Manual for Biological Sample Collection and Preservation for Genetic, Reproductive and Disease Analyses

Central Zoo Authority

&

Laboratory for the Conservation of Endangered Species (LaCONES)

CSIR- Centre for Cellular and Molecular Biology

Manual for Biological Sample Collection and Preservation for Genetic, Reproductive and Disease Analyses 2017

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CONTENTS

INDEX OF FIGURES

1. Introduction

The Indian subcontinent is home to over 91,000 animal species and 45,000 species of plants making it one of the mega-diverse countries in the world (IUCN, 2016). It is, however, disheartening that the rich diversity currently faces the dangers of extinction primarily caused by habitat destruction and reckless poaching. The depth of the problem can be assessed in the variety of applications of products derived from wildlife to develop cosmetics, culinary, medicines etc. It is estimated that the quantum of trade in illegal wildlife products is only surpassed by narcotics. In India, the first step towards the protection of wildlife took place with the enforcement of Wildlife (Protection) Act in the year 1972 and since then different government agencies have been imparted with the responsibility of monitoring wildlife offences.

The Section 38C(j) of the Wildlife (Protection) Act, 1972 has assigned a function to the Central Zoo Authority to provide technical assistance to zoos for their proper management and development on scientific lines. This manual is in partial fulfilment of the assigned function.

Among the numerous initiatives set on by the Centre for Cellular and Molecular Biology (CCMB) and Department of Biotechnology and the Central Zoo Authority (CZA) of the Government of India, Laboratory for Conservation of Endangered Species (LaCONES) located at Hyderabad is the most scientifically organized program which was set up in 1998. The project has uniquely got together the organizations, whose

names are mentioned above to commence a project targeting not only large carnivores but also herbivores, non-human primates, aves etc. The plethora of technologies applied to assess genetic diversity and assisted reproduction is being continuously manoeuvred to make them more efficient.

The LaCONES has been primarily aimed at conserving endangered animals in order to prevent their extinction and ensuring the numbers are sustainable into the future. The objectives were clearly stated as:

1. *Monitoring of genetic variation by modern techniques such as DNA fingerprinting.*
2. *Establishment of gene and cell banks by cryopreservation of semen, eggs and embryos and tissues of endangered species to be used in future for various purposes including cloning.*
3. *Fertility analysis based on semen profiles and hormonal profiles to facilitate captive breeding.*
4. *Artificial insemination: Although well established in case of domestic animals, this technique needs to be standardized for use in wild animals.*
5. *In vitro fertilization (IVF) and embryo transfer involves fusion of a spermatozoon with an oocyte in vitro and the transfer of the resulting embryo to the true or surrogate mother.*
6. *Cloning: The technology to be developed only for very rare species.*
7. *Disease monitoring using PCR based on techniques.*

The LaCONES has successfully developed Genetic Markers for the Asiatic lion (*Panthera leo*), Bengal tiger

(*Panthera tigris*) and Indian leopard (*Panthera pardus*) establishing that the three species are genetically healthy and diverse. Non-invasive methods of DNA extraction have been developed. The universal primer for species identification which is widely popular owing to its successful application in solving wildlife forensic cases since more than a decade has also been developed. Works on the Star tortoise (*Geochelone elegans*), Himalayan wolf, the Gangetic river dolphin (*Platanista gangetica*), Olive ridleys (*Lepidochelys olivacea*), Blackbuck (*Antilope cervicapra*), Spotted deer (*Rusa alfredi*) and Blue rock pigeon (*Columba livia*) has been completed. Besides, DNA based methods for bird species identification have also been developed. For the first time a method was standardised for semen collection from the White backed vulture. A baby Spotted deer ‘Spotty’ and baby Black buck 'Blacky' were produced using artificial insemination. Recently, non-invasive pregnancy detection and fertility analysis have been developed for a variety of wild animals.

2. Collection and Storage of Wildlife Samples for Genetic Analysis (Species Identification, Heterozygosity, Relatedness, etc.)

Humans have been directly or indirectly dependent on biodiversity for sustenance to a considerable extent. However, ever increasing biotic pressure and developmental activities have led to large scale depletion of the natural resources. Wildlife conservation is the attempt to protect endangered species along with their natural habitat. *In-situ* (within natural habitat) and *ex-situ* (outside natural habitat) are the two conservation measures in vogue. *In-situ* conservation enables a species population to self-replicate and maintain its potential for continued evolution. Protected Areas are the corner stone of *in-situ* conservation. Protected areas include Tiger Reserves, National Parks, Sanctuaries, Biosphere Reserve, Heritage Sites, etc. Management and monitoring of these areas are the main objectives of *in situ* conservation.

Ex-situ conservation is a complementary approach to *in situ* conservation as it preserves biological biodiversity outside their natural habitat. The techniques included in this category are storage of DNA, semen/ova, embryos, or captive breeding through the establishment of Gene Banks. Both approaches have played a critical role in preventing species extinction as well as increasing the population numbers.

The DNA-based analysis is an *ex situ* approach and ranges from development of DNA-based genetic markers like

Restriction Fragment Length Polymorphism (RFLP), Single Nucleotide Polymorphism (SNP), microsatellites, to their application in evaluation of genetic diversity in terms of heterozygosity levels, inbreeding, relatedness, etc. and examining the role of various factors in shaping up diversity. It is mainly used in the identification of species of animals and birds, population-level surveys (e.g. Tiger census), genetic disease identification, etc.

The first step, however, as long as biological research is concerned remains the collection and storage of specimen for laboratory analysis. Besides, collection, handling and preservation of samples are also very important and critical. Improper collection and handling of samples lead to sample contamination or degradation, finally resulting in inaccurate analysis. Cross-contamination of samples may also occur when different biological samples are handled at the same time which is also not preventable given a large number of cases and comparatively less infrastructure. To avoid cross-contamination of samples, instruments should be sterilised or should be cleaned in between the handling of different samples.

Genetic material is fragile and can suffer degradation if stored in inappropriate conditions. A detailed description of the different steps necessary for sample collection and preservation is explained hereunder.

1. **Blood**

   Name of Specimen: Fresh Blood

   Requirements: Disposable syringes, K_3EDTA (Ethylene Di-amine Tetra Acetic Acid) coated vials, cotton and

absolute alcohol/rectified spirit, ice-box, marker for labelling (**Fig. 1**).

Optimal amount of sample: As required depending on the size of the animal (0.2 ml to 2 ml).

Instructions for collection:

- The animal should be anaesthetized in the presence of a veterinarian before drawing blood, and only a trained technician/phlebotomist should be allowed to draw blood.
- A suitable body site should be first chosen to draw blood from.
- After drawing blood, it should be immediately transferred to a K_3EDTA coated vial and labelled specifying name, age, sex of the animal and date and location of collection.

Figure 1: Labelled vacutainer and tube with blood sample.

Storage and/ transport:

- The sample vial must be stored in an ice-box till it is transferred to a refrigerator.

- For transferring to another laboratory for analysis, it should be properly packed in a compartment that will prevent any mechanical damage during transport, and that will ensure a low temperature.

Precautions:

- Administering of anaesthesia and drawing of blood MUST be carried out in the presence of a veterinarian.
- Forceful restraint to the animal should be avoided at all times.
- Gloves and masks should be worn by the technician and other personnel.
- In the case of infected blood samples, personnel must use sanitizers before and after the collection.

2. Blood stains

Name of Specimen:

Blood stained material (for example soil, plant parts, knife or an axe (**Fig. 2**).

Required devices: Plastic vial (in case of soil or dried blood scraped from knife or axe), plastic.

Optimal amount of sample: 5 - 10 grams.

Instructions for collection:

- A few particles of blood stained soil matter can be placed in a clean and dry test tube.
- Blood stained parts of plants can be wrapped in a clean plastic zipper bag and stapled well for storage.

- Blood stained knife or axe can be scrapped (not vigorously) to remove the dried stain and the scrapings can be collected in a clean and dry test tube.

Storage and/ transport:
The sample tubes can be stored in a dry box containing an ice-pack for transport.

Precautions:
Personnel should wear gloves at all times to prevent contamination by touching the sample as it is very small in amount in case of blood stains.

Figure 2: Blood stained (A) sickle and (B) soil

3. Faeces or dung

Name of Specimen: Faeces / scats / dung / pellet

Required devices: Collection spoon, Silica gel beads, plastic zip lock (**Fig. 3**)
Optimal amount of sample: 50 grams

Instructions for collection:

- Fresh samples (<24 hours old) should be collected without contamination.
- A sterilised and dry collection spoon is used to collect the faecal material.
- The sample should be placed inside a clean and dry zip lock consisting of silica gel beads which will absorb moisture and prevent microbial contamination.

Figure 3: Scat sample and silica beads (blue turned pink) sealed in a zip lock pouch

Storage and/ transport:

The zip lock can be placed in an ice box for transport or kept at 4°C for short-term storage in the laboratory.
Precautions:

- The scat should have been defecated in the last 24 hours.
- Moisture should be prevented to avoid microbial contamination.

4. Hair

Name of specimen: Hair (**Fig. 4**)

Required devices: Forceps, Plastic pouch or zip lock or Petri dish

Optimal amount of sample: 15-20 strands

Instructions for collection:

- Hair should be plucked (and not cut) with forceps ensuring that the root of the hair shaft stays intact.
- The sample should then be transferred into a clean and dry plastic zip lock pouch (or Petri dish) and labelled accurately.

Storage and/ transport:

The plastic pouch containing hair samples can be kept in a refrigerator until use for analysis or in an ice box for transferring to another laboratory.

Precautions:

Hair sample should not be cut but plucked with the root of the hair shaft in tact.

Figure 4: Hair sample sealed in a zip lock pouch

5. Horn /Ivory/Bone

Name of specimen: Horn or ivory (**Fig. 5**)

Required devices: Driller, a filer, Petri dish, zip lock pouch.

Optimal amount of sample: 5-10 grams.

Instructions for collection:

- Take the piece of bone (or horn or ivory) and wipe it with a dry cloth to remove any impurities sticking to it.
- Wipe the filer with alcohol to sterilise it.
- Use the filer to cut small pieces of the horn or bone or ivory.
- Place the pieces obtained in a Petri dish or a zip lock.

Storage and/ transport:

The sample can next be stored at 4°C in a refrigerator for laboratory analysis.

Precautions:

- The personnel involved in handling the tissue should wear gloves at all times to avoid contamination.
- The filer should be cleaned with alcohol before use.
- In case of multiple samples, the filer should be cleaned each time.

Figure 5: (A) Idol made of ivory, (B) Horns (leftmost original and rest fake), (C) Animal Bones and (D) Horns

6. Meat/Cooked meat

Name of specimen: Meat/Cooked meat (**Fig. 6**).

Required devices: Sterilized blade, Petri dish, salt, zip lock pouch.

Optimal amount of sample: 5- 10 grams.

Instructions for collection:

- Cut a slice of meat/cooked meat with a sterilised blade.
- Place the slice in a jar containing common salt or alcohol as a preservative. Never Put The Sample In "Formalin".

Figure 6: Meat samples in (A) Jar with common salt and (B) dipped in alcohol

Storage and/ transport:

The container with the meat/cooked meat sample should be placed in an ice box for a short period or a 4°C refrigerator for a longer period.

Precautions:

- Personnel must wear gloves at all times.
- The blade used for slicing should be wiped with alcohol before and after use.
- In case of multiple samples, the blade should be wiped every time.

7. Skin

Name of specimen: Skin (**Fig. 7**).

Required devices: Scissors or blade, Plastic pouch or zip lock or Petri dish.

Optimal amount of sample: Five centimeters of skin piece.

Instructions for collection:

- Clean the scissors or blade with alcohol for complete sterilization.
- About 5 X 5 cm piece of skin (or leather hide) should be cut.
- Place the skin piece in a clean and dry zip lock or Petri dish.

Storage and/ transport: The sample can be kept in a refrigerator at 4°C or in an ice box for transferring to another laboratory for analysis.

Precautions:

- The scissors used for cutting the skin piece should be properly sterilized.

- Personnel must wear gloves.

Figure 7: Complete Skin and a skin piece in a zip lock pouch

3. Biological Samples for Reproductive (pregnancy and fertility) and Stress Assessment

Hormones are vital to regulate the reproductive success and to maintain the animal's health and well-being. They play an important role in regulating metabolism, preparing the body for mating, 'fight or flight' and various other behavioral activities. Hormones can be measured in biological products like blood, urine, feces, saliva, hair, feather and plasma of whole blood drawn from systemic circulation thus acting as an indicator of reproductive as well as other social behavior of the animal. However, blood sampling has its limitations as the circulating hormone levels are affected in response to the stress of handling and physical restraint of the animal, thus altering the physiological and behavioral parameters and increasing the glucocorticoid concentration which eventually affects reproductive fitness. Furthermore, repeated blood collection in free-ranging animals is not possible. In the recent years, non-invasive hormone analysis is being widely used to study the reproductive physiology, behavioral ecology, conservation biology etc. in a wide range of animals. Furthermore, non-invasive fecal hormone metabolites represent the cumulative hormone levels over several hours rather than actual steroid concentration, thus being less affected by episodic fluctuations. The choice of the biological sample depends on many factors like the steroid metabolism and excretion, assay procedure, ease of sample collection, type of study, etc.

1. Blood

Blood serum or plasma can be used to measure the hormone profiles and study the physiological status of an animal. Collecting blood samples (2 ml) involves less steps in sample preparation, provides a real time picture of hormone status and can be used to monitor short term changes in the circulating hormones of the animal. It can be used to measure steroids like progestogens, androgens, estrogens etc. apart from protein hormones like luteinizing hormone (LH), follicle stimulating hormone (FSH), relaxin, inhibin, prolactin etc. The animal is subjected to either physical or chemical restraint (anesthesia) for collection of samples and this can consequently affect the circulating hormone level resulting in inaccurate results. The samples should be frozen at -20°C immediately after collection and should not be subjected to repeated freeze thaw cycles. Longitudinal monitoring of steroids using blood has been studied in many species including sheep, rhinoceros and felids.

2. Saliva

Cotton swabs or absorbent materials also can be used to collect saliva from smaller animals. Saliva is collected by aspirating from the buccal cavity of the animal using a pipette or a syringe. Alternatively, samples can be collected by drool collection or swabbing the interior of the mouth with cotton. The samples should be passed through a syringe filter to remove bacteria and should be frozen until analysis. Only the unbound steroids are present in the salivary fraction as a result of which the steroid concentrations are low in the saliva. Saliva has been

previously used to study stress in primates and reproductive monitoring in rhinoceros.

3. Urine

Urinary hormone monitoring is an alternative to the invasive blood sampling procedures. Urine samples can be collected directly (2- 3 ml), midstream, when the animal urinates, followed by centrifugation to remove the particulate matter and debris. They are then frozen at -20°C until assayed. There is a lag time between steroid production and its appearance in the excreted urine and this can range from 6 to 14 hours. Thus the changes in urinary hormones reflect the physiological events that happened several hours earlier and this has to be kept in mind during sample collection.

4. Hair

Hair samples are easy to store and transport. They are also not subjected to short-term stress while collecting the sample (40 - 50 hairs) as is the case with blood or urine samples and are helpful in providing information on the long-term changes in the hormone profile. Hair can be cut from the body of the animal using clippers or scissors and stored at ambient temperatures for long periods of time until analysis. Hair samples have been used to assess stress in Polar bears reproductive hormones in cattle, Grizzly bears and felid species.

5. Faeces

Faeces are the most preferred biological sample due to their ease of collection under natural conditions and minimum contact with the animals. Fecal samples (5 - 10 gm) can be collected directly from the field or the animal enclosure after defecation (within hour), thus making it unnecessary to separate or restrain the animal. Steroids excreted in the faeces are unevenly distributed, so during sample collection the feces have to be homogenized with a spatula or gloved hands before storing them in appropriate containers. The faecal storage methods affect the steroid metabolite concentration in a species-specific manner. Previous studies show that freezing the faeces at -20°C immediately after collection is more preferred over the storage of samples in organic solvents. Khan et al. (2002) showed that the long term storage of faeces frozen in ethanol, altered the steroid hormone concentration. So it is prudent to store the faecal samples in a freezer immediately after sample collection. If freezers are not available in the field, the samples can be dried to remove the moisture completely, thus preventing bacterial contamination and steroid degradation. The extraction procedure depends upon the species being studied and the hormones being measured. The faecal hormones can be extracted by either using methanol with 5 to 20% water or boiling the samples in 90% ethanol.

4. Collection of Ovary for Germplasm Preservation

The new concept of *ex situ* genetic management of small populations of endangered species has gained interest among conservation scientists with a view to re-introduce them into the wild. Modern Reproductive Technologies such as gamete (sperm and egg) cryopreservation, artificial insemination, *in-vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI) and embryo transfer will play an important role in such newer conservation breeding programs that are necessary to propagate the rescued genes to the endangered populations for genetic management programs.

Wild animals in captivity as well as in the wild usually die unexpectedly, even many animals die before attaining their reproductive maturity. In such cases, recovery and cryo-storage/preservation of their valuable gametes from those endangered animals after death would be of great value in wildlife conservation and future studies. Testis is source of male gametes (i.e. sperm) while oocytes are female gametes produced in the ovary. After post-mortem, there is a very short time available for gamete rescue because tissue degeneration occurs rapidly in the field conditions where there is no storage facility. Thus it is crucial to collect gonadal tissues appropriately as soon as possible after death, keeping in mind, the purpose of assuring optimal quality of gametes and preserving their fertility potential for future offspring production.

Materials required:

1. A pair of scissors
2. Scalpel with BP blade
3. Sterile normal saline (0.9% sodium chloride) solution with antibiotics
4. Sterile phosphate buffered saline with antibiotics
5. Ziplock bags
6. Ice packs, storage and shipping box
7. Rectified spirit (70% ethanol)
8. Tissue paper roll
9. 50-ml collection tubes
10. Bottles of 100-200 ml capacity

Collection of Ovaries:

Ovary is the source of female gametes i.e. oocytes/eggs. Collection and cryopreservation of oocytes is equally important for conservation breeding programs because it is not always that spermatozoa of the same species will be available for IVF or ICSI at the same time as the females die (**Fig. 8**). Ovary of an adult cat contains several thousands of preantral follicles. The oocytes in the follicles are commonly cryopreserved in form of ovarian tissues. Follicles extracted from ovarian tissues can also be isolated and grown *in vitro* in a culture medium or grown *in vivo* using a xenotransplantation technique (Thuwanut et al. 2011).

1. As early as possible, a pair of whole ovary should be collected after death of animal.
2. Cut open the carcass by using sharp knife. Open the pelvic cavity and look for the reproductive tract that lies under the large intestine/rectum.

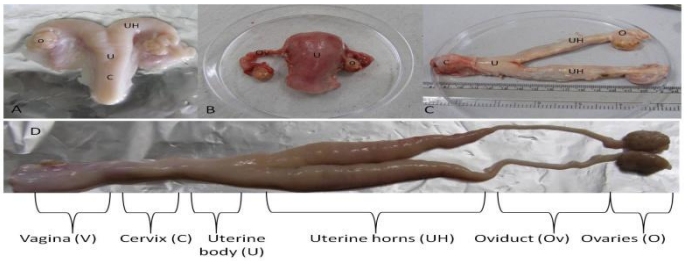


Figure 8: Female Reproductive tracts of Chousingha (A), sacred Baboon (B), Lion (C) and parts of the female reproductive tract of the Chousingha (D).

3. Collect the complete reproductive tract comprising a pair of ovaries, uterine body and oviduct. In small-sized animals, whole tract can be collected and shipped while in case of large animals, only paired ovaries would be enough to collect and ship on ice.
4. Ovaries should be collected in phosphate buffered saline or normal saline containing antibiotics in a plastic container and stored on ice.
5. Label all details about animal species, date of collection, fertility status, age and history of any reproductive disorder, if any, and ship to the laboratory on ice or dry ice.
6. On arrival in the laboratory, follicles will be collected either by aspiration method or whole follicle recovery. Immature or mature oocytes will then be recovered from follicles and either cryopreserved or used for *in vitro* maturation and fertilization procedures that develop into an embryo. Follicles or oocytes collected from the ovaries can also be used for basic studies such as transcriptomic and microRNA analysis.

5. Collection of Testis for Germplasm Preservation

Cryopreservation (freezing) of testis of dead wild animals provides a feasible option for the preservation of germplasm of wild animals. In cases of unsuccessful sperm retrieval through other means prior to the death of the adult animal, testis is the only source of sperm. This is especially of significance for germplasm preservation of valuable, rare and endangered animals whose dwindling population is affected by high mortality. To propagate and preserve the genetic potential of endangered wild animals, testis cryopreservation techniques associated with assisted reproductive technologies (ART) such as intra-cytoplasmic sperm injection (ICSI). Cryopreservation of testis of sexually immature animals in conjunction with testis tissue xenografting provides a powerful approach for the conservation of endangered species. For the successful freezing of testis, efficient collection of testis from dead wild animal is mandatory. However, the collection of testis can be quite a challenge from wild animals. This is because of the time delay between death and collection of testis can lead to loss of cell viability making the sample useless for future use. Therefore, it is essential that as soon as after the death of an animal, the carcass should be transferred to a cold room (if such a facility is available in the zoo) or shifted to a cool place away from sunlight. The procedure of *post-mortem* should be initiated immediately with the help of zoo veterinarian to prevent loss of crucial cell viability of the gonads.

A detailed history of the animal should be taken from the zoo veterinarian to assess if the animal had died of infectious cause. The testis from animals that had died due to infectious

reasons should be avoided as there can be a potential zoonotic infection threats. During the *post-mortem* procedure, care should be taken that all aseptic precautions are followed (**Fig. 9**). Proper protective barriers should be maintained such as gloves, mask, apron etc. Samples from large animals should be collected and stored in a container containing ice/cooling pack. Testis from small animals can be stored in Phosphate Buffered Saline (PBS) on ice in a sterile container.

Figure 9: Collection of testis from dead Gaur (*Bos gaurus*) from a zoo. (A) A 7-year-old animal died due to non-infectious cause. (B) The scrotal sac where testis are located. (C) Collection of scrotal sac. Note the ascetic precautions used by the veterinarian. (D) Collected scrotal sac of the animal. (E) Testes after removal from scrotal sac. (F) Transportation box for the testis. It consists of insulated box with cool pack and zip-lock bag (optional). (G) Transportation of testis on cool pack in the insulated container.

Some of the precautions that need to be taken during sample collection:

- Person with open wounds should not handle the samples.
- The sample should not be exposed to fixatives such as formalin.
- Sterility should be maintained as far as possible.
- The testis parenchyma should not be exposed at any cost to maintain sterility.
- After collection, the gonads should be placed on ice/cool pack in an insulated box and transported immediately to the laboratory.

Following procedure may be followed while collection:

1. At the earliest possible after death, both the testicles should be collected and stored on ice.
2. Before detaching from the body, the scrotum should be cleaned thoroughly with 70% ethanol and then with normal saline.
3. Testicles should be cut from the base of body using a scalpel and the scissors and apply tissue paper to remove blood from the cut portion of testis.
4. Cleaned testis will then be transferred in a plastic zip-lock bag and stored on ice or in a refrigerator till its shipment to the laboratory for sperm recovery.
5. Label the sample with proper animal details such as species, estimated age, fertility status, and probable cause of death.
6. On arrival in the laboratory, epididymal sperm will be recovered in a suitable sperm collection medium (that

needs to be optimized for every species), complete sperm analyses will be done and depending on the sperm viability, using species-specific sperm cryoextender, sperm will be preserved in liquid nitrogen to be used for future breeding program.

6. Collection of Tissue Samples For Establishing a "Frozen Zoo"

The development of species specific protocols for assisted reproductive technologies (ARTs) holds tremendous potential for conservation of wild/endangered species (Shivaji et. al. 2003). Lack of basic knowledge about reproductive functions and non availability of minimum number of individual animals of wild/endangered species is the one of the major concern to develop ARTs for species concerned (Andrabi and Maxwell, 2007). Preservation of species by cryobanking of semen, oocytes, embryos, tissues, and cell lines (genetic resource banks: GRBs) are also have potential implications in conservation (Wisely et. al. 2015). Regeneration of extinct Pyrenean Ibex (mountain goat) from cryopreserved cell lines using somatic cell nuclear transfer technology (SCNT) has indicated the importance of genetic resource banks in conservation programs. The ability to recover and preserve spermatozoa and oocytes (Rao et. al. 2010; 2011; 2013; 2015; Mahesh et. al. 2011), tissues and cell lines (Mahesh et. al. 2012; 2016) from wild/endangered animals which have died due to accidents or medical reasons is the one of the promising approach to build basic biology of gametes and establishment of GRBs for further development of ARTs. In addition to SCNT, conversion of somatic cells into pluripotent stem cells would give an additional strength to the existing reproductive technologies to increase the population size of the wild/endangered species.

Developing cell lines from the tissues of live or dead animals and preservation at sub-zero temperatures is a prerequisite for regeneration of any species. It is a routine practice in various laboratories for livestock species. In all these cases the tissues were processed immediately after collection. However, the processing of tissues immediately after collection of tissues, is not possible always, either from live animal or dead animal. In wild animals, developing cell lines from *post-mortem* tissues is a challenging task due to the delay in reaching of experts to conduct *post-mortem*. During this delay the tissues undergo various degenerative changes which may cause loss of tissue integrity that leads to cell death. In addition to *post-mortem* delay, transportation of collected tissues from field to laboratory for further process increases tissue damage, if the tissues are not transported in appropriate conditions. The detailed procedure for collection and transport of tissues for developing cell lines and preservation of oocytes is given below.

Materials required

1. Disinfectant/soap for cleaning of sampling area.
2. Shaving blades to remove hair on sampling area.
3. 70% alcohol for surface sterilization of sampling area.
4. Sterile tissue forceps, scissors and surgical blades for excision of tissues.
5. Sterile media to preserve tissue: Phosphate buffered saline /Dulbecco's Modified Eagle's medium/Normal Saline.

6. Sterile test tubes/vials/containers with sterile media to preserve tissues.

7. A cool box for sending tissues to the nearest laboratory.

Source of samples

In live animals, tissues can be obtained by excision of the hanging tissues from the injuries caused by accidents. A small piece of skin can be excised from ear, neck, flank or lower abdomen by sterile scalpel blade, scissors or biopsy needle under anaesthesia. The tissues can also be obtained from dead animals at the time of *post-mortem*.

Procedure

1. The skin samples should be collected as early as possible after the death of animal to preserve the tissue integrity. It is always better to shift the whole carcass to low temperature areas, such as cold room (4ºC) as early as possible.
2. Ear is the easiest place to collect skin tissue. But ear contains cartilage which limits the attachment of skin explants to the culture dish/flask. Thus the flank region or lower abdomen is the other choices for collection of skin sample.
3. The sampling area should be cleaned with disinfectant and sterilized by 70% alcohol before excision of the tissue.
4. The tissue samples should be excised using sterile tissue forceps and surgical blade or scissors.
5. After excision, tissue sample should be washed 3-4 times in sterile Phosphate buffered saline (PBS), once in 70% alcohol (30 sec) and again 3-4 times in PBS before being transferred into the test tube.

6. The containers or tubes that carry tissue should be labeled properly with the following information: Date and place of the collection, species, animal Id/name, age, sex, cause and time of the death.
7. Tissues should be preserved in appropriate medium immediately after collection. Phosphate buffered saline (PBS), Dulbecco's Modified Eagle's medium (DMEM) are commonly used media to preserve the tissue samples. PBS is available as tablets (1 tablet for 200 ml) and it is very easy to prepare. Normal saline also can be used for preservation of tissues. All the media should be supplemented with antibiotics (Gentamycin solution (0.1mg/ml).
8. If the medium is not readily available especially in field conditions, tissues (skin or ovaries) should be placed in sterile polythene zips lock cover and the cover should be kept in cool place.
9. Tissues should be kept in refrigerator before sending to laboratory.
10. Tissue should be sent to the nearest laboratory as early as possible for further processing.
11. During transportation the tissues should be placed in a cool box that is maintained at 4-8ºC.

7. Collecting Samples from Birds for Molecular Sexing and other Diagnosis

For genetic studies on birds such as molecular sexing, DNA can be obtained from various sources as discussed below. Method to be adopted would depend on size of the bird, biology of the bird and whether it is free ranging or captive bird.

DNA from Avian Blood

Bird blood is nucleated and contains a lot of DNA when compared to un-nucleated mammal blood. Thus sufficiently high quality and quantity bird DNA from blood samples can be obtained using relatively simple extraction techniques.

Blood for birds can be obtained from various body parts. Most common practice is to collect blood from Brachial vein. Amount of blood drawn would depend on size of bird. In any case, not more than 1% of body weight should be collected. For a sparrow sized bird (weight: 20-30g) 20-30 microlitre of blood should be sufficient.

Source: Sheffield Molecular Genetics Facility Protocols
(https://www.sheffield.ac.uk /nbaf-s/protocols list)

To collect blood from brachial vein, hold the bird on hand as shown in the image, extend its wing and wipe underwings with 70% ethanol. This is to disinfect the skin as well as to make brachial vein visible. Using a lancet/syringe a fine prick is made on brachial vein and blood drop which oozes out can be collected via fine capillary. Blood from the Capillary can be blown into 1.5 ml vial containing absolute ethanol.

Collected blood can be stored in several different buffers though ethanol is easily available solvent for storing blood. Once blood is mixed with ethanol it can be stored at room temperature. If Analytical Reagent grade ethanol is not available DMSO (Dimethyl Sulfoxide) can be used. However; DMSO is toxic and strict precautions are required when dealing with this buffer.

Approximately 50 microlitres (one drop) of blood should be added directly to 1ml (1000 microlitres) of absolute ethanol (must be Analytical Reagent grade) in an eppendorf. The screw top should be securely sealed and then the tube shaken well to mix the blood and ethanol. Eppendorfs are then stored at room temperature. The ratio of blood to alcohol should not exceed 1 in 10 - this also applies when collecting tissue.

The eppendorfs used should be the type which have rubber sealed lids. The eppendorfs and their lids should be clean and dry and need to have been autoclaved to ensure they are sterile. After adding ethanol and the blood sample, ensure the lids are secured as tight as possible. Otherwise, the ethanol will evaporate. Use autoclaved time tape (or autoclave tape) to label the eppendorfs and ensure to use a permanent marker to label the tube. If transporting eppendorfs containing ethanol and

blood, the lids should be double wrapped with parafilm to prevent leakage. It is important not to contaminate the blood sample with blood from other birds or with your own human DNA. So one should not blow blood out of capillary tubes. Blood can be released into the eppendorf by taking finger off the end of the capillary tube and letting it flow out gently with gravity or else a little puffer that fits over the end of the capillary tube can be used to blow blood into the vial.

Bird corpses

Blood is also the best sample to collect for DNA extraction from dead birds (if the bird is freshly deceased or has been frozen soon after death). If no blood is available, brain or liver are the best tissues to collect from bird corpses. The tissue should be diced and stored in ethanol as above (i.e. Analytical Reagent grade ethanol, not more than 1 to 10 ratio of tissue to ethanol and stored in rubber sealed tubes).

Feathers

The technique requires that a few feathers are plucked, and then preserved in 70% ethanol at room temperature. One advantage of this procedure is the ease with which samples can be collected and sent by post. Feathers with large rachises or vanes (contour or flight feathers) will yield the highest quantities of DNA, while freshly plucked feathers provide the highest quality DNA. Moulted feathers provide DNA with varying degrees of quality and quantity. Those feathers with no visible signs of degradation, a transparent calamus and intact barbs on the vane will yield the best results. DNA can be extracted from two parts of the feather:

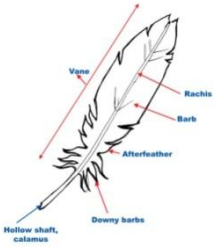
1) 5-10mm of the basal tip of the calamus

2) a small, usually visible blood clot found where the vane meets the calamus (superior umbilicus).

Higher quantity and quality DNA can usually be obtained from the latter. Where possible, several feathers (2-3) should be taken from each individual. Feathers from different nests or birds should be stored in separate bags/envelopes. Feathers do not need to be frozen and can be stored dry at room temperature.

Sampling procedure

With clean forceps, a few feathers are plucked with a single motion from the breast or from the top of the head, and placed into a sterile cryotube containing 70% ethanol. To avoid possible contamination, the feathers are plucked only with forceps, and the base must not be touched. Samples can be stored for several months at room temperature (or, more preferably, at 4°C).

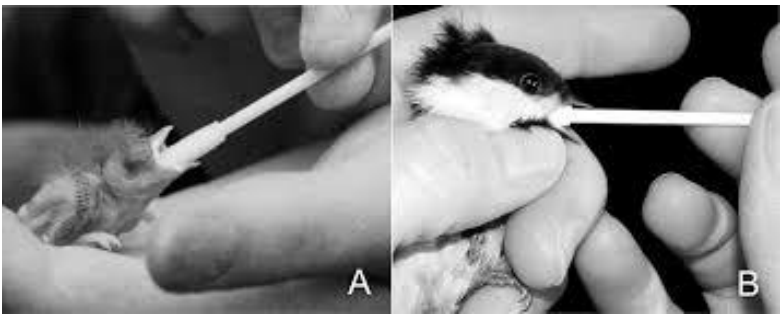


Source: Ornithol Sci 5: 139–143, 2006

Avian Buccal cells

Buccal cells can be used as a non-invasive sample for molecular studies in ornithology because sampling buccal cells is an easily performed and safe method taking less time than

sampling blood. It involves collecting buccal epithelial cells by holding the bird in one hand and gently rotating a sterile foam-tipped buccal swab with the other hand 3–5 times against the inner cheeks and across the tongue. Each sample require only 5–10 seconds to collect. Wash hands with an alcohol-based disinfectant before handling birds.



Source: Marrero et al. Biol Res 42, 2009, 147-151

Feces and regurgitated seeds

Avian DNA can also be extracted from alimentary tract cells found on external surfaces of feces and regurgitated seeds. Fecal samples from a target species contain epithelial cells from the digestive tract walls, but also other contaminant DNA, such as those from bacteria and prey. In herbivores, secondary compounds from plant foods also represent a source of inhibition of the PCR enzymatic reaction.

Fresh fecal cells and regurgitated seeds can be collected from roosting areas of bird. Transparent plastic sheets can be placed under perches, and only recent and fresh samples are to be collected and carefully stored at -20ºC prior to genetic analysis.

8. Collecting Samples from Reptiles for Molecular Diagnosis

How much Blood is needed?

As a general rule the blood volume for reptiles is thought to be between 5-8% of the total body weight. That means a 100 gm reptile will have 5-8 ml of total blood volume and approximately 10% (0.5-0.8 ml) of that total volume can safely be removed from the reptile of interest without harming it. Reptile blood is nucleated and contains a lot of DNA, therefore, small quantities of blood can yield sufficient quantity of DNA.

What are the sites for blood sample collection?

Common sites for blood collection in reptiles include the ventral coccygeal vein of the tail and cardiocentesis for snakes, ventral coccygeal vein, jugular vein and abdominal vein for lizards. In chelonians it includes the jugular vein, dorsal coccygeal sinus or brachial vein. For Crocodiles the ventral coccygeal vein and dorsal occipital sinus are the sites for venipuncture. Ventral Coccygeal Vein is most common, preferred and convenient site for venipuncture in reptiles is ventral coccygeal vein, located on the ventral midline of the tail between the coccygeal vertebrae.

Procedure of blood collection

Venipuncture site should be cleaned and aseptically prepared with 70% alcohol prior to blood collection. For medium and large reptiles, a 25-gauge needle of length 1–1.5 inch is attached to a 3-ml syringe and for small reptiles, a 27-gauge needle of length 0.5 inch is attached to a 0.5 to 1 ml syringe is used. The ventral coccygeal vein can be accessed either ventrally or laterally. Preferred site for blood collection is 1/4 to 1/2th

distance from the vent to the tip of the tail. Whether the approach is ventral or lateral, the needle should be inserted along the length of the tail at least 20% and no more than 80% of the distance from the base to the tip of the tail (or approximately 5 cm posterior to the vent). It is important to access the vein at a location caudal to the cloaca to avoid damaging the hemipenes in males. Needle placement is at a 45 degree angle between ventral scales and passed to the point of the vertebra. As the needle comes in contact with the bone, aspirate gently until blood flows into the syringe. The lateral approach to the same vein at the same distance on the tail, but the needle is placed laterally on the tail where there is a depression between the muscles of the tail and directed medially at a 45 degree angle toward the coccygeal vertebrae. For this lateral technique the needle will come in contact with bone again (lateral spinous process) where the needle is then guided just ventral to the bone until blood is noted in the syringe on gentle aspiration. For chelonians it is difficult to collect blood samples due to their ability to pull their bodies into the protective shell. In that case the tail is held and extended out and the needle is inserted on the dorsal midline in a cranial direction. The needle will contact the vertebrae and with slight aspiration blood will flow into the syringe. The blood is collected in 1.5ml tubes with lithium heparin. Anaesthesia is not necessary to collect blood from reptiles. Physically restraining the animal would be sufficient.

Collection of feacal samples

Chelonians, geckos, snakes and lizards can be kept individually inside a large airy cotton cloth bag for about 12 h, during which they usually defecate. The faeces of specific individuals can be collected and dried under a 40 W incandescent

lamp or oven at 40°C for 12 hours. The dried material can be stored in paper bags that are properly labelled. These paper bags can be kept inside an air tight container with silica gel at the base to keep the container free of moisture. The chances of extraction of DNA from these samples are extremely low, However, they can be used for hormone level monitoring.

Skin samples

Reptiles produce keratins on the skin, and they are resistant to degradation due to the rigidity added by disulphide bridges formed by the cysteine residues. Reptile scales or scutes grow in a very short time before they become metabolically inert. Therefore, the isotopic values of scales and scutes will reflect the animals' diet at the time of formation. For this purpose, isotopic values obtained from keratin molecules from feathers and scales are compared to the isotopic values obtained from the animal's diet. Notching is a common used technique to mark crocodiles and turtles in captivity to enable individual identification. Scute clippings can be obtained for the above analyses from the single-scuted and double-scuted portion of the tail and three caudal scutes are collected from each of the crocodile sampled. Crocodilians, in general, are hardy and studies have shown that caudal scute clipping do not have any adverse effects on them.

In the case of snakes, fresh slough skin yield reasonable amount of DNA. Fresh slough skin can be stored in a sterile plastic pouch or air tight box with silica gel for several months at 4°C.

9. Collecting Swab Samples from Amphibians for Disease Diagnosis

Swabbing is a non invasive and sensitive method for detecting chytrid fungus. For each frog, a new sterile cotton swab should be taken and 70 strokes should be done on frog's body (10 strokes over each of the following frog parts: dorsal surface, groin to armpit, ventral surface and underside of the thighs, five outward strokes on the underside of each foot

(**Fig. 10**).

What are the essential data required?

A	B	C	D	E	F	G	H	I	J	K	L	M
Serial Number	**Sample ID**	**Location**	**Name of collector**	**Elevation**	**Temperature (°C)**	**Date**	**Habitat**	**Genus**	**species**	**Snout_Vent Length**	**Weight(g)**	**Skin Deformitie**
MM0001	Mumi0001	Munnar	Milind Mutnale	1490	15	09-10-2014	Stream	Ghatixalus	asterops	24.5	2.3	No

A) Serial No: Start with Your initials and 0001 (For example, if your name is Robert Frost then your first sample should be labelled as RF0001).

B) Sample ID: This is a combination of columns A, B and C. First two alphabets of the C and B and the number on A will be the Sample ID.
C) Name of collector: Please enter your full name.
D) Location: As accurately as possible.
E) Altitude: Elevation in feet if available, if not try to give an estimate. Let this be a range. This will let us know that it is an estimate.
F) Max and Min Temp: The maximum and minimum temperatures on the day of collection in degrees Celsius, if available. If not remember to give a range of temperatures.
G) Date: Day, Month and year as per the collection.
H) Habitat: please use what works but whatever you do make sure that you provide us with a legends and that you remain consistent across all your samples.
(For example, secondary forest or eucalyptus plantation, or riparian zone) Since there are many possibilities, make around 5-6 types and then roughly distribute samples into them.
I) Genus: Give genus name.
J) Species: Give species name if sure, if not say and give the name of the species you feel that it might be. In such cases, take good pictures of the frog and send to experts for identification. Please do not try to guess, as this information is very crucial.
K) Snout-Vent length: Give the value of length from the snout to the cloaca of the frog in mm.
L) Weight: If possible weigh the individual and present data in grams.

M) Skin deformities: If present, type "yes" and give details, if not just type in "no".

Figure 10: Swabbing protocol for frogs

Culturing protocol for Bd from Toe clip

It is essential to culture Bd for identifying the strain of fungus responsible for infections. This step though tedious is very important in Bd infection studies. For this, wear gloves and hold the frog firmly at the top of its back legs. Clip off the top 1-

2mm of the 4th hind toe (counting from the inside to outside) using a sterile dissection scissors. The cut on the toe can be treated with Betadine™, and the frog should be kept for observation for one hour in a clean box with a wet tissue paper lined at the bottom. After one hour the frog can be released in the wild or into the enclosure. Change gloves or thoroughly wash/disinfect hands between each animal. If working in the field, place the toe in an agar plate for transport before moving onto step. Toes from the same location and species can be put on the same plate. Clean the toe *very thoroughly* by using the sterile needle to drag the toe through the mTghL + antibiotic agar, ensuring all of the toe has come into contact with the media, and moving the toe up and down through the body of the media. This is to remove contaminating bacteria and fungi on the skin of the toe. Up to 3 toes can be cleaned per plate. Place the toe in a sterile tube containing liquid media and preferably store in a cool and dry place. For each tube record: Species (if known), Location (as specific as possible) and any other additional notes (male/female/juvenile; any outward signs of illness etc). Transfer the toe clips in sterile cell culture plate having mTgL broth media and incubate the plates at 18°-20°C. Observe the plates after interval of 4-5 days and look for motile zoospores. Change the 2/3 liquid media every week and look for motile zoospores. This process should be repeated for a period of 8 weeks or until motile zoospores are visible.

Preparation of mTGhL agar plates with antibiotics:

Weigh 8g Tryptone, 2g Gelatin hydrolysate, 4g Lactose, 10g Agar into a 1 liter flask. Add 950ml of distilled water and autoclave it. Weigh out: 0.2g (200 mg/L) penicillin-G o 0.2g (200

mg/L). Streptomycin sulfate into a 50ml falcon tube. Add 50ml of distilled water and mix well. After autoclaving, place the media on magnetic stirrer. When the solution reaches ~50° C move it to a laminar hood and filter in the antibiotic mix through a 0.2 micrometer millipore filter. Mix for another 2 minutes on the magnetic stirrer. In a laminar hood, fill petri dishes to 2/3 full with media, leave the lids off to allow the media to set.

Preparation of mTGhL media broth tubes:

Weigh 8g Tryptone, 2g Gelatin hydrolysate, 4g Lactose in to a 1 Litre flask. Add 950ml of distilled water and autoclave it. Weigh 0.2g (200 mg/L) penicillin-G o 0.2g (200 mg/L) streptomycin sulfate into a 50ml falcon tube. Add 50ml of distilled water and mix well. After autoclaving the mixture it is placed on a magnetic stirrer. When the solution reaches ~50°C remove it to a fume hood and filter the antibiotic mix through a 0.2 micrometer filter. Mix for another 5 minutes on the magnetic stirrer. In the fume hood, fill the screw topped tubes 2/3 full of media.

10. Collection of Samples for Disease Diagnosis in Wild Animals

Disease has not traditionally been considered as a significant driver of species extinction but it can also cause, directly or indirectly, decline in wild populations (*Smith et. al. 2008*). Wild animals serves as reservoir for transmission of infectious diseases to humans as well as domestic animals (*Kruse et. al. 2004*). Approximately sixty percent of the human pathogens (1415) are zoonotic in nature and seventy percent of these zoonotic pathogens were originated from wildlife (*Taylor et. al. 2001*). Early detection of diseases in wild animals is very important for management/prevention of disease outbreaks in wild animals as well as emerging infectious diseases in human. The major limitations to diagnose wild life diseases are lack of information and availability of biological material from affected animals (*Smith et. al. 2008*). Thus collection and preservation of biological samples from free living and captive wild animals are very important to know the epidemiology of the diseases.

Determination of the cause of death in zoo animals is often difficult and may require number of investigations such as biochemical, bacterial, fungal, viral, parasitological and toxicological, and histopathology. These investigations require fresh samples/specimens of the affected live animals or dead animals to get accurate diagnosis. In live animals, blood, exudates of vesicles, scab, saliva and secretions of nasal, eye, genital organs, fecal materials and urine of infected animals should be collected for preliminary diagnosis. It is always

preferable to collect samples from infected animal or terminally sacrificed animal for diagnostic purpose.

In many times it is not possible to conduct a post-mortem immediately after death of animal. To prevent postmortem related autolytic changes, the carcass should be shifted to cool places immediately after the death is noticed. In small bodied animals the entire body should be packed in plastic bag covers and stored in a refrigerator. Air inside the plastic cover should be removed before packing. In big animals, samples should be collected as soon as possible because shifting and storage at low temperatures of large bodied animals is difficult. Systematic collection, storage and shipping of collected material increase the quality of diagnostic results tremendously. Samples should be collected as soon as the animal has died. Samples should be sent to the diagnostic laboratory as soon as possible under appropriate conditions with the following information: Place and date, species, animal id/name, sex, age, type of the sample and name of the preservation medium, if used. Detailed clinical history of the animals and a copy of the post-mortem findings should be enclosed with the samples.

Histopathological Examination

Post-mortem changes occur rapidly in the tissues or organs after death so specimens or samples collected for histotopathology should be fixed as soon as possible to preserve normal tissue integrity and contamination. The samples collected for histopathological examinations should be preserved in 10% neutral buffered formalin (10 times the tissue

volume). A small piece (0.5 cm^3) of all tissues should be taken, including portions of spleen, liver, lungs heart muscle, kidney, brain and lymph nodes. The affected organs and tissues and its associated lymph nodes should be collected. Tissues should be collected using a sharp knife/surgical blade/razor blade. Both the normal and abnormal (lesions) portion of the organ or tissue should be included while cutting the tissue piece. Loops of the gastrointestinal tract should be taken immediately after opening the abdomen to minimize contamination of post mortem invaders. Cut open the loop of the gastrointestinal tract longitudinally before it is transferred into formalin solution to penetration of the formalin.

Microbiological Specimens

Specimens for microbiological examination should be collected as aseptically as possible. The instruments used for collection of samples should be autoclaved or should be boiled for minimum 15 min or swabbing with 70% alcohol and then flaming until red hot before using them. All the samples intended for bacteriology should be placed in sterile containers separately and refrigerated immediately after excision of tissue. The swabs from exudates and scrapings of external lesions from the live animal should be collected. It is recommended to sear the surface of the organ or tissue with a flame heated spatula before incise the organ/tissue with sterile surgical blade and collect the material from the deeper portion of solid organs, abscess, or coagulated masses. Tissue smears should be also made from the fresh cut sections of all organs. Tissue smears of liver, lungs or lymph nodes may be used for the identification of protozoal parasites. The tissue smears should be dried

quickly in the air and wrapped in paper or a box to exclude insects. Sterile cotton swabs should be used to collect samples of feaces, pus, heart blood and other body fluids. These swabs should be transported under refrigerator or in the appropriate medium. Tissue fragments, and aspirates from body cavities should also be collected in a separate sterile containers and stored and transported under refrigeration for culture. The gastrointestinal tract are best handled by obtaining a loop tied at both ends and placed in a sterile polythene covers.

Viral

It is always preferable to collect the samples of live infected animals such as whole blood, serum, plasma, body fluids (saliva, urine, semen, milk, nasal, eye and vaginal secretions), sterile swabs of throat, rectum, ulcers, vesicles, eye secretions, cervical, genital, and skin lesions. Some viruses are very fragile and decompose rapidly after death of the host. Others are more robust and persist for a considerable time. Tissue pieces of spleen, lung, kidney, liver and associated lymph nodes should be collected as soon as possible after death and placed in a sterile container with a transportation medium. Feacal material (2-4 g) should be placed in a sterile container. The swabs of exudates or secretions of vesicles, ulcers should also be collected. Materials intended for virological studies should be collected as aseptically as possible and transported under refrigeration. If the refrigeration facilities are not available, they can be stored and submitted in 5 to 10 volumes of sterile 50% buffered glycerin solution.

Parasitological

During postmortem examination ectoparasites and endoparasites should be collected for identification. Ectoparasites should be collected as soon as before or after death of animal because these parasites leave the dead animal as it begins to cool. Ticks, fleas, lice and mites should be carefully removed from the fur/hair using a brush and preserved in 70% ethyl alcohol or methanol. Live ticks may be required for identification of some infectious agents. Mange mites should be collected by scrapping the affected skin deeply and put the scrapings in a glass slide with a drop of mineral oil. Roundworms and tapeworms should be collected from intestinal segments and placed in 70% ethyl alcohol or methanol immediately after collection. The scolex is important for species identification of tapeworms so it should not remove them from its attachment to avoid detachment of scolex. Both mature and immature segments should be included for tapeworm. Deep scrapings of affected intestinal mucosa may be useful for the diagnosis of coccidial infection. For nematode eggs the collection bottle should be filled with feces and tightly packed down to exclude air. The nematode eggs will not hatch in the absence of air. Samples can be refrigerated to further delay maturation of the nematode eggs.

Blood

It is always preferable to collect blood from live animals. Blood should be collected separately for biochemical analysis (serum, plasma), microbiological and molecular (DNA and

RNA) diagnosis. Use scalp vein sets or intravenous catheters for blood collection to avoid multiple venipunctures. Wet and thin blood smears should be made for hemoparasitic examination at the time of blood collection. Blood smears should be dried quickly, labeled and wrapped in a clean paper or placed in a box. Thin smears should be fixed immediately by dipping in methyl alcohol but thick smears should not be fixed. In some cases, blood samples may still be obtained from the heart before dissection of the heart chambers, if the animals have died three to four hours before collection of samples. The whole blood samples can be stored in refrigerator (4.0° C) for short time (1week). Do not freeze blood samples. Collect the blood into serum separated vacutainers for serum collection. Serum and plasma samples should be stored at 4° C before shipping to laboratory and shipping should be done in containers maintained at chilling temperature.

Swabs

Swabs are generally used to collect cells and fluid from the pharynx, nasal passages, throat, rectum, ulcers and vesicles, eye secretions, cervical, genital and skin lesions. Swab each collection site with a separate sterile swab and place each swab into separate tubes. Swab samples can be stored in the refrigerator (4°C) for up to one week. Swab samples can be stored frozen (≤ 60.0 ° C) indefinitely. Swabs must be sent in a sterile viral with appropriate transport medium such as phosphate-buffered saline or physiologic saline in chilled.

Serology

Generally, the blood for serum separation is collected from the veins of a live or recently killed animal. Blood for serum separation should be collected as aseptically as possible in sterile glass or plastic vials. Should not apply too much vacuum in the syringe while drawing blood, it causes hemolysis. Do not agitate, freeze, or heat blood to avoid hemolysis. Nowadays serum separation vacutainers are availble in the market. In the field, serum separation can also be possible from the heart blood of an animal which has died recently (2-3h). In these animals the heart is often a good source of a mixture of blood clot and serum. Such mixtures can be separated by centrifugation or sedimentation in a refrigerator. Serum collected from dead animal should be refrigerated immediately to avoid bacterial contamination. The cervico-vaginal mucus agglutination tests for vibriosis, brucellosis and trichomonosis do not require serum in such cases mucus should be collected by pipette or tampon and placed in a sterile tube and refrigerated. If serum is not available, a minimum of 50 g of lung tissue can be collected and frozen.

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