IMMUNOPROFILE
IN EFFUSION CYTOLOGY

"As we develop methods for extrapolating the secrets previously locked within the individual cells, it becomes evident that the cells were talking all along, we just did not know how to listen."

Abati A. 1998

SUMMARY

BACKGROUND: Cytology has a crucial role for diagnosing pleural and abdominal effusions. A prompt accurate diagnosis has both prognostic and therapeutic significance. However, cell morphology alone is not always sufficient to formulate such a diagnosis. In human medicine, immunocytochemistry of effusion cytology has now standardized procedures that provide reliable insights into various diagnostic dilemmas. **OBJECTIVE**: To describe the method of immunocytochemistry in effusion cytology and to estimate the value of a panel of markers in identifying cells in canine and feline effusions. **MATERIALS AND METHODS**: Human, feline and canine mesothelial cells were isolated in culture. Western-blot (WB) analysis was used to ascertain antibody cross-reactivity for all the markers, with the exception of HBME-1. Forty-four cytospined or smeared effusion specimens from dogs and cats with a cytological diagnosis of reactive effusion or malignancy of non-hematopoietic origin were stained with a standard panel of Vimentin, Cytokeratin (CK) AE1/AE3, CK 5/6 and HBME-1 as mesothelial cell markers; desmin as mesothelial cell malignancy marker; and CK7/CK20 as a marker of metastasis. Malignancy was confirmed by histologic evaluation; non-malignant conditions were confirmed by follow-up. Sensitivities, specificities and predictive values were calculated. **RESULTS**: The WB analysis confirmed the specific crossreactivity of the human antibodies for canine and feline proteins in mesothelial tissue. No significant differences were found between canine and feline results. Vimentin/cytokeratin coexpression had a sensitivity of 79% and a specificity of 92%, HBME-1 had 89% sensitivity and 23% specificity, and CK5/6 had 26% sensitivity and 100% specificity for mesothelial cells. Desmin had only 20% specificity for benign mesothelial cells, while CK7-/CK20+ had a specificity of 79% and sensitivity of 30% for metastatic cells on effusions. **CONCLUSION**: Immunocytochemistry can be applied in effusion samples, and valuable results can be obtained. The most useful marker, with the highest overall accuracy for the identification of mesothelial cells in effusion, is the Vim/CK coexpression, being CK5/6 the more specific and HBME-1 the more sensitive antibody. Desmin is not useful for discriminating between benign and malignant mesothelial cells. The coordinate expression of CK7-/CK20+ has not proved to be useful on the identification of metastatic cells on effusions.

INTRODUCTION

The mesothelium

The lining of serous cavities was first described by Bichat, in 1827, as a "single layer of flattened cells similar to those of the lymphatics". It was only 50 years later that Minot proposed the term "mesothelial", due to its function as "epithelial lining of mammalian mesodermic cavities". (Mutsaers and Wilkosz, 2007)

Mesothelial cells, embryologically developed from the mesodermal tissue, form a monolayer of specialized epithelial cells that line internal organs and serous cavities (peritoneal, pleural, and pericardial) (Yung and Chan, 2007).

Mesothelial Cell Morphology

Irrespective of species or anatomic origin, mesothelial cells constitute a homogeneous population that adopts a predominantly elongated, flattened, squamous-like morphology, approximately 25μm in diameter, with the cytoplasm raised over a central round or oval nucleus. Mesothelial cells rest on a thin basement membrane supported by connective tissue stroma. The cells contain microtubules and microfilaments, glycogen, vesicles and vacuoles, few mitochondria, a poorly developed Golgi apparatus and little rough endoplastic reticulum (RER). (Mutsaers, 2004; Yung and Chan, 2007)

Mesothelial cells of cuboidal morphology have also been identified in the septal folds of the mediastinal pleura; in close proximity to parenchymatous organs such as the spleen, liver, and diaphragm; and in the milky spots of the omentum. Similar cells are also seen in areas of mesothelial injury. A study on the histology of feline mesothelial cells also reported that the cuboidal mesothalial cells are especially characteristic of the visceral sheets, while the flat cells predominate in the parietal sheets (Michailova, 1996). Ultrastructural studies have identified abundant mitochondria and RER, a well developed Golgi apparatus, microtubules and a comparatively greater number of microfilaments, suggesting these cuboidal cells represent a more metabolically activate state (Mutsaers *et al.*, 2002; Mutsaers and Wilkosz, 2007) (Table 1). However, mesothelial cells originating from different anatomical sites present the same protein markers (Serre *et al.*, 2003).

The boundaries between mesothelial cells are tortuous, with well-developed cell–cell junctional complexes including tight junctions, adherens

junctions, gap junctions and desmosomes. The luminal surface of the mesothelial cell has a well-developed microvillous border that vary in length, density, and shape. The mesothelial cell apical surface also has occasional cilia that are typically five times longer than adjacent microvilli. (Yung and Chan, 2007) The microvilli markedly increase the functional mesothelial surface area, up to 40m2, for exchange between mesothelial cells and the peritoneal cavity. However, the number of microvilli expressed on each cell varies under different physiological and pathological conditions. (Michailova and Usunoff, 2006)

Table 1. Ultrastructural features of mesothelial cells

	Squamous morphology	**Cuboidal morphology**
Nucleus	Round or ovoid	Large; prominent nucleolus
Mitochondria	Few	Abundant
Rough endoplasmic reticulum	Sparse	Abundant
Golgi apparatus	Poorly developed	Well developed
Vesicles	Few	Numerous
Microfilaments	Present	Numerous
Microvilli	Abundant	Abundant

Microvilli protect the mesothelial surface from frictional injury by entrapment of water and serous exudates acting as lubricants for the cells. Cilia also protect the mesothelial surface from frictional injury by regulation of surfactant secretion and contribute to the cellular surveillance system by identifying humoral substances or microbial products within the peritoneal cavity during peritoneal injury or peritonitis. While microvilli are observed in proliferating mesothelial cells, cilia are lacking. (Bird, 2004; Mutsaers, 2004).

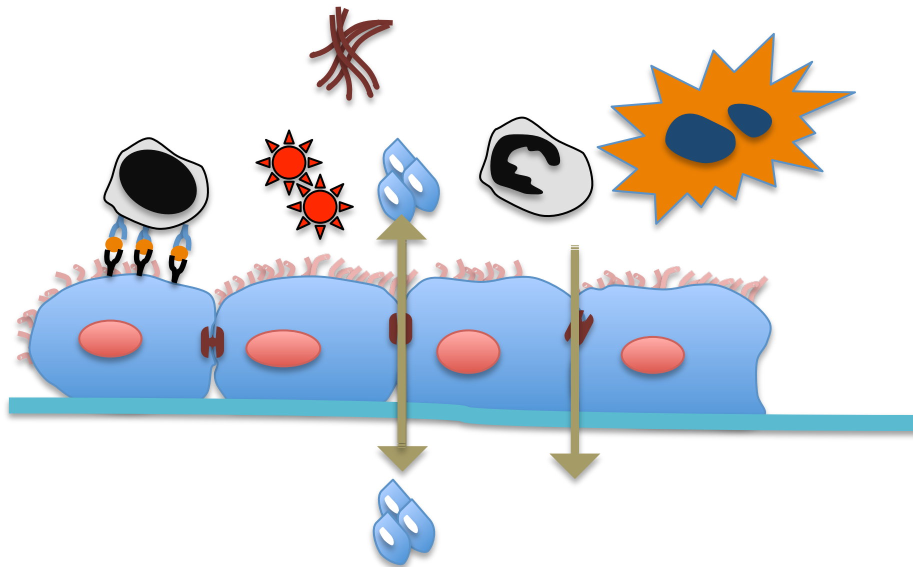
Mesothelial Cell Functions

The primary function of mesothelial cells is to provide a slippery non-thrombogenic surface that protects the viscera and allows their smooth friction. In addition, although previously considered solely to provide a protective surface that facilitates movement, compelling evidence now highlights the importance of the mesothelium in the control of fluid and solute transport, immune

surveillance, antigen presentation, inflammation, and wound healing (Figure 1). (Yung and Chan, 2007; Witowski *et al*., 2008).

In fact, once considered to be a passive tissue, the mesothelium is now seen as a dynamic membrane that plays a pivotal role in the structural, functional, and homeostatic properties of the peritoneum. (Yung *et al*., 2006; Yung and Chan, 2007).

Figure 1. Functions of mesothelial cells.



(adapted from Mutsaers, 2004)

The predominant role of the mesothelium is to preserve peritoneal homeostasis. However, more recent studies have begun to elucidate many different roles for mesothelial cells (Mutsaers, 2004; Herrick and Mutsaers, 2004; Yung *et al*., 2006)

1. Under physiologic conditions, mesothelial cells secrete numerous glycosaminoglycans, proteoglycans, and phospholipids that form a non-adhesive glycocalyx surrounding the cells. This protective barrier acts against infection, tumor dissemination and abrasion, allowing intracoelomic movement.

2. Mesothelial cells can synthesize a surplus of cytokines, chemokines, growth factors, and matrix components that regulate inflammation; initiate cell proliferation, differentiation, and migration; present antigen to T-cells (Mutsaers, 2004); and mediate tissue repair. For example, mesothelial cells modulate the microcirculation by their ability to secrete vasodilators, such as prostaglandins

and nitric oxide, or vasoconstrictors, such as endothelin (Yung *et al.*, 2006). By this way, mesothelial cells participate in both the induction and resolution of intracavitary inflammation (Jantz and Antony, 2008). Mesothelial cells also play an active role in local fibrin deposition and clearance in the serosal cavities. Their fibrinolytic activity is essential to the prevention and removal of fibrin deposits that form after mechanical injury and infection. (Yung and Chan, 2007; Herrick and Mutsaers, 2007)

3. Mesothelial cells are involved in the movement of cells and solutes across the peritoneal membrane, through pinocytic vesicles, intracellular junctions, and stomata. The mesothelial membrane has a net negative charge that acts as a selective permeability barrier to the passage of plasma proteins. (Aroeira *et al*., 2005)

4. Mesothelial cells are the first line of defence during bacterial peritonitis since they have phagocytic properties that participate in defence against infections (Yao *et al.*, 2003).

5. Mesothelial cells maintain a chemotactic gradient assisting in leukocyte infiltration during bacteria- or chemical-induced inflammation (Yung and Chan, 2009).

6. Mesothelial cells secrete hyaluronan and other glycosaminoglycans which may prevent tumour cell adhesion. (Mutsaers, 2004)

Changes in mesothelial cells during effusions

The number of mesothelial cells lining the peritoneum depends on the fine balance between cell proliferation and cell death. According to studies in human medicine, under normal peritoneal homeostasis, mesothelial cells exhibit limited cell proliferation, with only 0.1%–0.5% of cells in the mesothelium undergoing mitosis at any one time. However, injury to mesothelial cells significantly induces cell proliferation and cell death, thereby altering the structural integrity of the peritoneal membrane. When appropriately stimulated, the mitotic activity of mesothelial cells can be greatly increased. Within 48h of injury to the serosal surface, 30–80% of mesothelial cells at the wound edge and on the apposing surface begin synthesizing DNA. Contact inhibition may be one trigger for this rapid increase in proliferation but soluble mediators released from inflammatory

and injured cells are also potent stimulants (Mutsaers *et al*., 2002).

The effects on mesothelial cells of the accumulation of fluid in a serosal cavity include: cell enlargement, transformation from squamous to cuboidal or columnar morphology, increased nucleus/cytoplasmic (N/C) ratio, vacuolation, activation, and loss of microvilli (Di Paolo *et al.*, 1986; Williams *et al.*, 2003). Prolonged exposure of mesothelial cells to the effusion fluid also results in the collapse of cell–matrix interactions and abrogation of cell–cell contact inhibition with subsequent shedding of mesothelial cells into the peritoneal cavity. (Whitaker and Papadimitrou, 1985)

Injury to the mesothelium triggers events leading to the migration of mesothelial cells from the edge of the lesion towards the wound center and desquamation of cells into the serosal fluid. The fate of these cells after shedding into the peritoneal cavity is still under research. The most accepted hypothesis is that mesothelial cells are still viable in suspension, capable of maintaining function within the peritoneum, instead of being degenerative cells that are destined to apoptosis and removal from the peritoneal cavity by phagocytosis. Currently, most researchers agree that floating cells in effusions will attach and incorporate into the regenerating mesothelium. (Mutsaers *et al.*, 2000; Foley-Comer *et al.*, 2002; Mutsaers, 2004)

Epithelial-To-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) is a central feature of the normal development of tissues and organs by which epithelial cells acquire a mesenchymal, fibroblastic appearance, reduced intercellular adhesions, and increased motility (Yáñez-Mó *et al.*, 2003; Witowski *et al.*, 2008). In vitro, omentum-derived mesothelial cells adopt a fibroblastic appearance before reaching confluence (Connell and Rheinwald, 1983; Yung *et al.*, 2006). Mechanical injury of a mesothelial monolayer can also induce EMT in a proportion of cells situated at the periphery of the wound (Yáñez-Mó *et al.*, 2003). EMT also occur in the mesothelial tissue during effusion. Yanez–Mo *et al*. (2003) demonstrated the presence of elongated mesothelial cells positive for cytokeratin and ICAM-1 embedded in the peritoneal submesothelium.

The hability that mesothelial cells have to change their phenotype is comparable to changes seen in epithelial-to-mesenchymal transition (EMT) (Vargha *et al.*, 2008). This has implications both in normal repair and pathological processes. After several passages in culture, mesothelial cells lose their cytokeratin expression and adopt a fibroblast-like phenotype, consistent with ultrastructural observations in healing serosa. Upon re-establishment of an intact mesothelium, these cells return to an epithelial-like phenotype (Connell and Rheinwald, 1983; Foley-Comer *et al.,* 2002; Mutsaers *et al.*, 2002).

Mesothelial Cell Culture

The ability to propagate mesothelial cells in culture has resulted, over the past two decades, in an explosion of mesothelial cell research pertaining to peritoneal disorders. Particularly, in human medicine it has provided immense information about changes in the morphologic, structural, and functional properties of these cells during peritoneal dialysis. (Díaz *et al.*, 1998; Yung and Chan, 2007)

Diverse techniques have been described for the isolation of mesothelial cells. Direct explants, enzymatically degraded specimens of human omentum, as well as effluent-derived cells have been used as the source of mesothelial cells. (Stylianou *et al.*, 1990; Yung *et al.*, 2006) Direct explants of human omentum and cells obtained by enzymatic disaggregation have the advantage of avoiding contamination by other cells present on effusion fluids. Both of these approaches have successfully and reproducibly yielded homogeneous human mesothelial cell cultures, with identity confirmed by immunohistochemical and ultrastructural criteria (Stylianou *et al.*, 1990). Effluent-derived mesothelial cells in culture possess morphologic characteristics identical to those observed in mesothelial cells found in peritoneal biopsies. However, a limiting factor is the poor cell yield. (Yung *et al.*, 2006)

The proliferative potential of mesothelial cells is limited in culture since they can be maintained without significant loss in cell morphology up to the second or third passage. Thereafter, cells become enlarged and flattened, with numerous nuclei and vacuoles. Cells beyond the sixth passage fail to attach to their substratum. (Yung and Chan, 2007) As confirmed in human literature, mesothelial cells cultured in vitro possess the same immunohistochemical

markers as mesothelial stem cells, and thus they provide a pertinent in vitro model to study their morphologic, structural, and functional properties.

In veterinary medicine, the characterization of mesothelial cells could add valuable information on the establishment of an useful immuno marker profile for the diagnosis of reactive and neoplastic conditions of the coelomic cavities. Other than reports on mousse, porcine and bovine mesothelial cell cultures (Satoh and Prescott, 1987; Gotloib *et al.*, 1988; Ohan *et al.*, 1999; Bot *et al.*, 2003) we are unaware of studies regarding the isolation and culture of mesothelial cells in veterinary medicine, particularly on canine and feline species.

Effusion Cytology and Immunocytochemistry

Effusion cytologies are among the most difficult to evaluate objectively (Whitaker, 2000; Lin, 2009; Shidham and Falzon, 2010). Mesothelial cells may vary widely in their morphology, resulting in difficulty distinguishing between macrophages, mesothelial and carcinoma cells (Whitaker, 2000; Addis and Roche, 2009). Moreover, other types of tumors may exfoliate into the pleural or peritoneal cavity, being the correct interpretation of the cells dependent upon the cytologist ability to recognize and characterize them. (Davidson, 2004; Politi *et al.*, 2005)

Cytology is known to be a highly specific (99% for canine and 100% for feline effusions) but not very sensitive (64% for dogs and 61% for cats) technique on the diagnostic of effusions (Hirschberger *et al.*, 1999). The cytologic identification of neoplastic cells in effusions is a very clear indication that, almost certainly, the effusion is caused by cancer. However, the correct determination of the origin of the neoplastic cells is, by far, a very difficult issue for the cytopathologist. Often, even the histological picture itself is not helpful, because tumors of different primary sites may have metastasis with similar histological features (Tot, 1999). In these cases, immunohistochemistry has proven to be of value in human (Tot, 1999; 2001; 2002) and veterinary medicine (Espinosa de los Monteros *et al.*, 1999).

Nowadays, in human medicine, a high number of techniques are available to help the cytologist with the aim of increase the sensibility and specificity of the diagnostic. When the usual cytologic criteria do not allow one to form a definitive opinion, immunocytochemistry can be used as a reliable diagnostic arbiter. (Dalquen *et al.*, 1993; Delahaye *et al.*, 1997; Fetsch and Abati, 2001; Ko *et al.*, 2001; Zimmerman, 2005; Politi *et al.*, 2005; Ordonez, 2007).

In veterinary medicine, immunohistochemical procedures are well established (Ramos-Vara *et al.*, 2008). On the other hand, immunocytochemical studies, with the exception of hematopoietic neoplasia, have rarely been reported (Hoinghaus *et al.*, 2007). When compared to immunohistochemistry, immunocytochemistry is a more rapid, economical and non-invasive diagnostic method, providing the clinicians with useful information to design prompt rational therapeutic strategies (Vernau, 2005). Recently, studies on the

immunocytochemical differentiation of canine epithelial and mesenchymal tumors on imprint preparations were published (Höinghaus *et al.*, 2007b; Höinghaus *et al.*, 2008). Another paper commented also on the optimization of immunocytochemical technique on veterinary medicine, but a major focus was given to lymphoid neoplasias (Valli *et al.*, 2009). However, to our knowledge, no studies on immunocytochemistry of non-hematopoietic cells on effusions have been done so far.

In human histopathology (Tot, 1999; 2001; 2002; Chu *et al.*, 2000; Chu *et al.*, 2002) and cytopathology (Fetsch and Abati, 2001; Davidson *et al.*, 2001; Politi *et al.*, 2005; Sack and Roberts, 1997) markers like cytokeratin (CK) AE1/AE3, CK5/6, vimentin (Vim), desmin (Desm), and HBME-1 have been proven to be useful for the identification of mesothelial cells, others (CK7, CK20) have been used to identify epithelial neoplasms.

So far published information regarding the immunohistochemical diagnosis of mesothelioma in veterinary medicine mostly concerns the coexpression of the tumor cells with CKAE1/AE3 and vimentin (Reggeti *et al.*, 2005). Mesothelial cells are unique since although they are derived from the mesoderm and express the mesenchymal intermediate filaments vimentin and desmin, they also express cytokeratins, which are intermediate filaments characteristic of epithelial cells (Ferrandez-Izquierdo *et al.*, 1994; Afify *et al.*, 2002). An immunohistochemical study involving a larger panel of markers, in 10 cases of feline mesothelioma, revealed that all cases coexpressed cytokeratin and vimentin, six showed reactivity to HBME-1, but only two were positive for CK5/6 (Bacci *et al.*, 2006). In human medicine, it is proved that the addition of CK20 and CK7 to the standard panel of antibodies in the differential diagnosis of mesothelioma versus adenocarcinoma is useful, as CK20+/CK7- is a clear indicator of epithelial metastasis (Tot, 2001).

Thesis Hypothesis

The hypothesis of this PhD work is that immunocytochemistry can reliably help conventional cytology on the achievement of an accurate diagnosis on effusion samples, with the primary goal the identification of the origin of tumoral cells.

In this vein, the main purposes of this work are:

1) Confirm that the following anti-human antibodies: CKAE1/AE3, CK5/6, CK7, CK20, Vimentin and Desmin cross-react with their feline and canine counterparts in mesothelial cells. This could be proven by isolating and culturing pericardial mesothelial cells, and demonstrating protein expression by western blot analysis.

2) To describe and characterize the method of immunocytochemistry in effusion cytology.

3) To evaluate the usefulness of a panel of markers:

- Cytokeratin/Vimentin coexpression, CK5/6 and HBME-1 as mesothelial markers;
- Desmin as a marker of mesothelial cell malignancy;
- Coordinate expression of CK7-/CK20+ as a marker of metastasis.

MATERIALS AND METHODS

Isolation and characterization of mesothelial cells

Tissue Samples

Canine and Feline Mesothelial Cells. Segments of feline and canine *pericardium* (1g pieces) were collected during necropsy performed at the Department of Pathology, Microbiology and Immunology of the University of Davis, CA. The feline sample was from a crossbreed cat dead from an intestinal lymphoma. The dog sample was from a Labrador Retriever, dead from an histiocytic sarcoma. Both animals had no pericardial effusion.

The pericardium pieces were rinsed in PBS to remove contaminating RBC. Samples were incubated, with constant agitation at 37ºC, with pre-warmed 0,1% tripsin and 0,01% EDTA for 20 minutes, in Hanks' solution. Then, the solution was aspirated, and the internal surface of the partial pericardium was gently scraped with a scalpel blade and washed with 50 ml of Hanks' solution. All of the cell suspensions were combined and the cells were recovered by centrifugation at 350g for 10 min. They were washed once and resuspended again with Hanks' solution. After new centrifugation, cell pellet was resuspended in 10 ml of mesothelial cell culture medium containing M199 + 15% fetal bovine serum (FBS) + 0.4 mg/ml hydrocortisone + 10 ng/ml EGF + 100 U/mL of penicillin + 100 mg/mL streptomycin. The number of the cells in this final suspension was counted with an hematocytometer, and about $5x10^5$ cells/ml were found.

Human Mesothelial Cells. Human peritoneal mesothelial cells (primary cell line, LP9) were obtained from Dr. J. Rheinwald (HSDRC Cell Culture Core - Brigham and Women's Hospital, Harvard University, Boston, MA).

Cell Culture

About $5x10^5$ feline and canine cells were seeded into 25-cm^2 tissue-culture flasks with 5mL of mesothelial cell medium, prewarmed at 37ºC. The flasks were incubated at 37ºC in a humidified 5%-CO2 atmosphere. 1,5mL of culture medium was added every other day, and replaced every 4 days (6mL). When more than 75% cells were confluent in culture, cells were detached by trypsinization. Each flask was tripsinized with 1mL 0,1%trypsin, and remaining cells were washed with cell medium. The cell suspensions were transferred into one conical tube

and centrifuged at 200*g* for 10 min. Cell pellet was resuspended with 2mL cell medium and the content divided into two 25-cm^2 flasks (prewarmed with 5 ml of medium). This procedure was later repeated with 75-cm^2 flasks, till the 4th passage. Human mesothelial cells, acquired at the 4th passage, were subcultured till the 6th passage, with the same procedure described previously.

For each species, at the final passage, cells were trypsinized and the cell pellet from one flask was used to freeze and storage, while the other flasks were used for cell lysis. For cell freeze, the cell pellet was resuspended in 2mL of cryomedia - 80%FBS + 10% Dimethyl Sulfoxide (DMSO) + 10% Dulbecco's Modified Eagle Medium (DMEM) and divided into two 1mL cryovials. The vials were placed in a freezing box (Mr. Frosty - Nalgene, Sigma), filled with 200 ml of isopropanol at room temperature. The container was then placed in the -80ºC freezer. When the container reached -80ºC (overnight) the vials were removed and immediately placed in liquid nitrogen storage, where they can be maintained for several years.

The cell pellet from the remaining flasks was resuspended in 25mL º4C PBS and centrifuged at 400g for 5 minutes. After removing the supernatant, cells were resuspended again in 15 mL cold PBS and counted with an hematocytometer. $2,7x10^6$ cells/mL were found (a total of $4,0 \times 10^7$ cells in the 15mL suspention).

Immunofluorescent analysis

An immunofluorescent staining analysis (IFA) was performed for each cell line. For that, 2µm of the cell suspension were added to each well of a 12-well Teflon slide. The slide was fixed in cold acetone for 3 minutes and air dried. Then, it was placed in PBS bath for 2-5 minutes to rehydrate. When removed from the PBS bath, 15 µl of primary antibody, in an adequate dilution (table 2), were added to each well. After incubation at room temperature for 20 minutes, the slide was washed with PBS and placed in a PBS bath for 5 minutes. Then, slide was removed from bath and 15µl of appropriate secondary antibody, in a dilution of 1:250, was added to each well. For HBME-1, the secondary antibody was anti-mouse IgM - H&L *(*DyLight® 488), while for all the others markers the anti-mouse IgG - H&L *(*DyLight® 488) was used. Slide was incubated at room

temperature for 20 minutes and washed again in PBS, as described before. Slides were coversliped using Shandon Immu-mount (Thermo Scientific) and read/photographed on a IFA scope.

Cell lysis

For cell lysis, all steps were completed at 2-8ºC. In a 50mL conical tube, 20mL of cold PBS was added to the cell suspension. Cells were centrifuged as above. The supernatant was discarded and a cold lysis buffer was added, in a proportion of 10^6-10^7 cells/ml of lysis buffer (Mammalian Cell Lysis Kit, Saint Louis, Sigma). Lysis buffer was composed of 250mM Tris-HCl pH7,5; 5mM EDTA; 750mM NaCl; 0,5% Lauryl sulphate; 2,5% Deoxycholic acid; 5% Igepal CA-630 and proteinase inhibitor. In this vein, 4mL of lysis buffer were added to the cell pellet, which then was divided into four 1,5mL conical screw-cap tubes. Cells were incubated with the buffer for 15 minutes on an orbital shaker. The lysed cells were centrifuged for 10 minutes at 12000*g*, and the pellet was discarded. The protein-containing supernatant from the four tubes was removed and saved in different tubes: three Eppendourf vials, stored at -80ºC, and a chilled test tube, which was kept on ice for immediate use.

Protein concentration for each cell line was determined by the Bicinchoninic acid (BCA) method, using bovine serum albumin (BSA) as a protein standard. The reaction was analysed with a light spectrophotometer, with transmission set to 562nm. Briefly, a standard working solution (WS) was prepared by mixing reagente A and B in a proportion of 1:50.

- Reagent A: 1gm sodium bicinchoninate (BCA), 2 gm sodium carbonate, 0.16gm sodium tartrate, 0.4gm NaOH, and 0.95gm sodium bicarbonate, brought to 100 ml with distilled water; pH 11.25.
- Reagent B: 0.4gm cupric sulfate in 10 ml distilled water.

Then, 25μl of each sample were pipetted in replicate into a microplate well. 200μl of the WS were added to each well and mixed thoroughly on a plate shaker for 30 seconds. Plate was cover and incubated at 37°C for 30 minutes, and absorbance was measured. The 3 cell types rendered protein concentrations of ~2μg/μL each.

Table 2. List of clone; western blot (WB) applicability; molecular weight; dilution in WB, immunocytochemistry and immunofluorescence; and source of antibodies applied in this project.

Antibody	Vim	CK AE1/AE3	HBME-1	CK5/6	Desm	CK7	CK20
Clone	V9	AE1/AE3	-	D5/16 B4	DER II	OV-TL 12/30	Ks 20.8
Application on WB	Yes	Yes	No	Yes	Yes	Yes	Yes
Molecular Weight	53kDa	**AE1** - 56.5, 50, 48, and 40 kD; **AE3** - 65-67, 64, 59, 58,56, and 52 kD	?	(CK4 - 59KDa) CK5 – 58KDa CK6 – 48KDa	50KDa	54KDa	46KDa
Positive Controls	Esophagus	Bladder	-	Esophagus	Bladder	Bladder	Esophagus
Dilution In WB	1:400000	1:100000	-	1:50	1:400	1:200	1:200
Dilution in IHC and IFA	1:20000	1:10000	1:100	1:50	1:400	1:200	1:200
Company	Dako	Zymed	Dako	Dako	Novocastra	Dako	Dako

Legend: WB, Western Blot; IHC, Immunocytochemistry; IFA, Immunofluorescente analysis

SDS-PAGE and Western Blot analysis

SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) technique is a standard means for separating proteins according to their molecular weight (Kurien and Scofield, 2003; Kurien and Scofield, 2006). SDS-PAGE was performed on cell lysate and proteins were transfer onto a protein-binding membrane: Polyvinylidene fluoride (PVDF) membrane, using a semi-dry transfer system.

Briefly, ready gel 4-20% Tris-HCl (BioRad) was placed into the electrode assembly (Mini-Protean II, BioRad) and the chamber was filled with ~125ml running buffer (Tris-Glycine-SDS buffer). Meanwhile, the protein-containing sample was mixed with an equal volume of sample amplification buffer (SAB) with 2-Mercaptoethanol and boiled for 5 minutes. With special gel loading tips, 30uL of sample (corresponding to 15ul of protein) were loaded in each well. One

well was filled only with SAB, while the external lanes were filled with a molecular weight marker (Kaleidoscope, BioRad). The gel was ran at 40mA, for 45 minutes.

Gels were then placed in transfer buffer to be blotted. A PVDF membrane, for each gel, was immersed in ethanol, then in distilled water and then was allowed to equilibrate in transfer buffer. A sandwich of paper/gel/membrane/paper wetted in transfer buffer is placed directly between positive and negative electrodes (cathode and anode respectively). For that, extra-thick filter paper was immersed in transfer buffer (2/gel) and placed on electrode. The wet membrane was positioned directly on top of filter paper and the second filter paper (again, dripping with buffer) onto the top of the stack. After laying the lid on the unit, electrophoresis ran for 20min. Once electrophoresis of semi-dry blot was completed, the unit was disassembled and membrane was stained immediately.

Membranes were washed twice in water, then in TRIS and blocked for 1 hour with blocking buffer (5% Bovine Serum Albumin (BSA) and 0.05% Tween-20 in Tris-buffered saline) at room temperature and under constant agitation to minimize nonspecific binding. Membranes were then washed in Tris-0,05%Tween for 5 minutes and incubated with the primary antibodies at the asserted dilution (table 2) for 1 hour at room temperature. Antibodies had been tested previously at multiple dilutions in control samples to assess reactivity by western blot. Membranes were then washed again and incubated with the secondary antibody (goat anti-mouse IgG) in 5% milk for 1 hour with agitation at room temperature. PVDF membranes were washed 3 more times and the immunoreactive bands were visualized using the NBT/BCIP chromogenic substrate (Nitro Blue Tetrazolium / 5-Bromo-4 chloro-3 indolyl phosphate).

Concurrently, the same procedures were performed on human (HeLA cell lysate, ProSci), dog (esophagus and bladder lysates, Zyagen) and cat (esophagus and bladder lysates, Zyagen) samples, which acted as positive controls for antibody reactivity.

Effusion cytology

From November, 2007 till July, 2009, 44 cases of canine and feline intracavitary effusions sent to our Department for routine diagnosis were prospectively studied. Only cases that fulfilled the following criteria were selected: cytological diagnosis of reactive effusion; cytologic diagnosis of malignancy of non-hematopoetic origin (carcinoma or mesothelioma); complementary one year follow-up or histopathology; immunocytochemistry performed with a standard panel of markers.

Fluids were received in EDTA-containing tubes and refrigerated at 4°C. Within 4 hours of collection, samples were prepared with the smear technique and, for those nonturbid fluids with low to moderate cellularity (<5000 nucleated cells/uL), sediment smears were also performed. This was done by cytocentrifuging 2 drops of the fluid for 5 minutes at 450 rpm. Samples were air-dried and stained with May-Grünwald-Giemsa. Cytomorphological features were assessed. Cases diagnosed with a malignancy (of non-hematopoietic origin) as well as those with reactive changes (reactive mesothelial cells) were selected. Clinical information (age, gender, breed, clinical signs and other diagnostic procedures) was recorded. The cytological diagnoses were proved by histopathology or follow-up information. Necropsy was performed when possible.

Immunocytochemistry

Immunocytochemistry was performed with a standard panel of markers (table 3). For immunocytochemical examination, preparations were air-dried, fixed in cold acetone (4°C) for 3 minutes, and stored at -80°C until the procedure was done (maximum 1 year). A PAP pen was used to draw the barriers and confine the reagents to a defined area of interest on the slide. This area was chosen by visual and/or microscopic observation of the unstained slide. The specimens were placed for 30 minutes in 1% hydrogen peroxide to remove endogenous peroxidase activity, and rinsed in 3 changes of 0,1-M Tris-phosphate-buffered saline (Tris-PBS), pH 7,7, for 5 minutes. The selected areas of interest in the slides were covered with normal horse or goat serum (see table 3), for 30 minutes. During all the incubation times, the slides were maintained in an humid

chamber. Then, they were incubated with the primary antibodies at the specific working dilutions (table 3), previously determined in our laboratory, for 1 hour at room temperature. After 3x5-minute rinse with Tris-PBS, a biotin-labeled secondary antibody was applied in a dilution of 1:200 in PBS, for 30 minutes. This was followed by another 3x5-minute wash with Tris-PBS, after what the slides were incubated with the Avidin:Biotinylated enzyme Complex (ABC) (Vectastain®), for 30 minutes. Finally, the slides were washed in Tris-PBS, during 3x5-minutes, and developed with the aminoethylcarbazole (AEC) substract kit (Dako, Hamburg, Denmark). The slides were washed in Tris-PBS, for 3x5-minutes, and counterstained with Mayer's hematoxylin, for 3-5 minutes (exact time was monitored with microscopic visualization). Negative controls were made by omitting the primary antibody. Positive controls were made of touch imprints of tissues as described in table 3.

Table 3 – List of specificity, source, clone and dilution of antibodies applied in this project.

	Human Specificity	**Source***	**Clone**	**Dilution**	**Secondary antibody**
CK AE1/AE3	Broad spectrum epithelial cells	Zymed	AE1/AE3	1:10000	Horse anti mouse IgG
VIM	Mesenchymal cells	Sigma	V9	1:20000	Horse anti mouse IgG
Desm	Striated and smooth muscle cells; mesothelial cells	Novocastra	DER II	1:400	Horse anti mouse IgG
CK5/6	Stratified squamous epithelium Mesothelial cells	Dako	D5/16 B4	1:50	Horse anti mouse IgG
HBME-1	Mesothelial cells	Dako	-	1:100	Goat anti mouse IgM
CK 7	Ductal epithelium	Dako	OV-TL 12/30	1:200	Horse anti mouse IgG
CK 20	Merkel cell	Dako	Ks 20.8	1:200	Horse anti mouse IgG

Zymed Laboratories, San Francisco, CA; Sigma, St Louis, MO; Novocastra Laboratories Ltd, Newcastle UK; Dako, Glostrup, Denmark.

Immunohistochemistry

Immunohistochemical analysis was performed in 5-µm formalin-fixed and paraffin-embedded histologic sections. Samples were labelled by the avidin-biotin-peroxidase complex method with the primary antibodies, whose clone and dilution are summarized in table 3. Counterstaining was done with Mayer's hematoxylin. Negative controls were preformed as for the immunocytochemical procedure. Positive control tissues are described in table 3.

Both immunocytochemical and immunohystochemical staining were characterized based on pattern (membranar or cytoplasmic) and intensity, and classified as – (negative); + (faint); ++ (moderate); and +++ (strong), subjectively assessed in at least 10 mid-power fields (x40 objective) in random areas of the specimen. Staining found only in occasional dispersed cells was considered negative.

Coexpression of Vim+/CKAE1AE3+, and the individual markers HBME-1 and CK5/6 were evaluated as mesothelial markers, by calculating its sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Desmin was evaluated for its usefulness on the distinction between reactive mesothelium and mesothelioma. Finally, the coordinate expression of CK7-/CK20+ was investigated as a marker of carcinoma cells in effusion.

RESULTS

Cell Culture and Western Blot data

In order to ascertain the antibody crossreactivity of human antibodies in feline and canine mesothelial cells, protein expression within mesothelial cells from human tissue was compared with their feline and canine counterpart.

The cell culture conditions for canine and feline mesothelial cells provided an adequate grow and replication of cells. Feline and canine cells in culture had an elongated appearance and were similar to their human counterpart (figure 2). All cell lines were grown up in culture and cell lysis performed after the 6th passage for human, and 4th passage for feline and canine cells. At that time, an immunofluorescent analysis revealed that >90% of the cells were positive for both vimentin and cytokeratin (figure 3).

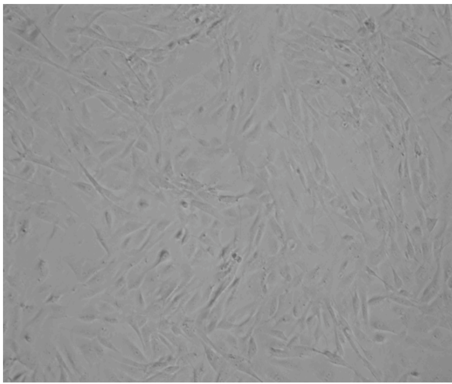


***Figure 2a**. Human Mesothelial cells culture at day 5, of 5th passage, in >75% confluency.*

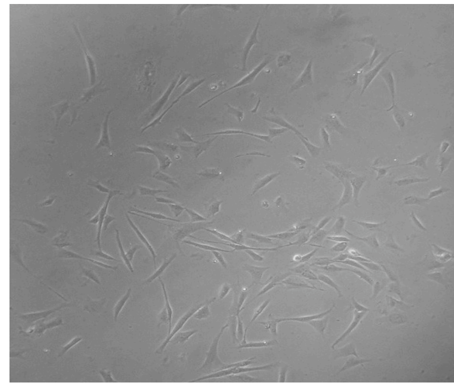


***Figure 2b**. Feline Mesothelial cells culture at day 4, of 2nd passage, in ~40% confluency.*

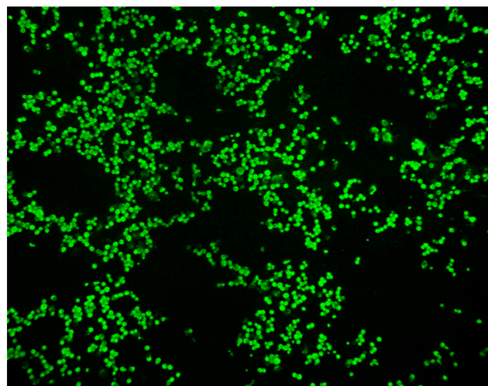


***Figure 3**. Immunofluorescent staining of feline mesothelial cells after culture till the 4th passage. Vimentin staining.*

After cell lysis, the protein supernatant of the three species was analysed by western blot. In both feline and canine mesothelial tissues, antibodies detected the target proteins. The same occurred in control tissues, confirming the crossreactivity of this human antibodies in feline and canine tissues. The protein to which the HBME-1 antibody reacts is still unkown and it does not work on western blot (Jirsova *et al*., 2010), so this antibody was not used in this study section.

Clinical Data

Forty four effusion samples were included in this study. There were 32 canine and 12 feline effusions, whose specific location and diagnostic group is reported in tables 4-5. Main represented breeds of dogs were German Shepherd, Golden Retriever, Yorkshire and crossbreed. Cats were mainly Domestic Shorthair and Siamese (table 6).

Table 4. Selected cases from **dogs**.

	Patients		**Samples**			
	Age (years) (median)	M:F	Pleural	Peritoneal	Cardiac	
Reactive Mesothelium	9 (5-13)	4,6:1	6	3	5	14
Carcinoma	11,5 (6-15)	0,2:1	9	4	0	13
Mesothelioma	6 (6-10)	2:1	5	0	O	5
			20	7	5	32

Legend: M, Male; F, Female

Table 5. Selected cases from **cats**.

	Patients		**Samples**			
	Age (years) (median)	**M:F**	**Pleural**	**Peritoneal**	**Cardiac**	
Reactive Mesothelium	-	-	0	0	0	0
Carcinoma	13,5 (3-18)	2:1	6	4	1	11
Mesothelioma	12 (10-14)	2:0	1	0	0	1
			7	4	1	**12**

Legend: M, Male; F, Female

Table 6. List of selected Cases

	Caso	Patient	Location	Cytologic Diagnosis	Histology / Follow-Up
1	C1734/07	Dog, Crossbreed, -	pleural	RE	Hypoalbuminemia /enteritis
2	C63/08	Dog, -	pleural	RE	Hemangiosarcoma
3	C132/08	Dog, Crossbreed, Female, 5yo	pleural	RE	Osteosarcoma
4	C146/08	Dog, German Shepherd, Male, 9 yo	cardiac	RE	Idiopatic pericarditis
5	C287/08	Dog, German Shepherd, Male, 11 yo	cardiac	RE	Hemangiosarcoma
6	C468/08	Dog, Beauceron, Male, 10 yo	pleural	RE	Hepatic Insufficiency
7	C534/08	Cat, Domestic Shorthair, Male, 18yo	peritoneal	Carc	Biliar carcinoma
8	C757/08	Dog, Crossbreed, Male, 12yo	cardiac	RE	Idiopatic pericarditis
9	C857/08	Cat, Domestic Shorthair, Male, 8yo	pleural	Carc	Carcinoma
10	C1008/08	Dog, Crossbreed, Male, 11yo	pleural	RE	Pneumonia
11	C1200/08	Dog, Crossbreed, Female, 10yo	pleural	Carc	Carcinoma NOS
12	C1214/08	Dog, Crossbreed, Male, 11yo	pleural	Carc	Carcinoma NOS
13	C1282/08	Dog, -	peritoneal	Carc vs Mesot	Carcinoma NOS
14	C1415/08	Dog, Crossbreed, Male, 11yo	pleural	Carc	Carcinoma NOS
15	C1419/08	Dog, Crossbreed, Female, 6yo	pleural	RE vs Carc	Mesotelioma
16	C1430/08	Dog, -	cardiac	RE	Idiopatic pericarditis
17	C21/09	Dog, Crossbreed, Female, 10yo	pleural	Carc	Mammary adenocarcinoma
18	C34/09	Dog, Basset Hound, Female, 15yo	pleural	Carc	Carcinoma NOS
19	C77/09	Dog, Cocker, Female, 13yo	pleural	Carc	Carcinoma NOS
20	C80/09	Cat -	pleural	Carc	Carcinoma NOS
21	C139/09	Dog, Labrador Retriever, Female, 9yo	peritoneal	Carc	Ovarian adenocarcinoma
22	C140/09	Dog, Golden Retriver, Male, 10yo	pleural	Carc vs Mesot	Mesotelioma
23	C144/09	Cat, Persian, Male, 14yo	peritoneal	Carc	Intestinal adenocarcinoma
24	C195/09	Dog, Yorkshire, Female, 13yo	peritoneal	Carc	Ovarian adenocarcinoma
25	C201/09	Cat -	pleural	Carc	Carcinoma NOS
26	C202/09	Dog, Collie, Female, 6yo	pleural	Carc	Ovarian adenocarcinoma
27	C205/09	Dog, Crossbreed, Female, 6yo	pleural	RE vs Carc	Mesotelioma
28	C263/09	Cat, Domestic Shorthair, Male	pleural	Mesot	Mesotelioma
29	C279/09	Cat, Domestic Shorthair, Female, 13yo	pleural	Carc	Carcinoma NOS
30	C289/09	Dog, German Shepherd, Male, 8 yo	peritoneal	RE	Hemangiosarcoma
31	C290/09	Dog, Labrador Retriever, Female, 12yo	pleural	Carc	Mammary adenocarcinoma
32	C305/09	Dog, Cocker, Female, 13yo	peritoneal	Carc	Transitional Cell Carcinoma
33	C322/09	Cat, Domestic Shorthair, Male, 15yo	peritoneal	Carc	Carcinoma NOS
34	C400/09	Cat, Domestic Shorthair, 16yo	pleural	Carc	Carcinoma NOS
35	C437/09	Cat, Domestic Shorthair, Female, 7yo	peritoneal	Carc	Carcinoma NOS
36	C468/09	Dog, Crossbreed, Female, 5yo	peritoneal	RE	Renal insufficiency
37	C502/09	Cat, Domestic Shorthair	cardiac	Carc	Bronchial carcinoma
38	C520/09	Dog, Crossbreed, Female, 6yo	pleural	RE vs Carc	Mesotelioma
39	C528/09A	Dog, Pyrenean Shepherd, Male, 8yo	pleural	RE	Bronchial cists
40	C528/09B	Dog, Pyrenean Shepherd, Male, 8yo	peritoneal	RE	Bronchial cists
41	C528/09C	Dog, Pyrenean Shepherd, Male, 8yo	cardiac	RE	Bronchial cists
42	C549/09	Dog, Crossbreed, Female,	pleural	Carc	Mammary adenocarcinoma
43	C566/09	Dog, Chiuahua, Female, 9yo	pleural	RE	Mesotelioma
44	C652/09	Cat, Domestic Shorthair, Male	pleural	Carc	Squamous carcinoma

Legend: RE, Reactive Effusion; Carc, Carcinoma; Mesot, Mesotelioma; NOS, Not Otherwise Specified.

Cytological Features

Reactive effusions were cytologically characterized by the presence of a moderate number of mesothelial cells isolated and/or dispersed in small groups. In rare cases they formed papillary structures with a core of eosinophilic matherial. These cells sometimes presented a characteristic eosinophilic "brush border" and were characterized by a moderate amount of basophilic cytoplasm and round to oval nuclei with prominent nucleoli. Marked reactivity features such as cytoplasmic hyperbasophilia and vacuolation, mitotic activity, multinucleation and megakaryosis were frequently present (fig. 4).

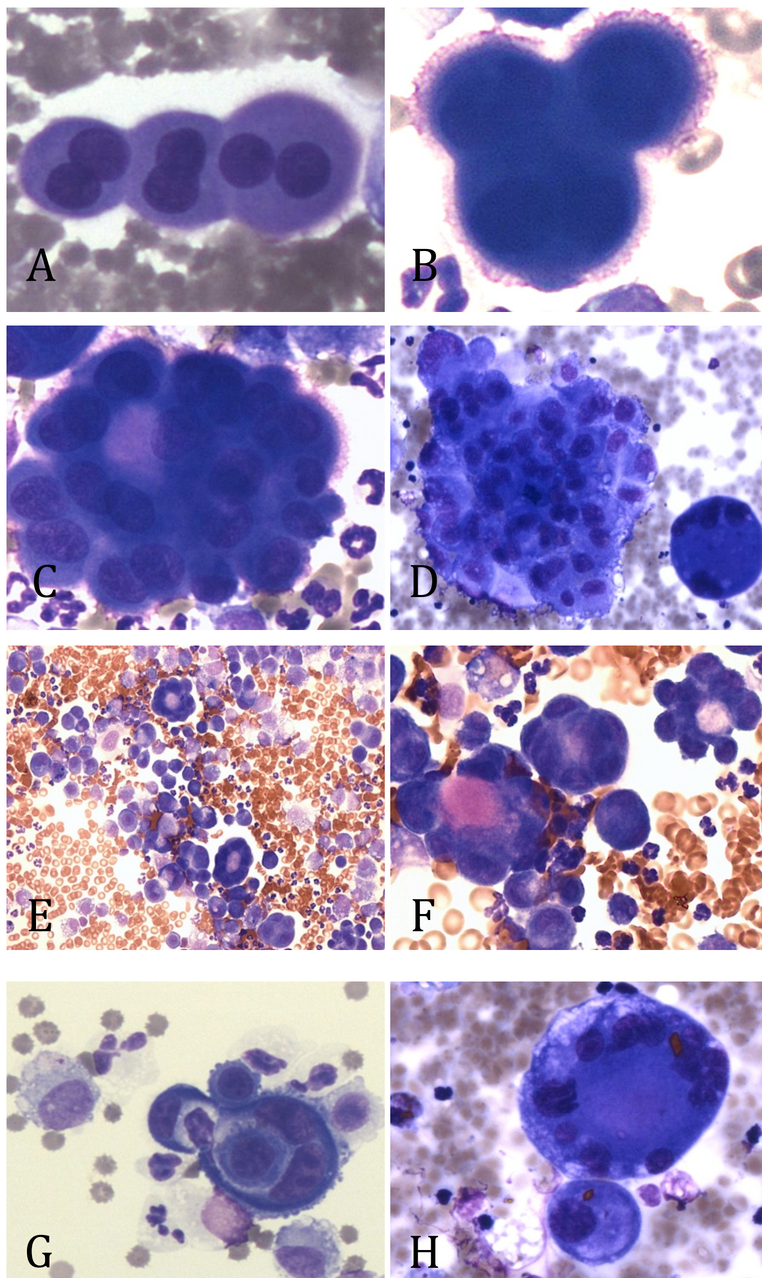


***Figure 4**. Morphologic spectrum of reactive mesothelial cells, with microvilous border (A-C), peripheral vacuolation (D), bi- and multinucleation (A, D, G, H), acinar arrangment (C, E), colagenous center (F), cell-in-cell arrangment (G) and phagocytosis (H).*

Along with reactive mesothelial cells, a variable number of macrophages and other hematopoietic cells were frequently present.

Mesothelioma cases varied in morphology. Some cases had highly atypical cells difficult to distinguish from carcinoma cells, while others had a prevalent population of well differentiated cells with moderate malignant criteria, difficult to distinguish from reactive mesothelium (fig. 5). A prominent features of some mesotheliomas was the existence of a prominent nucleolus.

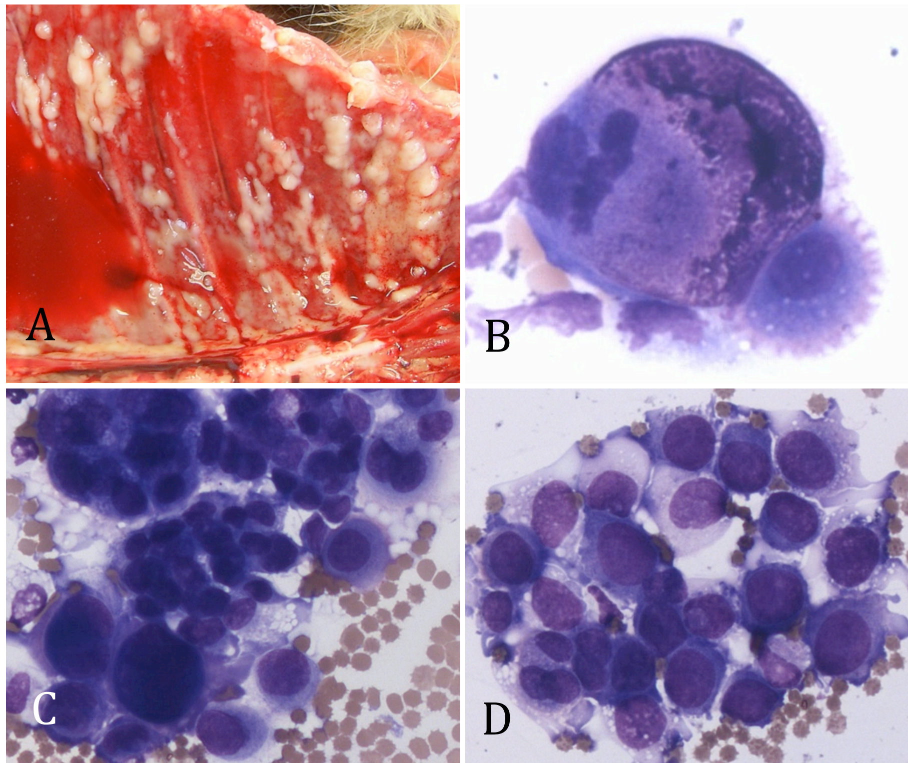


Figure 5*. Mesothelioma. A, macroscopic appearance of pleural cavity with numerous mesotelioma nodules. B-D, cytologic appearance of neoplastic mesothelial cells with moderate to marked malignant criteria.*

Carcinoma cases, mainly composed of mammary adenocarcinomas, were frequently characterized by large groups of cohesive cells in a acinar, papillary or tridimensional conformation. Large cells, occasionally with signet ring appearance, were often present. The N/C ratio was variable and nucleus varied from central to, most commonly, eccentrally located. Typical malignant criteria of

malignancy such as multinucleation with anisocaryosis, macrokaryosis and anisonucleolosis were seen (fig. 6).

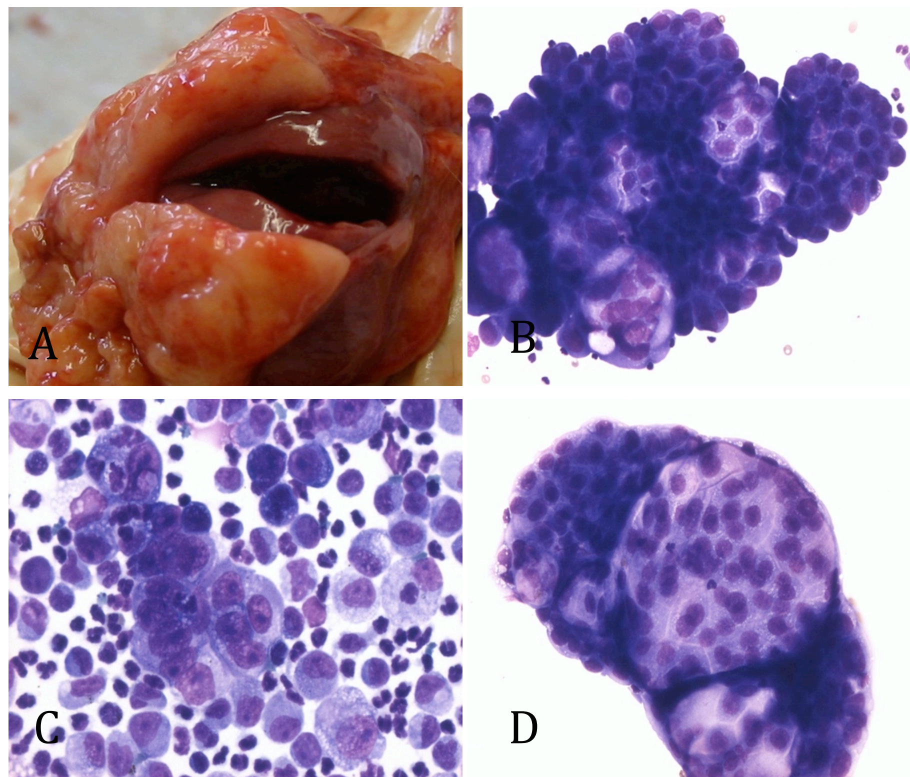


***Figure 6**. Carcinoma. A, macroscopic appearance of a pericardial sac carcinomatosis; B-D, cytologic appearance of carcinoma cells, in large tridimensional groups (B) or isolated (c), sometimes with evident intercelular windows, a feature that although tipically seen in mesothelial tissue can also be present on carcinoma cells (D).*

Immunocytochemistry

The reactivities found on cytological specimens to the individual antibodies are given in table 7. Staining intensity and percentage of stained cells are summarized in table 8 and 9.

Table 7. List of immunocytochemistry results performed in all cases

	Case	Cytological Diagnosis	CKAE	VIM	DESM	CK5/6	HBME-1	CK7	CK20
1	C1734/07	Reactive Effusion	+ ; 25%	+ ; 25%	+ ; 25%	+ ; 25%	+ ; 25%	++; 25%	+++; 25%
2	C63/08	Reactive Effusion	+ ; 25%	+++ ; 50%	+ ; 25%	-	++ ; 25%	-	+++; 75%
3	C132/08	Reactive Effusion	-	+ ; 25%	-	-	++ ; 25%	-	-
4	C146/08	Reactive Effusion	+ ; 25%	++;50%	-	-	+++ ; 25%	+; 25%	++; 25%
5	C287/08	Reactive Effusion	++ ; 50%	++ ; 75%	++; 25%	-	++ ; 25%	-	+; 25%
6	C468/08	Reactive Effusion	+ ; 50%	++ ; 50%	-	-	++ ; 50%	++;25%	++;25%
7	C534/08	Mammary carcinoma	++,90%	+ ; 50%	-	-	++ ; 75%	+++;90%	+++;90%
8	C757/08	Reactive Effusion	+ ; 25%	+ ; 25%	-	-	++ ; 25%	-	-
9	C857/08	Carcinoma NOS	-	+++; 75%	-	-	-	-	+++; 50%
10	C1008/08	Reactive Effusion	+++ ; 25%	+++; 90%	+ ; 25%	+ ; 25%	+++ ; 75%	+++; 25%	+++; 25%
11	C1200/08	Carcinoma NOS	-	+++; 90%	-	-	++; 75%	-	+; 25%
12	C1214/08	Carcinoma NOS	-	-	-	-	+; 25%	++ ; 25%	++ ; 25%
13	C1282/08	Carcinoma NOS	-	-	-	-	+; 25%	++ ; 25%	+++ ; 75%
14	C1415/08	Carcinoma NOS	-	-	-	-	++; 25%	+++; 75%	+++; 75%
15	C1419/08	Mesotelioma	-	++;50%	+;25%	-	++ 25%	+++; 75%	+++; 75%
16	C1430/08	Reactive Effusion	+++ ; 50%	+++; 75%	+++; 25%	-	++ 25%	-	++; 25%
17	C21/09	Mammary carcinoma	++; 25%	-	+++; 25%	-	-	++; 75%	+++; 25%
18	C34/09	Carcinoma NOS	++; 50%	-	-	-	-	-	+++; 50%
19	C77/09	Carcinoma NOS	+	-	++; 50%	-	++; 50%	+; 25%	+++; 25%
20	C80/09	Carcinoma NOS	-	-	-	-	++ 25%	-	+++; 50%
21	C139/09	Ovarian carcinoma	-	++; 75%	-	-	-	-	-
22	C140/09	Mesotelioma	++; 90%	+++; 90%	+++; 90%	-	+; 75%	+++; 90%	++; 90%
23	C144/09	Carcinoma NOS	++; 75%	+++; 90%	++; 25%	-	++; 25%	+; 25%	++; 50%
24	C195/09	Ovarian carcinoma	++; 50%	-	+++; 75%	-	-	-	++; 90%
25	C201/09	Carcinoma NOS	++,50%	-	-	-	++; 50%	-	+++; 75%
26	C202/09	Ovarian carcinoma	-	+; 50%	+++; 75%	-	++; 25%	++;75%	++; 50%
27	C205/09	Mesotelioma	-	-	-	++; 25%	+;25 %	-	++; 50%
28	C263/09	Mesotelioma	-	+++;90%	-	-	-	+++;25%	-
29	C279/09	Carcinoma NOS	+; 25%	+++; 25 %			-	-	-
30	C289/09	Reactive Effusion	+ ; 25%	++	-	-	+; 25%	++; 25%	++; 25%
31	C290/09	Mammary carcinoma	++, 75%	+++, 90%	+++; 25%	-	-	+++,75%	++; 75%
32	C305/09	TCC	-	-	-		+; 25%	-	+; 25%
33	C322/09	Carcinoma NOS	++; 75%	-	-	-	+; 25%	++; 25%	++; 25%
34	C400/09	Carcinoma NOS	+++; 25%	-	++; 25%	-	-	+++; 25%	+++; 50%
35	C437/09	Carcinoma NOS	++; 25%	-	-	-	-	++;25%	-
36	C468/09	Reactive Effusion	-	++ ; 75%	-	-	++ 50%	+; 50%	++; 25%
37	C502/09	Bronchic carcinoma	-	-	-	++;25%	++ ; 25%	++;25%	++;25%
38	C520/09	Mesotelioma	++;75%	+++;90%	+++	+++;90%	+; 25%	++;25%	+++;90%
39	C528/09A	Reactive Effusion	++; 90%	++; 90%	+++; 75%	-	-	+++; 90%	+++; 90%
40	C528/09B	Reactive Effusion	++; 90%	++; 90%	+; 75%	-	++; 25%	+++; 75%	+++; 75%
41	C528/09C	Reactive Effusion	++; 75%	++; 25%	++; 50%	-	+; 25%	++; 25%	++; 25%
42	C549/09	Mammary carcinoma	-	-	-	-	+++; 50%	++; 25%	-
43	C566/09	Mesotelioma rhabdoid	++, 90%	+++; 90%	++, 90%	+ ; 25%	-	++; 25%	++; 50%
44	C652/09	Carcinoma NOS	-	-	-	+ ; 25%	++; 25%	+++; 90%	++ ; 25%

Legend: NOS, Not otherwise specified; TCC, Transitional Cell Carcinoma.

Table 8. Immunocytochemical staining intensity and median (minimum-maximum) percentage of positive tumor cells for **canine** effusion specimens, based on histologic diagnoses.

	Reactive Mesothelium		Mesothelioma		Carcinoma	
n	14		5		13	
	Intensity	**% cells**	**Intensity**	**% cells**	**Intensity**	**% cells**
CKAE1/AE3	- / +++	25 - 90	++ / +++	75 - 90	- / ++	25 - 75
VIM	+ / +++	25 - 90	- / +++	50 - 90	- / +++	50 - 75
DESM	- / +++	25 - 75	- / +++	25 - 90	- / +++	25 - 75
CK5/6	- / +	25	- / +++	25 - 90	-	-
HBME-1	- / +++	25 - 75	- / ++	25 - 75	- / ++	25 -75
CK7	- / +++	25 - 90	- / +++	25 - 90	- / +++	25 - 75
CK20	- / +++	25 - 90	++ / +++	50 - 90	- / +++	25 - 90

Table 9. Immunocytochemical staining intensity and median (minimum-maximum) percentage of positive tumor cells for **feline** effusion specimens, based on histologic diagnoses.

	Reactive Mesothelium		Mesothelioma		Carcinoma	
n	0		1		11	
	Intensity	**% cells**	**Intensity**	**% cells**	**Intensity**	**% cells**
CKAE1/AE3	ND	ND	-	-	- / ++	25 - 90
VIM	ND	ND	+++	90	- / +++	25 - 90
DESM	ND	ND	-	-	- / ++	25
CK5/6	ND	ND	-	-	- / ++	25
HBME-1	ND	ND	-	-	- / ++	25 -75
CK7	ND	ND	+++	25	- / +++	25 - 90
CK20	ND	ND	-	-	++ / +++	25 – 75

Legend: ND, Not done.

Table 10 exposes the summary of each antibody reactivity for each diagnostic group. Vimentin reactivity was seen in all cases of benign mesothelial cells. Most of mesothelioma cases also expressed vimentin, but only 23% and 18% of the canine and feline carcinoma cases, respectively, showed staining with anti-vimentin antibody. The staining intensity was variable for all diagnostic groups, but consistently higher in benign mesothelial cells.

Table 10. Overall immunocytochemical reactivity of individual markers in canine and feline effusions for each cytological diagnosis.

	Reactive Mesothelium		Mesothelioma		Carcinoma	
	Dog, %	**Cat, %**	**Dog, %**	**Cat, %**	**Dog, %**	**Cat, %**
CKAE1/AE3	86 (12/14)	ND	60 (3/5)	0 (0/1)	46 (6/13)	55 (6/11)
VIM	100 (14/14)	ND	80 (4/5)	100 (1/1)	23 (3/13)	18 (2/11)
DESM	57 (8/14)	ND	80 (4/5)	0 (0/1)	38 (5/13)	18 (2/11)
CK5/6	14 (2/14)	ND	60 (3/5)	0 (0/1)	0 (0/13)	9 (1/11)
HBME-1	93 (13/14)	ND	80 (4/5)	100 (1/1)	77 (10/13)	27 (3/11)
CK7	71 (10/14)	ND	80 (4/5)	100 (1/1)	62 (8/13)	64 (7/11)
CK20	86 (12/14)	ND	100 (5/5)	0 (0/1)	85 (11/13)	64 (7/11)

Legend: ND, Not done.

Reactivity to cytokeratins was variable and dependent upon keratin subtype. Pancytokeratin (CKAE1AE3), CK7 and CK20 staining was present in a large number of cases in all diagnostic groups. As depicted in table 11, in canine samples, CKAE1AE3, when evaluated together with vimentin, had a moderately high sensitivy and high specificity for mesothelial cells. However, when present in carcinoma cells the reactivity was seen in a lower percentage of cells and with a low intensity (table 8).

Table 11. Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of antibodies for the detection of **canine** mesothelial cells

	CKAE1AE3+/ Vim+	HBME-1	CK5/6
Sensitivity, %	79	**89**	26
Specificity, %	**92**	23	**100**
PPV, %	94	63	100
NPV, %	75	60	100
Accuracy, %	84	63	56

CK5/6 was expressed in a reduced number of cases. In dogs, no carcinoma cases were reactive to CK5/6, whereas in cats only one showed reactivity. For canine samples, CK5/6 demonstrated a high specificity for mesothelium, being

expressed in a higher percentage and staining intensity in mesothelioma cases. Yet, it had a very low sensitivity (table 11), being present in only 16% of all canine samples.

Table 12. Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of CK7-/CK20+ for the detection of **canine** carcinomatous cells.

	CK7-/CK20+
Sensitivity, %	30
Specificity, %	**79**
PPV, %	43
NPV, %	60
Accuracy, %	56

The CK7-/CK20+ panel was only present in 22% of all canine cases. It had a very poor sensitivity (30%) but, when present, showed a moderate specificity (79%) for the detection of metastasis in canine samples. In all group samples the staining intensity and percentage of positive cells was variable.

Table 13. Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of Desmin for the detection of benign mesothelial cells in **dogs**.

	Desm
Sensitivity, %	57
Specificity, %	20
PPV, %	67
NPV, %	14
Accuracy, %	47

Desmin reactivity was present in all group samples. It was observed in 57% of the reactive mesothelial cases, and in 80% of mesothelioma cells, with a variable staining intensity. Moreover, it was also expressed in 38% and 18% of canine and feline carcinomas, respectively.

HBME-1 was invariably present in most benign and neoplastic mesothelial cells and in carcinoma cells too. So it is a very sensitive although a not specific marker (table 11). Its staining intensity was higher in reactive effusions. Of particular note, the staining pattern of mesothelial cells was predominantly membranar, as opposed to the cytoplasmic pattern seen in carcinoma cells (fig 7). However, this staining feature was not always present, or nor always easy to evaluate.

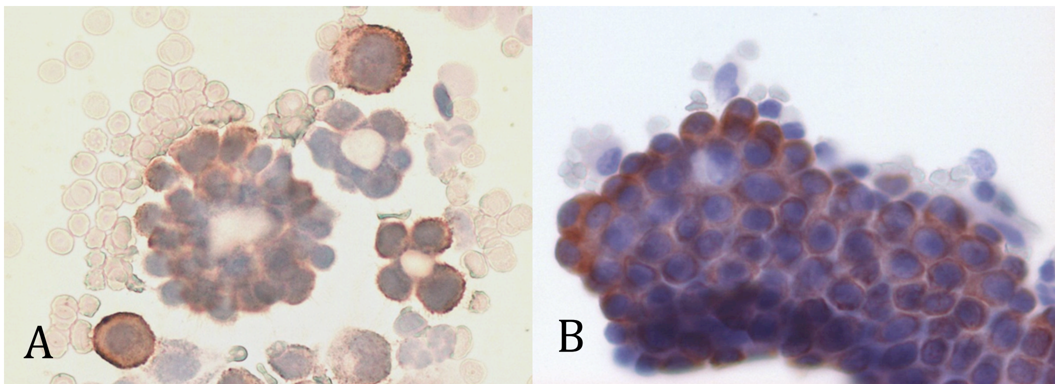


***Figure 7**. Immunocytochemical staining with HBME-1. In A, reactive mesothelial cells show positive cytoplasmic and thick membranar staining. Carcinomatous cells in effusion (B) predominantly express a cytoplasmic staining.*

Immunohistochemistry

In 16 cases, the immunocytochemical staining was compared with the immunohistochemical reactivity from the corresponding material. Comparison of the immunoreactivities between cytologic and histologic specimens from the same case is presented in table 14.

Table 14. Comparison of immunocytochemical and immunohistochemical reactivity from the same case.

	Histology > Cytology, %	**Cytology > Histology, %**
CKAE1/AE3	**25** (4/16)	6 (1/16)
VIM	6 (1/16)	**44** (7/16)
DESM	0 (0/16)	**50** (8/16)
CK5/6	6 (1/16)	6 (1/16)
HBME-1	6 (1/16)	0 (0/16)
CK7	0 (0/16)	**37** (6/16)
CK20	6 (1/16)	6 (1/16)

There was usually a good agreement between the two techniques, but some random discrepancies were observed (table 14). In general, CKAE1/AE3 staining was present in a higher number of histologic tissues, while for vimentin, desmin and CK7 antibodies, reactivity varied considerably, being predominantly lower in histologic sections.

Of particular notice, was the fact that vimentin positivity was infrequently seen in mesothelial cells with cuboidal morphology, when compared with flattened mesothelial cells (fig 8).

Staining intensities and patterns were similar, with random occasional differences, between histologic and cytologic material. Of note, HBME-1 maintained the same pattern of expression as seen in cytologic specimens, being more intense on the cell membrane (fig 9-A,B). In contrast, other markers usually demonstrated major cytoplasmic expression (fig 9-C,D).

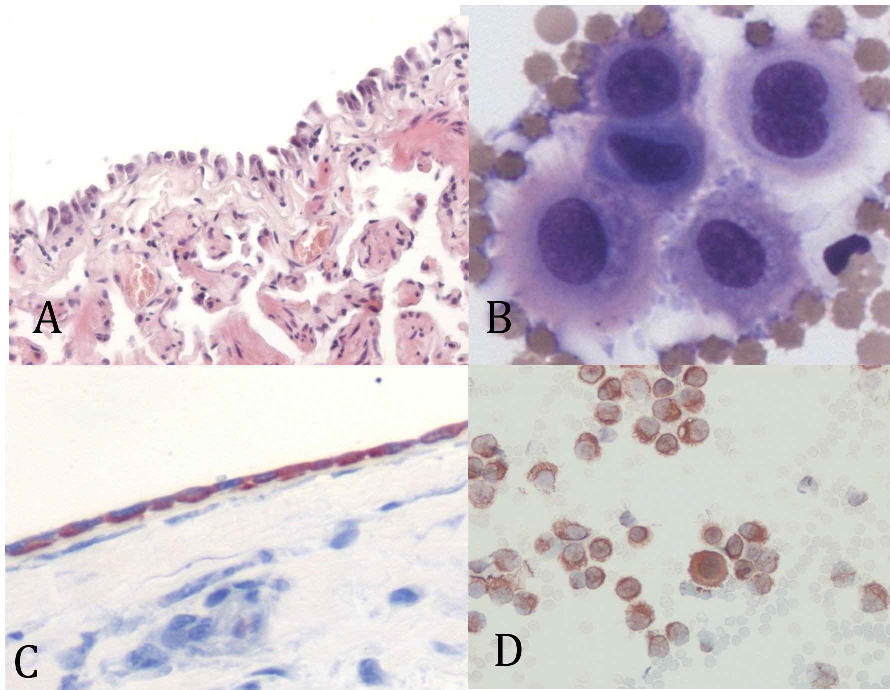


Figure 8. *Histologic (A), cytologic (B), immunohistocheminal (C) and immunocytochemical (D) appearance of benign mesothelial cells. Different morphologies of mesothelial cells are illustrated in A – cuboidal, and C – flattened cells. Vimentin staining of flattened mesothelial cells (C) and of detached cells in effusions. Note that in D also macrophages and neutrophils appear positive.*

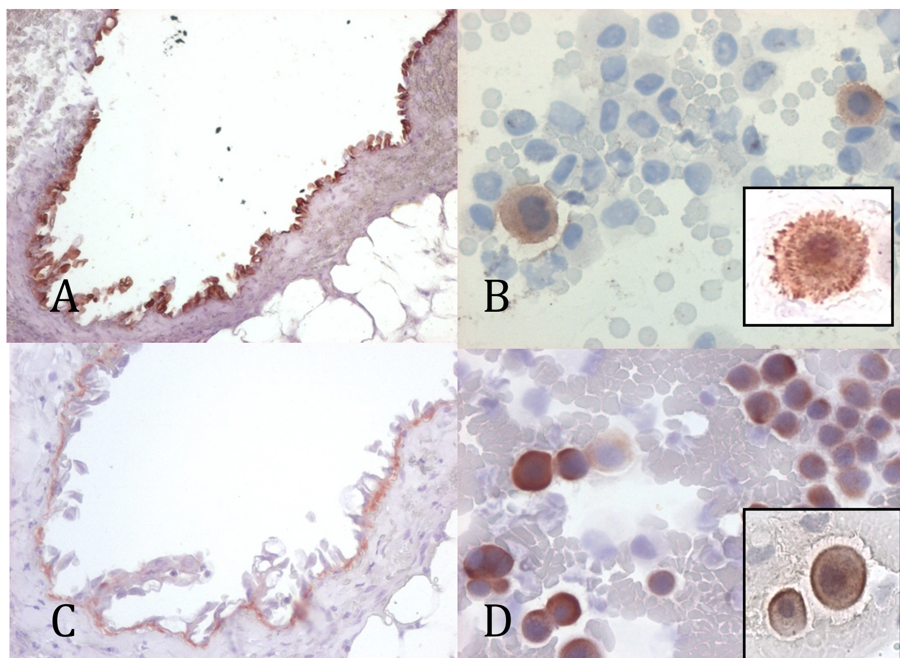


Figure 9. *Immunohistochemical staining of mesothelial tissue (A, C) and immunocytochemistry of a reactive effusion (B, D) with HBME-1 (A, B) and CKAE1/AE3 (C, D). Note the predominant membranar and vilous pattern of HBME-1 reactivity in mesothelial cells, as opposed to the cytoplasmic pattern of cytokeratin (inset).*

The profile of antibody reactivity was similar to that observed in cytologic specimens. All markers, with the exception of CK5/6 for canine tissues, were present in the 3 types of sample goups. HBME-1, a formerly considered specific marker for mesothelium, was present both on mesothelial and carcinoma cells (fig 10).

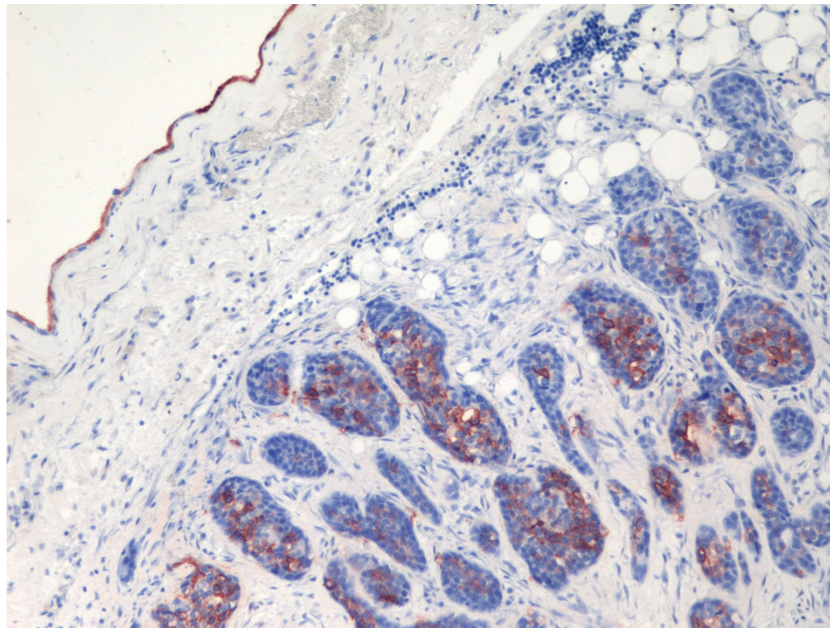


***Figure 10**. Immunohistochemistry with HBME-1. Immunoreactivity is seen both in the carcinoma part and in the intact mesothelium.*

DISCUSSION

Effusion cytologies are among the most difficult to evaluate objectively, mostly because effusion can occur in patients with cancer, although not containing neoplastic cells. The major problem related with body cavity fluids analyses is the possibility of the existence of a secondary inflammation that will modify the mesothelial cells. (Mohanty and Dey, 2003) These cells may vary widely in their morphology, resulting in difficulty distinguishing between macrophages, mesothelial and carcinoma cells (table 15) (Henderson *et al.*, 1998; Whitaker, 2000). Moreover, other types of tumors may exfoliate into the pleural or peritoneal cavity, being the correct interpretation of the cells dependent upon the cytologist ability to recognize and characterize them. (Delahaye *et al.*, 1997; Davidson, 2004; Politi *et al.*, 2005; Addis and Roche, 2009)

The basic cytological criteria of malignancy and tumor type differentiation can not be directly applied to effusion specimens for interpret them as positive for malignancy. In the effusion fluid, the surface tension causes cells to "round-up", and the native shape of cancer cells in traditional fine needle aspiration cytology (FNAC) cannot be useful to decipher the tumor type. Also, the nutrient-rich effusion fluid causes cells to divide and form proliferation spheres. (Whitaker, 2000)

A general approach of microscopic evaluation considered useful towards the identification of neoplastic cells in effusion cytology is the "two cell population theory" (Shidham and Falzon, 2010). Although mesothelial cells in effusion fluid present with a wide morphological spectrum (fig 1; table 10), all cells of the same family are similar and demonstrate subtle morphological continuum (Shidham and Falzon, 2010). In contrast, malignant effusions with metastatic tumors to the mesothelial cavities usually show a morphologically alien population. However, in some cases this distinction, although suggestive, can be difficult. In such cases, further evaluation with ancillary tools such as immunocytochemistry may objectively demonstrate the second neoplastic population. (Shidham and Falzon, 2010)

In human medicine, the ongoing development of immunostains has been keeping effusion cytology in the research spotlight (Zimmerman, 2005; Órdonez, 2007; Metzgeroth *et al.*, 2008). Several studies have shown that immunocytochemistry in combination with conventional cytology can improve

specificity and sensibility for the detection of malignant cells in body cavity fluids. (Delahaye *et al.*, 1997; Fetsch and Abati, 2001; Ko *et al.*, 2001; Zimmerman, 2005; Politi *et al.*, 2005; Ordónez, 2006; Ordónez, 2007)

Table 15. Cytologic features of Reactive Effusion versus Mesothelioma versus Carcinoma (Stevens *et al.*, 1992; Yu *et al.*, 1999; Davidson, 2004; Cakir *et al.*, 2009; Kimura *et al*,. 2009; Shidham and Falzon, 2010)

	Reactive Effusion	**Mesothelioma**	**Carcinoma**
Cytologic Pattern	- proliferative cell balls (fig 4.F) - monolayer cell aggregates - acini-like strutures (fig. 4C,E) - spheres with collagenous cores (fig. 4F) - occasional papillary groups - extensive morphologic variation		- variable, depending on tumor type
	- solitary cells (fig. 4E) - few cohesive clusters (fig. 4C)	- frequent cohesive clusters (usually > 50 cells)	
Citoplasmic Features	- faint staining thin halo along the edge (microvilli) (fig 4 A-C) - peripheral blebs (fig. 4G) - two-zone cytoplasm with peripheral vacuolation / foamy appearance (mainly at cell periphery) (fig. 4D) - phagocytic activity (fig. 4H)		- variable, depending on tumor type - absence of microvilli
Nuclear Features	- centrally placed nuclei (fig. 4A-C) - oval to round nuclei - hyperchromatic nuclei - bi- and multinucleation with anisonucleosis (fig. 4G,H) - mitotic features - high nucleo:cytoplasmic ratio (fig. 4C) - macrokaryosis		- usually eccentric located nuclei, touching the cytoplasmic membrane (no rim of cytoplasm between nucleus and cell membrane) (fig. 6B)
Other	- mesothelial windows between the cells (more typical of mesothelial origin, but also present on carcinomas – fig. 6C) - cell-in-cell configuration (more typical of reactive mesothelium) (fig. 4G)		

Large-scale cytology studies evaluating the use of antibodies in effusion cytology have been performed on a myriad of preparations (smears and cytospins, ThinPrep preparations and cell blocks), and focus mostly on the distinction of reactive mesothelium (RM) from mesothelioma (MM) and adenocarcinoma (AC). Despite all this research, no single antibody specific for an entity has been found (Fetsch and Abati, 2001). In immunohistochemistry, only few single markers show site specificity, such as prostatic specific antigen (Mai *et al*., 2008). Therefore, the use of a panel of antibodies with a high combined sensitivity and specificity is recommended for the identification of a tumor type (Fetsch and Abati, 2001; Davidson, 2004; Politi *et al*., 2005).

To our knowledge, no previous studies on immunocytochemistry of effusion have been published in veterinary medicine. In this vein, based on what is stated on human medicine, we started to study a basic panel of markers for the identification of mesothelial (benign and malignant) and carcinoma cells.

On the antibody crossreactivity on mesothelial cells

To our knowledge, no previous reports exist on the culture of feline or canine mesothelial cells. In this study, the culture conditions, and essentialy the isolation procedure, were adequate. Therefore, this isolation procedure can be used for the characterization of canine and feline mesothelial cells. Care is required to prevent contamination of mesothelial cell cultures with peritoneal fibroblasts. Theoretically, peritoneal fibroblasts can be distinguished by their elongated appearance, but it is not easy to distinghuish such features in culture. Most importantly, the negative staining for cytokeratin will definitely identify such cells as non-mesothelial (Stylianou *et al*., 1990; Yung *et al*., 2006).

Based on the western blot results, the specific cross-reactivity of the antibodies on the feline and canine mesothelial tissue was demonstrated. Moreover, it was demonstrated that these antibodies reacted with the same specific proteins also identified in other control tissues. So, feline and canine mesothelial cells do express Vimentin, CK AE1/AE3, CK5/6, CK7, CK20 and Desm, and the same results were found for human cells. This is in contrast with some reports that affirm that human mesothelial cells do not express CK20 (Ascoli *et al*., 1995; Tot, 2002; Han *et al.*, 2004). In fact, also in the results of

immunocytochemistry and immunohistochemistry performed in this study, it was found a high percentage of mesothelial cells positive to CK20. In this vein, the positivity to CK20 within a cell in a effusion can not be seen as a marker of metastasis, as the local mesothelial tissue can also express this protein.

On the immunocytochemistry staining method in effusion samples

With the results of the present study, it can be concluded that the immunocytochemical procedure implemented in this study is feasible and can be reasonably adopted in effusion cytology in veterinary medicine. The methodology described was able to produce viable results, and is recommended to cytologists aiming to add or enhance an immunocytochemical service on effusion samples.

Basic recommendations for achieving optimal results are the same as employed for the immunocytochemistry of other tissue samples (Lee *et al.*, 2000; Vernau, 2005; Ramos-Vara *et al.*, 2008; Valli *et al.*, 2009). Performing a thin monolayer preparation is of maximum importance. In our study, fixation was achieved with 100% acetone, for 3 minutes, at 4ºC. However, other methods such as 10% formalin for at least 1 minute, can be applied (Suthipintawong *et al.*, 1996). Fixation with acetone gives the most consistent results when cells have a well-spaced distribution on the slide. However, it is reported that cell clusters or tridimensional tissue fragments will be detached, with almost complete loss of cells if forceful jet washing occurs between stains, as it frequently happens with automated stainers (Valli *et al.*, 2009). In our experience, as the technique was all manual, the washing could be processed gently and cell lost was absent or minimal.

Cytologic specimens fixed in formalin, alcohol, or paraformaldehyde have excellent cell preservation but need to be processed for antigen-retrieval, as is routinely done for formalin-fixed paraffin-embedded tissues (Valli *et al.*, 2009).

An important point on immunocytochemistry evaluation is that attention must be taken on evaluating a case as positive. The positivity must be considered only in cells corresponding to the morphology identified as suspect, and should not be based on the positive staining of other unspecific cells, such as the expression of vimentin by neutrophils and macrophages (fig. 11). Also, reactive

mesothelial cells that are present on carcinomatous effusions can produce a positive staining and interfere with the adequate assessment of the tumor reactivity. This dilemma could be minimized by the use of cell-block preparations, where cell morphology can be assessed on Hematoxylin-Eosin (H&E) staining. Then, consecutive cuts of the paraffin-embebbed sample can be made, and the cells previously observed can be "objectively" tested. (Fetsch *et al.*, 2002) Advantages of cell blocks include good morphological interpretation, relative comparability of immunoreactivity results with formalin-fixed paraffin-embedded tissue sections, evaluation of many immunomarkers simultaneously, and an archival benefit with availability of material for other types of testing in the future. (Gong *et al.*, 2003; Kim *et al.*, 2009; Shidham and Falzon, 2010)

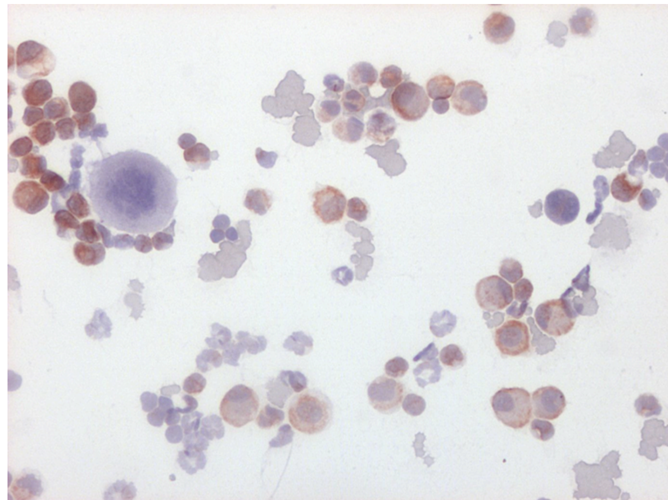


Figure 11. *Carcinomatous effusion. Immunocytochemical staining with. vimentin. Note that both macrophages and neutrophills are expressing vimentin, while the large neoplastic cells is not.*

Human medicine literature provides conflicting results regarding the usefulness of diverse techniques for the assessment of the immunoprofile in effusion cytology. Fetsch et al. (2000) reported that cytospin and ThinPrep samples provided similar results with a high background staining in 66% of cases. Also, the author referred that membrane staining patterns were difficult to interpret with these techniques and concluded that cell blocks provided the best option for morphologic interpretation, with less background staining and results that most closely approximated those reported in the surgical pathology literature. In human medicine,

the cost per test for the cell block technique can also be optimized and provide the most economical option. On the other hand, the immunoreactivity of ethanol-fixed and formalin-fixed cell-blocks can be significantly lower with most of the antibodies. For this reason, Ueda *et al*. (2006) concluded that smear preparations are more sensitive than cell-blocks for immunocytochemical studies.

On the value of a panel of markers

Regarding the evaluation of the utility of a panel of markers in effusion cytology, it can be concluded that the coexpression of cytokeratin and vimentin has the highest overall accuracy for the identification of mesothelial cells, whereas HBME-1 and CK5/6 are, respectively, highly sensitive and specific. Desmin has a very poor specificity for the identification of benign mesothelial cells. The panel CK7-/CK20+ is moderately specific for carcinoma cells, and has an overall accuracy of only 56%.

No significant differences in the proposed panel were found between canine and feline immunoreactivity, except for a lower expression of HBME-1 in feline carcinomatous effusions. The agreement between canine and feline immunoreactivity for mesothelial cells in reactive effusions and mesotheliomas could not be assessed due to the reduced number of feline cases.

The coexpression of Vim+/CKAE1AE3+ and the individual markers HBME-1 and CK5/6 were studied as mesothelial markers.

Cytokeratins (CKs) are intermediate-sized monofilaments found in the cytoplasm of nearly all epithelial cell types. Monoclonal antibodies to specific cytokeratin subtypes have been used in an attempt to classify tumor origin (Tot, 2002; Chu and Weiss, 2002b). CKAE1/AE3 is a mixture of two different clones of anti-cytokeratin monoclonal antibodies: AE1 (for cytokeratins 10, 14, 15, 16 and 19) and AE3 (for cytokeratins 1, 2, 3, 4, 5, 6, 7 and 8) (Chu and Weiss, 2002b). In the present study, CKAE1/AE3 expression was seen most frequently in reactive effusions and mesotheliomas, with a lower expression in carcinomatous cells.

Vimentin is a group III intermediate filament that primary identifies cells of mesodermal origin. It is expressed by both benign and malignant connective

tissue, as well as in benign mesothelial cells and mesothelioma (fig. 12D) (Afify *et al.*, 2002; Reggeti *et al.*, 2005). Some reactivity in adenocarcinomas has also been reported. Although frequently seen in mesotheliomas, it is less often detected in sections of the epithelial component (Dejmek and Hjerpe, 2000). Vimentin can also be expressed by macrophages and neutrophils (Mor-Vaknin *et al*, 2003), which are cells frequently present in effusions. The cytologist should not overestimate the positive staining of these cells with the specific staining of the cells in study. In this study, vimentin was expressed in all cases of reactive effusions and in most mesotheliomas. Considerably lower was the reactivity of carcinomas, which makes vimentin a potential useful marker for differentiating mesothelial from carcinoma cells, particularly if evaluated together with cytokeratin.

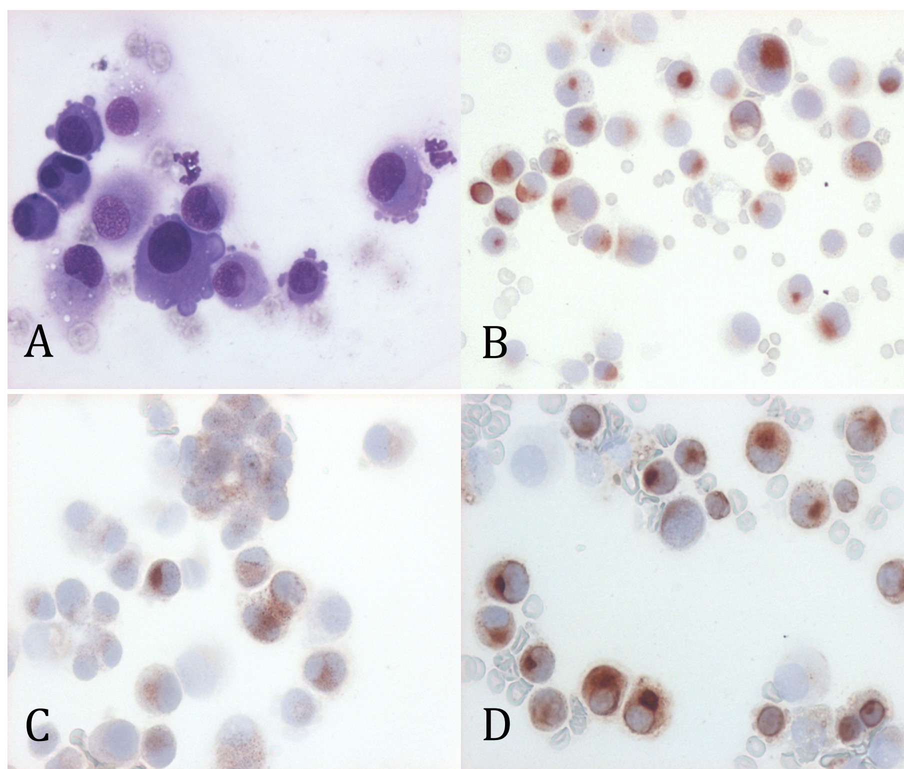


Figure 12. *Mesothelioma. Cytologic appearance (A) and immunocytochemical staining with CK20 (B), CKAE1/AE3 (C) and Vim (D), notably in a perinuclear pattern.*

Both in human (LaRocca and Rheinwald, 1984) and veterinary medicine (Moroni *et al.*, 2006), cytokeratin and vimentin coexpression within a cell is

suggestive of a mesothelial origin, particularly if the filaments are prominent and in a perinuclear distribution (fig. 8C-D) (King *et al.*, 2006). This coexpression, however, can be seen in other tumors including anaplastic carcinomas, amelanotic melanomas, renal carcinomas and Sertoli cell tumors (Reggeti *et al.*, 2005). In the present study, when analysed together, positivity to cytokeratin and vimentin was a very specific marker of mesothelial cell origin. Only 4 carcinomas (two mammary adenocarcinomas and two non specified) demonstrated such coexpression. The overall accuracy of this panel is moderately high, being the highest of the studied antibodies. Thus, results of this study corroborate previous studies in immunohistochemistry, and denote that the coexpression of cytokeratins and vimentin can be a useful parameter for the identification of mesothelial cells in effusion cytology.

CK5/6 specifically targets the CK5 moiety of the CK5/CK14 pair, which is almost exclusively expressed in the cytoplasm of mesothelial derivates, and therefore has the potential to distinguish between adenocarcinomas from mesotheliomas (Fetsh and Abati, 2001; Chu and Weiss, 2002a; Zimmerman, 2005). The presented results highlight the fact that CK5/6 is a very specific marker for mesothelial cell origin, but has poor sensitivity. This parallels what is stated also in human medicine (Shield and Koivurinne, 2008). Its value should be related with its use for confirmation of a mesothelial cell origin, after a prior staining with more sensitive markers or after a suspect is determined with microscopic examination.

HBME-1 (Hector Battifora Mesothelial Cell-1) is a recently available monoclonal antibody that reacts with an unknown antigen on microvilli of mesothelial cells (Politi *et al*., 2005; Bacci *et al*., 2006). HBME-1 was originally described as a specific marker of normal and malignant mesothelial cells (González-Lois *et al.*, 2001), but it was later shown to also stain many carcinomas (Longatto Filho *et al*., 2002; Papotti *et al*., 2005). In fact, it is reported that HBME-1 is very sensitive for human benign and malignant mesothelium, but reacts also with 15-100% of adenocarcinoma cells (Su *et al.*, 2010). Usually, HBME-1 is expressed as a thick membrane pattern in mesothelial cells due to abundant lung

microvilli on the surface of these cells, and cytoplasmic pattern in adenocarcinoma cells (Mocanu *et al.*, 2006). However, some overlap has been reported by researchers. This study showed that the sensitivity of HBME-1 for mesothelial cells (either benign or malignant) was 89%. The specificity was only 23%. Due to this low specificity and overllaped positive membranar pattern, it can be concluded that the value of HBME-1 for the distinction of mesothelial cells from carcinoma cells is limited.

Desmin is an intracellular intermediate filament characteristically demonstrated in smooth and skeletal muscle. It has also been described in non-myogenous tumors, including primitive neuro-ectodermal tumors. It is more commonly expressed in benign rather than malignant mesothelium, producing a cytoplasmic staining pattern (Gill *et al.*, 2000; Davidson *et al.*, 2001; Su *et al.*, 2010). For this reason is used in human medicine to differentiate reactive from malignant mesothelium (Su *et al.*, 2010; Hasteh *et al.*, 2010). Other authors consider that there is not a marker able to precisely differentiate reactive mesothelium from mesothelioma (Hurlimann, 1994). Along with desmin, other markers as epithelial membrane antigen (EMA), p53 and bcl-2 have been used but so far with confliting results (Cury *et al.*, 1999; Attanoos *et al*, 2003). In this study, desmin was not a valuable marker for identifying malignancy in mesothelial cells, being even expressed in a major percentage in mesotheliomas, compared with reactive effusions.

CK20 is a newly identified cytokeratin, remarkable for being unique to a small subset of epithelia and their corresponding tumors (ex, gastrointestinal tract) (Tot, 1999; 2002). In human literature several reports consider its reactivity almost absent with mesothelium (Ascoli *et al.*, 1995; Tot, 2002; Han *et al.*, 2004), a particular useful feature in effusion specimens (Sack and Roberts, 1997). By this way, finding reactivity to CK20 in an effusion sample can be an indication of metastasis. On the other hand, other articles report that benign and malignant mesothelial cells can also express CK20 (Garcia-Prats, 1998). His spectrum of activity is somewhat complementary to that characteristic of CK7,

which is mainly found in adenocarcinomas of breast and lung (table 16) (Ramaekers *et al.*, 1990; Tot, 1999; 2001; 2002).

In this study, the coordinate expression of CK7-/CK20+ was investigated as a marker of carcinoma cells in effusion. Our results suggest that this panel of markers is not sufficiently specific for the identification of carcinomatous cells, having also a poor sensitivity. In fact, mesothelial cells frequently expressed CK20, and occasionally CK7, making this panel of markers an unvaluable option for the identification of carcinomatous cells in effusion immunocytochemistry.

Table 16. Summary of predominant CK7/CK20 expression of human, canine and feline neoplasms.

	Human	**Dog**	**Cat**
CK7+/CK20+	Transitional Cell Carcinoma	-	Pancreatic adenocarcinoma
CK7-/CK20+	Gastrointestinal tumor	Gastrointestinal tumor	Thyroid carcinoma
CK7+/CK20-	Mammary adenocarcinoma Lung adenocarcinoma Ovary adenocarcinoma **Mesothelioma**	Mammary adenocarcinoma Cholangiocarcinoma **Mesothelioma**	Mammary adenocarcinoma **Mesothelioma**
CK7-/CK20-	Hepatocellular carcinoma Prostatic adenocarcinoma Adrenal gland tumor	Hepatocellular carcinoma Renal carcinoma Bronchioalveolar carcinoma Pancreatic adenocarcinoma	Gastric adenocarcinoma

On immunohistochemical studies, the coordinated profile of CK7/CK20 demonstrated useful in discriminating primary origins of metastatic tumors in human (Sack and Roberts, 1997; Tot, 1999; 2002; Chu *et al.*, 2000; Rubin *et al.*, 2001) and veterinary medicine (Espinosa de los Monteros *et al.*, 1999) (table 16). This feature can be useful in effusion cytology, as a possible clue to the primary site of origin of the carcinomatous cells (Longatto Filho *et al.*, 1997; Jang

et al., 2001; Stopyra *et al.*, 2001; Pomjanski *et al.*, 2005). In fact, in effusions it is not only useful to distinguish mesothelioma from carcinoma, but also to identify the origin of a given malignant neoplasm (Tot, 1999). The search for an unknown primary tumor is a common clinical problem. Extensive radiologic and endoscopic examinations are expensive, time consuming, labouring, and often unsuccessful. (Tot, 1999) Consequently, pathologists play an essential role on the search for a clue about the primary tumor site. Moreover, other than for therapeutic decision-making, a proper diagnosis of the primary site is important for correct epidemiologic registration, which in turn influences our knowledge about the natural history and prognosis of particular tumor types. (Tot, 1999; 2002)

In this study, we determined the exact primary location of 9 carcinomas. In these cases, the results of the coordinate expression of CK7 and CK20 were variable and no specific patterns could be correlated with a particular primary site, as those reported in table 11. However, in this study the number of cases with known primary site was reduced, and no preliminary conclusions should be drawn from these results.

The results obtained in this study with histological and cytological specimens were not always consistent. Our results show a lower reactivity of vimentin, desmin and CK7 antibodies in histologic sections, as compared with cytologic specimens, while the opposite happened with CKAE1AE3. In human literature, it is reported that a discrepancy rate of 10% can occur in various antibodies (Chin-Yang *et al.,* 1987). Tissue processing of histologic samples can influence antigenicity by causing protein loss or relocation of antigens. In fact, antigenicity is dependent on the physicochemical nature of the three-dimensional structure of the antigen. (Valli *et al.*, 2009) Varying degrees of antigens loss may occur with different fixatives. Alcohol and acetone are dehydrating and denaturating agents, which alter antigenicity minimally. However, translocation of antigens and even diffusion of antigens out of the tissue may occur. (Pettigrew, 1989) On the other hand, aldehydes fix primarily by creating covalent cross-links between proteins. For example, it is known that

formalin-fixed tissue has shown significant loss of vimentin reactivity (Azumi and Battifora, 1987).

Therefore, discrepant immunocytochemical results may be caused by the fact that the formaldehyde fixation used for histological material may have destroyed antigenic reactivity, giving false negative results. Lack of reactivity in cytological material compared with histological sections may be due to impaired penetration of the fixative or of the antibody to the central parts of large or multilayered complexes, or to possible alterations at the reaction site in cytological specimens (Dinges *et al.*, 1989). This is particularly true for immunocytochemical stains of uneven smears, where inconsistent results may be obtained in large cell groups with lack of reactivity in central portions of the cell complexes. This has been noted especially with antibodies to cytoskeletal proteins (Dinges *et al.*, 1989; Dejmek and Hjerpe, 2000). Also, uneven smear preparations may be cause of non-specific reactivity (Pettigrew, 1989).

Other than technique-related discrepancies, this inconsistency between cytologic and histologic immunostaining can be due to altered protein expression within a cell suspended in an effusion (Dejmek and Hjerpe, 2000). Adenocarcinoma cells, for example, may switch on the expression of vimentin when they are detached in a fluid (Ramaekers *et al.*, 1983). The same is described for mesothelial cells, as they undergo the phenomenon of epithelial-to-mesenchimal transition (EMT) (Yung *et al.*, 2006; Lee and Ha, 2007; Aroeira *et al.*, 2008). The EMT phenomenon is responsible for the convertion of mesothelial cells from an epithelial to a mesenchymal phenotype with a progressive loss of epithelial morphology and a decrease in the expression of cytokeratins and E-cadherin through an induction of the transcriptional repressor *snail (*Yáñez-Mó *et al.*, 2003; Aguilera *et al.*, 2005).

CONCLUSION

There is no single marker that appears sensitive and specific enough to correctly diagnose the cause of a body cavity effusion. From this study, it can be concluded that the chosen panel of human antibodies cross-react with canine and feline mesothelial cells. The most useful marker, with the highest overall accuracy for the identification of mesothelial cells in effusion, is the Vim/CK coexpression. CK5/6 is the most specific and HBME-1 the more sensitive mesothelial cell marker, but both have a poor overall accuracy. Desmin is not a valuable marker for the distinction between benign and malignant mesothelial cells. The coordinate expression of CK7-/CK20+ has not proved to be useful on the identification of metastatic cells on effusions.

Therefore, it is advised that the immunocytochemical procedure must be done after a careful microscopic examination. Specific mesothelial markers, such as CK5/6, can be used to confirm a suspected mesothelial cell origin, after more sensitive markers, such as HBME-1, are used. With this study, we found no specific markers for mesothelial cell malignancy or carcinoma cells on effusion.

Immunocytochemistry technique on effusion cytology can indeed be a reliable procedure able to produce viable results, if one do not forget the following golden principles (Fowler and Lachar, 2008):

1. Immunocytochemical analyses can only be interpreted in the context of an informed, carefully considered clinical and cytologic diagnosis.
2. Single immunostains are unlikely to provide a specific diagnosis, even within a limited differential diagnosis.
3. Even a panel of immunostains is not specific for a certain diagnosis. Rather, reproducible immunoprofiles have distinct relative predictive values for different diagnostic alternatives.

BIBLIOGRAPHIC REFERENCES

Abati A, Fetsch P, Filie A. If cells could talk. The application of new techniques to cytopathology. Clin Lab Med. 1998 Sep;18(3):561-83.

Addis B, Roche H. Problems in mesothelioma diagnosis. Histopathology. 2009 Jan;54(1):55-68.

Afify AM, Al-Khafaji BM, Paulino AF, Davila RM. Diagnostic use of muscle markers in the cytologic evaluation of serous fluids. Appl Immunohistochem Mol Morphol. 2002 Jun;10(2):178-82.

Aguilera A, Yáñez-Mo M, Selgas R, Sánchez-Madrid F, López-Cabrera M. Epithelial to mesenchymal transition as a triggering factor of peritoneal membrane fibrosis and angiogenesis in peritoneal dialysis patients. Curr Opin Investig Drugs. 2005 Mar;6(3):262-8.

Aroeira LS, Aguilera A, Selgas R, Ramírez-Huesca M, Pérez-Lozano ML, Cirugeda A, Bajo MA, del Peso G, Sánchez-Tomero JA, Jiménez-Heffernan JA, López-Cabrera M. Mesenchymal conversion of mesothelial cells as a mechanism responsible for high solute transport rate in peritoneal dialysis: role of vascular endothelial growth factor. Am J Kidney Dis. 2005 Nov;46(5):938-48.

Aroeira LS, Loureiro J, González-Mateo GT, Fernandez-Millara V, del Peso G, Sánchez-Tomero JA, Ruiz-Ortega M, Bajo MA, López-Cabrera M, Selgas R. Characterization of epithelial-to-mesenchymal transition of mesothelial cells in a mouse model of chronic peritoneal exposure to high glucose dialysate. Perit Dial Int. 2008 Nov;28 Suppl 5:S29-33.

Ascoli V, Taccogna S, Scalzo CC, Nardi F. Utility of cytokeratin 20 in identifying the origin of metastatic carcinomas in effusions. Diagn Cytopathol. 1995 Jun;12(4):303-8.

Attanoos RL, Griffin A, Gibbs AR. The use of immunohistochemistry in distinguishing reactive from neoplastic mesothelium. A novel use for desmin and comparative evaluation with epithelial membrane antigen, p53, platelet-derived growth factor-receptor, P-glycoprotein and Bcl-2. Histopathology. 2003 Sep;43(3):231-8.

Azumi N, Battifora H. The distribution of vimentin and keratin in epithelial and nonepithelial neoplasms. A comprehensive immunohistochemical study on formalin- and alcohol-fixed tumors. Am J Clin Pathol. 1987 Sep;88(3):286-96.

Bacci B, Moranti F, de Meo M, Marcato PS. Ten Cases of Feline Mesothelioma: an Immunohistochemical and Ultrastructural Study. Journal of Comparative Pathology, 2006;134, 347-354.

Bird SD. Mesothelial primary cilia of peritoneal and other serosal surfaces. Cell Biol Int 2004; 28:151–9.

Bot J, Whitaker D, Vivian J, Lake R, Yao V, McCauley R. Culturing mouse peritoneal mesothelial cells. Pathol Res Pract. 2003;199(5):341-4.

Cakir E, Demirag F, Aydin M, Unsal E. Cytopathologic differential diagnosis of malignant mesothelioma, adenocarcinoma and reactive mesothelial cells: A logistic regression analysis. Diagn Cytopathol. 2009 Jan;37(1):4-10.

Chin-Yang L, Lazcno-Villareal O, Pierre RV, Yam LT. Immunochemical identification of cells in serous effusions. Am J Clin Pathol 1987; 88: 696–706.

Chu P, Wu E, Weiss LM. Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. Mod Pathol. 2000;13, 962-72.

Chu PG, Weiss LM. Expression of cytokeratin 5/6 in epithelial neoplasms: an immunohistochemical study of 509 cases. Mod Pathol, 2002a;15, 6-10.

Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. Histopathology, 2002b;40, 403-39.

Connell ND, Rheinwald JG. Regulation of the cytoskeleton in mesothelial cells: reversible loss of keratin and increase in vimentin during rapid growth in culture. Cell. 1983 Aug;34(1):245-53.

Cury PM, Butcher DN, Corrin B, Nicholson AG. The use of histological and immunohistochemical markers to distinguish pleural malignant mesothelioma and in situ mesothelioma from reactive mesothelial hyperplasia and reactive pleural fibrosis. J Pathol. 1999 Oct;189(2):251-7.

Dalquen P, Sauter G, Epper R, Kleiber B, Feichter G, Gudat F. Immunocytochemistry in diagnostic cytology. Recent Results Cancer Res. 1993;133:47-80.

Daly LE, Bourke GJ. Interpretation and uses of medical statistics, 5th edn. Oxford: Blackwell Science 2000;412–5.

Davidson B, Nielsen S, Christensen J, Asschenfeldt P, Berner A, Risberg B, Johansen P. The Role of Desmin and N-Cadherin in Effusion Cytology A Comparative Study Using Established Markers of Mesothelial and Epithelial Cells. The American Journal of Surgical Pathology. 2001;25, 1405–1412.

Davidson B. Malignant Effusions: From Diagnosis to Biology. Diagnostic Cytopathology. 2004; 31, 246-252.

Dejmek A, Hjerpe A. Reactivity of six antibodies in effusions of mesothelioma, adenocarcinoma and mesotheliosis: stepwise logistic regression analysis. Cytopathology. 2000 Feb;11(1):8-17.

Delahaye M, van der Ham F, van der Kwast TH. Complementary value of five carcinoma markers for the diagnosis of malignant mesothelioma,

adenocarcinoma metastasis, and reactive mesothelium in serous effusions. Diagn Cytopathol. 1997 Aug;17(2):115-20.

Di Paolo N, Sacchi G, De Mia M, Gaggiotti E, Capotondo L, Rossi P, et al. Morphology of the peritoneal membrane during continuous ambulatory peritoneal dialysis. Nephron 1986; 44:204–11.

Díaz C, Selgas R, Castro MA, Bajo MA, Fernández de Castro M, Molina S, Jiménez C, Ortiz A, Vara F. Ex vivo proliferation of mesothelial cells directly obtained from peritoneal effluent: its relationship with peritoneal antecedents and functional parameters. Adv Perit Dial. 1998;14:19-24.

Dinges HP, Wirnsberger G, Höfler H. Immunocytochemistry in cytology. Comparative evaluation of different techniques. Anal Quant Cytol Histol. 1989 Feb;11(1):22-32.

Espinosa de los Monteros A, Fernández A, Millán MY, Rodríguez F, Herráez P, Martín de las Mulas J. Coordinate expression of cytokeratins 7 and 20 in feline and canine carcinomas. Vet Pathol. 1999;36, 179-90.

Ferrandez-Izquierdo A, Navarro-Fos S, Gonzalez-Devesa M, Gil-Benso R, Llombart-Bosch A. Immunocytochemical typification of mesothelial cells in effusions: in vivo and in vitro models. Diagn Cytopathol. 1994;10(3):256-62.

Fetsch PA, Abati A. Immunocytochemistry in Effusion Cytology - A Contemporary Review. Cancer Cytopathology. 2001;93, 293-308.

Fetsch PA, Simsir A, Brosky K, Abati A. Comparison of three commonly used cytologic preparations in effusion immunocytochemistry. Diagn Cytopathol. 2002 Jan;26(1):61-6.

Foley-Comer AJ, Herrick SE, Al-Mishlab T, Prêle CM, Laurent GJ, Mutsaers SE. Evidence for incorporation of free-floating mesothelial cells as a mechanism of serosal healing. J Cell Sci. 2002 Apr 1;115(Pt 7):1383-9.

Fowler LJ, Lachar WA. Application of immunohistochemistry to cytology. Arch Pathol Lab Med. 2008 Mar;132(3):373-83.

Garcia-Prats MD, Ballestin C, Sotelo T, Lopez-Encuentra A, Mayordomo JI. A comparative evaluation of immunohistochemical markers for the differential diagnosis of malignant pleural tumours. Histopathology. 1998 May;32(5):462-72.

Gill SA, Meier PA, Kendall BS. Use of desmin immunohistochemistry to distinguish between mesothelial cells and carcinoma in serous fluid cell block preparations. Acta Cytol. 2000 Nov-Dec;44(6):976-80.

Gong Y, Sun X, Michael CW, Attal S, Williamson BA, Bedrossian CW. Immunocytochemistry of serous effusion specimens: a comparison of ThinPrep vs cell block. Diagn Cytopathol. 2003 Jan;28(1):1-5.

González-Lois C, Ballestín C, Sotelo MT, López-Ríos F, García-Prats MD, Villena V. Combined use of novel epithelial (MOC-31) and mesothelial (HBME-1) immunohistochemical markers for optimal first line diagnostic distinction between mesothelioma and metastatic carcinoma in pleura. Histopathology. 2001 Jun;38(6):528-34.

Gotloib L, Shostack A, Jaichenko J. Ruthenium-redstained anionic charges of rat and mice mesothelial cells and basal lamina: the peritoneum is a negatively charges dialyzing membrane. Nephron 1988; 48:65–70.

Han J, Kim MK, Nam SJ, Yang JH. E-cadherin and cytokeratin subtype profiling in effusion cytology. J Korean Med Sci. 2004 Dec;19(6):826-33.

Hasteh F, Lin GY, Weidner N, Michael CW. The use of immunohistochemistry to distinguish reactive mesothelial cells from malignant mesothelioma in cytologic effusions. Cancer Cytopathol. 2010 Apr 25;118(2):90-6.

Henderson DW, Shilkin KB, Whitaker D. Reactive mesothelial hyperplasia vs mesothelioma, including mesothelioma in situ: a brief review. Am J Clin Pathol. 1998 Sep;110(3):397-404.

Herrick SE, Mutsaers SE. Mesothelial progenitor cells and their potential in tissue engineering. Int J Biochem Cell Biol. 2004 Apr;36(4):621-42.

Herrick SE, Mutsaers SE. The potential of mesothelial cells in tissue engineering and regenerative medicine applications. Int J Artif Organs. 2007 Jun;30(6):527-40.

Hirschberger J, DeNicola DB, Hermanns W, Kraft W. Sensitivity and specificity of cytologic evaluation in the diagnosis of neoplasia in body fluids from dogs and cats. Veterinary Clinical Pathology. 1999;28, 142-146.

Höinghaus R, Hewicker-Trautwein M, Mischke R. Immunocytochemical differentiation of neoplastic and hyperplastic canine epithelial lesions in cytologic imprint preparations. The Veterinary Journal. 2007b; 173, 79–90.

Höinghaus R, Hewicker-Trautwein M, Mischke R. Immunocytochemical differentiation of canine mesenchymal tumors in cytologic imprint preparations. Veterinary Clinical Pathology. 2008;37, 104-11.

Höinghaus R, von Wasielewski R, Hewicker-Trautwein M, Freund M, Mischke R. Immunocytological detection of lymph node metastases in dogs with malignant epithelial tumours. J Comp Pathol. 2007a; 137, 1-8.

Hurlimann J. Desmin and neural marker expression in mesothelial cells and mesotheliomas. Hum Pathol. 1994 Aug;25(8):753-7.

Jang KY, Kang MJ, Lee DG, Chung MJ. Utility of thyroid transcription factor-1 and cytokeratin 7 and 20 immunostaining in the identification of origin in malignant effusions. Anal Quant Cytol Histol. 2001 Dec;23(6):400-4.

Jantz MA, Antony VB. Pathophysiology of the pleura. Respiration. 2008;75(2):121-33.

Jirsova K, Neuwirth A, Kalasova S, Vesela V, Merjava S. Mesothelial proteins are expressed in the human cornea. Exp Eye Res. 2010 Nov;91(5):623-9

Kayser K, Böhm G, Blum S, Beyer M, Zink S, André S, Gabius HJ. Glyco- and immunohistochemical refinement of the differential diagnosis between mesothelioma and metastatic carcinoma and survival analysis of patients. J Pathol. 2001 Feb;193(2):175-80.

Kim JH, Kim GE, Choi YD, Lee JS, Lee JH, Nam JH, Choi C. Immunocytochemical panel for distinguishing between adenocarcinomas and reactive mesothelial cells in effusion cell blocks. Diagn Cytopathol. 2009 Apr;37(4):258-61.

Kimura N, Dota K, Araya Y, Ishidate T, Ishizaka M. Scoring system for differential diagnosis of malignant mesothelioma and reactive mesothelial cells on cytology specimens. Diagn Cytopathol. 2009 Dec;37(12):885-90.

King JE, Thatcher N, Pickering CA, Hasleton PS. Sensitivity and specificity of immunohistochemical markers used in the diagnosis of epithelioid mesothelioma: a detailed systematic analysis using published data. Histopathology. 2006 Feb;48(3):223-32.

Ko E, Jhala NC, Shultz JJ, Chhieng DC. Use of a Panel of Markers in the Differential Diagnosis of Adenocarcinoma and Reactive Mesothelial Cells in Fluid Cytology. American Journal of Clinical Pathology. 2001; 116, 709-715.

Kurien BT, Scofield RH. Protein blotting: a review. J Immunol Methods. 2003 Mar 1;274(1-2):1-15.

Kurien BT, Scofield RH. Western blotting. Methods. 2006 Apr;38(4):283-93.

LaRocca PJ, Rheinwald JG. Coexpression of simple epithelial keratins and vimentin by human mesothelium and mesothelioma in vivo and in culture. Cancer Res. 1984 Jul;44(7):2991-9.

Lee A, Baloch ZW, Yu G, Gupta PK. Mesothelial hyperplasia with reactive atypia: diagnostic pitfalls and role of immunohistochemical studies-a case report. Diagn Cytopathol. 2000 Feb;22(2):113-6.

Lee HB, Ha H. Mechanisms of epithelial-mesenchymal transition of peritoneal mesothelial cells during peritoneal dialysis. J Korean Med Sci. 2007 Dec;22(6):943-5.

Lin O. Challenges in the interpretation of peritoneal cytologic specimens. Arch Pathol Lab Med. 2009 May;133(5):739-42.

Longatto Filho A, Alves VA, Kanamura CT, Nonogaki S, Bortolan J, Lombardo V, Bisi H. Identification of the primary site of metastatic adenocarcinoma in serous effusions. Value of an immunocytochemical panel added to the clinical arsenal. Acta Cytol. 2002 Jul-Aug;46(4):651-8.

Longatto Filho A, Bisi H, Alves VA, Kanamura CT, Oyafuso MS, Bortolan J, Lombardo V. Adenocarcinoma in females detected in serous effusions. Cytomorphologic aspects and immunocytochemical reactivity to cytokeratins 7 and 20. Acta Cytol. 1997 Jul-Aug;41(4):961-71.

Mai KT, Ahmed I, Robertson SJ, Belanger EC, Veinot JP, Islam S. Immunocytochemical study of urine cytological preparations from secondary prostatic adenocarcinoma involving the urinary bladder. Diagn Cytopathol. 2008 Oct;36(10):715-20.

Marchevsky AM. Application of immunohistochemistry to the diagnosis of malignant mesothelioma. Arch Pathol Lab Med. 2008 Mar;132(3):397-401.

Metzgeroth G, Kuhn C, Schultheis B, Hehlmann R, Hastka J. Diagnostic accuracy of cytology and immunocytology in carcinomatous effusions. Cytopathology. 2008 Aug;19(4):205-11.

Michailova KN, Usunoff KG. Serosal membranes (pleura, pericardium, peritoneum). Normal structure, development and experimental pathology. Adv Anat Embryol Cell Biol. 2006;183:i-vii, 1-144.

Michailova KN. The serous membranes in the cat. Electron microscopic observations. Ann Anat. 1996 Oct;178(5):413-24.

Mocanu L, Cîmpean AM, Raica M. Value of antimesothelioma HBME-1 in the diagnosis of inflammatory and malignant pleural effusions. Rom J Morphol Embryol. 2006;47(4):351-5.

Mohanty SK, Dey P. Serous effusions: diagnosis of malignancy beyond cytomorphology. An analytic review. Postgrad Med J. 2003 Oct;79(936):569-74.

Mor-Vaknin N, Punturieri A, Sitwala K, Markovitz DM. Vimentin is secreted by activated macrophages. Nat Cell Biol. 2003; 5:59-63.

Moroni M, Bettini G, Morandi F, Burdisso R, Marcato PS. Deciduoid Peritoneal Mesothelioma in a Dog. Veterinary Pathology. 2006; 43, 198–201.

Mutsaers SE, Whitaker D, Papadimitriou JM. Mesothelial regeneration is not dependent on subserosal cells. J Pathol. 2000 Jan;190(1):86-92.

Mutsaers SE, Whitaker D, Papadimitriou JM. Stimulation of mesothelial cell proliferation by exudate macrophages enhances serosal wound healing in a murine model. Am J Pathol. 2002 Feb;160(2):681-92.

Mutsaers SE, Wilkosz S. Structure and function of mesothelial cells. Cancer Treat Res. 2007;134:1-19.

Mutsaers SE. The mesothelial cell. Int J Biochem Cell Biol. 2004 Jan;36(1):9-16.

Ohan J, Gilbert MA, Brouland JP, Rougier JP, Trugnan G, Wassef M, Leseche G, Drouet L. Phenotypic and functional characteristics of porcine peritoneal mesothelial cells. In Vitro Cell Dev Biol Anim. 1999 Nov-Dec;35(10):625-34.

Ohsawa M, Naka N, Tomita Y, Kawamori D, Kanno H, Aozasa K. Use of immunohistochemical procedures in diagnosing angiosarcoma. Evaluation of 98 cases. Cancer. 1995; 15; 75(12):2867-74.

Ordóñez NG. The diagnostic utility of immunohistochemistry and electron microscopy in distinguishing between peritoneal mesotheliomas and serous carcinomas: a comparative study. Mod Pathol. 2006 Jan;19(1):34-48.

Ordonez NG. What are the current best immunohistochemical markers for the diagnosis of epithelioid mesothelioma? A review and update. Human Pathology. 2007; 38, 1–16.

Papotti M, Rodriguez J, De Pompa R, Bartolazzi A, Rosai J. Galectin-3 and HBME-1 expression in well-differentiated thyroid tumors with follicular architecture of uncertain malignant potential. Mod Pathol. 2005; 18:541-6.

Pettigrew NM. Techniques in immunocytochemistry. Application to diagnostic pathology. Arch Pathol Lab Med. 1989 Jun;113(6):641-4.

Poblet E, Gonzalez-Palacios F, Jimenez FJ. Different immunoreactivity of endothelial markers in well and poorly differentiated areas of angiosarcomas. Virchows Arch. 1996; 428, 217-21.

Politi E, Kandaraki C, Apostolopoulou C, Kyritsi T, Koutselini H. Immunocytochemical Panel for Distinguishing Between Carcinoma and Reactive Mesothelial Cells in Body Cavity Fluids. Diagnostic Cytopathology. 2005; 32, 151-154.

Pomjanski N, Grote HJ, Doganay P, Schmiemann V, Buckstegge B, Böcking A. Immunocytochemical identification of carcinomas of unknown primary in serous effusions. Diagn Cytopathol. 2005 Nov;33(5):309-15.

Ramaekers F, van Niekerk C, Poels L, Schaafsma E, Huijsmans A, Robben H, Schaart G, Vooijs P. Use of monoclonal antibodies to keratin 7 in the differential diagnosis of adenocarcinomas. Am J Pathol. 1990 Mar;136(3):641-55.

Ramaekers FC, Haag D, Kant A, Moesker O, Jap PH, Vooijs GP. Coexpression of keratin- and vimentin-type intermediate filaments in human metastatic carcinoma cells. Proc Natl Acad Sci U S A. 1983 May;80(9):2618-22.

Ramos-Vara JA, Kiupel M, Baszler T, Bliven L, Brodersen B, Chelack B, Czub S, Del Piero F, Dial S, Ehrhart EJ, Graham T, Manning L, Paulsen D, Valli VE, West K; American Association of Veterinary Laboratory Diagnosticians Subcommittee on Standardization of Immunohistochemistry. Suggested guidelines for immunohistochemical techniques in veterinary diagnostic laboratories. J Vet Diagn Invest. 2008 Jul;20(4):393-413.

Reggeti F, Brisson B, Ruotsalo K, Southorn E, Bienzle D. Invasive epithelial mesothelioma in a dog. Veterinary Pathology. 2005; 42, 77-81.

Riera JR, Astengo-Osuna C, Longmate JA, Battifora H. The immunohistochemical diagnostic panel for epithelial mesothelioma: a reevaluation after heat-induced epitope retrieval. Am J Surg Pathol. 1997 Dec;21(12):1409-19.

Rubin BP, Skarin AT, Pisick E, Rizk M, Salgia R. Use of cytokeratins 7 and 20 in determining the origin of metastatic carcinoma of unknown primary, with special emphasis on lung cancer. Eur J Cancer Prev. 2001 Feb;10(1):77-82.

Sack MJ, Roberts SA. Cytokeratins 20 and 7 in the differential diagnosis of metastatic carcinoma in cytologic specimens. Diagn Cytopathol. 1997; 16, 132-6.

Satoh K, Prescott S. Culture of mesothelial cells from bovine pericardium and characterization of their arachidonate metabolism. Biochim Biophys Acta. 1987 930: 283-296.

Serre V, Tézenas du Montcel S, Kontula L, Simon M, Perret E, Nédellec P, Vicart P, Duron JJ. In vivo protein markers of human peritoneal mesothelial cells: do they differ according to their anatomical sites? Eur J Surg Suppl. 2003 Jul;(588):40-5.

Shidham VB, Mary Falzon. Serous effusions. In: Gray W, Kocjan G, eds. Diagnostic Cytopathology, 3rd Edition. London: Elsevier. 2010: 115-175.

Shield PW, Koivurinne K. The value of calretinin and cytokeratin 5/6 as markers for mesothelioma in cell block preparations of serous effusions. Cytopathology. 2008 Aug;19(4):218-23.

Stevens MW, Leong AS, Fazzalari NL, Dowling KD, Henderson DW. Cytopathology of malignant mesothelioma: a stepwise logistic regression analysis. Diagn Cytopathol. 1992;8(4):333-41.

Stopyra GA, Warhol MJ, Multhaupt HA. Utility of CK7 and CK20 immunohistochemistry in the detection of synchronous breast and colon carcinoma in a pleural effusion: a case report and supporting survey of archival material. Diagn Cytopathol. 2001 Jul;25(1):54-8.

Stylianou E, Jenner LA, Davies M, Coles GA, Williams JD. Isolation, culture and characterization of human peritoneal mesothelial cells. Kidney Int. 1990 Jun;37(6):1563-70.

Su XY, Li GD, Liu WP, Xie B, Jiang YH. Cytological differential diagnosis among adenocarcinoma, epithelial mesothelioma, and reactive mesothelial cells in serous effusions by immunocytochemistry. Diagn Cytopathol. 2010 Sep 10. [Epub ahead of print]

Suthipintawong C, Leong AS, Vinyuvat S. Immunostaining of cell preparations: a comparative evaluation of common fixatives and protocols. Diagn Cytopathol. 1996 Aug;15(2):167-74.

Swanson PE. Diagnostic immunohistochemistry: a practical primer for the benighted bureaucrat. Am J Clin Pathol. 1993;99:530–532.

Tot T. Adenocarcinomas metastatic to the liver: the value of cytokeratins 20 and 7 in the search for unknown primary tumors. Cancer, 1999; 85, 171-7.

Tot T. Cytokeratins 20 and 7 as biomarkers: usefulness in discriminating primary from metastatic adenocarcinoma. Eur J Cancer. 2002; 38, 758-63.

Tot T. The value of cytokeratins 20 and 7 in discriminating metastatic adenocarcinomas from pleural mesotheliomas. Cancer. 2001; 92, 2727-32.

Ueda J, Iwata T, Ono M, Takahashi M. Comparison of three cytologic preparation methods and immunocytochemistries to distinguish adenocarcinoma cells from reactive mesothelial cells in serous effusion. Diagn Cytopathol. 2006 Jan;34(1):6-10.

Valli V, Peters E, Williams C, Shipp L, Barger A, Chladny J, Hoffmann W. Optimizing methods in immunocytochemistry: one laboratory's experience. Vet Clin Pathol. 2009 Jun;38(2):261-9.

Vargha R, Bender TO, Riesenhuber A, Endemann M, Kratochwill K, Aufricht C. Effects of epithelial-to-mesenchymal transition on acute stress response in human peritoneal mesothelial cells. Nephrol Dial Transplant. 2008 Nov;23(11):3494-500.

Vernau W. The use of immunocytochemistry in the diagnosis of neoplasia. Proceedings of the 50° Congresso Nazionale Multisala SCIVAC. 2005.

Whitaker D, Papadimitrou J. Mesothelial healing: morphological and kinetic investigations. J Pathol 1985; 145:159–75.

Whitaker D. The cytology of malignant mesothelioma. Cytopathology. 2000 Jun;11(3):139-51.

Williams JD, Craig KJ, von Ruhland C, Topley N, Williams GT, for The Biopsy Registry Study Group. The natural course of peritoneal membrane biology during peritoneal dialysis. Kidney Int 2003; 64(Suppl 88):S43–9.

Witowski J, Ksiazek K, Jorres A. New insights into the biology of peritoneal mesothelial cells: the roles of epithelial-to-mesenchymal transition and cellular senescence. Nephron Exp Nephrol. 2008;108(4):e69-73.

Yáñez-Mó M, Lara-Pezzi E, Selgas R, Ramírez-Huesca M, Domínguez-Jiménez C, Jiménez-Heffernan JA, Aguilera A, Sánchez-Tomero JA, Bajo MA, Alvarez V, Castro MA, del Peso G, Cirujeda A, Gamallo C, Sánchez-Madrid F, López-Cabrera M. Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. N Engl J Med. 2003 Jan 30;348(5):403-13.

Yao V, Platell C, Hall JC. Role of peritoneal mesothelial cells in peritonitis. Br J Surg. 2003 Oct;90(10):1187-94.

Ylagan LR, Zhai J. The value of ThinPrep and cytospin preparation in pleural effusion cytological diagnosis of mesothelioma and adenocarcinoma. Diagn Cytopathol. 2005 Mar;32(3):137-44.

Yu GH, Sack MJ, Baloch ZW, DeFrias DV, Gupta PK. Occurrence of intercellular spaces (windows) in metastatic adenocarcinoma in serous fluids: a cytomorphologic, histochemical, and ultrastructural study. Diagn Cytopathol. 1999 Mar;20(3):115-9.

Yung S, Chan TM. Intrinsic cells: mesothelial cells - central players in regulating inflammation and resolution. Perit Dial Int. 2009 Feb;29 Suppl 2:S21-7.

Yung S, Chan TM. Mesothelial cells. Perit Dial Int. 2007 Jun;27 Suppl 2:S110-5.

Yung S, Li FK, Chan TM. Peritoneal mesothelial cell culture and biology. Perit Dial Int. 2006 Mar-Apr;26(2):162-73.

Zimmerman RL. Effusion cytology: Keeping researchers and journals in business for the past 20 years—and it is not over yet. Curr Diagn Pathol, 2005;11, 194–202.

APPENDIX I

Diagnostic Sensitivity (Sens), Specificity (Spec), Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Accuracy (Acu) were calculated using the following method.

Table A. Sens, Spec, PPV, NPV and Acu of a diagnostic test.

Test result	Disease present (number of cases)	Disease absent (number of cases)	Total number of cases tested
Positive	TP	FP	TP + FP
Negative	FN	TN	FN + TN

Sensitivity = TP / (TP + FN)

Specificity = TN / (TN + FP)

PPV = TP / (TP + FP)

NPV = TN / (TN + FN)

Accuracy= (TN + TP) / (TN + FN + TP + FP)

PUBLICATIONS DURING THE PhD PERIOD

- PAPERS IN INTERNATIONAL SCIENTIFIC PERIODICALS

PROSTATIC SARCOMATOID CARCINOMA IN A DOG: CYTOLOGIC AND IMMUNOHISTOCHEMICAL FINDINGS

N. Pinto da Cunha, G. Ghisleni, S. Romussi, M. Caniatti

Veterinary Clinical Pathology 2007; 36(4):368–372

Abstract:

An 8-year-old neutered male Boxer was presented with tenesmus, hemorrhagic urethral discharge, and dysuria. Abdominal ultrasound and radiographic examinations revealed irregular prostatic enlargement. Laparotomy was performed and intraoperative cytology was done on imprint smears of a biopsy specimen obtained from a prostatic mass. The cytologic preparation was highly cellular and contained a predominant population of atypical, large, loosely cohesive spindle cells, with rare multinucleated cells and mitotic figures. The cytologic findings were consistent with undifferentiated sarcoma. At necropsy, a large cystic prostatic mass and numerous satellite nodules in the soft tissues around the pelvis were was found. On histologic examination the tumor was composed primarily of bundles of neoplastic spindle cells. Rare pseudo-acinar structures and signet-ring cells also were observed. On immunohistochemical examination, the neoplastic cells co-expressed cytokeratin and vimentin. Based on histologic and immunohistochemical findings, the tumor was diagnosed as primary prostatic sarcomatoid carcinoma. This is a rare tumor in dogs, in which biphasic morphology of epithelial and mesenchymal cells can complicate the diagnosis, requiring immunochemical stains for confirmation.

ANALYTICAL VALIDATION OF THE HAEMATOLOGY ANALYZER SYSMEX XT-2000iV ON CANINE AND FELINE EFFUSIONS AND CONCORDANCE WITH CYTOLOGICAL DIAGNOSIS

N. Pinto da Cunha, A. Giordano, M. Caniatti, S. Paltrinieri

Veterinary Clinical Pathology 2009; 38(2):230-241

Abstract:

Background: Sysmex XT-2000iV is a hematology analyzer that combines laser and impedance technology. Its usefulness on canine and feline intracavitary effusions has not yet been studied. **Objective:** to evaluate the analytical performance of this analyzer on effusions; to assess the concordance between instrumental and cytological diagnoses. **Methods**: Seventy-two effusions were analyzed. Hct, RBC and total nucleated cell count (TNCC) were compared with those of an impedance counter. Imprecision, inaccuracy and limit of detection (LOD) were determined. An algorithm was designed to classify the effusions as acellular/transudate, reactive/inflammatory, feline infectious peritonitis, chylous, round cell tumor (RCT), neoplastic (non-RCT). Results were compared with those of cytology. **Results:** The agreement with the impedance counter showed a proportional error for TNCC. The LOD was negligible; imprecision and inaccuracy were low except for fibrin-rich effusions, on which fibrin clots entrap the cells. The application of the algorithm had a low inter-observer variability and in 43/72 cases provided the same diagnosis obtained by cytology. Discordant diagnoses, mostly due to the presence of cells with similar morphology, were within the same category of disease (e.g. non-neoplastic, neoplastic), except in 12/72 cases. In 6 out of these 12 cases the instrument did not detect cells suggestive of specific diseases. **Conclusion:** The Sysmex XT-2000iV provides precise and accurate TNCC and detects fibrin-rich samples. A moderate level of concordance with cytology was found. Cytology is thus mandatory to achieve a diagnosis, but the analysis with the Sysmex counter can provide to the cytopathologist preliminary information on the type of effusion.

ULTRASOUND-GUIDED CYTOLOGY OF SPLEEN AND LIVER: A PROGNOSTIC TOOL IN CANINE CUTANEOUS MAST CELL TUMOUR

D. Stefanello, S. Faverzani, P. Valenti, V. Bronzo, V. Fiorbianco, N. Pinto da Cunha, S. Romussi, M. Cantatore, M. Caniatti

Journal of Veterinary Internal Medicine 2009; 23(5): 1051-7

Abstract:

Background: in the clinical staging of cutaneous mast cell tumors (cmct), the diagnosis of metastasis is controversial based on cytological examination of lymph nodes, spleen, liver, bone marrow, and blood.

Objectives: to define the prognostic role of ultrasound-guided cytology of spleen and liver in cmct. the results of cytological evaluation were compared in relation with survival time.

Animals: fifty-two client-owned dogs with a diagnosis of cmct.

Methods: selection of cases was based on cytological evaluation of liver and spleen to detect infiltration at distant sites. the kaplan meier method was used to compare survival in dogs with and without infiltration of spleen and liver (log-rank test $p < .05$).

Results: ten dogs with cmct had mast cell infiltration of spleen, liver, or both and 4 of these dogs had involvement of the regional lymph nodes. the majority of dogs had 2 or more ultrasonographically abnormal findings simultaneously in spleen and liver. nine dogs had grade ii cmct, and 1 had grade iii cmct. dogs with positive evidence of mast cell infiltration to spleen, liver, or both had shorter survival times (34 versus 733 days) compared with dogs negative for mast cell infiltration at distant sites.

Conclusion and clinical importance: dogs with evidence of mast cell infiltration at distant sites have a shorter survival times than dogs without evidence of infiltration at distant sites. this study suggests that cytology of spleen and liver is indicated either for ultrasonographically normal or for ultrasonographically abnormal spleen and liver in dogs with cmct.

ELECTROPHORETIC FRACTIONATION OF CREATINE KINASE ISOENZYMES AND MACROENZYMES IN CLINICALLY HEALTHY DOGS AND CATS AND PRELIMINARY EVALUATION IN CENTRAL NEUROLOGIC DISEASE.

S. Paltrinieri, S. Cazzaniga, N. Pinto da Cunha, A. Giordano

Veterinary Clinical Pathology 2010; 39(3):329-336

Abstract:

Background: Information about the electrophoretic distribution of CK-MM, CK-MB, and CK-BB, serum creatine kinase (CK) isoenzymes that are indicators of skeletal muscle, cardiac muscle, and brain lesions, respectively, and CK macroenzymes (macro-CK1 and macro-CK2) in dogs and cats with and without central neurologic disease is scant and equivocal. Objectives: The objectives of this study were to describe the electrophoretic distribution of CK isoenzymes and macroenzymes in healthy dogs and cats and to provide a preliminary assessment of the utility of CK enzymatic electrophoresis in dogs and cats with central neurologic disease. Methods: Electrophoretic separation of serum CK isoenzymes and macroenzymes was performed on freeze-thawed serum samples from 20 healthy dogs and 3 dogs with central neurologic disease and from 14 healthy cats and 6 cats with neurologic feline infectious peritonitis (FIP). Electrophoretic separation was also performed on supernatants of homogenized brain, skeletal muscle, and cardiac muscle from both species, to assess the tissue distribution of isoenyzmes in dogs and cats. Results: CK-MM was the predominant isoenzyme in the serum of healthy dogs and cats, followed by macro-CK2 and CK-BB in dogs and by both macroenzymes in cats. In dogs, CK-MB was essentially absent from both serum and homogenized hearts. CK-BB increased in dogs with neurologic disease. In cats, CK-BB was essentially absent from serum, but was present in brain homogenates. Two of 6 cats with FIP had increased macro-CK1 and increased CK-BB activity. Conclusions: This study identified the electophoretic distribution of CK isoenzymes and macroenzymes of dogs and cats and provided encouraging data about the possible use of CK-BB as a biomarker for canine neurologic disorders, but not for FIP.

SUBCUTANEOUS EMBRYONAL RHABDOMYOSARCOMA IN A DOG: CYTOLOGIC, IMMUNOCYTOCHEMICAL, HISTOLOGIC, AND ULTRA- STRUCTURAL FEATURES.

G. Avallone, N. Pinto da Cunha, C. Palmieri, L. Della Salda, D. Stefanello, P. Roccabianca, M. Caniatti

Vet Clin Pathol. 2010 [Epub ahead of print]

Abstract:

A subcutaneous mass on the left antebrachium of an 11-year-old intact female English Pointer dog was evaluated presurgically by cytologic examination and immunocytochemical staining. The sample consisted of discrete, variably sized, markedly pleomorphic neoplastic cells that expressed vimentin with diffuse cytoplasmic staining, desmin with focal paranuclear staining, and myoglobin with diffuse cytoplasmic staining, consistent with a diagnosis of rhabdomyosarcoma. Lymphocytic and histiocytic markers were negative. Aspirates of the enlarged ipsilateral prescapular lymph node were positive for metastatic disease. Surgical excision of the tumor and lymph node were followed by histologic and electron microscopic examination. Histomorphologic appearance of neoplastic cells from the mass and the lymph node paralleled cytologic findings; the histologic diagnosis was round cell variant of embryonal rhabdomyosarcoma. By ultrastructural evaluation, cells contained numerous mitochondria and masses of cytoplasmic tangled myofilaments, features typical of rhabdomyoblasts. The dog received doxorubicin (30 mg/m(2)) every 3 weeks for 5 treatments. Local recurrence developed 6 months after resection but was not treated. Despite a guarded prognosis and untreated local recurrence, the dog was still alive 18 months after surgery. Cytologic evaluation and immunocytochemical staining were pivotal for the presurgical diagnosis of rhabdomyosarcoma.

PAPERS IN INTERNATIONAL CONFERENCE PROCEEDINGS

- Pinto da Cunha N, Giordano A, Caniatti M, Paltrinieri S: Flow Cytometric analysis of pleural and ascitic effusions in dogs and cats with the Sysmex XT-2000iV. *ESVCP* 9th Annual Congress in conjunction with the *ECVIM-CA* 17th Congress: **Budapest, Hungary**. September 12-15, 2007. In: *Veterinary Clinical Pathology* 2007; 36: 382–398

- Stefanello D, Faverzani S, Valenti P, Bronzo V, Pinto da Cunha N, Romussi S, Caniatti M. Ultrasound-guided cytology of spleen and liver: a prognostic tool in 52 dogs with cutaneous mast cell tumor. World Veterinary Cancer 1st joint meeting of ESVONC and VCS, **Copenhagen**, **Denmark**, feb. 28-mar 1, 2008. Proceedings of the 1st World Vet Cancer Meeting, 2008; 58-58.

- Paltrinieri S, Cazzaniga S, Pinto da Cunha N, Giordano A. Creatine Kinase (CK) isoenzymes in dogs, cats, sheep and horses. *ESVCP* 10th Annual Congress: **Barcelona**, **Spain**. September 30th – October 3rd, 2008. In: V*eterinary Clinical Pathology* 2008; 37: 3-40

- *Pinto da Cunha N, Ghisleni G, Mortellaro CM, Caniatti* M: Role of brush cytology in the diagnosis of canine chronic intranasal disease. 19th *ECVIM* Annual Congress: **Porto, Portugal**. September 8-10, 2009. In: *Journal Veterinary Internal Medicine* 2009; 23: 1319–1350

- Pinto da Cunha N, Palmieri C, Della Salda L, Stefanello D, Roccabianca P, Caniatti M, Avallone G. Subcutaneous embryonal rhabdomyosarcoma in a dog. Proceedings of the 27th ESVP/ECVP Meeting. **Olsztyn – Kraków, Poland.** 8th -12th September, 2009. In: *Veterinary Pathology 2009; 46:1084-1089*

- Ghisleni G, Pinto da Cunha N, Santos M, Florenti M, Caniatti M, Roccabianca P. The utility of fine-needle aspiration cytology in the differential diagnosis of cutaneous masses containing keratin. ESVCP Annual Congress. **Thessaloniki**, **Greece**. 7-9 October 2009. In: *Veterinary Clinical Pathology* 2009; 38:E16-E41.

PAPERS IN NATIONAL MEETINGS PROCEEDINGS

- De Francesco B, Pinto da Cunha N. Versamento pericardico e pleurico in un Golden Retriever. Incontro SICIV (Società Italiana di Citologia Veterinaria), **Cremona, Italia.** 9 Novembre, 2008. In: Atti di "L'esame dei versamenti cavitari: citologia e non solo", pp 12.
- Sforna M, Brachelente C, Lepri E, Capuccini S, Pinto da Cunha N, Mechelli L. Istiocitosi Progressiva Felina: Aspetti morfologici ed immunocitochimici di un caso. Incontro SCIONCOV. **Cremona, Italia.** 18-19 Febbraio, 2009.

INVITED ORAL PRESENTATIONS

- Pinto da Cunha N. "L'esame citologico dei versamenti cavitari: concetti vecchi e nuovi " Incontro SICIV (Società Italiana di Citologia Veterinaria), **Cremona, Italia**. 9 Novembro 2008. In: Atti di "L'esame dei versamenti cavitari: citologia e non solo", pp 7-10.