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Simple method of blood sampling from Indian freshwater turtles for genetic studies

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Abstract. Biological material is now commonly employed during the characterization of species. Superficial blood vessels are the most appropriate sites in reptiles for blood sampling. In this study we describe a method of blood collection from various species of Indian freshwater turtles for genetic characterization and discuss its advantages over the previously described techniques. The method proved most suitable, easy, and less harmful. The quantity of blood samples obtained is enough for different applications such as karyotyping, cytological examinations of cell types, isozyme analysis from RBCs and plasma, DNA isolation for PCR-RAPD and mitochondrial gene specific amplification. We conclude that the femoral vein is probably the most suitable site for blood collection from Indian freshwater turtles for various experimental procedure and methodologies related to genetic characterization.

Keywords. Femoral vein, blood sampling, Indian freshwater turtles.

Genetic characterization of endangered species is essential for proper genetic conservation and management in their respective habitat. Studies on various aspects of genome characterization require blood or tissue samples. For this purpose, sampling from substantial number of individuals is required, but the classical methods of hematological, biochemical (e.g., allozyme) cytogenetic, or DNA analysis use materials from blood cells, spleen, liver, muscle, kidney, intestine or cell cultures of heart and skin fibroblast (Baker et al., 1971; Bickham, 1975; Frye, 1991; Mader, 2000). All the above methods involve killing or surgery, and this is generally not feasible in the case of endangered species, where the source as well as number of animals, both is limited.

Under such conditions, the development of new and simple methods becomes highly desirable. Thus, we have optimized a safe, sterile and efficient blood sampling method, which is equally applicable to a variety of reptilian species, including Indian freshwater chelonian species.

A variety of sites in the chelonian body have been used to obtain blood, including heart, veins (e.g., jugular, brachial, ventral coccygeal or scapular), brachial artery, orbit-

al sinus, cervical sinus, sub-carapacial venipuncture site and trimmed toe-nails, (Gandal, 1958; Dessauer, 1970; McDonald, 1976; Maxwell, 1979; Owens and Ruiz, 1980; Taylor and Jacobson, 1981; Stephens and Creekmore, 1983; Avery and Vitt, 1984; Jacobson, 1987; Ulsh et al., 2000; Hernandez-Divers et al., 2002; Rogers and Booth, 2004; Gregory and Gabriel, 2006). Each sampling method has certain advantages and disadvantages. Some methods have even higher risk of infection (Gandal, 1958; Nicole et al., 2007). In turtles and tortoises, orbital sinus sampling method has been used for collecting small volumes of blood through capillary tubes (Nagy and Medica, 1986). This method, however, results in the dilution of blood sample with extra cellular fluids and secretions, which may alter the composition of plasma and affect percent volume of cellular components. Since lymphatics are well developed in chelonian forelimbs (Ottaviani and Tazzi, 1977), obtaining blood samples from veins and arteries may result in hemodilution with lymph. Sub-carapacial venipuncture site is located at the angle where cervical vertebrae join the shell and is formed by the junction of the common intercostals and the caudal cervical branch of the external jugular veins. Blood is readily obtained from a post occipital venous plexus, that is located dorsally to the cervical vertebrae, behind the occipital protuberance of the skull of tortoises (Gottdenker and Jacobson, 1995).

Most of the published techniques have been applied in adult turtles and hatchlings of long-tailed species such as *Graptemys geographica, Trachemys scripta* and marine turtle species (Gregory and Gabriel, 2006; Wibbels et al., 1998; Bennett, 1986; Ulsh et al., 2000). However, the tail is very short in Indian freshwater turtle species like *Asperidetes gangeticus, Geoclemys hamiltoni, Kachuga* sp.*, Lissemys punctata,* and thus, blood collection from the coccygeal vein is very difficult. The tail cannot be held firmly by any recommended device as described for *Trachemys scripta* (Ulsh et al., 2000). In addition, the amount of blood obtained from such species is very low, not enough for lymphocyte separation and culture for cytogenetic characterization. We collected blood samples from juveniles (less than 20 g body mass) and adults (weighing up to 5 kg) of both, hard shell *K. dhongoka* (n = 5) and *G. hamiltoni* (n = 1) and soft shell turtle species *L. punctata* (n = 10) and *A. gangeticus* (n = 1).

Adult individuals of soft shell turtles were collected from ponds and dams located in the Gwalior-Chambal region with the help of local fishermen and also purchased from local fish markets. The animals were reared in an artificial pond of dimension 5×2×1 m near the animal house of the Department of Zoology. Animals were fed on freshwater frozen fishes, live earthworms, snails, and aquatic weeds. The animal care procedures followed in this study were as recommended by ethical committee of Jiwaji University and as per guide lines of the Forest Department of Madhya Pradesh Government (Rohilla et al., 2006).

The hind limbs were cleaned with distilled water-soaked cotton and after complete drying swabbed twice with absolute ethanol. The femoral vein site was identified for accurate vein puncture for collection of blood. It is located in the midventral part of the femur above the knee joint. A small muscle depression appears which was identified as the correct site for venipuncture (Fig. 1). Before inserting the needle in the skin, 50-100 µl of anticoagulant was taken into the syringe. The plunger of the syringe was withdrawn gently (up to the 0.1 to 0.3 ml mark) to create a vacuum for easy withdrawal of blood. A 2-6 mm deep insertion of the needle is required, which, however, depends on the musculature and age of the animal, generally being 2-3mm in hatchling or juvenile. The correct insertion

Fig. 1. Position and site (femoral vein) of blood collection from the hind leg of adult freshwater soft shell turtle *L. punctata* (Inset: enlarged view to locate the exact position).

of needle requires a 30º-40° angle downward, between the thigh and the needle. If blood does not appear in the syringe immediately, the syringe is gently rotated and the angle is slowly decreased until blood enters into the syringe.

From a healthy 5 kg animal, we could easily withdraw about 4-5 ml blood through the femoral vein with the help of 5 ml syringe equipped with 25G, 5/8-inch needle pre-flushed with sodium heparin, but keeping 250 µl heparin saved in the syringe to prevent immediate clotting. Similarly, about 0.2-0.3 ml of blood was collected from one month old juveniles of three *Kachuga* species with the use of a 13 mm insulin or tuberculin syringe. After blood collection the skin was again swabbed with absolute ethanol and medicated with Betadine solution (Tincture Iodine) or Sofaramycin cream to prevent microbial infection. Finally the turtle was left on the sand to recover for about one to two hours before transferring to water. None of the adults, hatchling and juvenile turtles used to collect blood showed any sign of diseases or health related problems, even after three months.

Our procedure on soft shell turtle species of *Lissemys* and *Kachuga* allows to collect enough blood for cytology (see Mader, 2000), genetic characterization, including identification of cell types for cell culture, gene-specific PCR amplification, RAPD fingerprinting and allozyme analyses from RBCs or whole blood. This method appeared more appropriate, effective and easy with several advantages. The femoral vein is located in the peripheral part of the body, hence, there is no chance of injury to vital organs of the body and

sufficient amount of blood can easily be withdrawn from both large and small-sized animals. The method demonstrated an easy mode of holding the animal, expected to be less painful, does not require anesthesia and provides sterile conditions. This method can be adopted for several other reptilians also with some modifications (see Frye, 1991). On a wild lizard, *Varanus bengalensis*, it proved to be equally effective (our unpublished observation). However, this technique may not be equally applicable for Indian tortoise species (i.e., *Geochelone* sp.) due to hard scales on the legs, which can damage the tip of the needle and unlike freshwater turtles, the leg protrusion is difficult to obtain easily.

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