust the sampling temperature to higher avian body temperature and acknowledged that consequently the results produced were likely not reflective of in vivo values despite the relatively minor temperature differences.[6] Because sea turtles are poikilothermic, water temperature approximates the internal body temperature; therefore, core temperature can vary extensively.[33] Arguably more samples should be performed at varying temperatures even lower than 30°C to assess the possible effects of hypothermia on coagulation.[34 35 36 37] For future research TEGs from cold-stunned individuals could be performed at their core temperature at the time of rescue to provide a representative sample.

This pilot study demonstrates that TEG has the potential to become a viable diagnostic test for use in sea turtles and potentially other reptiles. By modifying a protocol to work specifically in these species and adjusting the temperature, the results may reflect in vivo conditions. Extrapolation of TEG reference ranges across species is not recommended due to interspecies variation. Further research will facilitate establishing methodology-specific and species-specific reference intervals and enabling comparison with sick individuals, improving knowledge of coagulopathies in sea turtles in clinical and research settings.

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