Royal College of Veterinary Surgeons Trust

Title of the Blue Sky Project

"Liquid-based cytology and immuno-cytochemistry for canine body cavity effusions"

(a) Project background, context and need

Immunocytochemistry is a technique which detects antigens (immunomarkers) in cytological specimens by applying antibodies specific for those antigens. In human medicine, the use of liquid-based cytology (ThinPrep methodology) has improved the quality of cytological preparations and this technique is now the procedure of choice in many institutions as it can be applied to urine, body cavity fluids, mucosal brushings, lavages and fine needle aspirates. The main advantages of the ThinPrep technology over the conventional cytological (Cytospin) preparation are a) the preparation of slides with a cell monolayer and evenly distributed cells b) elimination of air-drying and spreading artefacts c) removal of obscuring elements such as erythrocytes, inflammatory cells and proteinaceous debris and d) ability to prepare multiple slides for immunocytochemical stains. As a result, with most immunoreagents the staining patterns and intensity on ThinPrep slides are excellent.
In human medicine the distinction between mesothelioma and adenocarcinoma is challenging on the basis of cytology alone and therefore various immunomarkers are routinely applied on ThinPrep cytology slides from neoplastic body cavity fluids. In addition to cytological features, the presence or absence of positive cells, grading of the immunostaining according to the percentage of positive cells and different staining patterns (e.g. cytoplasmic, nuclear, along cell membrane) all aid greatly in the distinction between these two entities. Commonly used markers with their typical immunoreactivity for mesothelioma and adenocarcinoma are presented below:

	Calretinin	Cytokeratin	Vimentin	CEA	EMA	ESA
Mesothelioma	+	+	+/-	-	+*	-
Adenocarcinoma	-	-	-/+	+	+**	+

CEA: carcinoembryonic antigen, EMA: epithelial membrane antigen; *strong cell membrane staining, **diffuse cytoplasmic staining, ESA: epithelial specific antigen; various monoclonal antibodies available (MOC31, Ber-EP4, AUA1)

In canine medicine, various studies have evaluated and utilised a number of immunomarkers in the investigation and diagnosis of epithelial and non-epithelial tumours. Utilising a variety of commercially available antibodies, immunoreactivities to cytokeratins, vimentin, desmin, CEA or thyroid transcription factor-1 (TTF-1) have been evaluated and reported only in tissue samples from skin, or organs in the thoracic and abdominal cavity. In dogs, body cavity carcinomatosis can be difficult or impossible to distinguish cytologically from mesothelioma. Immunochemical staining aiding in the diagnosis of mesothelioma or carcinoma has been employed in a limited number of studies and only in tissue biopsies. Studies on immunochemical staining of cytological specimens made from body cavity effusions have not been published.

Study population: Fifty body cavity effusions (30 malignant and 20 benign) will be included in the study. The effusions will be collected from patients referred to the Small Animal Hospital. Effort will be made to actively recruit cases by publishing a relevant letter in the veterinary press, by contacting the local general practitioners, other referral centres and institutions as well as contacting representatives of the Small Animal Medicine Society (SAMSoc) and Veterinary Oncology Society (VONC).
The neoplastic effusions will be from dogs with carcinoma or mesothelioma while the non-neoplastic effusions will be from animals with pyothorax, peritonitis, heart failure, liver failure, lung-lobe torsion, diaphragmatic hernia, protein losing enteropathy or protein losing nephropathy.
Samples: Pleural and/or peritoneal effusions will be drained and samples will be collected into an EDTA tube, a plain tube without anticoagulant and a CytoLyt® solution wash vial (methanol based, haemolytic & mucolytic, preserves cells for at least 8 days, prevents protein precipitation) which will be submitted to the School's Clinical Pathology Diagnostic Laboratories. Samples in the plain tubes will be used for biochemical analysis.

Cytospin cytology slides will be prepared from the EDTA samples by centrifugation (1500g for 5 mins) (Cytospin 3 centrifuge, Shandon) and will be stained automatically with Wright's modified stain (Haema-Tek® 2000, Bayer). The CytoLyt® vials will be centrifuged at 2000 rpm for 5 minutes and the re-suspended cell pellet will be added to a PreservCyt® solution vial (methanol based, preserves cells for 3 weeks at room temperature, bactericidal). Cytospin slides will be prepared from the PreservCyt® solution vial by centrifugation (1500g for 5 mins) (Cytospin 3 centrifuge, Shandon).

The PreservCyt® solution vials will be then transported/posted to the Cytology Laboratory, Department of Cellular Pathology, NHS Southmead Hospital where ThinPrep slides will be prepared from the vials automatically using the ThinPrep® 2000 processor (Cytyc). Additional ThinPrep slides will be prepared and transported/posted to the School of Clinical Veterinary Science at Langford.

Methods

Immunocytochemistry: A labelled streptavidin-Horse Radish Peroxidase-biotin complex method will be employed; slides will be covered with peroxidase to block any endogenous peroxidase activity. Primary monoclonal antibodies at different dilutions will be applied fro calretinin, cytokeratin, vimentin, desmin, CEA, EMA and ESA (clone AUA1). ThinPrep and Cytospin slides will be then incubated with the biotinylated secondary antibody and then the Streptavidin – Horse Radish Peroxidase (HRP) will be applied. The final step will be the visualisation of the previously detected antigens using Diaminobenzidine (DAB) which detects the streptavidin – biotin The end result is that the antigens will be stained in a brown colour. For each slide (manual and automated mehod), staining intensity will be evaluated and graded subjectively as negative (-), weak (+), moderate (++) and strong (+++). The percentage of immunostained cells and the staining distribution (nuclear, cytoplasmic, membranous) will also be recorded. Positive histological sections will serve as controls for the primary antibodies while omission of the primary antibody in the reaction above will provide a negative control.

The above Immunocytochemical method will be performed twice:

Automatically, at the Cytology Laboratory, NHS Southmead Hospital, on ThinPrep slides using the Vision Biosystems immunocytochemical staining machine, and

Manually at the School of Clinical Veterinary Science, Langford, on Cytospin and ThinPrep slides.

Final diagnosis: For the neoplastic cases the final diagnosis will be established through histopathological examination of biopsies collected via thoracoscopy/ laparoscopy / exploratory surgery or post-mortem. For the non-neoplastic cases, the final diagnosis will be based on evaluation of the clinical history, clinicopathological data, routine cytological examination, diagnostic imaging, histopathology and follow up of the patients.

(b) Expected outcomes

-Develop automated and manual methods for the 7 commercially available immunocytochemical markers

-Compare the results generated by the two methods (automated vs manual) using ThinPrep slides

-Compare the results generated by the manual method using ThinPrep and Cytospin slides

-Identify the immunomarkers with the best sensitivity and specificity for the diagnosis of mesothelioma and carcinoma

(c) Benefits and beneficiaries

-The immunocytological markers will be applicable to samples collected minimally/non-invasively this is of benefit as many of these patients are higher anaesthetic risk particularly those with pleural space disease.

-Reliable immunocytological staining in combination with conventional cytology will be suitable for differentiating carcinoma from mesothelioma aiding in an accurate diagnosis. If this can be done in the longer term by obtaining a centesis sample only then the morbidity of these patients may be reduced.

-Accurate diagnosis will result in appropriate selection of treatment/management options without the need for invasive diagnostic procedures and without the patient having increased morbidity and or mortality as a consequence of invasive diagnostics. Also if chemotherapy is suitable, time will not be required for surgical healing before therapy is started.

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