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Topic of General Interest
Wildlife Medicine

Veterinary disease-oriented Biobanking for biomolecular analysis based on frozen tumor biopsies, cell culture and forensic tissues[1]

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ABSTRACT.- Montoya-Flórez L.M., Missen-Tremori T. & Rocha N.S. 2024. **Veterinary disease-oriented Biobanking for biomolecular analysis based on frozen tumor biopsies, tumor cell culture and forensic tissues.** *Pesquisa Veterinária Brasileira 44:e07292, 2024.* Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional de Colombia, Grupo de investigación en Patología, Bogotá, Colombia. E-mail: lmontoyaf@unal.edu.co

Collecting and analyzing biological specimens leads to unprecedented opportunities for assessing and understanding the complex networks of interactions between biomolecules, and the functional and clinical consequences of their alterations. In this context, the Tissue Bank plays a key role in life science research and development, making its implementation in veterinary medicine essential for developing new research, especially in the forensic and tumor fields. In total, 52 tissues were collected, 15 forensic samples, 25 neoplasms, and 12 cell cultures. The storage, freezing, data management protocols, and the quality of these samples were analyzed. In the same way, the quality of the RNA and DNA in the short and long term was evaluated. The quality of the genetic material was confirmed by polymerase chain reaction. For data comparisons, non-parametric tests were used, with a significance level of $p<0.05$. The measures adopted in the Tissue Bank for obtaining and handling the samples and for controlling and guaranteeing the quality were considered adequate, as they enabled optimal preservation of the integrity of the genetic material, a relevant factor for the use of tumor samples and forensic samples stored in the Biobank, thus contributing to future molecular research in the area of pathology and investigations of crimes against fauna.

INDEX TERMS: Crime, cancer, molecular, Biobanking, tissue bank.

RESUMO.- [**Biobanco orientado nas doenças veterinárias para a análise biomolecular com base em tecidos congelados, cultura celular e tecido forense.**] A coleta e análise de espécimes biológicos é necessária porque abre oportunidades sem precedentes para avaliar e conhecer as complexas redes de interações entre biomoléculas, e as consequências funcionais e clínicas das suas alterações. Nesse contexto, o Banco de Tecidos desempenha um papel fundamental na investigação e desenvolvimento das ciências da vida, tornando a sua implementação na área da medicina veterinária essencial para o desenvolvimento de novas pesquisas em especial na área forense e tumoral. Foram coletadas 52 amostras de tecidos, 15 de amostras forenses, 25 neoplasias e 12 culturas celulares. Nesses tecidos, foram analisados os protocolos de armazenamento, congelamento, gerenciamento de dados e qualidade das amostras. Da mesma forma, foi analisada a qualidade do RNA e do DNA a curto e longo prazo e a qualidade do material genético foi confirmada pela reação em cadeia da polimerase. Para comparação dos dados, foram utilizados testes não paramétricos, com nível de significância $p<0,05$. As medidas adotadas no Banco de Tecidos para obtenção e manuseio das amostras, controle e garantia da qualidade foram adequadas, pois possibilitaram a preservação ideal da integridade do material genético, fator relevante para a utilização de amostras tumorais e forenses armazenadas no Biobanco, contribuindo assim para futuras pesquisas moleculares na área de patologia e investigação de crimes contra a fauna.

TERMOS DE INDEXAÇÃO: Crime, câncer, molecular, Biobanco, banco de tecidos.

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INTRODUCTION

The Biobank or Tissue Bank (TB) is an organized bio-repository in which responsible technicians and pathologists collect, catalog, store, and make available tissue samples, tumor cells, and biological fluids, which may be of neoplastic or non-neoplastic origin and that can later be used for current and future translational research (Hewitt & Watson 2013, Liu 2014, Doucet et al. 2017, Coppola et al. 2019, Diffalha et al. 2019, Lhousni et al. 2020, Müller et al. 2020, Dagher 2022).

In animals and humans, biological specimens have been used for decades to investigate disease pathogenesis, test scientific hypotheses, and assess biomarkers identified in experimental studies. Unfortunately, a significant roadblock to these investigations is the lack of available quality animal biospecimens in veterinary medicine. TB is a possible solution in this context, especially in the era of personalized and precision medicine. Collecting and analyzing biological specimens leads to unprecedented opportunities for assessing and understanding the complex networks of interactions between biomolecules, and the functional and clinical consequences of their alterations. For this and other reasons, TB plays a key role in life science research and development (NCI 2016).

TB requires the organization of adequate protocols for the collection and handling of samples, design of research, and teaching and assisting academic processes within universities (Shickle et al. 2010, Hewitt & Watson 2013, Liu 2014). Thus, the function of a Biobank is to facilitate the collection, preservation, storage, and supply of material for use in research and the clinic (Simeon-Dubach & Watson 2014, Haregu et al. 2019, Lhousni et al. 2020). In human medicine, the protocols for harmonization are standardized and validated, guaranteeing high-quality biosamples; in veterinary medicine, especially in developing countries, the introduction of these procedures or protocols is not uniform, and it is necessary to standardize the processes of quality assessment, consent, sample collection, and storage in order to guarantee the availability of high-quality samples, favoring research advances.

Biobanks are not recent but are considered one of the ten solutions that have transformed scientific research worldwide (Park 2009). This is because, at present, there is a relevant need for research projects to provide high-quality materials that facilitate the answers to scientific questions, as well as to guarantee a sufficient number of samples from a statistical point of view (Paskal et al. 2018, Lommen et al. 2020, Dagher 2022). In this sense, TBs play a fundamental role in the era of precision medicine (Carey et al. 2016, Coppola et al. 2019, Kim & Milliken 2019, Dagher 2022, Parra et al. 2022), since the absence of a sample with adequate quality and quantity condemns the study from the beginning, while its presence guarantees the essential and appropriate raw material to answer the scientific questions (Dhir 2008, Liu 2014).

There are two types of TBs: Population-based and disease-oriented (Paskal et al. 2018, Coppola et al. 2019). As a rule, samples of biological tissues, RNA and/or DNA, and liquids, among others, are confidentially stored in the TB (Shickle et al. 2010, Hewitt & Watson 2013, Majidzadeh-A et al. 2013, Coppola et al. 2019). Surgical samples are acquired from surgical procedures such as biopsies, and fluids from cytopathological exams, as well as from necropsies, sent by the Pathology Service (Oosterhuis et al. 2003, Balaguer et al. 2006, Carvalho et al. 2007).

It is important to emphasize that fragments of altered or normal tissues are used to satisfy diagnostic procedures, and the residual sample is the input stored in the TB, making it a valuable resource for future research (Riegman et al. 2006).

Tissue storage has already been implemented in human medicine as a tumor bank (Simeon-Dubach & Watson 2014), which allows the replacement of cell lines and experimental animals with tumor samples in cancer research (Livolsi et al. 1993). In the same way, it is currently used for the development of the cancer atlas, classification of tumor subtypes, specific therapies with antibodies (Weinstein et al. 2013, Coppola et al. 2019), and infectious diseases such as Ebola (Hayden 2015, Paskal et al. 2018). It is proposed as an essential tool in responding to pandemics (Miyauchi et al. 2020). This is due to the possibility of applying genomic techniques, such as gene sequencing, polymerase chain reaction (PCR), microarrays, transcriptome, proteomic studies, and other "omics" in order to obtain a molecular signature that aids in the identification of the stage of the disease, the individual's prognosis, and individual or collective adjustments to treatments (Brimo et al. 2012, Foster 2011, Liu 2014, Dagher 2022).

The first country to implement TB was the United States in the 70s. This was followed by other high-income countries, such as Spain, Croatia, the United Kingdom, the Netherlands, Germany, and Portugal (Carvalho et al. 2007), and their implementation has recently occurred in developing and underdeveloped countries (Mendy et al. 2014). In Latin America, the National Cancer Institute (INCA) of Brazil implemented a TB, with the following tumors selected for storage: Esophagus, lung, prostate, head, and neck; in addition, other experiences of creating TB have been reported in other countries in the region (Campos et al. 2017).

In veterinary medicine, techniques established in the human field can also be used and extrapolated in order to constitute a fundamental database for the development of genomic, proteomic, or other "omics" analyses, together with bioinformatics and big data analyses, thus promoting high-quality scientific research and the effective transfer of information (Grizzle et al. 1998, Carvalho et al. 2007).

When one intends to implement a TB in oncology, it is necessary to understand several ethical concepts and the biology of the disease; for example, from a legal point of view, in human medicine, a Biobank is only started when the patient has been clarified and informed as a tissue donor (Simeon-Dubach & Watson 2014). A TB can help in neoplastic situations, ranging from biological behavior to therapeutic protocols (Carvalho et al. 2007).

In the veterinary area, neoplastic processes are currently gaining more relevance because, in pets, one of the main current causes of mortality is cancer. This is because pets have started to lead longer lives than previously due to the improvement in the quality of life, availability of veterinary medical care, more sophisticated medicines, and industrialized feed, among others (Dobson 2013, Salas et al. 2015, Stromberg & Meuten 2017); which leads to pets becoming more vulnerable to senile diseases, such as tumors. This area requires ongoing study to develop a better diagnostic and therapeutic approach (Simeon-Dubach & Watson 2014). In this sense, a TB can facilitate the validation of molecular pathways in a large-scale study and their validation from the point of view of translational medicine.

Tumor development is a multistep process, progressing from normal tissue to a neoplastic lesion, which may or may not be malignant. In this sense, there is a need to obtain tumor tissues at different stages of carcinogenesis in order to decipher the molecular mechanisms of each stage of tumor progression (Gerlinger et al. 2012). Thus, in current practice, a TB constitutes an indispensable instrument (Balaguer et al. 2006).

Regarding forensic tissues in legal veterinary medicine, this field includes a wide range of areas, such as environmental conservation, animal identification, genealogy, combating smuggling and illegal trafficking of animals, injuries resulting from mistreatment and other crimes against animals, products of animal origin, and expertise in general. In this sense, a TB can be very useful in properly storing the biological samples needed in cases of counter-evidence, new expertise, or even future investigations of a given case.

Establishing a TB involves the standardization of different protocols for collection, storage, and analysis, among others, that help in the homogenization of protocols in veterinary medicine, including providing some useful information from a comparative point of view in human medicine (Ransohoff & Gourlay 2010, Langhof et al. 2018). Considering the aforementioned and in order to serve as a subsidy for other veterinary research centers in the development of a TB, the current study aimed to unify protocols for collection, storage, data management, and quality analysis of TB samples.

MATERIALS AND METHODS

Ethical approval. The study was submitted to the Ethics Committee on the Use of Animals (CEUA) of the "Faculdade de Medicina Veterinária e Zootecnia" (FMVZ), "Universidade Estadual Paulista 'Júlio de Mesquita Filho'" (Unesp), of Botucatu and the "Sistema de Autorização e Informação em Biodiversidade" (System of Authorization and Information on Biodiversity – SISBIO), "Ministério do Meio Ambiente"; obtaining favorable opinions (Protocol No. 139/2015) and (Protocol No. 51628-1), respectively.

With the approval of the CEUA and the consent of the animal's owner, the excess material from the analysis was sent to the TB.

Animals and tissue collection. The project included the collection of transmissible venereal tumor (TVT) fragments, TVT cell culture (immortalized samples), and tissues from forensic examinations.

The population sample size followed the criteria established by Dohoo et al. (2009). In total, 52 samples were analyzed, 25 of which were TVT tumor tissues, 12 TVT primary cultures, and finally 15 samples of specimens from the following wild animals: howler monkey (*Alouatta* spp.), marsh deer (*Blastocerus dichotomus*), caiman (*Caiman crocodilus*), guinea pig (*Cavia porcellus*), forest fox (*Cerdocyon thous*), chinchilla (*Chinchilla chinchila*), nine-banded armadillo (*Dasypus novemcinctus*), opossum (*Didelphis albiventris*), agouti (*Dasyprocta aguti*), giant anteater (*Myrmecophaga tridactyla*), jaguar (*Panthera onca*), cougar (*Puma concolor*), southern tamandua (*Tamandua tetradactyla*), and tapeti (*Sylvilagus brasiliensis*).

Obtaining and handling samples, quality control and assurance. To standardize the collection, storage, and distribution of samples, and based on international standards and the International Society for Biological and Environmental Repositories (ISBER 2012, 2018), the following program was implemented:

Collection time: Maximum of 30 minutes, in order to minimize alterations in gene expression, until the time of freezing.

Material dissection: Use sterile equipment and materials, as well as disposable nitrile gloves, to conserve the integrity of the RNA that is particularly sensitive to environmental RNases.

Size of the samples: All samples were collected through an incision after total asepsis of the site, and fragments of approximately $1cm^3$ with the absence of necrosis, blood, fat, etc., were stored for macroscopic analysis. For the extraction and analysis of the quality of RNA and DNA, 1.5mL microtubes free of DNase and RNase were used. The selection of samples always followed the order of priorities: 1st Diagnosis, 2nd Prognosis, 3rd Investigation.

Identification of the sample: Before collection, using a permanent pen, each microtube was identified with a sequential number that allowed identification of the animal's number, collection date, and topography. After the sample was obtained, the registration forms were completed, with the patient's history data, for the entry of the material in the Biobank and tabulated in MS Excel®.

Freezing of the sample. The microtubes containing the samples for RNA and DNA analyses were quickly placed in 2L containers with gel at -10°C. The microtubes contained RNAlater Stabilization Solution (Life Technologies) for RNA conservation.

The samples frozen in the gel at -10°C were transported to the Biobank and stored in the freezer at -80°C in plastic boxes, with numerical sequences, in an ordered manner.

Freezer security system. The freezer has a CO_2 "backup" system and an alarm system that monitors any drop in temperature above or below 10% to prevent damage caused by temperature fluctuations or power outages.

Data management. A data management system that allows the identification and easy selection of samples when necessary to facilitate the selection and follow-up of samples for research was developed.

Preparation of technical operating protocol. Work instructions were created on the operational dynamics of the Biobank so that in the absence of a responsible person, another qualified person can replace them without prejudice.

Microscopic analysis of sample representativeness. The samples included in the study were processed using hematoxylin and eosin (HE) staining techniques and independently analyzed by two pathologists using a Carl Zeiss optical microscope, Lab.A1, Germany (Zeiss Axio Lab.A1) in 10x and 40x objectives, in order to define the quality of the tissue architecture.

Primary culture and karyotypes in transmissible venereal tumor. Once the diagnosis of TVT was confirmed in the patient, after anesthesia of the animal and total asepsis of the site, the tumor sample was collected by incisional biopsy of fragments of approximately $1cm^3$. All collections were performed before the animals received chemotherapy. The samples were stored in saline and phosphate solution (PBS) pH 7.4, in RNAlater (Qiagen) and buffered formalin until the material was processed.

Culture isolations were performed according to the protocol of the "Laboratório de Patologia Veterinária" (Laboratory of Veterinary Pathology), Unesp-Botucatu. For this, the tumor fragments were placed in a saline solution of PBS pH 7.4 and taken to the "Laboratório de Fertilização *In vitro* e Cultivo Celular" (*In vitro* Fertilization and Cell Culture Laboratory) of the "Departamento de Reprodução Animal e Radiologia Veterinária" (Department of Animal Reproduction and Veterinary Radiology) at FMVZ-Unesp, Botucatu Campus.

They were transferred to trypsin solution (TrypLE Select – Invitrogen 12563-029) at 37.5°C and kept for 60 minutes with a magnetic homogenizer. Next, the solution was centrifuged, the supernatant discarded, and the *pellet* resuspended and placed in $25cm^2$ flasks (Sarstedt – 83.1810.300) with 5mL of DMEM high glucose culture medium (Dulbecco's modified

essential medium – Gibco 11995-065), supplemented with 10% fetal bovine serum (FBS) (Gibco 12657-029), and with the combination of 100U/mL penicillin with 100mg/mL streptomycin (Gibco 15140) and 3µg/mL Amphotericin B (Gibco 15290).

Subsequently, the samples were incubated in a 5% CO_2 oven at 95% humidity, and a temperature of 37.5°C; cell viability and concentration were determined by the trypan blue exclusion test, and cells were resuspended in DMEM high glucose culture medium (Dulbecco's modified essential medium – Gibco 11995- 065).

It is worth mentioning that the immunocytochemistry (IHC) technique was used to verify the cells as being from TVT.

In addition, five samples were randomly selected for cytogenetic analysis at the "Laboratório de Genética e Melhoramento Animal" (Animal Genetics Laboratory) of the "Instituto de Biociências" (Institute of Bioscience), Unesp-Botucatu. For this, the supernatant was discarded, and the cells were detached, added to 5mL of hypotonic KCl solution (0.075M), incubated for 40 minutes at 38ºC, and then centrifuged at 1500g for 5 minutes. Next, the cells were washed several times in 3:1 methanol and acetic acid fixative solution until the supernatant was clear and resuspended in 0.5ml of fixative solution to prepare the slides. Three to four drops of the suspension obtained were placed on a clean histological slide and maintained in ice-cold distilled water. The prepared slides were dried at room temperature and kept in a refrigerator until the individual chromosomal analysis of each sample was obtained.

Cell count. After cell trypsinization, cell counts were performed using a NeuBauer Chamber with a Leica® DM IRB microscope.

Cryopreservation of cultures. For cryopreservation of cultures with two or three passages, a trypsinization procedure was performed with counting, and later, the number was adjusted to $1x10^6$ cells per mL. Then, using a solution with 10% dimethyl sulfoxide (DMSO) (Sigma C6164), 20% FBS, and 70% DMEM high glucose with penicillin, streptomycin, and amphotericin-B. The material obtained was distributed homogeneously and placed in cryotubes (Sarstedt 72,694,006) for 24 hours in a freezer at 80ºC and then placed and kept in a cryogenic cylinder (-196ºC) (Flórez et al. 2017).

Immunohistochemistry of transmissible venereal tumor. The slides were rinsed 3x with Tris pH 7.0 and incubated "overnight" with primary antibodies for anti-vimentin, anti-lysozyme, anti-alpha-1-antitrypsin, anti-CD3, and anti-CD79α. The reaction product was visualized using a polymer-based technology: HiDef® Detection HRP System (Cell Marque®). The slides were revealed with chromogen 3,3´-diaminobenzidine (DAB) and counter-stained with Harris hematoxylin. Immunocytochemical analyses were performed on cell cultures, which were first hydrated and permeabilized. Then, after blocking the endogenous peroxidase, immunochemical steps were conducted.

Negative controls were prepared by substituting specific primary antibodies with antibody diluent (Novocastra®). Positive controls were set with the liver for anti-lysozyme, anti-alpha-1-antitrypsin, the lymphatic node for anti-CD3, CD79 α, and the heart for anti-vimentin (Table 1).

Samples were subjected to a semi-quantitative method to identify and quantify the expression of the reaction rate, as follows: 1 = <25% labeled cells; 2 = 26-50%; 3 = 51-75%; 4 = >75%; were considered, respectively, 1 = unmarked; 2 = slightly marked; 3 = moderate, and finally, 4 = with intense staining. One hundred cells were counted for each slide. Images were captured with a Carl Zeiss optical microscope, Lab.A1, Germany, 10x and 40x objectives, and processed by AxioVision 4.8 software. In all cases, the immunohistochemistry evaluation was performed by two pathologists.

Evaluation of the quality of the samples. According to ISBER (2012, 2018) standards, the quality of the fragment was evaluated by quantifying the RNA. Samples were analyzed one month after collection and eight months after storage.

RNA extraction. The *RNeasy* Mini kit (Qiagen) was used for RNA extraction, according to the manufacturer's instructions. Subsequently, it was treated with RQ1 RNase-free DNase (Promega, Madison/WI, USA) for 30 min at 37ºC to avoid false positive results from genomic DNA amplification for RNA purification.

The quality of the extracted RNA was evaluated on a 2% agarose gel stained with ethidium bromide; quantification was performed using NanoVue equipment (GE Healthcare). Likewise, in 10 samples selected at random, the quality was analyzed through the bioanalyzer; the samples were processed according to the manufacturer's instructions and subjected to an automated system based on electrophoretic separation. In the process, the RIN is calculated by applying an algorithm to the radius of the 18S/28S ribosomal unit, in which an RIN ≥5 is expected to be of good quality. Subsequently, the RNA was stored in a freezer at -80ºC.

RNA quality over the long term. After extraction, it was stored at -80ºC for a period longer than eight months to assess the long-term quality of the RNA, after which 12 RNA samples were randomly selected and analyzed again for concentration and quality in NanoVue equipment (GE Healthcare).

DNA extraction and purification. The ReliaPrep™ gDNA Tissue Miniprep System kit from (Promega) was used for DNA extraction. The samples were transferred to 1.5ml Eppendorf tubes containing PBS (Invitrogen) for this process. They were then manually homogenized with micropistils in the tubes and subsequently added to the lysis buffer and proteinase k solution from the kit, after which the complex was incubated at 56ºC for 1 hour. RNase was added for DNA purification, and the complex was incubated under the same conditions. The kit columns were used according to the manufacturer's instructions to obtain the DNA. Finally, the DNA was diluted in 100µl of the kit's diluting solution.

The quality of the extracted DNA was evaluated on 2% agarose gel stained with ethidium bromide; quantification was performed using NanoVue equipment (GE Healthcare). The DNA was stored in a freezer at -80ºC.

Confirmation of RNA quality by real-time reverse transcription-polymerase chain reaction (RT-qPCR). Due to the greater instability of RNA compared to DNA, the quality of the first molecule was analyzed using RT-qPCR, and cDNA synthesis was performed using the High

Table 1. Specific antibody for the characterization of transmissible venereal tumor (TVT)

Antibody	Code	Dilution ratio	Manufacturer	Type of antibody
Anti-CD79a	A045201-2	1:100	Dako, EUA	Monoclonal
Anti-CD3	A045201-2	1:100	Dako, EUA	Policlonal
Anti-lysozyme	A009902-2	1:300	Dako, EUA	Policlonal
Anti-alpha-1-antitrypsin	N153330-2	1:300	Dako, EUA	Policlonal
Anti-vimentin	U7034	1:100	Dako, EUA	Monoclonal

Capacity Kit (Applied Biosystems) and produced using 1µg of RNA. The reaction was performed with 6µL of Random Primer (10x), 6µL of RT buffer (10x), 2.5µL of dNTPs (25x), 3µL of Multiscribe (50u/mL) and H_2O RNase free, according to the manufacturer's protocol. The reaction was incubated at 25°C for 10 min, then at 37°C for 120 min, before being kept at 4°C. The samples were maintained at the temperature of -20°C.

The PCR steps were performed in an automatic thermocycler (ABI Prism 7500 FAST Sequence Detection System, Applied Biosystems). For amplification of the primer sequences detailed in Table 2, SYBR Green and universal PCR Master Mix (Promega, USA) were used according to the manufacturer's instructions.

Quantification by RT-qPCR. The qPCR reaction consisted of 4µL of cDNA from the sample, 200nM of each *primer*, 10µL GoTaq qPCR Master Mix (Promega, USA), and nuclease-free water, to a final volume of 20µL.

The reaction conditions for all genes were: An initial denaturation of 95°C for 2 minutes, followed by 40 cycles of amplification (95°C for 15 s denaturation, 60°C for 1 min for annealing and extension) and the dissociation curve (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). As a negative control, nuclease-free water was used instead of the sample. The relative standard curve for each gene was generated using serial dilutions of cDNA from a reference sample. The lowest standard dilution was considered as a relative value of 100, and following serial dilutions of 1/10, the three points were 10, 1, and 0.1.

The relative concentration of the genes studied was normalized according to Larionov et al. (2005), and highly stable endogenous genes in canine tissue (RPS5, RPS19, and ACTB) were used, already tested by our group (Table 2). All reactions were performed in duplicate.

Statistical analysis. The purity and concentration of DNA and RNA were related to the different epidemiological parameters. In addition, these parameters were evaluated for RNA between tissue and culture and the degradation after storage. For this, the respective comparisons were performed using nonparametric Wilcoxon and chi-square analyses at a significance level of $p<0.05$. Other data are presented using descriptive statistics (Pagano & Gauvreau 2004). For all cases, the GraphPad Prism 5.0 program was used.

Table 2. Sense and antisense of the genes used in real-time reverse transcription-polymerase chain reaction (RT-qPCR)

Gene	Sense	Antisense
RPS5*	GAGGCGTCAGGCTGTCGAT	AGCCAAATGGCCTGATTCAC
RPS19*	GGGTCCTCCAAGCCCTAGAG	CGGCCCCCATCTTGGT
ACTB*	GGCATCCTGACCCTCAAGTA	CTTCTCCATGTCGTCCCAGT

* Brinkhof et al. (2006).

RESULTS

Epidemiological and clinical data of the animals

In total, 52 samples were analyzed, 40 of which were tissues (25 from TVT, 15 from wild animals), and 12 were primary cultures of TVTs. Considering the neoplastic and wild animal tissues, all were from animals from Botucatu and neighboring cities, which include the Veterinary Hospital of FMVZ-Unesp, Botucatu and the "Centro de Medicina e Pesquisa em Animais Selvagens" (Wild Animal Research Center – CEMPAS, Unesp, Botucatu). More details about the animals can be found in Table 3 and 4.

Table 3. Clinical data of patients treated at the Veterinary Hospital of Unesp who were transmissible venereal tumor (TVT) tissue donors for Tissue Bank (TB)

Animal	Sex	Age years	Breed	Location of the mass	Classification	Phase	Culture isolation*
1	Male	9	MB	Penis	P	Initial Regression	
2	Female	9	Teckel	Vagina	P	Progression	
3	Male	7	MB	Penis	P	Regression	*
4	Male	9	MB	Penis	P	Regression	
5	Male	-	MB	Penis, skin	P	Progression	
6	Male	10	MB	Penis	P	Progression	
7	Male	-	MB	Penis	P	Progression	
8	Female	10	MB	Vagina	P	Progression	*
9	Male	5	MB	Penis, skin	P	Initial Regression	*
10	Male	6	MB	Penis	P	Progression	
11	Male	-	MB	Penis	P	Progression	
12	Male	8	Poodle	Gingiva	P	Progression	*
13	Male	10	MB	Penis	P	Progression	
14	Male	10	MB	Penis	P	Progression	*
15	Male	16	MB	Penis	P	Progression	*
16	Male	6	Bull terrier	Penis	P	Progression	*
17	Male	-	MB	Penis	P	Progression	
18	Male	-	MB	Penis	P	Progression	*
19	Female	2	MB	Vagina	M	Progression	
20	Male	5	MB	Penis	M	Progression	*
21	Female	2	Border collie	Vagina	M	Progression	*
22	Female	3	MB	Vagina	P	Initial Regression	
23	Male	3	MB	Penis	P	Progression	*
24	Female	4	MB	Perivulvar	P	Progression	*
25	Male	5	MB	Penis	P	Progression	

MB = mixed breed, P = plasmacytoid morphology, M = mixed morphology, R = resistant; * TVT isolated cultures.

In relation to TVT, the primary diagnosis was performed through cytological examination and classified as to morphology (differentiating between plasmacytoid and lymphocytoid morphologies). The histopathological analysis enabled the classification into different stages of evolution. For this, the relationship between cellularity and number of fibroblasts was used.

Macroscopy of tumors

The tumors appeared as traditional, single or multiple lesions with a cauliflower appearance, sometimes multilobulated, adherent, or pedunculated. The tumors had varying sizes and relatively firm consistency, in addition to heavy bleeding for TVT. The color ranged from white through light pink to intense red.

Isolation and characterization of TVT primary culture

From the total tissues collected from TVT, 12 cultures were isolated, respectively, of which, after the establishment of the subculture and immunophenotyping, aliquots were stored at -196oC and later analyzed for the quantity and quality of RNA, Table 5.

Immunophenotyping of TVT cells

Cells showed positive staining for lysozyme, alpha-antitrypsin, and vimentin and negative for CD3 and CD79α.

Cytogenetics of primary cultures

Five of the total number of cultures analyzed were selected to count the number of chromosomes. All cells showed a variable number from 56 to 70. None of the cultures showed a number equal to the dog's somatic cells.

RNA extraction

RNA was extracted from 44 samples (tissue and culture), considering that it was impossible to extract RNA in some wild animal tissues. They showed good integrity of 18s and 28s ribosomal RNA in agarose gel. Later, 10 samples were selected for analysis using the bioanalyzer. In these samples, the RIN was always greater than six (Fig.1). Table 5 details the concentration and quality of the extracted RNA.

Table 4. Data from wild animals from CEMPAS tissue donors for the Tissue Bank (TB) from forensic samples

Animal	Species	Tissue
1	*Dasypus novemcinctus*	Skin
2	*Sylvilagus brasiliensis*	Skin
3	*Cavia porcellus*	Skin
4	*Chinchilla chinchila*	Skin
5	*Panthera onca*	Skin
6	*Puma concolor*	Skin
7	*Puma concolor*	Skin
8	*Didelphis albiventris*	Skin
9	*Myrmecophaga tridactyla*	Skin
10	*Tamandua tetradactyla*	Skin
11	*Cerdocyon thous*	Skin
12	*Dasyprocta aguti*	Skin
13	*Caiman crocodilus*	Skin
14	*Alouatta* spp.	Skin
15	*Blastocerus dichotomus*	Skin

Long-term quality of RNA

Comparatively, all the analyzed cases showed concentrations lower than the initial concentration, which was statistically significant ($p<0.05$). The absorbance parameters A 260/280 and A260/230 were biologically and statistically similar ($p>0.05$) (Table 6).

Table 5. RNA concentration and quality analysis of tumor samples (tumor tissue, cell culture and wild animals) belonging to the Tissue Bank (TB) of FMVZ-Unesp, Botucatu

Tissue sample	ug RNA/ul sample	A260/A280	A260/A230	Culture*
1	0.170	2.15	0.603	
2	0.07	2.299	0.069	
3	0.317	2.107	0.817	
4	0.397	2.083	2.130	
5	0.432	2.133	1.939	
6	1.511	2.116	2.091	
7	0.839	2.166	0.822	
8	0.077	2.166	0.822	
9	0.246	2.132	1.915	
10	0.631	2.080	2.096	
11	1.240	2.115	1.356	
12	0.454	2.052	2.096	
13	1.035	2.094	2.081	
14	0.100	2.287	1.206	
15	0.90	-	-	
16	0.083	2.396	0.527	
17	0.096	2.218	0.595	
18	0.055	2.517	0.696	
19	0.093	2.263	1.665	
20	0.164	2.094	1.513	
21	0.143	2.1	1.144	
22	0.129	2.196	1.464	
23	1.519	2.036	2.085	
24	1.203	2.115	1.069	
25	1.533	2.106	1.885	
26	0.203	2.128	1.687	
27	0.140	2.152	1.176	
28	0.142	2.153	1.196	
29	0.102	2.237	1.110	
30	0.275	2.129	1.811	
31	0.291	2.085	1.470	
32	0.694	2.087	1.892	
33	0.128	2.216	1.560	*
34	0.253	2.186	0.174	*
35	0.163	2.15	1.548	*
36	1.754	2.087	2.134	*
37	0.393	2.128	1.519	*
38	0.369	2.109	1.749	*
39	0.542	2.100	1.855	*
40	0.964	2.094	1.62	*
41	0.103	2.203	0.886	*
42	0.104	2.213	1.03	*
43	0.427	2.088	1.851	*
44	0.702	2.105	1.936	*

* RNA concentration and quality analysis in TVT isolated cultures.

RNA quality by RT-qPCR

The gene expression of the tested *primers* was obtained from the total analyzed tissues in 26 samples. Of the 12 cultures, all showed expression for the genes tested.

DNA extraction

In total, DNA was extracted from 15 samples from wild animals. In agarose gel, they showed good integrity. Table 7 details the concentration and quality of the extracted DNA.

DISCUSSION

The TB is an indispensable instrument in the study of diseases, using new techniques within pathological anatomy, as sample analysis enables different approaches to establish the prognosis, evolution, and treatment of a disease (Boudou-Rouquette et al. 2010, Crowley et al. 2013, Lommen et al. 2020, Dagher 2022). In addition, the TB constitutes a fundamental element in translational veterinary medicine. For this reason, developing an operational tissue Biobank needs to take into account the norms of international operation processes, including approval by the ethics committee, obtaining the free and informed consent form before performing any intervention on the animal, distribution of procedures for sample collection in the different departments, and standardization of the storage of the surplus sample of the microscopic diagnosis with its respective clinical history (Yu & Zhu 2010, ISBER 2012, Guerrera et al. 2016, Bossert et al. 2017, Glimelius et al. 2017, Lhousni et al. 2020, Lommen et al. 2020, Dagher 2022).

The collection of tumor samples was performed in specific areas in the "Laboratório de Cirurgia Experimental" (Experimental Surgery Laboratory) in "Universidade Estadual Paulista 'Júlio de Mesquita Filho'" (Unesp). In contrast, forensic samples were collected during necropsies or forensic examinations in the "Serviço de Patologia Veterinária" (Veterinary Pathology Service) of the "Faculdade de Medicina Veterinária e Zootecnia" (Faculty of Veterinary Medicine and Animal Science – FMVZ). It is worth noting that, in all cases, epidemiological and clinical data were recorded and that the storage of the sample in the Biobank was always the surplus of the material used for diagnosis; in addition, for the TVT samples, each case was accompanied by the histopathological slide to verify the integrity of the material. Finally, to preserve the privacy of the owner and the animal, a serial number was used in each sample to replace the name; in this way, an attempt was made to preserve the bioethical processes already well documented in the human area (Dhai 2016**,** Ballantyne 2019).

Given the above, we highlight that TB is a tool that successfully adapts to internationally recommended standards for the storage of samples for use in research and molecular analysis (Yu & Zhu 2010, ISBER 2012, Guerrera et al. 2016, Glimelius et al. 2017, Lommen et al. 2020).

Regarding the cell culture samples, we chose to store and analyze tissue from tumors currently of wide scientific interest. TVT is a neoplasm of transmissible origin, which attracts scientific interest due to its complex mechanisms of evolution, biological behavior, and interaction with the host (Flórez et al. 2016). It is considered that the study of this neoplasm may provide a unique opportunity in research on the evolution of cancer (Murchison et al. 2014, Ujvari et al. 2016, Baez-Ortega et al. 2019).

Due to the future importance of the samples in the Biobank, the cell cultures were characterized before their storage, as described by (Moore & Rosin 1986, Sandusky et al. 1987, Mozos et al. 1996, Marchal et al. 1997, Pereira et al. 2000,

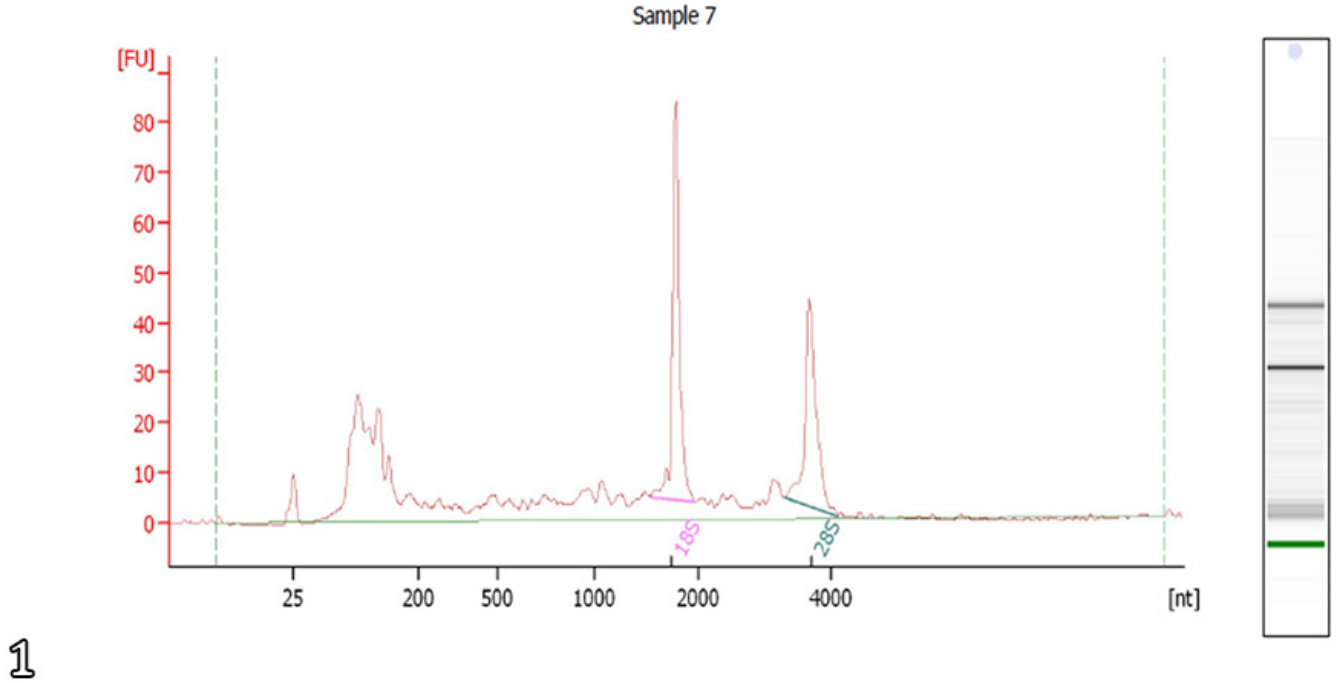

Fig.1. Tumor sample. Image of RIN 7.4, obtained from electropherogram and virtual gel.

Morris et al. 2002, Mukaratirwa & Gruys 2003, Araújo et al. 2012, Mascarenhas et al. 2014, Flórez et al. 2016).

The importance of TBs for future analysis of samples using new techniques is indisputable, so it is crucial to guarantee the optimal quality and quantity of stored tissues and to use different techniques to verify the quality (Coppola et al. 2019, Lhousni et al. 2020); thus, temporal analyses of DNA and RNA are necessary (Sweart et al. 2009, Lommen et al. 2020).

Regarding RNA, the quality of expression in tissue samples depends on multiple factors, such as tissue type, patient intrinsic factors, ischemia time, fixation time, and storage method (Lalmahomed et al. 2017, Coppola et al. 2019). In addition, the molecule is highly unstable because tissues, saliva, and the environment have high concentrations of lytic enzymes (RNases) that rapidly degrade RNA (Sandusky et al. 2007, Yu & Zhu 2010). Therefore, for its manipulation and storage in Biobanks, it is necessary to employ several measures, especially when the RNA will be used in techniques that involve gene expression (Morente & Alonso 2005, Annaratone et al. 2013, Galissier et al. 2016, Malentacchi et al. 2016, Dagher 2022). On the other hand, DNA is a more stable molecule than RNA (Tang et al. 2012).

In the present study, different measures were established to guarantee the optimal conservation of the quality of the samples and to avoid the degradation of RNA and DNA, among which the following stand out: During the collection, the use of microtubes free of RNase and DNase in order to avoid the degradation of the samples in the short and long term, tubes containing EDTA to conserve blood in forensic cases (necropsy or expert), and the quick storage of samples in 2L containers containing gel at -10°C; according to recommendations established by ISBER (2012) and researchers such as Lommen et al. (2020); with subsequent quick storage in a freezer at -80°C as already suggested in other works (Paskal et al. 2018) and liquid nitrogen at -196°C in the case of cell culture.

In the case of cell culture, the samples were divided into aliquots. The time between collection and final storage of the sample in the freezer or liquid nitrogen cylinder was always less than 20 minutes, as recommended in other studies (ISBER 2012, Lalmahomed et al. 2017, Coppola et al. 2019, Lommen et al. 2020).

Table 6. Concentration and analysis of RNA purity and concentration of samples stored for a period of eight months in the Tissue Bank (TB) of FMVZ-Unesp, Botucatu

Sample	ug/ul	A260/A280	A260/A230
1	0.038	1.9	1.37
2	0.070	2.00	0.176
3	0.042	1.83	1.77
4	0.442	2.079	2.075
5	0.113	2.029	1.763
6	0.103	1.9	1.4
7	0.154	2.042	1.739
8	0.267	2.034	1.577
9	0.101	2.04	1.68
10	0.103	2.031	1.792
11	0.180	2.032	2.013
12	0.258	2.067	1.14

It was necessary to evaluate the purity and concentration of the RNA and DNA of the samples, starting with good conservation of the tissues and cultures. For this, the absorbance ratios 260nm/280nm and 260nm/230nm were used. For the measurement of these parameters, absorbance spectrometry is a fast and reliable way to measure small amounts and consume little of the sample (Glasel 1995, OGT 2012).

The absorbance ratio 260nm/280nm is used to assess the contamination by proteins in the sample, and a reference value greater than or equal to 1.8 (free of protein) is accepted. The 260nm/230nm absorbance ratio is used as a secondary measure of nucleic acid purity, and factors such as dilution can influence the accuracy of the ratio (Glasel 1995, OGT 2012, Karlsson et al. 2016).

When analyzing the 260nm/230nm ratio, 64% (29/45) and 13% (2/15) of the RNA and DNA samples, respectively, presented values lower than 1.8. Despite this finding, this parameter is secondary in the purity analysis (OGT 2012). In this sense, results close to ours have already been reported by Viana (2013), who emphasized that the relationship between sample weight and amount of phenol can also influence this parameter. RIN was developed in 2006 to assess RNA integrity (Schroeder et al. 2006) and is now widely used for sample selection for molecular analysis (Lalmahomed et al. 2017). RIN variations between five and seven are considered optimal for these tests (Hong et al. 2010, Bao et al. 2013, Viana et al. 2013). In the current study, according to previous experiments, the cut-off point was established as ≥5, a range that was exceeded by 100% of the selected samples. In this way, we demonstrate the high quality of the RNA, which can be used in complex analyses such as transcriptomes or other techniques.

Regarding purity and concentration, the results match data reported in the human area, in which 90% of stored tissues are sustainable for molecular analysis (Lalmahomed et al. 2017). RNA quality is high in tumor tissues (Bao et al. 2013, Musella et al. 2013, Galissier et al. 2016, Guerrera et al. 2016). These findings indicate that the measures adopted for collecting and transporting tissues and cultures were adequate,

Table 7. Concentration and analysis of purity and DNA concentration of wild fauna samples stored in the Tissue Bank (TB) of FMVZ-Unesp, Botucatu

Sample	ug/ul	A260/A280	A260/A230
1	0.092	1.878	2.165
2	0.272	1.978	2.185
3	0.285	1.857	2.280
4	0.269	1.898	2.246
5	0.213	1.885	2.393
6	0.091	1.936	1.838
7	0.027	2.053	1.794
8	0.141	1.88	2.169
9	0.069	1.878	0.396
10	0.101	1.915	2.071
11	0.237	1.870	2.330
12	0.050	2.128	3.115
13	0.173	2.000	2.505
14	0.139	1.972	2.505
15	0.045	2.093	3.103

as they allowed evidence with high quality and integrity of the RNA extracted in the analyzed samples.

In the same way, we emphasize that the storage temperature of the samples was adequate, in agreement with what has been described and established in other works that describe that low sample storage temperatures are one of the keys to guarantee the integrity of the quality of molecular material (Qualman et al. 2004, Micke et al. 2006, Suh et al. 2009, Morente & Alonso 2005, Yu & Zhu 2010, Galissier et al. 2016, Guerrera et al. 2016, Lalmahomed et al. 2017, Lhousni et al. 2020, Wieser et al. 2022).

In the case of wild animals (forensic samples), for the extraction of RNA and DNA, it is important to highlight that all samples came from animals that had died previously. In many cases, it was not possible to control the time between death and sample collection, as it was always variable and greater than 1 hour. According to Lalmahomed et al. (2017), the factors that are decisive in the quality of the sample are the ischemia time and the time elapsed between collection and freezing; in this way, it is possible that by not being able to strictly comply with these parameters, due to the nature of the samples, the concentration, homogeneity, and quality of their genetic material were affected because, in 45% of these samples, a low concentration of genetic material was observed. In some samples, it was not possible to extract the RNA.

Thus, given the RNA and DNA analyses, we can infer that the protocols established for the Biobank, especially for obtaining RNA in tumor tissues and cultures, allowed the obtention of samples with excellent quality to advance studies of gene analysis, using techniques such as PCR, qPCR, database, transcriptome, etc.

Long-term preservation of organs and tissues depends on low temperatures that block all biochemical and biophysical processes at the cellular level (Yu & Zhu 2010). However, the real impact on RNA quality from parameters such as type of surgery, temperature, and duration of storage is still uncertain (Galissier et al. 2016). Similarly, research in veterinary medicine has not yet described the variation in the quality and integrity of extracted RNA with storage time and temperature. The current study seeks answers to these questions.

When analyzing the samples, we observed that the 260nm/280nm absorbance ratio was always greater than 1.8, so the purity was maintained, but the RNA concentration significantly decreased when compared to the initial sample. Despite this, it was considered that the concentration was not adequate to perform molecular analyses, such as PCR, in only 25% (3/12) of the samples.

In this regard, there is no comparative value in the scientific literature in the area of TBs for veterinary medicine. For this reason, our result was compared with that reported in human medicine. In this case, when contrasting the results, the RNA's quality and integrity differed from those of authors such as Viana (2013). It is important to highlight that it is possible that there are comparative differences between the species regarding the presence of small proteins that degrade RNA, whereby continuous activity of these RNases is likely. Therefore, it is necessary to establish new studies to analyze the origin of these proteins, if they exist, as well as their concentrations and their control, in order to contribute to the conservation of RNA in the long term and to ensure biological samples for translational research.

When using the RP5, RP19, and ACTB primers to confirm the quality of the RNA by RT-qPCR in the case of tumors, expression of endogenous genes was observed in all the analyzed samples, thus confirming that the material under analysis presented excellent quality to be used in short-term analysis.

In the case of animals for forensic purposes, even if low quality, forensic samples can be very useful when considering species identification processes, genealogy, or even understanding the geographical origin of animals since there are worldwide databases that facilitate the comparison of these materials. In addition to making an interface with what already exists in some forensic centers to identify criminals, DNA is a rather valuable piece of evidence due to its sensitivity and specificity.

To improve the quality of the genetic material, modifiable factors related to the quality of the sample, the time of ischemia, and the time between collection and storage should be as rigorous as possible, as well as the research on extraction methods and sample conservation, among others, to obtain better quality RNA/DNA, seeking new solutions and market advances.

CONCLUSIONS

It is important to articulate new research related to the conservation of material in Biobanks that allow the implementation of translational medicine programs in veterinary medicine as an essential tool for the research of precision medicine.

Likewise, it is important to highlight that in order to optimize the Biobank in the area of veterinary medicine, cooperation is needed between pathologists, oncologists, surgeons, biologists, epidemiologists, experts, statisticians, and technicians in the areas, guaranteeing that the correct follow-up and formulation of different measures according to the situation or case in hand. Publications and standards to create a tissue bank (TB) with a forensic approach are still scarce. However, this could represent a potential tool to provide the genetic material necessary for analyses of expert interest, contributing to future molecular research in pathology and investigations of crimes against wildlife.

Finally, we can summarize that the measures adopted in the TB for obtaining and handling the samples and for controlling and guaranteeing the quality were adequate and are in accordance with what was highlighted in works in the human area, since they allowed the optimal preservation of the integrity of the genetic material.

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