PLEURAL EFFUSION IN A DOG

Presenter: Martina Piviani

Contributors: Paolo Silvestrini, Riccardo Finotello, Lorenzo Ressel, Luis Mesquita, Jeremy Mortier

Small Animal Division, Small Animal Teaching Hospital, and Section of Veterinary Pathology, School of Veterinary Science, University of Liverpool, Neston, UK

Specimen: Sediment smear of pleural effusion

Signalment: 10-year-old, female spayed, cross-breed dog (medium size)

History and clinical findings: The dog was admitted by the Internal Medicine Service of the Small Animal Teaching Hospital (SATH) of the University of Liverpool for further investigation of tachypnea, dyspnea, and anorexia. Clinical signs appeared 10 days prior to referral when a bilateral pleural effusion was detected on thoracic radiographs by the referring veterinarian and drained. No response to oral furosemide and antibiotics (amoxicillin-clavulanate) was recorded. An automated full blood count performed prior to the referral revealed leukocytosis (17.76 x10^9/L; Reference Interval (RI) 5.5-16.90) apparently due to neutrophilia (14.08x10^9/L; RI 2-12) and monocytosis (2.30x10^9/L ;RI 0.30-2), and thrombocytosis (688 x10^9/L; RI 175-500). No blood smear review was performed. The biochemistry profile revealed hypokalemia (3.2 mmol/L; RI 3.5-5.8), hypochloremia (103 mmol/L; RI 109-122), and a proportional increase of urea (12.6 mmol/L; RI 2.5-9.6) and creatinine (169 mmol/L; RI 44-159). On presentation to the SATH, the patient appeared markedly dyspneic and had a restrictive respiratory pattern but was alert and responsive. The body weight was 21.4 Kg with a normal body condition score (BCS 4/9). Mucous membranes were pink, dry and had a prolonged capillary refill time (3 seconds). Thoracic auscultation revealed muffled heart sounds.

Thoracocentesis was performed and 810 mL of sero-hemorrhagic pleural fluid was removed from the right hemithorax and 600 mL from the left side. Total nucleated cell and erythrocyte counts were 2.9x10^9/L and 0.09x10^12/L, respectively. Total protein concentration was 25 g/L. Direct and sediment smears prepared from the fluid submitted in EDTA were air-dried and stained with Wright-Giemsa using an automated stainer.

Representative pictures of the stained sediment smear of pleural effusion are shown below (Figure 1a and 1b). Wright–Giemsa stain, 400 x magnification (picture taken with IPhone5).

Question 1: What is your interpretation?

Question 2: What additional tests would help you refine the cytological interpretation?
Answer 1: Slides contained moderate numbers of vacuolated macrophages with occasional erythrophagocytosis, fewer small lymphocytes, and occasional neutrophils, admixed with moderate numbers of atypical cells amid a background of moderate numbers of erythrocytes. Atypical cells were round to vaguely polygonal, mostly individualized but also seen in loose aggregates occasionally surrounding scant pink material. Nuclei were round, central to paracentral, with granular chromatin and one to multiple variably prominent nucleoli. Cells had moderate to abundant blue cytoplasm with distinct vacuoles and occasional peripheral blebs. A small proportion of these cells had a pericellular pink fringe. Anisocytosis and anisokaryosis were moderate with frequent binucleation and occasional multinucleation. Mitoses were frequent, including atypical figures. The cytologic interpretation was modified transudate with low-grade chronic inflammation and mild hemorrhagic component with proliferation of atypical cells interpreted as reactive and dysplastic mesothelial cells, although neoplasia (mesothelioma, carcinoma, histiocytic sarcoma) was not completely ruled out and further investigation was recommended.

Answer 2: To further characterize the cells in the effusion, a cell pellet of the fluid was prepared, embedded in paraffin and processed for immunohistochemistry (IHC) using commercially available antibodies for vimentin (monoclonal, clone V9, Dako, Glostrup, Denmark), pancytokeratin (monoclonal, clone AE1/AE3, Dako, Glostrup, Denmark), Iba1 (polyclonal, LS-B2402, LifeSpan BioSciences, Seattle, WA), CD18 (clone CA16.3C10, Peter Moore, Davis, CA), MUM1 (clone MUM1, Dako, Glostrup, Denmark), and S100 (polyclonal, Z0311, Dako, Glostrup, Denmark). Another pellet was re-suspended in a solution of 2.5% glutaraldehyde in 100 mM phosphate buffer at pH 7.0 and submitted for transmission electron microscopy (TEM, Phillips EM 208, FEI UK, Cambridge, UK). IHC of the pellet of the pleural effusion revealed that the large atypical cells were positive for vimentin and negative for pancytokeratin (Figure 2A-2C), thus consistent with mesenchymal but not mesothelial cells. IHC for all the other markers was negative (ruuling out histiocytic and lymphoid proliferation). On TEM, atypical cells had abundant rough endoplasmatic reticulum and hyperplastic Golgi apparatus (Figure 2D).
A computed tomography (CT) was performed with the patient under sedation. Images of the thorax and abdomen were acquired pre and post-contrast medium administration. The CT revealed a rounded, mineralized mass centered on the dorsal part of the left scapular spine measuring 1.7 cm (width) x 1.3 cm (high) x 4 cm (long) with destruction of the cortical and medullary bone and an ill-defined transition zone consistent with a primary bone tumor. There was also bilateral pleural effusion with diffuse pleural thickening and multiple pleural and pulmonary nodules and masses compatible with metastatic disease or a mesothelioma. The sternal lymph nodes were mildly enlarged with areas of mineralization. The CT of the abdomen revealed no abnormalities.

Fine-needle aspiration (FNA) of the left scapular and pleural masses was performed and squash smears made. Slides contained many neoplastic cells, seen mostly individualized and occasionally embedded in scant light pink material reminiscent of osteoid, intermingled with frequent osteoclasts and few macrophages. Several fragments of mineral material were noted in the background. Neoplastic cells were round to oval with an eccentric oval to round nucleus, stippled chromatin, single to multiple prominent nucleoli and abundant blue cytoplasm with a paracentral clearing (Figure 5). Criteria of malignancy included moderate anisocytosis and anisokaryosis, rare binucleation, prominent nucleoli and occasional mitoses (five per 10 fields at 400x magnification). Cytologic findings were highly suggestive of an osteosarcoma.
Figure 5: Squash preparation of fine needle aspiration of a scapular mass from a dog with dyspnea. A: There are many oval neoplastic cells occasionally surrounding scant amounts of pink material reminiscent of osteoid (arrow). Wright–Giemsa stain, scale bar 50 microns. B: Neoplastic cells have moderate anisocytosis and anisokaryosis, and multiple prominent nucleoli. Note the particle of slightly refractile material compatible with mineral (arrowhead). Wright–Giemsa stain, scale bar 50 microns.

To corroborate the cytological interpretation of scapular osteosarcoma with pleural metastasis, the substrate 5-bromo, 4-chloro, 3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA) was applied to one prestained slide of FNA from each site and to one archived canine liver FNA cytology slide (used as positive control), following published guidelines. Slides were then counterstained with a rapid romanowsky staining kit (TCS Biosciences Ltd, Botolph Claydon, UK) for 1 second in each solution cup, and rinsed with distilled water. A brown granular material, indicative of alkaline phosphatase activity, was evident within the cytoplasm of neoplastic cells (Figure 6) and along the cytoplasmic membrane of the hepatocytes but absent in leucocytes.
Figure 6: Fine-needle aspirate of a scapular mass in a dog. Neoplastic cells express alkaline phosphatase activity. Blood leucocytes (arrow) served as negative control. Wright–Giemsa, followed by 5-bromo, 4-chloro, 3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) and rapid romanowsky stain counterstaining, scale bar 35 microns.

The BCIP/NBT substrate was also applied to one of the prestained sediment smears of pleural fluid available and revealed ALP activity in the majority of the atypical cells (interpreted as neoplastic osteoblasts) but, as expected, not in erythrocytes and leucocytes, including macrophages (used as internal negative control) (Figure 7). A small proportion of the atypical cells (those that were more cohesive and often showed a peripheral pink fringe) did not show ALP activity and were interpreted as reactive mesothelial cells (arrow).
Based on the combination of imaging, cytology, cytochemistry, fluid pellet IHC and TEM findings, the patient was diagnosed with a neoplastic pleural effusion because of intrathoracic metastasis of a scapular osteosarcoma. Given the poor prognosis, owners elected euthanasia. A post-mortem examination was not authorized.

DISCUSSION

Cavitary effusions may be caused by disturbances of hydrostatic or oncotic pressure, inflammation, impaired lymphatic drainage, hemorrhage, organ rupture or neoplasia. Laboratory evaluation of cavitary fluid, including cell counts, total protein and cytology, is useful in determining the cause of the effusion and to identify neoplastic cells in many cases. In this case routine fluid cytology alone did not allow a confident interpretation of neoplasia as the atypical cells were still in the morphological spectrum of reactive and dysplastic mesothelial cells. The reported sensitivity of cytology for the diagnosis of malignant tumors in canine and feline effusions is 60%. Achieving a definitive diagnosis using a fluid sample harvested during
therapeutic thoracocentesis, avoiding invasive procedures such as thoracotomy and pleural or pulmonary biopsy, would be ideal but it is often not possible. The mesothelium lining the body cavities becomes hyperplastic and cells exfoliating into the fluid may mimic neoplasia as the nucleus to cytoplasm ratio increases, binucleation is frequent and mitotic figures may be seen. Thus, an interpretation of malignancy requires caution. Mesothelial cells often have a peripheral pink fringe but this feature is not consistent. In addition, mesothelial and epithelial cells exfoliating into an effusion often lose their cell-to-cell adhesion and can mimic round cells. This further limits the ability of a definitive identification of the cell phenotype.

Immunocytochemistry, flow cytometry and cell pellet IHC are all useful tools to characterize cells present in effusions and to refine the cytologic interpretation. Fluid pellet IHC is a simple, fast and effective diagnostic tool which can be performed in most histopathology laboratories with a wider panel of validated markers compared to that available for the two former techniques. In this case atypical cells were vimentin positive but pancytokeratin negative. These findings were consistent with a mesenchymal proliferation, refuting the initial interpretation of mesothelial hyperplasia and excluding the possibility of carcinoma and mesothelioma, as epithelial and mesothelial cells are expected to be pancytokeratin positive. A histiocytic proliferation, plasma cell tumor and melanoma were ruled out based on negativity for Iba1 and CD18, MUM1, and S100, respectively. A lymphoid origin was unlikely given cell morphology and further excluded based on negative immunostain for CD18. Cell pellet IHC findings were consistent with a neoplastic effusion due to a sarcoma. TEM confirmed that the atypical cells in the effusion were not mesothelial as they lacked the densely stippled, regular, long microvilli typical of mesothelial cells, while myocyte and endothelial origin were ruled out based on the absence of contractile myofilaments and Weibel Palade bodies, respectively.

Radiography is considered the first line diagnostic imaging technique for animals with thoracic disease but findings are often nonspecific or limited by the presence of pleural fluid. According to the referring veterinarian, the thoracic radiographs revealed only pleural effusion. In addition to the pleural effusion, the thoracic CT also revealed pleural thickening, pleural nodules and masses, pulmonary nodules, possible sternal lymphadenopathy and a scapular mass. Several studies have demonstrated the utility of CT to identify other abnormalities beyond pleural effusion, including lesions in the pleura, lung and mediastinum, or extra thoracic lesions. The presence of pleural nodules and masses is frequently associated with neoplasia. However, similar lesions may also be present in patients with chylothorax, pyothorax, and foreign body migration, with overlapping CT features. The mineralised mass in the left scapular spine detected in the CT was not mentioned in the clinical history provided by the referring veterinarian, although it is uncertain if this anatomical region was included in the chest radiographs performed before referral as the images were not available for review. The CT features of the scapular osteosarcoma described in this case report are consistent with the current published veterinary literature. Compared to radiography, the main advantage of CT is a clearer delineation of the internal and extracortical tumor margins. Osteosarcoma is the most common neoplasm of the scapula in dogs, followed by soft tissue sarcoma, chondrosarcoma, hemangiosarcoma and histiocytic sarcoma. Although less than 15% of dogs with osteosarcoma have radiologic evidence of pulmonary nodules or masses at the time of the diagnosis, more than 85% of patients develop gross metastases despite effective control of the primary tumor, suggesting that micrometastases arise early in the course of the disease. Considering just
osteosarcoma of extracranial flat and irregular bones, the incidence of thoracic metastases is less defined, although it is thought to be higher than in osteosarcoma of long bones. Other reported metastatic sites include bones, visceral organs, lymph nodes and eye.\textsuperscript{14-18} Despite the high incidence of intrathoracic metastasis, a neoplastic pleural effusion due to osteosarcoma has never been reported before.

The FNA of the scapular mass and one of the pleural nodules harvested a population of cells with features very reminiscent of osteoblasts (eccentric nuclei, abundant deep-blue cytoplasm with paranuclear clearing) and several criteria of malignancy (moderate anisocytosis and anisokaryosis, binucleation, mitoses), leading to an interpretation of osteosarcoma. Histology is considered the gold standard for the diagnosis of osteosarcoma upon demonstration of a neoplastic mesenchymal population producing osteoid. The scant pink material seen in the FNA was highly suggestive of osteoid and, although different types of extracellular matrix cannot be reliably distinguished in cytology, histology has similar limitations, as osteoid may sometimes be difficult to differentiate from fibrin or collagen and its presence may be dependent on tumor subtype, inconspicuous in small biopsies or absent in metastases. In a recent study, preoperative FNA of scapular lesions was performed in eleven of 42 dogs included in the study.\textsuperscript{13} The cellular yield was high and cytologic interpretation was confirmed by histopathology in all cases. The authors strongly recommended FNA for investigation of bone lesions, including those of the scapula, as a diagnosis may be achieved more quickly with less risk for the patient and lower cost compared to trucut biopsy. In our case, obtaining a biopsy sample of the scapular and thoracic masses for histological examination would have been an unnecessary and invasive procedure considering that cytology, combined with ALP cytochemistry, was already diagnostic.

The presence of a scapular osteosarcoma with intrathoracic metastasis suggested a possible osteoblastic origin for the neoplastic mesenchymal population identified by cell pellet IHC in the effusion. In the diagnosis of osteosarcoma, immunohistochemistry is most often used to rule out other types of neoplasia rather than definitively confirm osteosarcoma. IHC for alkaline phosphatase is used in human pathology but is unreliable in dogs. A recent study demonstrated the utility of osteocalcin as a marker for osteosarcoma in dogs was published.\textsuperscript{19} Immunostain for osteocalcin could have been useful to confirm osteosarcoma in our case albeit its specificity is limited by the frequent positivity in chondrosarcomas and the positive cytochemical reaction for ALP was already confirmatory, as bone is the only connective tissue shown to produce ALP in dogs. The BCIP/NBT substrate can be applied to cytology slides, even if prestained, as in this case, with a reported sensitivity and specificity for a diagnosis of osteosarcoma of 88 and 94\%, respectively.\textsuperscript{1} Positive cytochemical reaction for alkaline phosphatase was found in one of 2 and in one of 4 chondrosarcomas in two different studies.\textsuperscript{21,22} The unexpected result was attributed to a possible misdiagnosis of chondroblastic or periosteal osteosarcoma for chondrosarcoma in histology due to the presence of cartilage and lack of convincing osteoid in the section examined, or expression of ALP by undifferentiated neoplastic chondroblasts. Alkaline phosphatase activity was also detected in one of two and in one of eight cases of amelanotic melanoma.\textsuperscript{1,21} In our case, the lack of S100 expression in the fluid cell pellet IHC further supports a diagnosis of osteosarcoma versus chondrosarcoma or melanoma, as this marker is usually positive in the latter two types of neoplasia.\textsuperscript{23,24}
In conclusion this case report shows that metastasis of osteosarcoma is a possible consideration for a cavitary effusion containing oval cells with atypia in a patient with a bone tumor, highlights the importance of an integrated interpretation of CT findings and cytology, and further demonstrates the utility of cytochemistry, cell pellet IHC and TEM to refine a cytological diagnosis when obtaining a histology sample is not feasible.

REFERENCES