**Immunohistochemistry in equine pathology: a brief overview.**

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Immunohistochemistry (IHC) is a technique which revolutionised the world of pathology when introduced in the 1941 from Coons *et al*. (1941). One of the first immunohistochemical studies in horse was published in 1966 (Nairn & De Boer, 1966). Since then, there are 3965 PubMed-indexed articles at the time of writing this commentary. IHC uses primary antibodies to highlight any type of antigen expressed by normal host cells, viruses, bacteria and tumoural cells, and such versatility made IHC an invaluable tool for research focussing at the same time on protein expression and morphology. While IHC more deep technical aspects are beyond the scope of this manuscript and will therefore be not discussed (for additional data, check Ramos Vara & Miller, 2014), we will briefly discuss the importance IHC in equine diagnostic pathology, condensed in few paragraphs. All the IHC markers mentioned in the present study refers to IHC performed on formalin-fixed paraffin-embedded (FFPE) tissue, as this represent the most diffuse IHC used in veterinary pathology.

**Infectious agents**:

As in humans and other animal species, IHC can be used to highlight and locate pathogens in tissue sections. A broad number of viruses, bacteria, fungi and parasites are detectable via IHC, and in this section the most relevant will be discussed.

Equine herpesviruses (EHVs) are common pathogens able to induce numerous clinical syndromes and lesions in the horse. IHC targeting EHVs is a useful diagnostic method, but such technique is particularly important for the equine herpesviral encephalomyelopathy (EHM). This condition, usually induced by EHV-1, is characterised by viral replication within the CNS endothelial cells leading to thrombotic vasculitis and subsequent neural damage. While molecular tests (e.g. PCR) are able to identify the EHVs in any fluid and tissue, IHC (together with *in situ* hybridization) has the advantage to confirm clinical suspect of equine herpesviral encephalomyelopathy, when viral antigens are highlighted in endothelial cells (Pusterla *et al.*, 2022).

Bovine papillomavirus (BPV) type 1, 2 and 13 are considered the aetiological agents of equine sarcoids, the most common cutaneous tumour. Light microscopical features of equine sarcoids are characteristically enough to make a diagnosis; nevertheless, IHC targeting multiple viral proteins has been performed for research purposes and better understanding the biology of these tumours. According to literature, papillomaviral proteins E5, E7 were observed in both epidermal and dermal neoplastic cells while L1 labelling was observed only on the most superficial keratinocytes (Brandt *et al*., 2011).

Equine coronavirus is a recently described pathogen associated with enterocolitis in horses. (Zappulli *et al.*, 2020). Among the diagnostic tool used, immunohistochemistry is rarely reported, with only a single study highlighting pathological features (Giannitti *et al*., 2015). In such paper, IHC was carried using as a primary antibody targeting bovine coronavirus, due their antigenic similarity.

Bacteria can also be highlighted via IHC. While bacterial culture and isolation from equine tissue is a solid principle of equine medicine, IHC can come handy to help with most of them highlighting specific bacterial antigens for specific agents, such as Leptospira spp. (Szeredi & Haake, 2006) in the context of the tissue lesions.

Similar concept applies to parasites: nonetheless, for most parasites, IHC is seldom used for diagnosis in the equine species with few records for *Sarcocystis neurona* (Henker *et al.*, 2020), *Neospora caninum* (Anderson *et al*., 2021) and *Leishmania spp.* (Ortega-Garcia, 2021). This is likely due to reduced sensibility of such technique compared to serology or other PCR methods (personal observation of GR).

**Tumours and other endogenous antigens**:

IHC, as highlighted by the manuscript in this issue by Hoerdemann et al. (2023), is an invaluable tool to reach a final diagnosis in the majority of tumoural and proliferative lesions: identifying structural and/or inducible protein expressed by neoplastic cells indicates indeed the embryologic origin of the clonal neoplastic cell population. Numerous antibodies can be used as markers for equine neoplasms and are working in FFPE equine tissues. In figure 1, the most used markers and the most frequent tumours are schematised.

Cytokeratins are a large group of cytoskeletal proteins expressed in any epithelial cells. The most used antibody is a monoclonal one, the AE1/AE3, which is characterised by a mix of antibody reacting against high and low molecular weight, labelling almost all epithelia (and therefore commonly called “pan-cytokeratin” antibody). Despite the lack of a strict specificity for some epithelia, the pan-cytokeratin antibody is a very useful one as it can highlight any type of epithelial cells (either normal or neoplastic), even when tumours are poorly differentiated. This antibody can label all adenomas, carcinomas (including squamous cell carcinoma) and thymoma but it cannot identify which type of epithelial tumour (e.g. distinguishing an intestinal adenocarcinoma from mammary carcinoma): for this reason, in case of carcinomas of unknown primary (a.k.a. CUPs), this antibody can help poorly in determining the primary site. IHC targeting pan-cytokeratin is also incredibly useful when assessing metastasis of carcinomas in lymph node, where no epithelial cells are normally present.

Vimentin, on the other hand, is a cytoskeletal protein present in all mesenchymal cells, which usually label all cells not labelled by the pan-cytokeratin antibody. This feature makes this protein expressed by a large number of cells and tissue, with only few exceptions. For this reason, we consider that vimentin is an antibody useful only when in combination with pan-cytokeratin (when distinguishing poorly differentiated tumours): vimentin IHC doesn’t indeed give much more information apart from confirming the mesenchymal origin of tumour.

Mesotheliomas (and normal mesothelial cells) are somehow an exception from other tumours (and cell) as they both express cytokeratin and vimentin antigens. Rare reports of this tumour have been reported in the equine species (Dobromylskyj *et al.*, 2011).

Equine sarcoids are difficult (if not impossible) to distinguish from other mesenchymal cutaneous tumours. The difficulties reside in that bovine papillomaviruses’ DNA can be detected in normal skin (Bogaert *et al.,* 2005) and also in other mesenchymal equine tumours (Epperson & Castleman, 2017); furthermore, there are no specific IHC markers able to distinguish equine sarcoids from other mesenchymal tumours. Equine sarcoid are vimentin positive and are known to express also variable IHC markers including alpha-smooth actin and, rarely, S-100 (Martano *et al.,* 2016 ; Ogłuszka *et al.,* 2021).

The most common markers for muscular tumours remain alpha smooth muscular actin (ASMA) and desmin. Both should be able to label smooth muscle and myoepithelial cells (ASMA) and any type of muscular cells, respectively (Knottenbelt a *et al*., 2016a); nonetheless, distinction between smooth muscle tumours (leiomyoma/leiomyosarcoma) and striated muscle ones, is usually more reliable using morphology than immunohistochemistry. There are indeed numerous other mesenchymal tumours able to express ASMA (e.g. equine sarcoid), while ASMA/desmin expression in rhabdomyosarcoma can be very sparse.

Factor VIII is a key marker able to highlight any endothelial cell in the tissue. This antibody not only labels tumoural endothelial cells arising from blood vessels’ (e.g. haemangioma, haemangiosarcoma) but also the ones from lymphatic vessel (lymphangioma, lymphangiosarcoma), in addition to normal endothelial cells: therefore, factor VIII alone is not able to distinguish from vascular tumour arising from blood or lymphatic vessels. In this regard, a recent paper suggests that PROX-1 could represent as a good candidate for labelling lymphatic endothelial cells (Junginger *et al.,* 2010).

Melanomas are common neoplasm in horses. While in the majority of cases this type of tumour is characterised by heavily melanised neoplastic cells, in some cases, they can be “amelanotic” (i.e. without melanin) and showing a morphology overlapping with other tumours. The classic markers for melanocytes used for decades are S-100 (whose positivity should be present in both nucleus and cytoplasm) and Melan A; nonetheless, a paper describes PNL-2 as the most sensible and specific marker for equine melanocytic tumour and raises some doubts regarding the Melan-A usefullness in the equine species (Ramos-Vara *et al.,* 2014).

There are multiple nervous system markers in equine pathology. One of the most used ones remains the glial fibrillary acidic protein (GFAP) which labels glial cells. This antibody is usually employed when dealing with CNS tumour, especially to confirm astrocytomas. Olig-2 is an antibody used to label oligodendrocytes (and oligodendrogliomas) in multiple animal species; in the horse, Olig-2 IHC is seldom reported, suggesting it is a valid antibody also in the equine species (Cavasin *et al.,* 2020). Neuronal markers valid in equine pathology include S-100 (which is also considered a melanocytic one), PGP 9.5, synaptophysin and neuronal specific enolase (NSE). Among these, synaptophysin seems the most reliable for neurons as S-100, PGP 9.5 and NSE can be expressed by other cell types (especially S-100) (Ramos vara *et al.,* 2014). Similar concepts are applicable to the peripheral nervous system, where neurons and axons can be highlighted via NSE, synaptophysin, S-100 and PGP 9.5, while Schwann’s cells and perineurial cells can be highlighted via S-100 and GFAP (Knottenbelt *et al.,* 2016b). Synaptophysin (and also other neuronal markers) are also considered very useful to aid diagnosis of equine dysautonomia (a.k.a. grass sickness): indeed, synaptophysin is able to highlight neurons’ cytoplasm enhancing the ability of the pathologist to properly assess the neuronal shape and cytoplasm (Figure 2) (Wagget *et al.,* 2010). As supported by Hoerdemann *et al.* study (2023), the researchers reached a diagnosis in a ganglioneuromatosis case, stressing the importance of IHC in equine pathology: the tumour presented by the authors, in absence of IHC, would have been indeed diagnosed as a generic mesenchymal tumour (such as gastro-intestinal stromal tumour/fibrosarcoma, peripheral nerve sheath tumour) with no possibility of understanding its cellular origin.

Lymphoid markers are commonly used in equine pathology, not only to characterise lymphomas and other lymphoid neoplasia, but also to label immune cells in any study equine immunology. CD3 (T cell marker), Pax5, CD 79a, CD 20 and anti-BLA-36 (B cell markers) are reported to work on equine tissue (Durham *et al*., 2013), although, in our experience, the best one for B marker remains Pax5. It is always a good practice to include, for any lymphoma suspect case, to include CD3 and at least two different B cell markers, as B cell lymphomas can arise from a specific stage of B cell maturation and, therefore, express only a fraction of B cell proteins. In our lab, we prefer Pax5 and CD20, as CD79a is commonly giving us faint signal on equine tissue. Take in consideration, that in horses, as well as in other animal species, lymphomas may be negative to both T and B cell marker, complicating such diagnosis.

Rarer round cell tumours include mast cell tumour and histiocytic neoplasm. For these tumours, classic antibody such as IBA-1 (histiocytic neoplasms) and c-kit (mast cell tumour) are working very well in the equine species. C-kit is also used to diagnose gastro-intestinal stromal tumours (GISTs), which are mesenchymal neoplasms arising from the interstitial cells of Cajal, normally present within the gastro-intestinal musculature. The key features of this tumour should be the positivity to c-kit and vimentin, while the tumour shows variable labelling to other mesenchymal markers, including ASMA and S-100 (Knottenbelt et al., 2016c).

Despite the great utility, IHC doesn’t represent a “*deus ex machina*” able to solve any diagnostic challenge, having some limits: as an example, inflammation-driven dysplasia in epithelia can closely mimic carcinomas and there are no reliable IHC markers able to distinguish these two entities. IHC should be requested when a specific question needs an answer (e.g. an undifferentiated tumour is an epithelial or a mesenchymal one) and when such answer cannot be answered via histological features. Considering that IHC in the equine species “started” approximately 50-60 years ago, we have already a broad “arsenal” of antibodies employable to unravel many diagnostic dilemmas and we believe that many more IHC markers are awaiting to be discovered, as the pace of multidisciplinary scientific progress accelerates.

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Figure Legends:

Figure 1: Schematic drawing of main equine tumours (black characters) and immunohistochemical markers (white characters on blue background). Continuous line indicates diffuse positive labelling, while dashed line indicates variable positive labelling. SCC = squamous cell carcinoma; Pan-CK = pan-cytokeratin ; ASMA = Alpha smooth muscular actin ; GIST = gastro-intestinal stromal tumour ; MCT = Mast cell tumour.

Figure 2: Myenteric plexus from a horse with acute grass sickness. Synaptophysin immunohistochemistry highlights chromatolytic neurons. 40X. Courtesy of Dr. Emanuele Ricci.