The Pharma Innovation Journal 2023; SP-12(11): 2046-2055

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(11): 2046-2055

www.thepharmajournal.com
Received: 18-09-2023
Accepted: 23-10-2023

P Anupama
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

BP Shivshankar
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

B Kavitha Rani
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

SS Manjunatha
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

P Ravikumar
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

GM Jayaramu
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

U Sunil Chandra
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

BE Shambulingappa
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

Shivaraj
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

MS Rudraswamy
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

KN Brunda
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

Javoor Akash
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

CV Santhosh Kumar
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

Sunkad Meghana
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

CS Niranjana Murthy
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

Corresponding Author:
P Anupama
Department of Veterinary Pathology, Veterinary College, Shivamogga, Karnataka, India

Immunohistochemistry and cytopathology as an aid in tumor diagnosis

P Anupama, BP Shivshankar, B Kavitha Rani, SS Manjunatha, P Ravikumar, GM Jayaramu, U Sunil Chandra, BE Shambulingappa, Shivaraj, MS Rudraswamy, KN Brunda, Javoor Akash, CV Santhosh Kumar, Sunkad Meghana and CS Niranjana Murthy

Abstract
Neoplasia is the aberrant growth of cells or tissues which has an uncontrolled multiplication that is not under physiological control, and can be either benign or malignant. The majority of benign tumors have a slow growth rate and do not spread to adjacent body tissues. Malignant neoplasms can grow at unpredictable rates, invading nearby tissues as they spread to other areas of the body. Most of the neoplasms will be always lethal in nature, therefore, a correct diagnosis given at the appropriate time will aid in the animal's survival. The two most often utilized techniques for diagnosing tumors are immunohistochemistry and cytology. The principle of IHC is to use certain antibodies (Abs) to detect antigens (Ag) within tissue specimens. A colored histochemical response shows that antigen-antibody (Ag-Ab) binding has taken place. The presence of particular biomarkers in the tissue sections helps to diagnose tumors. On the other hand, cytology focuses on studying and diagnosing diseases at the cellular level. The microscopic examination of cells to identify benign, pre-malignant, and malignant processes would help in the grading of neoplasms, which in turn will help in planning a suitable therapeutic regimen and also indicates the prognosis. These techniques are widely used in the diagnosis sector.

Keywords: Tumor, antigen-antibody, tumor markers

Introduction
Neoplasm are new growth, comprising of abnormal collection of cells, the growth of which exceeds and is uncoordinated with that of normal tissue and persists in the same excessive manner after cessation of the stimuli which evoked the change. Cancer is a disease of the genome that develops from DNA changes that disrupt the structure or function of genes. Almost all neoplasms share the characteristic of cellular genome damage or altered gene expression (Cullen and breen, 2016) [14]. Tumour can be either benign or malignant. A tumour is said to be benign when its gross and microscopic appearances are considered relatively innocent, implying that it will remain localized, will not spread to other sites, and is amenable to local surgical removal.

Cancer, common name for malignant tumors is derived from the latin word for crab, which was previously known as 'karkinoma' in Greek. (Gupta *et al*., 2021) [19]. Malignant tumours can invade and destroy adjacent structures and spread to distant sites (metastasize) to cause death (Kumar, 2015) [30]. Cancer is a huge public health problem worldwide. Diagnosis of cancer, which is a prime requirement to take up treatment, is achieved in the field of oncology mainly by morphological and microscopic examinations including cytology and immunohistochemical techniques (Chandrashekaraiah *et al*., 2011) [9].

IHC and Tumour Markers
Immunohistochemistry (IHC) is a vital adjunct technique for pathologists as it uses a specific antigen-antibody reaction to specifically visualize the distribution and amount of a specific molecule in the tissue. Tissue (Magaki *et al*., 2019) [36]. It (IHC) is a diagnostic method that, where suitably labeled antibodies attach selectively to their target antigens in situ, which facilitate the visualization of individual tissue components. In IHC, staining of antibodies is done by either an enzyme or a fluorophore technique (Immunofluorescence or Immunoperoxidase, respectively) which is used to identify proteins and other compounds in cells (Kabiraj *et al*., 2015) [27].

Antibodies selected for immunohistochemical testing are based on their tumor specificity and likelihood of reacting with the tumor under investigation. Tissue sections are incubated with the respective antibodies and the positive reactions (tumour antigen- antibody binding) are detected through the application of one of several identification systems (Kabiraj *et al*., 2015) [27]. Both monoclonal and polyclonal antibodies are used in IHC.

Mice are the primary source of monoclonal antibodies (Kabiraj *et al*., 2015) [27]. Purified immunogen (Ag) is injected to mice and the B lymphocytes (cells that produce Ab) are isolated from the spleen once an immune response has been attained. These B cells are united with mouse myeloma cells due to their limited lifespan. (Vara *et al.* 2005) [46]. Since monoclonal antibodies binds to only one antigen epitope, they are having more specificity (Kabiraj *et al*., 2015) [27].

Numerous animal species, including the rabbit, horse, goat, and chicken, produce polyclonal antibodies. When compared to monoclonal Abs, polyclonal Abs have a lower specificity but a higher affinity (Kabiraj *et al*., 2015) [27].

In the traditional direct technique

The primary antibody is labeled either to enzyme or a fluorochrome and applied to the tissue (Fig 1). However, this approach is less frequently used because it does not amplify the signal, necessitating higher antibody concentrations and the labeling of every primary antibody (Magaki *et al*., 2019) [36].

Indirect method

Involves use of an unlabeled primary antibody against the target antigen in tissue and a labeled secondary antibody (Fig 2). Indirect method is much more sensitive than direct method because the detective signal is amplified (Chen *et al*., 2010) [10].

Strepavidin biotin technique

Compared to the simple indirect- or PAP/APAAP approaches, the ABC method is more sensitive. The protein avidin has a stronger affinity for biotin.Biotin can only attach to one avidin molecule while there are four biotin binding sites on avidin (Fig 3). The ratio of the two proteins is chosen so that the secondary antibody will have at least one avidin binding site for biotin. Tissue sections are treated with the main antibody, biotin- conjugated secondary antibody, and AB complex in that order (Janardhan *et al*., 2018) [24].

IHC includes the following steps

1. Deparaffinization of tissue sections on polylysine coated slides (or else the aqueous solutions do not penetrate).
2. Quenching of endogenous enzymes: This is usually done by 3% H_2O_2 or with free avidin.
3. Antigen retrieval: Blocking of nonspecific binding sites.
4. Binding of primary antibody
5. Binding with biotinylated secondary antibody.
6. Detection methods using peroxidases-ntiperoxidase methods, avidin biotin conjugates, peroxidases complexes or the more recently used polymer labelling two step method.
7. Addition of chromogen substrate, usually DAB.
8. Counterstaining, dehydrating and cover slipping the slide (Kabiraj *et al*. 2015) [27].

Antigen retrieval

This method is necessary due to poor antigen-antibody recognition during IHC staining, which is primarily the result of formalin fixation and masking of epitopes which is brought on by the chemical alteration of proteins by formaldehyde (Shi and taylor 2014) [54]. So this technique increases accessibility to the antigens. During this procedure cross-links are broken, diffusible blocking proteins are extracted, proteins are precipitated, calcium is chelated, and paraffin is removed. The rehydration of tissue improves antibody penetration and increases antigen accessibility (Kabiraj *et al*. 2015) [27].

Blocking endogenous enzymes

Some normal and malignant tissues contain peroxidase and chemicals that cause a pseudo-peroxidase response. The procedure that is most frequently employed is the pre-incubation of the sections in pure methanol with hydrogen peroxide. (Kabiraj *et al*., 2015) [27]. The most popular and commonly used method for inhibiting endogenous peroxidases in formalin is to treat the sample with diluted H_2O_2. (Bussolati and Radulescu, 2011) [8].

Blocking background staining

Endogenous enzyme activity, hydrophobic and ionic interactions, are the main sources of background staining in IHC. Adipocytes, epithelium, and connective tissues such as collagen all contribute for the background staining. By including a blocking protein or a detergent like Triton X, or a high salt concentration like 2.5% NaCl in the buffer, hydrophobic bonding can be reduced to a minimum. (Kabiraj *et al*., 2015) [27].

Primary antibody addition

The monoclonal or polyclonal primary antibody, which are labelled or unlabelled (if unlabelled, secondary antibodies are required), is titrated to maximize contrast between positively stained tissue and nonspecific background staining. (Lin and Chen, 2014; Taylor, 2014) [34, 62]. In general, polyclonal antibodies, which can bind a variety of epitopes, are more sensitive and monoclonal antibodies which bind to a single epitope are more specific (Taylor *et al*., 2013) [63].

Detection system

Without labeling, the Ag-Ab reaction cannot be seen under a light microscope. To enable visualization of the immune response, labels (reporter molecules) are consequently added to the primary, secondary, or tertiary Abs of a detection system. Fluorescent substances, enzymes, and metals have all been employed as labels, among others. (Mandel *et al*. 1994; Shi *et al*., 1997) [37, 53]. Enzymes, such as peroxidase, alkaline phosphatase, and glucose oxidase, are the most frequently used labels. At the site of the Ag-Ab reaction, enzymes in the presence of a certain substrate and a chromogen will form a colorful precipitate (Vara, 2005) [46].

Tumor Markers

These are biochemical indicators of presence of a tumour (Bhatt *et al*., 2010) [6]. It is described as a molecule that can be found in body fluids and plasma in clinical practice. (Virji *et al*. 1988) [68]. Tumor markers are quantifiable biochemicals linked to malignancy. Either tumor cells themselves (tumour-derived) or the body's immune system (tumour-associated) produce these indicators. Usually, these are chemicals that are delivered into the bloodstream and then can be measured (Sotiriou *et al*., 2004; Wu, 2001) [59, 70]. Tumour markers can be used as a laboratory test to support the diagnosis of cancer

rather than being the primary method. (Bhatt *et al*., 2010) [6]. They can be mainly classified into, Hormones: (Table 1) Tumour associated antigens. (Table 2). Serum enzymes and proteins (Table 3)

Prognostic Markers and IHC

The gold standard clinical endpoints for prognostic studies are metastasis, recurrence, disease-free interval, and overall survival. The following list includes a number of IHC markers that have prognostic significance. (Vara and Borst, 2016) [47].

Proliferation/cell cycle markers

Ki67, PCNA (Proliferating cell nuclear antigen), AgNOR (Argyrophilic nucleolar organizing regions), IMVD (intratumoral microvessel density), cyclins, and telomerase Ki67 are the most common biomarkers used to evaluate cellular proliferation (growth fraction).

PCNA

Is an essential DNA polymerase cofactor for cell replication and DNA repair? It is expressed in the nucleus and surrounds DNA. (Moldovan *et al*., 2007) [39].

Ki67

Is a nuclear protein that is expressed in all phases of the cell cycle with a peak in the M phase and has a half-life of less than one hour (Klopfleisch *et al*., 2011) [29] Ki67 is absent in noncycling cells (Webster *et al*., 2011) [69]. Cells that are not cycling lack Ki67. Both the PCNA and Ki67 indices in canine and feline mammary cancers correlate with the tumor phenotype (greater indices in carcinomas than in benign tumors). In canine subcutaneous MCTs Ki67 index correlates with local recurrence and metastasis and in felines, with histologic type and unfavorable outcome (Sabattini and Bettini, 2010; Thompson *et al*., 2011) [50, 61].

The number of cycling cells (growth fraction) and the rate of cell cycle progression (generation time) both have an impact on cellular proliferation. The latter is measured using a histochemical reaction that counts argyrophilic nucleolar organizing regions (AgNORs) (Vara and Borst, 2016) [47]. High transcription activity causes argyrophilic proteins to segregate, which increases the quantity and surface area of AgNORs that are silver-impregnated. AgNORs can be measured in terms of their quantity per cell, their area per cell, or their proportion to the size of the nucleus (Klopfleisch *et al*., 2011) [29]. In dogs, mammary neoplasms are classified as benign (lower count) or malignant (higher count) based on AgNOR number per cell and area per cell (Klopfleisch *et al*., 2011) [29].

IMVD

Is being used as a prognostic indicator of numerous human and animal tumors (Hughes *et al*., 2012; Lavalle *et al*., 2009) [22, 32]. The balance between proangiogenic (like members of the vascular endothelial growth factor receptor [VEGFR] family) and antiangiogenic (like tissue inhibitor of metalloproteinase) factors controls angiogenesis, a crucial mechanism in the evolution of cancer. (Kerbel, 2008; Nishida *et al*., 2006) [28, 43]. IMDV is considerably higher in high-grade than in low-grade tumors in canine MCTs (mast cell tumors). (Abbondati *et al*., 2013) [1] Compared to soft tissue sarcomas, IMVD is more common in histiocytic sarcomas. (Ordóñez, 2013) [44].

Telomeres are protein complexes made of non- coding DNA (many repeats of TTAGGG) that protect chromosomal ends from fusing together thus maintaining chromosomal integrity (Nasir *et al*., 2001; Nasir, 2008) [41, 40]. The reverse transcriptase enzyme telomerase creates telomeric DNA. IHC detection of Telomerase is correlated with the proliferation index (Renwick *et al*. 2006) [49], tumor grade (Zavlaris *et al.* 2009) [72].

Malignancy Markers

Different biomarkers have been employed to differentiate between benign and malignant neoplasms. These markers typically show only minimal or localized expression in benign lesions while being overexpressed in malignant neoplasms. For instance, it has been observed that CK19 and galectin-3 are overexpressed in many thyroid carcinomas but are not diffusely expressed in benign lesions. (Liu *et al*., 2017) [33]. Trophoblastic cell surface antigen 2 (TROP2) is linked to the growth and advancement of certain epithelial neoplasms, including tumor formation. (Shvartsur and Bonavida, 2015) [56] With no to very little detection in normal tissues, TROP2 is also overexpressed in numerous carcinomas. (Liu *et al*., 2017; Stepan *et al*., 2011) [33, 60].

Prostate-specific antigen (PSA)

Is a serineprotease of the glandular kallikrein family? Its primary purpose is to release spermatozoa by liquefying the seminal fluid coagulum. (Netto *et al*., 2010) [42]. More than 95% of prostatic carcinomas in people will be labeled when PSA and PSMA are combined (Bhargava and Dabbs, 2010; Netto *et al*., 2010)) [5, 42].

Prostatic-specific membrane antigen

(PSMA) is a membrane glycoprotein expressed in the prostatic epithelium, as well as to a lesser level in the neurological system, small intestine, and salivary gland. Both benign and malignant prostatic tumors in humans exhibit PSMA, with the latter showing a stronger expression. (Netto *et al*., 2010; Silver *et al*., 1997) [42, 57]. In 50% of canine prostatic carcinomas, PSMA expression was found. (Lai *et al*., 2008) [31].

One of the transcription factors involved in lymphatic endothelial development is Prox1 (Prospero-related homeobox gene 1). (Schulte-Merker *et al*., 2011; Francois *et al*., 2011) [51, 17]. Prox1 has been employed in veterinary medicine as an IHC marker of lymphatic endothelium in a variety of animals (dog, cat, horse). Endothelial cells in arteries do not display this marker, although other cells and malignancies can. (Sleeckx *et al*., 2013; Galeotti *et al*., 2004) [58, 18].

Somatostatin

(Cytoplasmic reactivity) is found not just in the endocrine cells of the pancreas but also in other endocrine tissues. The presence of this protein, which may also be accompanied by the expression of other peptides including calcitonin, ACTH, or gastrin, serves as a marker for somatostatinomas. (DeLellis *et al*., 2010) [16]. Villin (cytoplasmic or membranous reactivity) is a cytoskeletal protein that binds actin and is found in the brush border of enterocytes and proximal renal tubular epithelial cells. A fraction of lung carcinomas and the majority of colorectal carcinomas express it. (Bhargava and Dabbs, 2011) [5].

Advantages of IHC

Both fresh and frozen tissue samples can be used for IHC; Specific localisation of antigens insitu can be done; Staining lasts for years; Affordable and simple procedure that can be performed with few resources.

Disadvantages of IHC

a) Restricted capacity to measure protein content.
b) Problems with antibody types, can only identify very few protein changes.
c) Limited or devoid of criteria based on evidence.
d) Single or dual identification ability.
e) Different scoring procedures and reproducibility.
f) No standardization techniques.
g) Limited throughput (Kabiraj *et al*., 2015) [27].

Cytopathology

Branch of pathology that studies and diagnoses diseases on cellular level/interpretation of cells that has either exfoliated spontaneously or are obtained from various organs by different methods. The main features used in cytopathological diagnosis are: Variation in size of nuclei (nuclear pleomorphism); Changes in chromatin quality (nuclear hyperchromatism); The ratio of nuclear area to cytoplasmic area (by subjective assessment). There are two main branches of cytology: Exfoliative cytology and Intervention cytology (Sharkey *et al*., 2007) [52].

Types of Cytological Techniques

Fine needle aspiration techniques: This is the most effective and widely used technique for sampling proliferative masses and lesions. Typically, a 2-5 ml syringe and a 22-25 gauge needle are used (Fig 4). Generally smaller needle and syringe are needed for the softer tissue. Longer needles are typically needed to acquire a sufficient sample from organs like the liver or spleen. The investigation of soft tissue masses, such as cutaneous lesions, lymph nodes, intra- thoracic or intra-abdominal masses, and effusions from bodily cavities, can be done well with this approach. (John, 2014) [25].

The non-aspiration technique

Is used to reduce blood contamination when sampling any highly vascular masses or organs (such as the spleen or liver). For getting samples from tiny lesions like pustules, the "needle alone technique," which avoids aspiration, is helpful. Under the supervision of ultrasound, the spleen of a dog with ascites and icterus is sampled using the non-aspiration approach with the syringe linked to the needle. (Fig 5). (John, 2014) [25].

Impression techniques

Are used to obtain sample from tissues at necropsies, surgically excised tumors, cutaneous ulcers, or exudative lesions. It is important to imprint, clean, and re-imprint ulcers. The tissue to be imprinted should first be split in half to have a fresh surface in order to acquire impression smears from tissues collected at surgery or necropsy. The remaining blood and tissue fluid are then wiped off of the clean surface (Fig 6). A clean glass slide is then touched to the brand-new surface. (Tseng and waddell, 1999) [64]. The most frequent issues with touch imprints are insufficient specimen blotting and "non-exfoliative lesions. (Sharkey *et al*., 2007) [52]. Impression smears have the drawback of only collecting cells from the surface of the lesion, which makes them potentially unrepresentative of underlying pathology. (John, 2014) [25].

Swabs are typically only used when other methods of sample collection (such as ear canal, vaginal cytology, and fistulous tracts) are impractical. It is recommended to moisten the swab with sterile saline if the area is dry. Additionally, a sterile cotton swab should be utilized if culture is to be performed. The cotton swab is simply rolled on a glass slide after swabbing the region, and the smear is then left to air dry (Fig. 7) (John, 2014) [25].

Scrapings

In contrast to impression smears of comparable lesions, scrapings are utilized for superficial skin lesions and may be a better indicator of deeper abnormalities. First, gently wipe away any exudates or crusts that are on the surface of the lesion. Samples are then taken by scraping a scalpel blade across the lesion's surface, transferring the material to a clean glass slide, smearing it, and letting it air-dry (Fig. 8). Although this method can be employed in situations where imprinting is anticipated to produce insufficient cells for a thorough evaluation (such as ocular and mesenchymal neoplasia) (Hodges *et al*., 2013) [21].

Tape preparation

Is the method for evaluation of skin disease for detecting malessezia dermatitis and skin mites. On the skin lesion, a piece of clear pressure-sensitive tape is applied. A drop of blue (counter) stain is placed on a microscopic slide after the tape strip has been removed and fixed (Pereira *et al*., 2012) [45].

Fluid cytology (exfoliative cytology) sample collection techniques

Catheterization

Catheterization is the process of collecting samples while carrying out various washes (Fig. 8), such as bronchoalveolar lavage and transtracheal wash. Smears should be done when sampling. (Villiers and Dunn, 1998; Meyer, 2015) [67, 38].

Centesis is the aspiration of fluid or from a body cavity using a trocar, aspirator, or needle (Tseng and Waddell, 2000) [65].

Fluid cytology (exfoliative cytology) slide preparation techniques

Blood smear technique

Near one end of a glass slide (smear slide), the collected material is placed. When the material has spread across the width of the spreader slide, it is drawn backwards into the slide at an angle of about 45 degrees A material edge with feathers depicts the smear's end. In general, the spreader slide should be advanced more slowly when the material that is on the specimen slide is more, and the more acute the angle between the spreader and specimen slide, the longer the smear will be. The blood smear method results in less cell rupturing and shearing than squash preparation. However, blood smearing does not spread cells as well as using slide over slide (Allison and Velguth, 2010) [2].

Star fish preparation (needle spread) technique

A tiny needle biopsy sample is inserted in the center of a glass slide, and the sample is then dragged or teased outward in various directions to create a star- or starfish- shaped smear with numerous projections. On the smear, there are typically several cell monolayer sections that can be used for cytological analysis. If only a tiny amount of material needs

to be aspirated, this method is helpful since it reduces the amount of stress to delicate cells. It is utilized in the creation of both solid and liquid cytology slides (Hajdu and Ehya, 2008) [20].

Cytological Stains

Romanowsky stains (Wright's, Giemsa and Diff-Quick stain)

They are affordable, simple to use, and easily accessible to veterinary professionals. They offer good cytoplasmic and nuclear detail, and the infectious organisms are clearly visualised. The Diff-Quick stain is the most affordable, quickest, and easiest stain to use in clinical practice. (Cowell *et al*., 2007) [11].

Papanicolaou stains

Papanicolaou stains are time-consuming and unsuitable for use in clinics, but they offer good nuclear detail and acceptable cytoplasmic information. (Joudeh *et al*., 2016) [26].

Supravital stains

They are often used to assess reticulocyte identification (peripheral blood smear) or to determine the presence of weakly granulated mast cells. They also show excellent nuclear information but low cytoplasmic detail. (Joudeh *et al*., 2016) [26].

Cytological Diagnosis of Tumor

Nuclear criteria of malignancy include

- Nuclear size variation (Anisokaryosis) with larger nuclei (>10 m) (macrokaryosis) and increased nuclear/cytoplasmic ratio.
- A large nucleus and little cytoplasm (although this is typical for some types of cells, including tiny lymphocytes).
- Variable-shaped nucleoli that are abnormally prominent,
- Nucleoli are angular instead of round or oval.
- Improper chromosomal alignment, a coarse chromatin pattern (ropy or cordlike chromatin), and nuclear molding, in which the nucleus is deformed (molded) around other nuclei in the same cell or other cells and signifies a loss of contact inhibition, are all signs of abnormal mitosis.. (Cowell *et al*., 2008) [12].

Mast cell tumors

They are among the most prevalent cutaneous neoplasms in canines and felines, and they can develop both primary and secondary neoplastic disorders in any anatomic site. Any age animal is susceptible, however they are most frequently observed in dogs and cats that are around middle age. (Yager and Wilcock, 1994) [71].

Interpretation

Mast cells can be identified by their unique tiny intracytoplasmic red-purple granules. Most mast cell tumors produce cells that have enough granules for them to be easily identified. The greatest distinguishing characteristic of mast cell malignancies is these granules. Many loose granules may be present in the background as a result of some cells rupturing. The circular to oval nucleus of the cells may be hidden by the granules (Fig 9). There are also several eosinophil counts. (Cowell *et al*., 2008) [13].

Histiocytoma

Benign tumors in young dogs with a macrophage origin. They are small, benign-looking cells that are spherical and discretely orientated. (Tyler *et al*., 1999) [66].

Interpretation

They typically stain lighter than the background tissue fluid because they include a modest quantity of homogenous clear to grey to blue grey cytoplasm. These cells feature a round to oval nucleus, chromatin that is finely stippled, and nucleoli that are difficult to distinguish. Any number of lymphoid cells could be present (Fig 10). Small lymphocyte counts that range from moderate to high indicate an immunological response. (Raskin and Meyer 2016) [48].

Transmissible venereal tumors (TVT)

The benign reticuloendothelial (histiocytic) tumor of dogs, also known as infectious sarcoma, venereal granuloma, transmissible lymphosarcoma, or Sticker tumor, primarily affects the external genitalia. (Bloom *et al*., 1951) [7]. CTVT is shaped like a cauliflower and may be pendular, nodous, papillary, or multilobular. (Das and Das, 2000) [15]. They are sexually transmitted tumors, found typically on mucous membranes including the penis, vagina, and nose. They are often malignant in behavior, but they react effectively to chemotherapy and radiation treatment (Cowell *et al*., 2008). [13].

Interpretation

Large round cells known as CTVT cells have a round nucleus, coarse chromatin, one to two conspicuous nucleoli, an abundance of cytoplasm that is only mildly basophilic, and numerous punctate vacuoles (Fig 11) (Raskin and meyer, 2016) [48].

Mesenchymal cell tumor

Benign mesenchymal tumors typically exfoliate few cells, making it challenging to get samples that are diagnostic-grade. On the other hand, aspirates from malignant mesenchymal tumors may be quite cellular. Large clusters of cells may be present, especially if they are kept together by an extracellular matrix, even if the cells are typically individually orientated. (Igor *et al*., 2012) [23].

Interpretation

Contraryto discrete and epithelial cells, mesenchymal cells frequently have cytoplasmic borders that are difficult to detect (i.e., the cytoplasm appears to progressively blend into the backdrop), making it difficult to determine the boundaries of the cell membrane. However, the nuclear membrane is typically damaged in these traumatized cells, whereas the nuclear outline of intact mesenchymal cells is clearly defined. Ruptured cells may also have fuzzy cytoplasmic borders. (Baker and Lumsden, 2000) [3].

Advantages of Cytology

- The amount of sample needed is less and is a less invasive procedure.
- Cost effective procedure and less painful
- Can help in early screening of abnormal and neoplastic cells.
- Less time consuming

Disadvantages of Cytology

- Sometimes the sample collected will not be from the area of lesion, so can result in false negatives.
- At times there will be low cellularity and contamination.
- Not always specific.

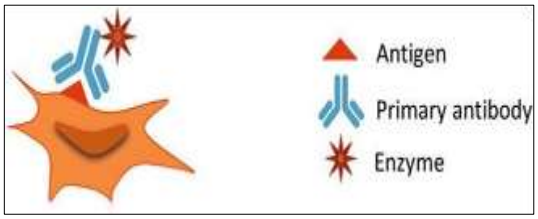


Fig 1: Using a direct immunostaining approach, the enzyme is directly attached to the primary antibody. (Shojaeian *et al*., 2020) [55]

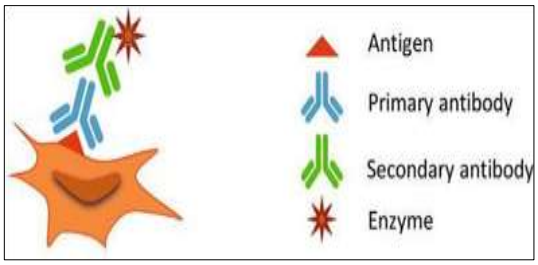


Fig 2: An indirect method uses a labelled secondary antibody generated against the primary antibody. Secondary antibody can be labelled with different flourophores or enzymes. (Shojaeian *et al*., 2020) [55]

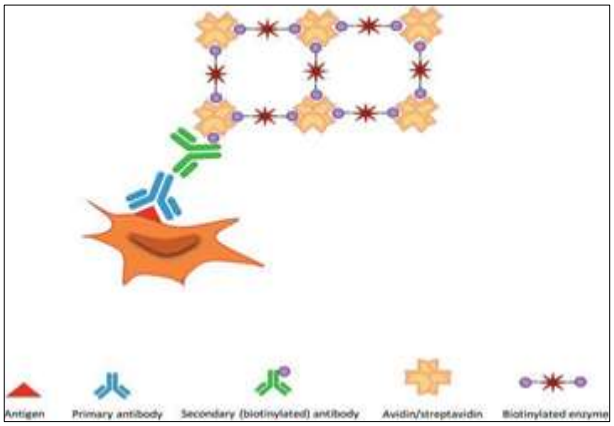


Fig 3: An unconjugated primary antibody, a biotinylated secondary antibody, and a sizable complex of enzyme that are avidin and biotinlabeled are used to create three distinct layers using the avidin-biotin complex technique. (Shojaeian *et al*., 2020) [55]

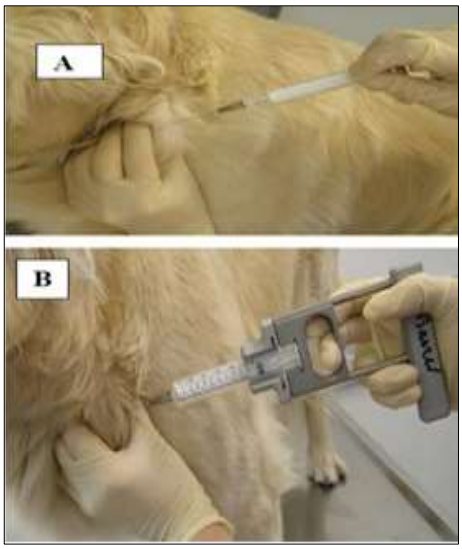


Fig 4: (A) Fine-needle aspiration in a dog using a needle and syringe. (B) Fine needle aspiration using an aspiration gun (John, 2014) [25]

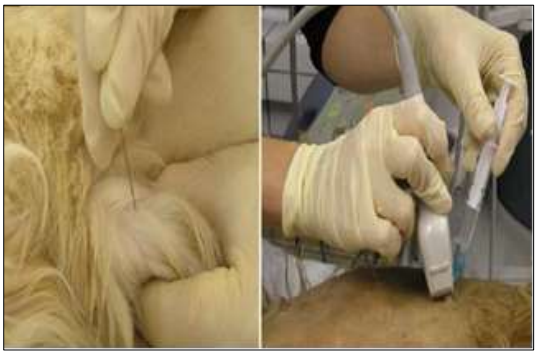
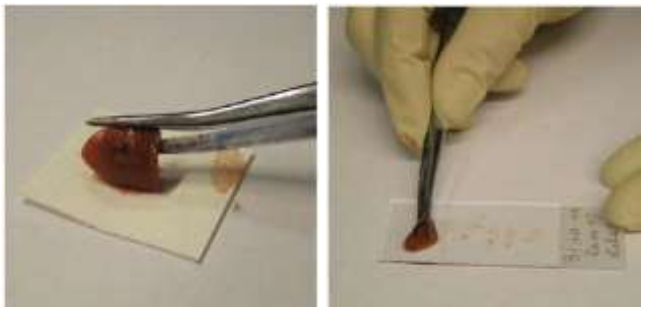


Fig 5: The "needle alone technique," a non- aspiration method. Ultrasound guided non aspiration technique with the syringe attached to the needle (John, 2014) [25]



(A) (B)

(C)

Fig 6: Impression smear of a liver biopsy. (A) Wiping off extra fluid on tissue gently on a filter paper. (B) and (C) By making multiple contacts between the slide's surface and the surface of the biopsy, the imprint smear is created. (John, 2014) [25]

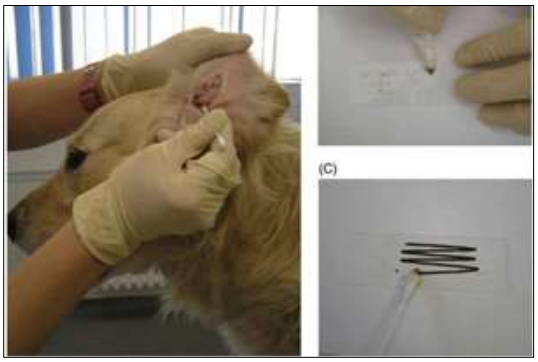


Fig 7: Collection of an ear swab for cytological investigation. (A) Using a cotton bud, the cerumen or discharge is collected. (B) After that, the material is spread out onto a slide. (C) Note the meandrical movement of the cotton bud (John, 2014) [25]

Fig 8: Sellotape Imprint. (A) A strip of Sellotape is pressed several times onto the skin lesion. This technique is ideal to show micro-organisms (bacteria, yeasts) on the skin surface. Parasites such as lice or Cheyletiella mites can also be detected with an unstained Sellotape preparation (John, 2014) [25]

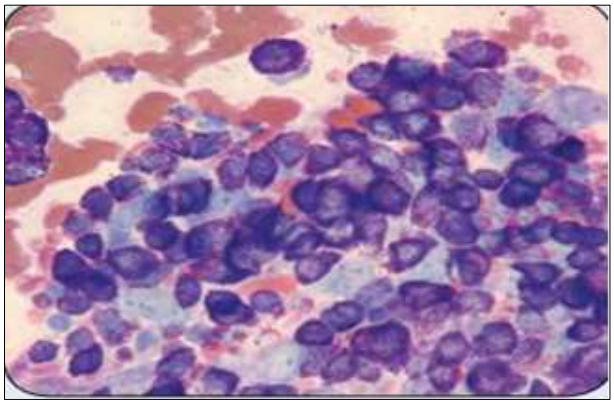


Fig 9: Canine mast cell tumor: The cells' cytoplasm is packed with many metachromatic granules. (Wright-Giemsa stain; 500×) (Barger, 2012) [4]

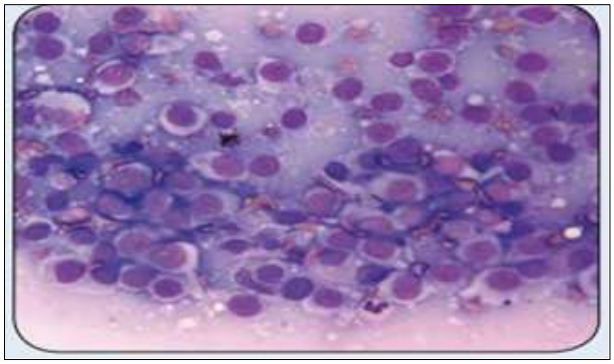


Fig 10: Histiocytoma: A diagnosis of histiocytoma is consistent with the peripheral clearing of the cytoplasm in many of the cells. (Wright-Giemsa stain; 500×) (Barger, 2012) [4]

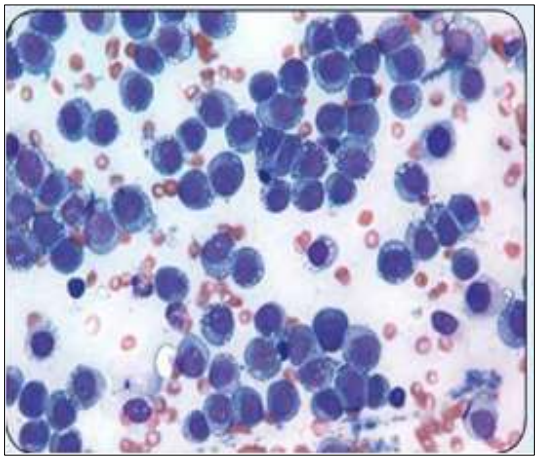


Fig 11: Canine transmissible venereal tumor: A large number of the cells had numerous tiny, distinct cytoplasmic vacuoles. (Wright- Giemsa stain; 500×) (Barger, 2012) [4]

Table 1: (Lokich, 1978) [35]

Associated antigen	Tumour associated
Carcinoembryonic antigen (CEA)	Tumour of liver, pancreas, lung, stomach and heart
α fetoprotein	Liver cancer, testicular cancer and non-trophoblastic germinal cell tumours

Table 2: (Lokich, 1978) [35]

Enzymes and proteins	Tumour
Prostatic acid phosphatase	Prostate cancer
Neuron specific enolase	Neuroblastoma
Immunoglobins	Multiple myeloma
PSA, PSMA	Prostate cancer
Calcium binding protein MRP 14	Occular squamous cell carcinoma (OSCC)
CD 59	OSCC
Profilin 1	OSCC
Catalase	OSCC
Cancer 125	Ovarian cancer
Cancer 19.9	Colon cancer
Cancer 15.3	Breast cancer

Table 3: (Lokich, 1978) [35]

Tumour marker	Common tumour association
ACTH (Adrenocortico tropic hormone)	Lung
Calcitonin	Thyroid follicular carcinoma
PTH (Parathyroid hormone).	Lung
Human chorionic gonadotrophin	Trophoblastic tumour, Non seminomatous testicular tumor
Insulin	Pancreatic tumor
Gastrin	Stomach
Placental proteins	Lung
Renin, erythropoeitin	Kidney
MSH (Melanocyte stimulating hormone), TSH (Thyroid stimulating hormone), ADH (Antidiuretic hormone).	Lung, ovary
Catecholamine	Pheochromocytoma
Glucagon	Pancreas

Conclusion

One of the leading causes of death in dogs is cancer, a deadly malignant disturbance. In the discipline of oncology, the diagnosis of cancer is a crucial prerequisite for beginning of treatment protocol and is mostly made by morphological and microscopic examinations including cytology and immunohistochemical procedures. The technique of immunohistochemistry has demonstrated its worth in identifying antigens present in tissues or cytologic preparations. The advent of computer-assisted quantification techniques for immunohistochemistry has improved not only the precision of marker identification but also the consistency of the obtained results. Hence these techniques will be useful both for diagnosis and as well as to know the prognosis of the treatment.

References

1. Abbondati E, Del-Pozo J, Hoather TM, Constantino-Casas F. Dobson JM. An immunohistochemical study of the expression of the hypoxia markers Glut-1 and Ca-IX in canine sarcomas. Veterinary Pathology. 2013;50(6):1063-1069.
2. Allison RW, Velguth KE. Appearance of granulated cells in blood films stained by automated aqueous versus methanolic Romanowsky methods. Veterinary Clinical Pathology. 2010;39(1):99-104.
3. Baker R, Lumsden JH. Color atlas of cytology of the dog and cat. Mosby, Inc.; c2000.
4. Barger A. Cytology of neoplasia an essential component of diagnosis. Today's Veterinary Practice. 2012;2(5):12-18.
5. Bhargava R, Dabbs DJ. Immunohistology of metastatic carcinomas of unknown primary. In Diagnostic Immunohistochemistry, WB Saunders; c2011. p. 206-255.
6. Bhatt AN, Mathur R, Farooque A, Verma A, Dwarakanath BS. Cancer biomarkers - current perspectives. Indian Journal of Medical Research. 2010;132(2):129-49.
7. Bloom F, Paff GH, Noback CR. The transmissible venereal tumor of the dog: studies indicating that the tumor cells are mature end cells of reticulo-endothelial origin. The American Journal of Pathology. 1951 Feb;27(1):119.
8. Bussolati G, Radulescu RT. Blocking endogenous peroxidases in immunohistochemistry: A mandatory, *yet al*so subtle measure. Acpplied Immunohistochemistry & Molecular Morphology. 2011 Oct 1;19(5):484.
9. Chandrashekaraiah GB, Rao S, Munivenkatappa BS, Mathur KY. Canine squamous cell carcinoma: A review of 17 cases. Brazilian Journal Of Veterinary Pathology. 2011;4(2):79-86.
10. Chen X, Cho DB, Yang PC. Double staining immunohistochemistry. North American Journal of Medical Sciences. 2010 May;2(5):241.
11. Cowell RL, Tyler RD, Meinkoth JH, DeNicola DB. Diagnostic cytology and hematology of the dog and cat-E Book Elsevier Health Sciences; c2007 Dec 21.
12. Cowell RL, Tyler RD, Meinkoth JH, DeNicola DB. Diagnostic Cytology and Hematology of the Dog and Cat, 3rd edn. Mosby; c2008.
13. Cowell RL, Tyler RD, Meinkoth JH, DeNicola DB. Diagnostic Cytology and Hematology of the Dog and Cat, 3rd edn. Mosby; c2008.
14. Cullen JM, Breen M. An overview of molecular cancer pathogenesis, prognosis, and diagnosis. Tumors in domestic animals. 2016 Dec 27:1-26.
15. Das U, Das AK. Review of canine transmissible venereal sarcoma. Veterinary Research Communications. 2000;24(8):545-556.
16. DeLellis RA, Shin SJ, Treaba DO. Immunohistology of endocrine tumors. In Diagnostic Immunohistochemistry.

Theranostic and Genomic Applications, 3rd edn. (ed. D.J. Dabbs). Saunders, St. Louis, MO; c2010. p. 291-339.
17. Francois M, Harvey N L, Hogan B M. The transcriptional control of lymphatic vascular development. Physiology. 2011;26(3):146-55.
18. Galeotti F, Barzagli F, Vercelli A, Millanta F, Poli A, Jackson DG, *et al*. Feline lymphangiosarcoma–definitive identification using a lymphatic vascular marker. Veterinary Dermatology. 2004 Feb;15(1):13-18.
19. Gupta Y, Sharma B, Gupta G. Importance of mother tinctures and tissue remedies in the management of tumours. Cancer, 2021, 13(22).
20. Hajdu SI, Ehya H. Foundation of diagnostic cytology. Annals of Clinical and Laboratory Science. 2008;38(3):296-299.
21. Hodges J. Using cytology to increase small animal practice revenue. Veterinary Clinics of North America: Small animal practice. 2013;43(6):1385-140.
22. Hughes K, Dobson JM. Prognostic histopathological and molecular markers in feline mammary neoplasia. The Veterinary Journal. 2012;194(1):19-26.
23. Igor U, Irena C, Ksenija I, Elena A, Goran N, Plamen T. Cytological diagnostic of Canine Transmissible Venereal Tumor-case report. Macedonian Veterinary Review. 2012;35(2):91-6.
24. Janardhan KS, Jensen H, Clayton NP, Herbert RA. Immunohistochemistry in investigative and toxicologic pathology. Toxicologic pathology. 2018 Jul;46(5):488-510.
25. John D. Manual of Diagnostic Cytology of the Dog and Cat. Axiom Vet. John Wiley & Sons; c2014. p. 288.
26. Joudeh AA, Shareef SQ, Al-Abbadi MA. Fine-needle aspiration followed by core-needle biopsy in the same setting: modifying our approach. Acta cytologica. 2016;60(1):1-3.
27. Kabiraj A, Gupta J, Khaitan T, Bhattacharya PT. Principle and techniques of immunohistochemistry: A review. International Journal of Biological and Medical Research. 2015;6(3):5204-5210.
28. Kerbel RS. Tumor angiogenesis. New England Journal of Medicine. 2008;358(19):2039-2049.
29. Klopfleisch R, Euler VH, Sarli G, Pinho SS, Gärtner F, Gruber AD. Molecular carcinogenesis of canine mammary tumors: News from an old disease. Veterinary pathology. 2011;48(1):98-116.
30. Kumar A, Abbas AK, Jon C. Aster: Robbins and Cotran pathologic basis of disease. Professional Edition; c2015.
31. Lai CL, Ham VDR, Leenders VG, Lugt VDJ, Mol JA, Teske E. Histopathological and immunohistochemical characterization of canine prostate cancer. The Prostate. 2008;68(5):477-488.
32. Lavalle GE, Bertagnolli AC, Tavares WL, Cassali GD. Cox-2 expression in canine mammary carcinomas: correlation with angiogenesis and overall survival. Veterinary Pathology. 2009 Nov;46(6):1275-1280.
33. Liu H, Shi J, Lin F. The potential diagnostic utility of TROP-2 in thyroid neoplasms. Applied Immunohistochemistry & Molecular Morphology. 2017 Sep;25(8):525.
34. Lin F, Chen Z. Standardization of diagnostic immunohistochemistry: Literature review and geisinger experience. Archives of Pathology & Laboratory Medicine. 2014;138(12):1564-1577.
35. Lokich JJ. Tumor markers: hormones, antigens, and enzymes in malignant disease. Oncology. 1978;35(2):54-57.
36. Magaki S, Hojat SA, Wei B, So A, Yong WH. An introduction to the performance of immunohistochemistry. Bio-banking: Methods and Protocols; c2019. p. 289-298.
37. Mandel U, Dabelsteen E, Gaggero B, Reibe LJ, Therkildsen MH, Clausen H. Oncofetal fibronectins in oral carcinomas: correlation of two different types. Apmis. 1994 Jul;102(7-12):695- 702.
38. Meyer DJ. The acquisition and management of cytology specimens. Atlas of Canine and Feline Cytology. Edn 3, Saunders, Philadelphia, PA USA; c2015. p. 1-14.
39. Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. Cell. 2007;129:665-679.
40. Nasir L. Telomeres and telomerase: Biological and clinical importance in dogs. The Veterinary Journal. 2008 Feb 1;175(2):155-163.
41. Nasir L, Devlin P, Mckevitt T, Rutteman G, Argyle DJ. Telomere lengths and telomerase activity in dog tissues: A potential model system to study human telomere and telomerase biology. Neoplasia. 2001;3(4):351-359.
42. Netto GJ, Epstein JI. Immunohistology of the prostate, bladder, kidney, and testis. In Diagnostic immunohistochemistry. Edn 6, Elsevier Inc,; c2011. p. 593-661.
43. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M. Angiogenesis in cancer. Vascular health and risk management. 2006 Sep 30;2(3):213-219.
44. Ordóñez NG. Application of immunohistochemistry in the diagnosis of epithelioid mesothelioma: A review and update. Human pathology. 2013;44(1):1-9.
45. Pereira AV, Pereira SA, Gremião ID, Campos MP, Ferreira AM. Comparison of acetate tape impression with squeezing versus skin scraping for the diagnosis of canine demodicosis. Australian Veterinary Journal. 2012;90(11):448-450.
46. Ramos-Vara JA. Technical aspects of immunohistochemistry. Veterinary pathology. 2005;42(4):405-426.
47. Amos-Vara JA, Borst LB. Immunohistochemistry: fundamentals and applications in oncology. Tumors in domestic animals. 2016 Dec 27:44-87.
48. Raskin RE, Meyer DJ. Canine and Feline Cytology: A color atlas and interpretation guide, Elsevier Health Sciences; 2015.
49. Renwick MG, Argyle DJ, Long S, Nixon C, Gault EA, Nasir L. Telomerase activity and telomerase reverse transcriptase catalytic subunit expression in canine lymphoma: correlation with Ki67 immunore activity. Veterinary and comparative oncology. 2006 Sep;4(3):141-150.
50. Sabattini S, Bettini G. Prognostic value of histologic and immunohistochemical features in feline cutaneous mast cell tumors. Veterinary pathology. 2010;47(4):643-653.
51. Schulte-Merker S, Sabine A, Petrova TV. Lymphatic vascular morphogenesis in development, physiology, and disease. Journal of Cell Biology. 2011 May 16;193(4):607-618.
52. Sharkey LC, Dial SM, Matz ME. Maximizing the diagnostic value of cytology in small animal practice. Veterinary Clinics of North America: Small Animal Practice. 2007; 37(2):351-372.
53. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. Journal of Histochemistry & Cytochemistry. 1997 Mar;45(3):327-343.
54. Shi SR, Taylor CR. Antigen retrieval in immunohistochemistry. Pathobiology of Human Disease. 2014 Jan 1:3817-3828.
55. Shojaeian S, Maslehat Lay N, Zarnani AH. Detection Systems in immunohistochemistry. Immunohistochemistry- The Ageless Biotechnology. Intech open; c2020.
56. Shvartsur A, Bonavida B. Trop2 and its overexpression in cancers: regulation and clinical/therapeutic implications. Genes & cancer. 2015 Mar;6(3-4):84.
57. Silver DA, Pellicer I, Fair WR, Heston WD, Cordon-Cardo C. Prostate-specific membrane antigen expression in normal and malignant human tissues. Clinical cancer research: An official journal of the American Association for Cancer Research. 1997;3(1):81-85.
58. Sleeckx N, Brantegem VL, Fransen E, Eynden VDG, Casteleyn C, Kroeze EV, *et al*. Evaluation of immunohistochemical markers of lymphatic and blood vessels in canine mammary tumours. Journal of Comparative Pathology. 2013 May 1;148(4):307-317.
59. Sotiriou C, Lothaire P, Dequanter D, Cardoso F, Awada A. Molecular profiling of head and neck tumors. Current opinion in oncology. 2004;16(3):211-214.
60. Stepan LP, Trueblood ES, Hale K, Babcook J, Borges L, Sutherland CL. Expression of Trop2 cell surface glycoprotein in normal and tumor tissues: potential implications as a cancer therapeutic target. Journal of Histochemistry & Cytochemistry. 2011;59(7):701-710.

61. Thompson JJ, Yager JA, Best SJ, Pearl DL, Coomber BL, Torres RN, *et al*. Canine subcutaneous mast cell tumors: cellular proliferation and KIT expression as prognostic indices. Veterinary Pathology. 2011 Jan;48(1):169-181.
62. Taylor CR. Immunohistochemistry in surgical pathology: principles and practice. Histopathology: Methods and Protocols; c2014. p. 81-109.
63. Taylor CR, Shi SR, Barr NJ, Wu N. Techniques of immunohistochemistry: principles, pitfalls, and standardization. Diagnostic immunohistochemistry. 2013 Oct 11;2:1-42.
64. Tseng SH, Chen YT, Huang FC, Jin YT. Seborrheic keratosis of conjunctiva simulating a malignant melanoma: An immunocytochemical study with impression cytology. Ophthalmology. 1999 Aug 1;106(8):1516-1520.
65. Tseng LW, Waddell LS. Approach to the patient in respiratory distress. Clinical techniques in small animal practice. 2000;15(2):53-62.
66. Tyler RD, Cowell RL, Baldwin CJ. Introduction in Diagnostic cytology and haematology of the dog and cat. (3rd Edn.), St. Louis: Mosby, USA; c1999. p. 1-19.
67. Villiers E, Dunn J. Collection and preparation of smears for cytological examination. In Practice. 1998 Jul;20(7):370-377.
68. Virji MA, Mercer DW, Herberman RB. Tumor markers in cancer diagnosis and prognosis. CA: A cancer journal for clinicians. 1988;38(2):104-126.
69. Webster JD, Dennis MM, Dervisis N, Heller J, Bacon NJ, Bergman PJ, *et al*. Recommended guidelines for the conduct and evaluation of prognostic studies in veterinary oncology. Veterinary pathology. 2011 Jan;48(1):7-18.
70. Wu JT. In: Diagnosis and management of cancer using serologic tumor markers. Clinical diagnosis and management by laboratory methods. 20th ed. Henry JB, editor. Philadelphia, PA: WB Saunders Company; c2001. p. 1028-1042.
71. Yager JA, Wilcock BP. Color atlas and text of surgical pathology of the dog and cat. Volume 1: Dermatopathology and skin tumors. Wolfe Publishing Ltd; c1994.
72. Zavlaris M, Angelopoulou K, Vlemmas I, Papaionnou N. Telomerase reverse transcriptase (TERT) expression in canine mammary tissues: A specific marker for malignancy Anticancer Research. 2009;29:319-326.