



Considerations for extracting blood from turtles and tortoises: venipuncture sites and anticoagulants

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ABSTRACT

Objective. Evaluate different venipuncture points and the use of two anticoagulants to obtain blood samples in turtles. Materials and methods. Eighty-two turtles of the species Trachemys callirostris, Podocnemis unifilis and Chelonoidis carbonaria were sampled. Three venipuncture points were evaluated: subcarapacial venous sinus, dorsal coccygeal vein, and jugular vein. Two anticoagulants were tested: sodium heparin and EDTA. Results. The jugular vein was the best place to practice venipuncture as the blood samples obtained were free of hemodilution and enough volume to carry out a blood profile. In contrast, samples from the other venipuncture points were usually hemodiluted. Blood samples from *C. carbonaria* stored with EDTA (40 µl/ml of blood) showed haemolisis, which was not observed using sodium heparin (100 UI/ml of blood) as anticoagulant. **Conclusions.** The jugular vein is the most recommended venipuncture site for the extraction of blood samples for clinical purposes. Sodium heparin was the best anticoagulant to store blood samples due to the fact that it does not induce haemolisis in any sample.

Keywords: Anticoagulants; Blood chemistry; Hematology; *Chelonoidis carbonaria; Podocnemis* unifilis; Trachemys callirostris (Source: CAB).

RESUMEN

Objetivo. Evaluar diferentes puntos de venopunción y el uso de dos anticoagulantes para la obtención de muestras sanguíneas en tortugas. Materiales y métodos. Se muestrearon 82 individuos de las especies Trachemys callirostris, Podocnemis unifilis y Chelonoidis carbonaria. Los

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puntos de venopunción evaluados fueron: seno venoso subcaparacial, vena coccígea dorsal y vena yugular; y se compararon dos anticoagulantes: heparina de sodio y EDTA. **Resultados.** A partir de la vena yugular se obtuvieron muestras sanguíneas sin hemodilución y en un volumen suficiente para realizar análisis hematológicos. Por el contrario, a partir de los otros sitios de venopunción se obtuvieron mayoritariamente muestras hemodiluidas. Las muestras de sangre obtenidas a partir de *C. carbonaria* se hemolizaron tras su almacenamiento en EDTA (40 µl/ml de sangre), lo que no se evidenció al utilizar heparina de sodio (100 UI/ml de sangre) como anticoagulante. **Conclusiones.** La vena yugular es el sitio de venopunción más recomendable para la extracción de muestras sanguíneas con fines clínicos. La heparina de sodio fue el anticoagulante de elección para almacenar dichas muestras al no inducir hemólisis en estas.

Palabras clave: Anticoagulante; Química sanguínea; Hematología; *Chelonoidis carbonaria; Podocnemis unifilis; Trachemys callirostris (Fuente: CAB)*.

INTRODUCTION

Turtles, like crocodiles, are the most threatened group of reptiles in Colombia since some species are used as a source of food, pets, or for leather exploitation (1). Added to the impact of habitat loss, environmental pollution, illegal trafficking, and infectious diseases that generally affect reptiles, this contributes to their drastic population decreases (2). According to The Reptile Database, Colombia has 36 species of turtles, of which 94.4% have aquatic habits. By 2015, 37% of the turtle species in Colombia were under some category of threat; and, unfortunately, several species are not listed (1).

Due to the high diversity of turtles in Colombia and the constant threat of illegal trafficking, many individuals are often sheltered in wildlife rescue centers. The Unidad de Rescate y Rehabilitación de Animales Silvestres (URRAS) of the Universidad Nacional de Colombia, carries out the clinical assessment and respective treatment of rescued animals. Clinical assessment of these reptiles is carried out through physical examinations, imaging, hematological tests, and blood biochemistry (3). Ideally, reference values should be previously established for the specimens analyzed; however, there is a lack of standard ranges and values for most of the turtle species in Colombia since the only species in this country that has hematological reference parameters is *Trachemys callirostris* (4,5). In the Neotropics there are hematological reports for only five turtle species: *Podocnemis expansa*, Phrynops geoffroanus, Eretmochelys imbricata, Chelonoidis chathamensis and C. denticulata (6,7,8,9,10,11,12).

A determinant factor for obtaining hematological reference values by laboratory tests is the

quality of the sample. However, extracting blood samples in turtles can be difficult due to anatomical variations among the species, the presence of protective dermal structures such as carapace and scales, the difficulty of manipulation due to the strength of individuals, the difficult location and visualization of veins and venous sinuses, and the proximity of venipuncture sites to different lymphatic vessels (13). Lymph hemodilution is one of the most frequent obstacles for obtaining blood samples for laboratory analyses (14). Another extrinsic factor that affects the quality of the sample is the anticoagulant used (15).

The objective of this article was to evaluate different venipuncture points, as well as the appropriate anticoagulant for the extraction and conservation of blood samples from chelonians of the species *Podocnemis unifilis, Trachemys callirostris,* and *Chelonoidis carbonaria* that allow an optimal analysis of hematological parameters and blood chemistry.

MATERIALS AND METHODS

Ethical statements. The ethics committee of the Faculty of Sciences of Universidad Nacional de Colombia endorsed this study by act 03-2019 of April 1, 2019. Sampling was carried out under the supervision of veterinary doctors of wildlife, to guarantee the safety of the sampled individuals. Additionally, this research was carried out following Law 84 of 1989 of the Congreso de la República de Colombia that defines the national statutes for animal protection. The guidelines stipulate in Resolution 8430 of 1993 of the Ministerio de Salud that the regulates biomedical research involving animals were followed. **Sampling.** We sampled turtles of the species *Podocnemis unifilis* (N=8), *Trachemys callirostris* (N=43) and *Chelonoidis carbonaria* (N=31). The sample size and the species chosen relied on the availability of individuals at the Unidad de Rescate y Rehabilitación de Animales Silvestres (URRAS) from the Facultad de Medicina Veterinaria y de Zootecnia of the Universidad Nacional de Colombia, Bogotá campus (2,555 MASL, average temperature: 14°C). The turtles were conditioned to an average artificial ambient temperature of 30°C.

Evaluation venipuncture of points. Venipuncture was carried out following the recommendations proposed by Naguib (13) and Perpiñan (16) for blood sampling in turtles. In all cases, non-anticoagulant syringes coupled to 25-gauge needles one inch in length were used. Each point was previously disinfected using chlorhexidine. The volume of blood taken was calculated from the individual's weight and did not exceed 1% of the weight/volume ratio (w/v) of each animal. Once the sample was obtained, moderate pressure was applied to the venipuncture sites to avoid bruising. The venipuncture points evaluated are listed below:

(A) *The subcarapacial venous sinus* is a point of convergence of numerous vessels. This is located on the animal's midline, where the eighth cervical vertebra joins the carapace (17) (Figure 1A). To access this point, it is necessary to retract the individual's head into the carapace or pull out the head and bend it down (17). The needle should be inserted over the dorsal midline of the neck, near the boundary of the skin and the carapace in the caudodorsal direction (Figure 1B). If the vertebra is touched, the needle should be moved slightly in the cranial direction (16).

(B) *The dorsal coccygeal vein* is located in the midline of the turtle's tail (Figure 1C). For accessing this point, the needle must be introduced tilted between 45° and 60° (17). Since these veins are adjacent to the vertebra, we recommend inserting the needle until it touches the vertebra and then slowly removing it and applying negative pressure on the syringe until blood is drawn (Figure 1D) (18).

(C) *The jugular vein* is found through the neck of the animal in an anteroposterior position, at the level of the eardrum (Figure 1E) (19). The needle should be inserted superficially, parallel to the neck in a caudal direction (Figure 1F) (16).

Storage of blood samples. Once the blood sample was taken, it was deposited in plastic vials containing the anticoagulant. Two anticoagulants were evaluated: sodium heparin (Liquemine[®], Roche), at a concentration of 100 IU/ml of blood (20) and ethylenediaminetetraacetic acid (EDTA, Químicos Albor), at a concentration of 40 μ l/ml of blood, suggested by the company. The blood was stored at 4°C before the haematological analysis and processed within a maximum period of 18 hours. The anticoagulant was evaluated, making blood smears after 12h of storage and then observed under the microscope to determine the integrity of the blood cells. Blood macroscopical observation was also done for this.

Hematological analysis. To evaluate the influence of the venipuncture site on sample quality, 15 individuals of *Trachemys callirostris* were sampled from the three venipuncture points. The haematological parameters analyzed were hematocrit, haemoglobin, and red blood cell count (RBC). The hematocrit percentage was determined by blood centrifugation in a microhematocrit at 12,000 g for 5 minutes. Haemoglobin was quantified using a BTS-350 spectrophotometer (BioSystem S.A., Barcelona - Spain). The red blood cell count was manually done in a Neubauer chamber using the Natt-Herrick solution at a 1:100 dilution (21).

Statistical analysis. Statistical analysis of the data obtained was performed using the R commander package version 2.6-2 of the R software version 4.0.3 (2020). The Shapiro-Wilk normality test was used. Subsequently, analysis of variances was carried out using a one-factor ANOVA, at a significance of 95% to compare the dependent variables (hematocrit, hemoglobin, and RGR) and the independent variables (venipuncture sites).



Figure 1. Venipuncture sites evaluated. A. Location of the subcarapacial venous sinus, B. Blood extraction from the subcarapacial venous sinus in a turtle of the species *Chelonoidis carbonaria*. C. Location of the dorsal coccygeal vein, D. Blood extraction from the dorsal coccygeal vein in a turtle of the species *Podocnemis unifilis*, E. Location of the jugular vein (Modified from Innis & Knotek (17)), F. Blood extraction from the jugular vein in a turtle of the species *Trachemys callirostris*.

RESULTS

Evaluation of venipuncture points. The venipuncture sites evaluated showed the following characteristics:

Subcarapacial venous sinus. This was easily accessible to obtain blood, especially in very active individuals where manipulation of limbs or head was complex. However, given its proximity to lymphatic vessels, the probability of hemodilution with lymph was increased; this was noticeable macroscopically since the hemodiluted samples showed a pale and light reddish coloration, unlike the dark deep red coloration of the non-hemodiluted specimens. Such dilution made those samples unsuitable for hematological and blood chemistry analysis.

Dorsal coccygeal vein. Some samples could be obtained from this point; however, its manipulation is complex due to the mobility and strength of the turtle's tail. About 50% of the samples analyzed throughout the study were found hemodiluited in some proportion (Figure 2).



Figure 2. Hematocrit percentage of samples obtained from each venipuncture site in *T. callirostris.****: *p*<0.001

Jugular vein. Due to the turtles' retraction of their head and the strength they apply to do this, access to this vein was difficult, particularly in those of the Cryptodira suborder. However, once the head was controlled, the vein was easily observable and palpable. Blood samples obtained from this site usually had good quality. They were rarely diluted with lymph, as evidenced after the RBC and the hemoglobin concentration analysis. The values obtained from this point of venipuncture were significantly higher than those of the other sites (Figures 3 and 4). The jugular vein showed good blood flow and was easy to localize but favored the formation of bruises. To avoid this, it was necessary to apply moderate pressure at the puncture point for about two minutes after blood extraction. Some of the advantages and disadvantages observed at each venipuncture site are summarized in table 1.

Data behave normally (p>0.05 by performing the Shapiro-Wilk test). We performed an ANOVA test to compare the variables measured against the different sampled sites. ANOVA test showed a significant difference between the puncture sites evaluated (p<0.01). The post-hoc test showed that the laboratory results obtained from the jugular vein differed from both the subcarapacial sinus and coccygeal veins in the three variables analyzed (Figures 2, 3, and 4). The hemoglobin was the exception, where no significant differences were observed between the samples taken from the jugular vein and the subcarapacial sinus (Figure 3). Additionally, Figures 2, 3 and 4 showed how the results obtained from the subcarapacial venous sinus (Ht: 16.6% ± 8.8; Hb: 5.6 mg/dL ± 2.2; RBC: $3.96 \times 10^5 \pm 2.0$) and coccygeal vein (Ht: 18.6%) \pm 8; Hb: 4.4 mg/dL \pm 2; RBC: 4.42 x 10⁵ \pm 1.9) tended to be lower than those obtained from the jugular vein (Ht: $30\% \pm 3.6$; Hb: 7.5 $mg/dL \pm 2.7$; RGR: 6.97 x 10⁵ ± 0.9).



Haemoglobin



Venipun sites	Species		
	Trachemys callirostris	Podocnemis unifilis	Chelonoidis carbonaria
	Advantages	Advantages	Advantages
	Easily visible vein. No hemodilution	Good blood volume. No hemodilution.	Thin neck skin. Easily visible vein. No hemodilution
Jugular vein			Disadvantages
	Disadvantages	Disadvantages	
	Complex head manipulation (Cryptodira) It is an aggressive species.	It has thick skin, which makes it challenging to visualize the vein.	Difficult manipulation of the head (Cryptodira). It has great strength in the neck. Bruising may form.
Dorsal coccygeal vein	Advantages	Advantages	Advantages
	Easy access point.	Easy access point. The tail is usually large.	The tail is manipulable in adults. Easy access point.
	Disadvantages	Disadvantages	Disadvantages
	Some samples have hemodilution. The tail is usually small. The vein is not visible. Could cause trauma to the spine	Some samples have hemodilution. The vein is not visible. Could cause trauma to the spine	In small individuals, the manipulation of the tail is complex. The vein is not visible. Could cause trauma to the spine
Subcarapacial venous sinus	Advantages	Advantages	Advantages
	Easy access point.	Easy access point.	Easy access point.
	Disadvantages	Disadvantages	Disadvantages
	Most samples have hemodilution. Non-visible sinus.	Most samples have hemodilution. Non-visible sinus	Most samples have hemodilution.
			Non-visible sinus.

Table 1. Advantages and disadvantages of venipuncture sites evaluated on the three species of turtles.



Figure 4. Red blood cell count (RBC) of samples obtained from each venipuncture site in T. callirostris. ANOVA and Post-hoc Test:***:p<0.001 **Blood sample storage.** Despite having used syringes without anticoagulant during the procedure, there was no evidence of rapid coagulation of the samples that allowed the sample to be divided *a posteriori* into different containers, either in microtubes with serum separation gel (for biochemical analysis) or vials with EDTA or sodium heparin (for hematological analysis).

EDTA lysed all blood samples obtained from *C. carbonaria* tortoises and prevented their posterior analysis. Microscopically, after 12 hours from the sampling, free nuclei and erythrocyte death was evidenced in the samples stored in EDTA, clear indicators of cell lysis (Figure 5A). Conversely, blood smears made from blood samples stored in sodium heparin show red blood cells with morphological integrity slightly compromised, shown by irregular cytoplasmic and nuclear borders, as well as poor coloration (Figure 5B).



Figure 5. Chelonoidis carbonaria's blood 12 hours after sampling. A. Blood was anticoagulated with EDTA, B. Blood anticoagulated with sodium heparin. Bar: 20 μm.

DISCUSSION

The jugular vein was the most favorable venipuncture point of the three evaluated since it allowed us to obtain a high volume of nondiluted blood samples, enough for blood profile analysis (Figure 2, 3, and 4). Restricting head movement can be a difficult task because of the strength of those animals; additionally, it could be dangerous as well, given the aggressive behavior and the strong bite of some turtle species like *T. callirostris*. Limb amputation due to chelonian bites have been reported, and subsequent infections are also a risk to the operator (22). The easily observed vein and the high blood flow make this venipuncture site a favorable one for blood samples. This site allowed the extraction of high-quality blood and enough volume for laboratory diagnostic purposes. So, this blood collection site is recommended over the others. A critical observation of this study reported as well by Redrobe and MacDonald (18) was that the jugular vein on the right side had a larger size, making it easier to observe and palpate. Additionally, the vein becomes more visible by tilting the anterior part of the turtle's body about 45° downwards.

Like the work of Redrobe and MacDonald (11), previous reports expose how a fast collection of a good volume of blood, and a low risk of secondary infections constitute some of the main advantages of taking blood samples of turtles from the jugular vein. (18,23). In various studies from Naguib (13), Perpiñan (16), Mans (19), and Eatwell et al (24), this site is recommended, given the low or no possibility of hemodilution with lymph. The dorsal coccygeal vein can be a good alternative for blood sampling when working with large individuals since they have a great retractive force in their head, making it difficult to access the jugular vein. Nonetheless, the dorsal coccygeal vein could be a problematic venipuncture site, as its proximity to lymphatic vessels can increase the risk of hemodilution. Tail manipulation in small individuals is more challenging, and an improper puncture could cause a dorsal spinal trauma, motion and sensibility loss, and epidural secondary infections (18), so taking samples from this site is not recommended.

Previous studies hiahliaht also the disadvantages of using the coccygeal vein as a site of venipuncture in turtles. López-Olvera et al (25) observes a significant hemodilution in samples obtained from this point in the Greek marginated tortoise (Testudo marginata) that negatively affects the hematological and blood chemistry parameters analyzed. A pronounced hemodilution of samples taken from the dorsal coccygeal vein, compared with samples from the occipital sinus, was also seen by Medeiros et al (26) in *T. scripta elegans* that produces a significant decrease in the leukocyte count and the concentration of total plasma proteins. In contrast, Perpiñan et al (27), did not find statistically significant differences between the hematological values obtained from the dorsal coccygeal vein compared to the subcarapacial venous sinus of the spiny softshell turtle (Apalone spinifera).

The easy access of the subcarapacial venous sinus is an advantage. However, like the dorsal coccygeal vein, a higher probability of hemodilution occurs compared to use of the jugular vein. In some cases, a liquid whose coloration varied from colorless to slightly yellow, probably corresponding to lymph or interstitial fluid without traces of blood, was extracted in the first instance. In those cases, a second sampling was required, submitting the animal to increased stress. Blood samples for hematological and biochemical analysis taken from this site are not recommended because of the alteration of blood parameters induced by lymph contamination (14,25). If a blood sample is required for genetic analysis, where the presence of lymph or interstitial fluid does not pose an inconvenience, the subcarapacial venous sinus is very favorable for blood extraction, due to the ease of the individuals' handling and the extraction of its blood.

In addition to the venipuncture site and the quality of the sample, the anticoagulant chosen for proper blood storage is essential. For this reason, the relevance of sodium heparin and EDTA as anticoagulants was analyzed to maintain the blood in optimal conditions for hematological analysis. Although EDTA is commonly used as the anticoagulant of choice for hematological analysis, in certain species of chelonians it causes lysis of blood cells; for this reason some authors recommend lithium heparin to preserve turtle blood (15). The results obtained here show the haemolysis of *C. carbonaria* blood cells stored with EDTA. The storage of blood samples of *P. unifilis* and *T. callirostris* in the same anticoagulant did not lead to haemolysis.

Our findings support Muro et al (15) and Perpiñan et al (27) statements about hemolytic effects of EDTA in blood samples from *Testudo hermanni* and Apalone spinifera, respectively. Studies carried out by Lyman (28) show the importance of calcium in erythrocytes permeability of *Chelydra serpentina* (Chelydridae), determining that, at low concentrations of this ion, a greater permeability of cations is observed inside the erythrocytes, leading to hemolysis (28). EDTA is a calcium chelating agent that could cause cation permeability into erythrocytes and lead to hemolysis. However, it does not occur in all turtle species. According to Lyman, C. serpentina is considered a primitive chelonian whose erythrocytes are dependent on calcium for ionic control of permeability. This feature may be lost in other species throughout their evolutionary history (28). This could explain the hemolytic behavior observed in *C. carbonaria*.

Most studies mention lithium heparin as the anticoagulant of choice for storing chelonian blood. Despite this, sodium heparin showed favorable results by preserving the blood samples of all the species analyzed in optimal conditions. These results supported other reports where sodium heparin has also been used for hematological analyses in turtles of T. scripta elegans, T. dorbingyi, Podocnemis expansa, Chelonoidis chathamensis, Caretta caretta, and Geochelone radiata (11,29,30,31,32,33). Unlike EDTA, heparin acts by inhibiting thrombin, decreasing the risk of hemolysis due to calcium chelation. However,

it can interfere with Romanowsky stains by promoting agglutination of thrombocytes and leukocytes, as well as generating a bluish effect in blood smears (24,33). After 12 hours of storage, the blood cells of *C. carbonaria* showed a slight morphological alteration that may be accentuated by the lapse and effects of heparin in the stain used. So, it is recommended to make blood smears using fresh blood without anticoagulant and, as soon as possible, to avoid morphological alterations. According to our results and previous reports, the use of sodium heparin is recommended to store blood samples from turtles.

Our study referred to strategies for extracting blood samples that allow the development of research to establish hematological and blood chemistry parameters, both in wildlife and captive specimens. We recommend extracting blood from the jugular vein and the use of sodium heparin for subsequent storage and hematological analysis. Additionally, we recommend the extraction of blood from the subcaparacial sinus for studies oriented towards genetics in wild populations when blood tissue can be obtained in a non-invasive way, the manipulation of individuals is facilitated, and the quality of the analysis is not affected by lymph hemodilution. This work was carried out from sampling three species of turtles: *Podocnemis unifilis, Trachemys callirostris,* and Chelonoidis carbonaria; but we believe that, given the anatomical and habitat differences of these turtles, our results can be extrapolated to a wider range of continental turtles.

In conclusion, for the turtle species studied here, the venipuncture site with the lowest risk of hemodilution was the jugular vein, allowing the extraction of blood samples of optimal quality that can be used in hematological analyses and clinical assessment of these individuals. Likewise, EDTA did not show hemolyzing effects on the blood cells of *T. callirostris* and *P. unifilis*. However, hemolyzing effects were observed in the blood samples obtained from *C. carbonaria*. Blood samples stored with sodium heparin were adequately preserved in all species of turtles evaluated, facilitating their subsequent analysis in the laboratory. Our data provide useful basic information for wildlife researchers and veterinarians of rehabilitation centers unfamiliar with the extraction of chelonian blood samples for laboratory analysis.

Conflict of interest

The authors state that there is no conflict of interest with the publication of this article.

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