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Technical Note

Minimally invasive blood collection techniques as a source of gDNA for genetic studies on turtles and tortoises

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Abstract: This technical note aims to describe the venipuncture procedure used to collect blood from Southern River terrapins via the subcarapacial venous plexus (SVP) and jugular vein. On uncooperative terrapins, SVP was applied while the jugular vein was reversed. 1.5 ml blood was preserved in 0.5 ml EDTA and stored at -20°C. ReliaPrep™ Blood genomic DNA Miniprep was used to extract DNA. Thermo Scientific™ NanoDrop 2000c was used to determine the concentrations of extracted DNAs. The greatest concentration of DNA is 136.3 g/L, and the highest purity is 1.90. The treatment is safe, minimally invasive, and effective.

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Introduction

Oral scrapings and cloacal swabs have been used to recover DNA from living reptiles (turtles, terrapins, and tortoises) (Wendland et al., 2009; García–Feria, 2015). Although whole blood is the ideal supply, numerous blood extraction procedures have been developed (Gottdenker and Jacobson, 1995). However, Chelonian members vary considerably in their vascular anatomy, degree of cooperation, and the possibility to cause injuries (Mans, 2008). Blood collection techniques formerly employed on freshwater turtles included highly invasive and fatal operations such as heart puncture and decapitation (McDonald, 1976). Non-lethal and less invasive blood collection methods include venipuncture of prominent veins (e.g., jugular, brachial, femoral, and iliac) (Mader, 2005), the subcarapacial venous plexus (Hernández–Divers et al., 2002), the occipital sinuses (Hernández–Divers et al., 2002), and cardiac puncture (Fowler, 1995).

The jugular vein is the blood collection site for Chelonians and should be used whenever possible. In most mammals, the jugular vein is precise and has a lower chance of lymph infection than other locations. This sample site is inappropriate for uncooperative or aggressive species without prior chemical restriction to enable adequate head extension (Kuchling, 2012). The (external) jugular vein is a superficial vein that runs from the dorsal part of the tympanum to the coelomic cavity on both sides of the neckline in a caudodorsal path. Some animals have a dorsal and ventral external jugular vein (Barrows et al., 2004; McArthur et al., 2008).

The venous contact of the cranial arteries from the azygous veins and the cervical anastomosis of the left and right jugular veins forms the subcarapacial venous plexus (SVP). This blood sampling site is frequently used in uncooperative species where jugular venipuncture without sedation is impossible, in neonates and other small Chelonians, and in individuals too ill to withstand the burden of restriction or sedation (Barrows et al., 2004). The SVP anatomy is found near the midline of the cranial side of the carapace, where the cervical vertebrae enter the shell. Several vessels, including the normal intercostal vessels and the caudal cervical branch of the external jugular veins, form a venous sinus at this site. As a result, there is a higher risk of lymph contamination (Barrows et al., 2004; Mans, 2008).

This blood collection study in *Batagur affinis* aimed to explain and demonstrate the efficacy of two minimally invasive venipuncture approaches until the

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Table 1. The venepuncture technique and procedures.

Step	SVP	Jugular Vein
1	The target area is swabbed with an alcohol swab to minimise the risk of infection - the alcohol swab we use contains 70% Isopropyl Alcohol and 30% water.	The head is slowly pulled out, and the neck is swabbed with an alcohol swab to minimise the risk of infection - the alcohol swab we use contains 70% Isopropyl Alcohol and 30% water.
2	The venipuncture person either extends the head and bends the neck ventrally or pushes the head back into the shell.	The head is placed in the appropriate position by slowly pulling it out from its retracted position underneath the carapace until the sampling site is easy to see.
3	The needle is directed dorsocaudally and inserted on the midline at the point where the skin attaches to the ventral aspect of the cranial carapace.	The needle is introduced in a craniocaudal direction, maintaining mild negative pressure.
4	The needle is bent upwards at a right angle of about 60 degrees (Fig. 2a) and advanced in a caudodorsal direction, creating intense negative pressure.	The needle is directed dorsocaudally and inserted on the midline at the point where the skin attaches to the ventral aspect of the cranial carapace.
5	If no blood is aspirated, the needle is slightly withdrawn and redirected until blood appears in the syringe hub.	Immediately after piercing the skin, the syringe is lightly aspirated. Then the needle is slowly inserted further into the neck until blood is seen to enter the syringe hub (Fig. 2b).
6	More blood is obtained by gentle suction throughout the operation than by strong suction, probably because the latter causes the vein to collapse.	More blood is obtained by gentle suction throughout the operation than by strong suction, probably because the latter causes the vein to collapse.
7	Swab the area of the needle puncture wound again with the alcohol swab.	Swab the area of the needle puncture wound again with the alcohol swab.

quality of genomic DNA (gDNA) was assessed. As a result, we are the first to provide a complete methods and protocols note on *Batagur* sp. blood collection. Until the gDNA quality was confirmed, it was the most comprehensive technical discovery from blood draws. The capacity to manage a sample volume without considerable sample contamination solved one of the primary concerns in the field after the invasive approach issue (Avataneo et al., 2019). Several research studies have described the use of gDNA sequences to examine genetic variation in *B. affinis* (Çilingir et al., 2019).

Materials and Methods

This study comprised 120 *B. affinis* individuals from four populations across the East and West Malaysia Peninsula: Pasir Gajah, Kemaman (KE), Terengganu (4.2524°N, 103.2957°E); Bukit Pinang (BP), Kedah (4.2221°N, 100.4370°E); Bota Kanan (BK), Perak (4.3489°N, 100.8802°E); and Bukit Paloh, Kuala Berang (KB), Terengganu (5.0939°N, 102.7821°E). A total of 30 individuals of *B. affinis* were sampled at each location. The arena permit approval number is B-00335-16-20, which was rewarded by the Department of Wildlife and National Parks, Peninsular Malaysia. The venipuncture blood collection technique and procedures consist of the following steps (Table 1).

Blood storing method: The vial should be shaken vigorously before being stored away from direct sunlight and preferably in cool place to ensure proper mixing. Although drawn-out preservation is best accomplished by keeping samples at +4 to –8°C, samples in the buffer can be kept up at encompassing temperature for quite a long time if necessary (Dutton, 1996) (Fig. 1).

DNA extraction protocol: Nucleic acids were extracted from 200 µL of each EDTA whole blood sample. After cell lysis and protein denaturation, extractions were performed with automated systems using ReliaPrep™ Blood gDNA Miniprep System (Promega, Madison, USA) with Binding Column technology. A final extraction volume of 200 µL was obtained from the 200 µL input volume of the EDTA whole blood sample.

The stages were as follows: The blood sample was shaken for at least 10 min at room temperature in a rotisserie shaker. 20 µL of Proteinase K (PK) solution, 200 µL of blood, and 200 µL of Cell Lysis Buffer (CLD) were added to a 1.5 ml microcentrifuge tube, vortexed for at least 10 seconds. The solution was incubated at 56°C for 10 minutes. 250 µL of Binding Buffer (BBA) was added and mixed by vortexing for 10 seconds. The contents were added to the Binding Column and located in a microcentrifuge at maximum speed for one minute. The flow-through in the collection tube was removed and discarded. 500 µL of Column Wash Solution (CWD) was added to the new Binding Column and centrifuged at maximum speed for 3 minutes. This washing procedure was repeated for a total of three washes. The column was placed in

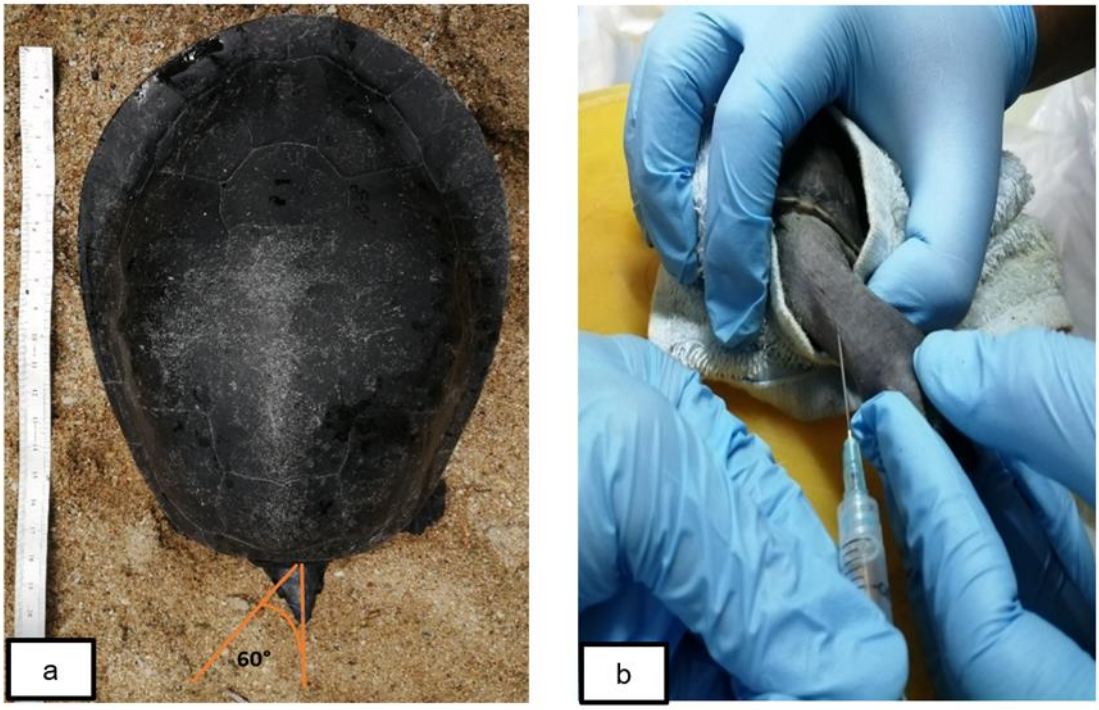

Figure 1. (a) SVP blood sampling angle is approximately 60 degrees, and (b) blood collection from the right jugular vein of a Malaysian Southern river terrapin (*Batagur affinis*).

a clean 1.5 ml microcentrifuge tube. 200 µL of Nuclease-Free Water was added to the column and centrifuged at maximum speed for one minute. The Binding Column was discarded. This study obtained a 200 µL volume of extracted DNA from the 200 µL input volume of whole blood.

DNA quality assessment: The concentration of the isolated DNAs was determined using the Thermo Scientific™ NanoDrop 2000c spectrophotometer model ND-2000 (Thermo Fisher Scientific, Waltham, USA). The automated systems ability to extract nucleic acids of high purity was proven by direct gel loading. Following NanoDrop quantification of the isolated nucleic acids, the results were put directly into the 1% agarose gel with molecular markers. Loading the extracted DNAs from the same sample was used to test the repeatability of each system.

Results and Discussions

Our approach is comparable to that outlined for sea turtles (Owens and Ruiz, 1980). Blood sampling methods such as ours have been defined as drawing blood from the cervical sinus, dorsal cervical sinus, occipital sinus, and subcarapacial vein. But true blood sinuses do not exist in jawed vertebrates (Randall et al., 1997), and these techniques draw blood from the external jugular vein or one of its branches.

Because of the varying proximity of the external jugular vein to the dorsal surface and other morphological aspects, some variant in blood collection practice is required. Therefore, we choose two blood collection techniques (collection from the jugular vein and SVP) and recommend them because they are more successful than other procedures (Mans, 2008). At the same time, we used two different-sized needles. First, we successfully collected more blood via the lateral jugular vein using a 23g x 1¼ inch needle. Then, we also tried a 3 ml syringe with a 23g x 1¼ inch needle and a 21g x 1¼ inch needle. Mans (2008) recommends using the smallest needle size appropriate for the animal's size and sample volume. Utmost Chelonian species require a 22- to 27-gauge hypodermic needle of sufficient length. The amount of blood to be obtained will determine the size of the syringe used. To avoid excessive negative pressure, which may cause the sampled vein to collapse, it should be used the smallest needle possible.

This minimally invasive blood sample procedure, successfully applied to *Batagur* species, has proven effective. Even for people who have only received a single training session and have little or no previous expertise with animals, the entire technique takes no more than two minutes to complete. The tension experienced by the animal is maintained to a bare

Table 2. Results of gDNA extraction from whole blood samples.

Sample Number	DNA concentration (ηg/μL)	Purity (OD 260/280)
1	120.8	1.82
2	39.3	1.80
3	42.7	1.90
4	35.2	1.83
5	71.4	1.88
6	136.3	1.87

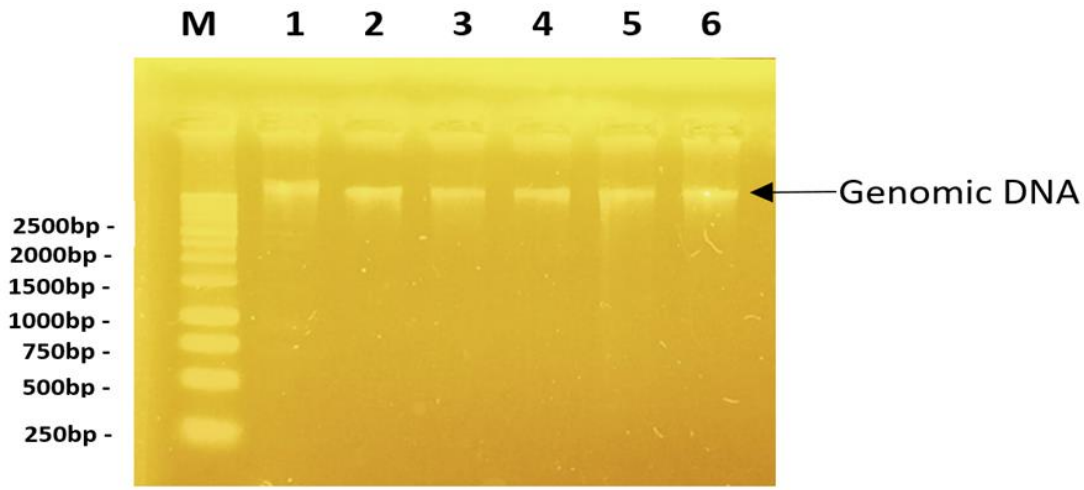

Figure 2. Genomic DNA was loaded into a 1% agarose gel and separated by electrophoresis for 40 minutes at 75 volts, Lane M: 1 kb ladder size standard marker, lanes 1-3: *Batagur affinis affinis* gDNA, lanes 4-6: *Batagur affinis edwardmolli* gDNA.

minimum, and there is virtually little risk of hurting the river terrapin as a result of this procedure. Furthermore, the current strategy has reduced the risk of sample contamination, which has been identified as one of the most pressing challenges in the field (Avataneo et al., 2019).

After sampling from the external jugular vein, there is generally a little superficial damage or scarring of terrapins actual bleeding of the needle puncture site, but this stops 5-10s after the region is swabbed with 70% alcohol. As a result, no physical harm or scaring of terrapins has been recorded. Some extracellular fluid contamination is acceptable when assessing isotope dilution and osmolality of bodily fluids. When evaluating the haematological qualities of blood, however, it is unacceptable. If blood contamination is intolerable, another sample can be collected from the opposite side of the head. As an alternative, we suggested a tissue collection procedure (about 2-4 mm of clipped scute of the shell), which is also a successful way of obtaining gDNA for genetic studies on turtles and tortoises (Praschag et al., 2009; Ismail et al., 2016).

The most commonly used solution for terrapin blood preservation consists of 100mM Tris-HCI, pH 8; 100mM EDTA, pH 8; 10mM NaCl and 1-2% Sodium Dodecyl Sulphate (SDS). Bowen et al. (1996), Encalada et al. (1996), and Seutin et al. (1991) recommend a 1:10 dilution of blood to buffer, and White and Densmore, (1992) suggest a 1:5 ratios. However, higher blood concentrations up to a 1:1 ratio may be employed, mainly if a higher concentration (2%) of SDS is employed to achieve sufficient cell lysis (Dutton, 1996). We collected 1.5 ml of blood and added 0.5 ml of EDTA for preservation in a 2 ml microcentrifuge tube at a ratio of 1:3 and stored at -20°C.

The DNA extraction protocol was efficient in extracting gDNA from whole blood based on an analysis of total gDNA in the agarose gel (Fig. 2). Spectrophotometer measurements showed DNA concentration and purity differences depending on the population (Table 2). Higher concentrations and purity were obtained from samples number 6 and 3, and vice versa for samples 4 and 2.

The concentration and 260/280 ratio of the extracted DNA samples corresponded to the recommended amount of 1 µg of suitable quality DNA

(concentration ≥ 20 ng/µl according to the protocol 'Illumina Library Construction Services - Sample Requirements' (Brajković et al., 2018; UC Davis Genome Center, 2018). Furthermore, the values of purity ratio Optical Density (OD) 260/280 followed the values proposed by Biase et al. (2002), ≥ 1.8.

Conclusions

This protocol proved that the blood collection method is advantageous due to its simplicity, rapidity and affordable reagents, apart from the high molecular weight DNA and purity achieved in all samples.

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