

Minimally invasive blood sampling method for genetic studies on *Gopherus* tortoises

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Abstract

Minimally invasive blood sampling method for genetic studies on Gopherus tortoises.— Obtaining good quality tissue samples is the first hurdle in any molecular study. This is especially true for studies involving management and conservation of wild fauna. In the case of tortoises, the most common sources of DNA are blood samples. However, only a minimal amount of blood is required for PCR assays. Samples are obtained mainly from the brachial and jugular vein after restraining the animal chemically; or from conscious individuals by severe handling methods and clamping. Herein, we present a minimally invasive technique that has proven effective for extracting small quantities of blood, suitable for genetic analyses. Furthermore, the samples obtained yielded better DNA amplification than other cell sources, such as cloacal epithelium cells. After two years of use on wild tortoises, this technique has shown to be harmless. We suggest that sampling a small amount of blood could also be useful for other types of analyses, such as physiologic and medical monitoring.

Key words: Blood extraction, DNA source, Tortoises

Resumen

Método de extracción de sangre mínimamente invasivo para estudios genéticos en tortugas terrestres del género Gopherus.— La obtención de muestras de tejido de buena calidad es la primera dificultad en cualquier estudio molecular. Esto es especialmente cierto en los estudios de manejo y conservación de la fauna silvestre. En el caso de las tortugas terrestres, la fuente más habitual de ADN son las muestras de sangre obtenidas principalmente de las venas braquial y yugular por contención química, o de individuos conscientes mediante métodos de manipulación y sujeción que pueden causar estrés en el animal. Se requiere una cantidad mínima de sangre para los ensayos del PCR. A continuación, presentamos una técnica mínimamente invasiva que ha resultado eficaz para extraer pequeñas cantidades de sangre apropiadas para realizar análisis genéticos. Además, las muestras obtenidas producen una amplificación de ADN mejor que otras fuentes celulares, como las células epiteliales cloacales. Después de dos años de aplicación en tortugas terrestres silvestres, esta técnica ha demostrado ser inofensiva. Sugerimos que el muestreo de pequeñas cantidades de sangre con esta técnica podría ser útil para otro tipo de análisis, como el seguimiento fisiológico y médico.

Palabras clave: Extracción de sangre, Fuente de ADN, Tortugas terrestres

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Introduction

Many behavioral, ecological, physiological, and medicinal studies for conservation purposes require the use of blood samples. To reduce physical risk during animal handling, a minimal invasive method has been tested to obtain blood samples for these as well as for other kinds of studies (e.g., genetic, systematic, toxicological and stable isotope analyses). In many cases, the use of molecular markers is currently one of the prime tools in wildlife research, management, and conservation (DeWoody, 2005; DeYoung & Honeycutt, 2005). Gathering good quality samples has sometimes been a problem under field conditions. In the past, collectors used to sacrifice individuals for museum collections and sometimes for obtaining accessory material such as tissue samples. The rapid development of PCR methods and kits for DNA extraction has made it possible to obtain suitable genetic material from minuscule samples (e.g., one hair). In many instances; however, collecting samples requires restraining the animals by physical or chemical means (*i.e.*, invasive techniques), resulting in prolonged stress and even injuring the subject. New non-invasive methods have been developed for specific taxa groups (García-Feria, 2008); nonetheless, for many species or for field conditions, these techniques are not an option. Isolation of DNA from stool samples is among the common non-invasive methods used in many wildlife studies (Dalén et al., 2004; Lukacs & Burnham, 2005), with the DNA source being the epithelial cells. However, these cells are usually scarce, and the feces may contain PCR inhibitory substances. Besides, there is a high risk of contamination from alien DNA (Taberlet et al., 1999; Broquet et al., 2007).

For reptiles, particularly for live turtles and tortoises, DNA has been extracted by means of oral scrapes and cloacal swabs (Nagy & Medica, 1986; Mautino & Page, 1993; Van der Kuyl et al., 2005; Wendland et al., 2009). Even so, whole blood is the best source, and several blood extraction techniques have been developed (Gandal, 1958; Avery & Vitt, 1984; Gottdenker & Jacobson, 1995; Knotková et al., 2002; López-Olvera et al., 2003; Rohilla & Tiwari, 2008). The sample is usually extracted either from the main veins (e.g., jugular, brachial, femoral, iliac vein) (Mader, 2005), the subcarapacial venous plexus (Hernández-Divers, et al., 2002), or the occipital sinuses, or by cardiac puncture (Mautino & Page, 1993; Fowler, 1995). Nonetheless, the anatomy and behavior of tortoises (Testudinidae) makes blood extraction rather difficult by any of these methods. The thick scales on the skin and the characteristic retraction of the limbs and the head within the shell block access to the veins. Sometimes the use of forceps and anesthetics for safe handling of the animals is necessary (Fowler, 1995). Additionally, lymphatic vessels running beside the main body veins may be damaged (Wendland et al., 2009). These methods are therefore excessive for PCR purposes. Turtles, like other reptiles, have nucleated erythrocytes (Knotková et al., 2002), so small quantities of blood are sufficient to obtain good quality genomic DNA.

Material and methods

When disturbed, *Gopherus flavomarginatus* (Bolson tortoise) can strongly retract its head and limbs, blocking access to the vessels used to draw blood. While in the retracted position, the exposed soft parts are covered with dense and thick scales (fig. 1). However, between the fingers and the dorsal area of the forelegs there is a characteristic thin line of almost naked skin (fig. 2). This is the recommended area for obtaining small amounts of blood. We have used the following method for over two years in a study that assesses the genetic variations of 76 wild individuals of the Bolson tortoise (Ureña-Aranda & Espinosa de los Monteros, 2012). According to Germano (1994), maturity is attained once the carapace reaches at least 28 cm; therefore all the handled specimens can be considered adults. Before the present field work, the blood sampling method was tested on two species of captive tortoises, *G. berlandieri* ($n = 1$) and *G. agassizii* ($n = 5$), also sampled by means of cloacal swabs. Blood was sampled as follows. First, we cleaned the dorsal side of the hand with a cotton swab soaked in 75% ethanol to avoid any possible infection or contamination of organic material. Then, a puncture was performed using a sterile hypodermic needle (27G x 13 mm; Becton, Dickinson & Company, Franklin Lakes, NJ) in the bare line of skin located at the distal edge of the hand just before the fingers. The needle should be introduced between the second and third fingers in an angle of 45° (approximately) toward the third finger. There may be no bleeding if the needle is introduced elsewhere or at a different angle. Without practice, no more than three puncture attempts were required to obtain the blood sample. Immediately after removing the needle, the blood can be collected in a borosilicate glass capillary tube that does not contain heparin. Finally, we placed a cotton swab with 75% ethanol on the puncture for a few seconds, applying little pressure as to stop any extra bleeding. The cloacal swabs were taken after cleaning the cloacal area with a cotton swab soaked in 75% ethanol. We then introduced and softly spun a rayon swab (Medical Wire and Equipment, 100–100 MW, Biomerieux) in the cloaca to obtain the epithelium cells.

Results

In the field, we usually collected up to 30 µl of whole blood and transferred this to 500 µl vials containing 100 µl lysis buffer (Longmire et al., 1997); the animals were released immediately after manipulation. The samples preserved in this buffer do not require refrigeration, which is an advantage for field conditions. However, if the genetic study involves protein analyses, blood samples must be stored by different means (e.g., liquid nitrogen). Once in the laboratory, we extracted DNA from the cloacal swabs, and dried tissue from shells samples and from the small aliquots of the blood-buffer mixture (10–20 µl) using Chelex 100® resin (Walsh et al., 1991). We obtained greater yields of high molecular weight DNA from the blood



Fig. 1. Bolson tortoise in retracted position. Note the inaccessibility to the blood vessels commonly used to draw blood.

Fig. 1. Tortuga del Bolsón en posición retraída. Nótese la imposibilidad de acceder a los vasos sanguíneos que habitualmente se utilizan para la extracción de sangre.

samples than from other tested tissue sources (*i.e.*, cloacal swabs, and dry tissue from shells; fig. 3). DNA amplification was conducted in Peltier-effect thermocyclers (ABI GeneAmp PCR system 2400) using the following parameters: one initial cycle at 95° for 120 s, followed by 32 cycles of 95° for 20 s, 47° for 20 s, 74° for 60 s, with one final cycle at 72° for 180 s.

This minimally invasive blood sampling method used on *Gopherus* species has been extremely useful. Performing the whole procedure takes no more than two minutes, even for those people who have been trained only once, and have little or no experience in animal handling. Using this technique, stress for the animal is kept to a minimum, and there is practically no risk of injuring the tortoise.



Fig. 2. Suggested puncturing site for the minimally invasive blood sampling method.

Fig. 2. Lugar sugerido para la punción como método de extracción de sangre mínimamente invasivo.

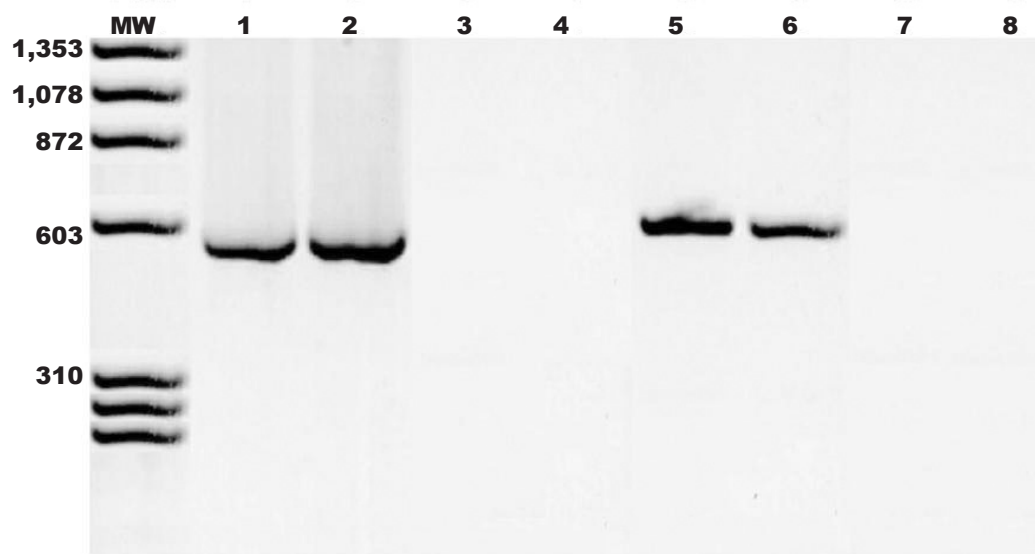


Fig. 3. Ethidium bromide stained 2% agarose gel showing PCR products from two regions of the mitochondrial D-loop gene. Amplifications from several specimens were attempted from forelimb puncture (lines 1, 2, 5 and 6), cloacal swab (lines 3 and 7), and carcass tissue (lines 4 and 8): MW indicates the molecular weight bands in base pairs.

Fig. 3. Productos de PCR de dos regiones del gen mitocondrial D-loop mostrados en un gel de agarosa teñido con bromuro de etidio al 2%. Se intentó realizar amplificaciones de diferentes muestras obtenidas a partir de la punción de la pata delantera (líneas 1, 2, 5 y 6), hisopos cloacales (líneas 3 y 7) y tejidos de carcasas (líneas 4 y 8): MW indica las bandas del peso molecular en pares de bases.

A remarkable characteristic of this species is its burrowing behavior. Several nerves and ligaments are located in the hand area, and some concerns may result from puncturing around this area. We have used this method for two years (*i.e.*, between 2009 and 2011), to collect blood samples from 76 individuals of the Bolson tortoise. This species is highly philopatric, and the adults do not have natural predators. In recent visits to the study area, we have been able to verify the health status of every manipulated individual, and none of them showed any apparent problem. Therefore, we are confident that this technique of foot puncture is harmless compared to other invasive blood sampling methods (*e.g.*, Gandal, 1958; Avery & Vitt, 1984; Fowler, 1995). Several analyses for physiological and medical surveys require larger volumes of blood than those extracted with the foot puncture method (from 0.5 ml to microtainer until 1.8 ml or more). However, the amount of blood that is obtained with the suggested method ($\approx 30\text{--}40\ \mu\text{l}$, approximately two-thirds to three-quarters of a capillary tube; Kerr, 2002) is adequate for implementing laboratory and clinical analyses other than PCR. This minimally invasive method can be applied to obtain blood for a morphological characterization of peripheral blood cells from blood smears (Knotková et al., 2002), packed cell volume (PCV) measurement by microhaematocrit, refractometry to assess

the total protein level, specific gravity and refractive index of serum, glucose level by blood glucose strips or pocket glucose meter (Kerr, 2002). We therefore recommend the use of this minimally invasive method before attempting more aggressive blood extraction techniques for genetic analysis or for any other survey that requires only small amounts of blood.

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