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CLINICAL COMMENTARY

Immunohistochemistry in equine pathology: A brief overview

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INTRODUCTION

Immunohistochemistry (IHC) is a technique which revolutionised the world of pathology when introduced in 1941 by 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 not be discussed (for additional data, see Ramos-Vara & Miller, 2014), we will briefly discuss the importance of IHC in equine diagnostic pathology, condensed in a few paragraphs. All the IHC markers mentioned in the present study refer to IHC performed on formalin-fixed paraffin-embedded (FFPE) tissue, as this represents 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 a technique is particularly important for 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 hybridisation) 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) types 1, 2 and 13 are considered the aetiological agents of equine sarcoids, the most common equine 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 a better understanding of the biology of these tumours. According to the literature, papillomaviral proteins E5 and 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 tools 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 to 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 in handy 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., 2019) and *Leishmania* spp. (Ortega-García et al., 2021). This is likely due to the reduced sensibility of such techniques compared to serology or molecular assays (e.g. PCR) (Guido Rocchigiani, personal observation).

TUMOURS AND OTHER ENDOGENOUS ANTIGENS

Immunohistochemistry, 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 antibodies reacting against high and low molecular weight cytokeratins, 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 (CUPs), this antibody can help poorly in determining the primary site. IHC targeting pan-cytokeratin is also very useful when assessing metastasis of carcinomas in lymph nodes, where no epithelial cells are normally present.

Vimentin, on the contrary, is a cytoskeletal protein present in all mesenchymal cells, which usually labels 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 a 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 does not 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 cells) as they both express cytokeratin and vimentin antigens. Rare reports of this tumour have been reported in the equine species (Dobromylskyj et al., 2011). It should be noted that in very poorly differentiated carcinomas, vimentin can be re-expressed in a mechanism called epithelial to mesenchymal transition, which is a feature of the anaplastic transformation of a tumour.

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 sarcoids are vimentin-positive and are known to also express some IHC markers variably 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 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

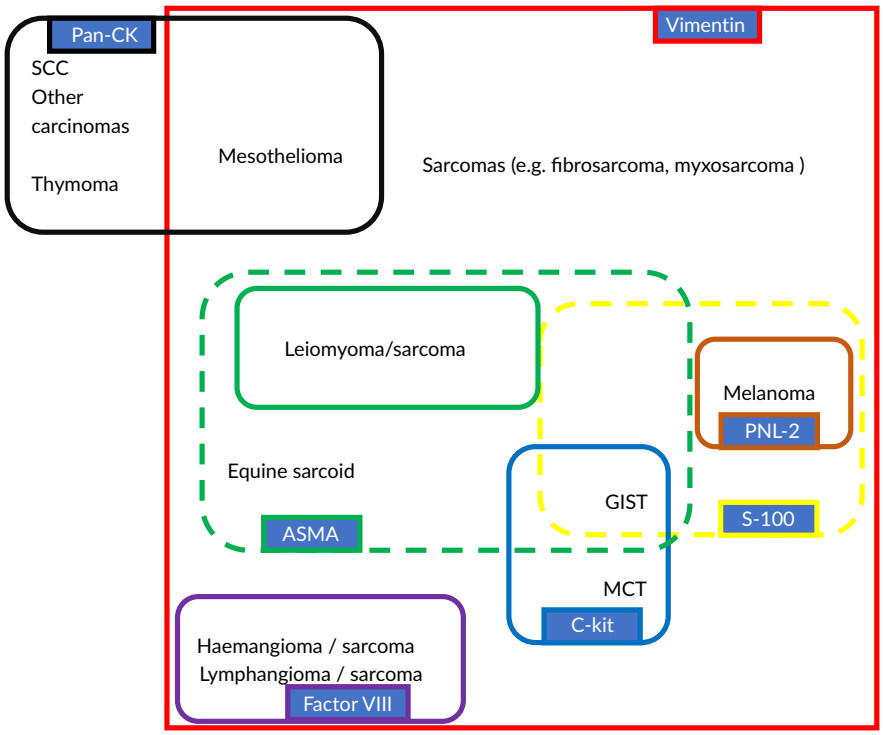

FIGURE 1 Schematic drawing of main equine tumours (black characters) and immunohistochemical markers (white characters on blue background). Continuous line indicates diffuse positive labelling, while the dashed line indicates variable positive labelling. ASMA, alpha smooth muscular actin; GIST, gastrointestinal stromal tumour; MCT, mast cell tumour; Pan-CK, pan-cytokeratin; SCC, squamous cell carcinoma.

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 vessels (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 a good candidate for labelling lymphatic endothelial cells (Junginger et al., 2010).

Melanomas are common neoplasms 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 show 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 usefulness 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 tumours, 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 the diagnosis of equine dysautonomia (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) (Waggett et al., 2010). As supported by the Hoerdemann et al. (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 gastrointestinal 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 of 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 signals on equine tissue (likely due to low cross-reactivity). Take into consideration, that in horses, as well as in other animal species, lymphomas may be negative to both T and B cell markers, complicating such diagnosis.

Rarer round cell tumours include mast cell tumours and histiocytic neoplasm. For these tumours, classic antibodies 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 gastrointestinal stromal tumours (GISTs), which are mesenchymal neoplasms arising from the interstitial cells of Cajal, normally present within the gastrointestinal 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 does not 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, and decision remains in most cases, a domain of the old-fashioned morphology. 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 an answer cannot be answered via histological features alone. 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.

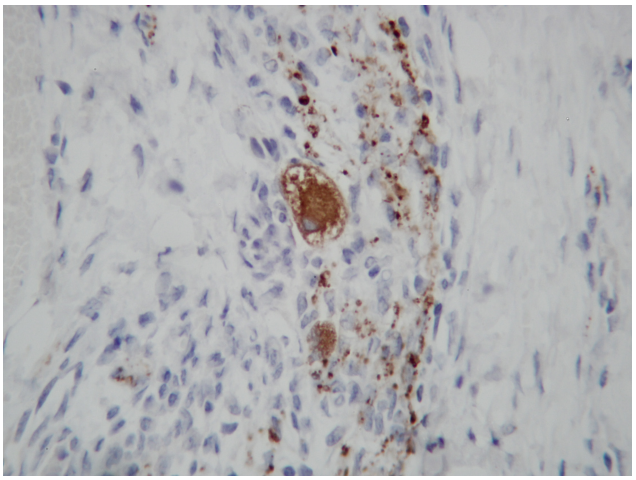

FIGURE 2 Myenteric plexus from a horse with acute grass sickness. Synaptophysin immunohistochemistry highlights chromatolytic neurons. 40×. Image courtesy of Dr Emanuele Ricci.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the preparation of this manuscript.

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No conflicts of interest have been declared.

ETHICS STATEMENT

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