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Immunohistochemistry in diagnostic veterinary pathology: a critical review

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Imuno-histoquímica na patologia veterinária diagnóstica: uma revisão crítica

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key words

Immunohistochemistry

Veterinary pathology

Tumor markers

abstract

Immunohistochemistry has become a practical and widely used tool for diagnosis in human pathology since the 70's. However, its application in veterinary diagnostic pathology has not been so common, especially due to the lack of specific antibodies. To overcome this drawback, antibodies which present cross reactivity with human and animal antigens have been applied. The purpose of the present study was to test the cross reactivity of some antibodies intended for the human pathology, which may be used in animal tissues, with the help of antigen retrieval and amplification systems. In the present study it was confirmed that many of the antibodies produced for use in human histopathology might be applied in veterinary pathology. Further studies are needed to increase the list of applicability of these antibodies to different animal species. It must be stressed that in this type of study some variables, such as clone of antibody, dilution, antigen retrieval method, and detection system, have to be evaluated.

resumo

A técnica de imuno-histoquímica é usada na rotina diagnóstica e na pesquisa em patologia humana desde 1970, porém seu uso na patologia veterinária é relativamente recente, principalmente com objetivo diagnóstico. A maior dificuldade no uso da imuno-histoquímica na patologia veterinária tem sido a falta de anticorpos específicos para os tecidos animais. Na falta de anticorpos específicos para as espécies domésticas, a patologia veterinária freqüentemente faz uso de anticorpos que apresentam reatividade cruzada entre antígenos humanos e animais. O objetivo deste trabalho foi testar a reatividade cruzada de diversos anticorpos feitos para uso humano em tecido parafinado de algumas espécies animais, utilizando-se dos novos métodos de recuperação antigênica e amplificação da reação imuno-histoquímica. No presente estudo foi possível confirmar a aplicabilidade de que muitos anticorpos produzidos para diagnóstico imuno-histoquímico em patologia humana podem ser utilizados em patologia veterinária. Novos estudos são necessários a fim de se ampliar a lista de aplicabilidade desses anticorpos em diferentes espécies animais, levando sempre em consideração as variações de clones, diluições, métodos de recuperação antigênica e de revelação.

unitermos

Imuno-histoquímica

Patologia veterinária

Marcadores tumorais

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Introduction

Immunohistochemistry has become a practical and widely used tool for diagnosis in human pathology since the 70's. However, its application in veterinary diagnostic pathology has not been so common, especially due to the lack of specific antibodies. To overcome this drawback, antibodies which present cross reactivity with human and animal antigens have been applied. Other limitations of the method are represented by antigen masking by formalin fixation, reducing specific antigen-antibody binding[4, 17, 21]. In this respect, an extraordinary progress has been achieved with the production of a wide variety of monoclonal antibodies to epitopes resistant to formalin fixation, thanks to the hybridoma technology, with the use of antigen retrieval methods and powerful amplifying systems. These are generally streptavidin-biotin based systems (e. g., labelled streptavidin biotin [LSAB], catalysed signal amplification [CSA]) or polymer-based systems (e.g., EnVision), which may amplify the signal of immunohistochemical reaction up to 200 fold[19]. All this has allowed cross reactivity of some antibodies produced for human antigens with formalin-fixed, paraffin-embedded animal tissues.

Veterinary medicine has progressively improved its therapeutic spectrum, especially in oncology, demanding a more accurate diagnosis. This fact has brought to the attention of veterinary pathologists the need to improve the application of immunohistochemistry in their daily routine, following the tendency of human diagnostic pathology.

The purpose of the present study is to test the cross reactivity of some antibodies intended for the human pathology, which may be used in animal tissues, with the help of antigen retrieval and amplification systems.

Tabela 1 Animal tissue and tumors used in the present study

Tissue	Animal	Number of cases
Melanoma of the skin	Dog	4
Mastocytoma	Dog	9
TVT	Dog	2
Seminoma	Dog	1
Lymphoma	Dog	15
Cutaneous histiocytoma	Dog	10
Neuroendocrine tumor	Dog	3
Hemangiosarcoma	Dog	1
Mesothelioma	Bruin (*Ursus arctos middendorffi*)	1
Reactive lymph node	Dog, bovine, monkey (*sagüi una*)	10
Normal lung	Dog	1

TVT: transmissible venereal tumor.

Material and methods

All cases tested in the present study were obtained from the files of the Department of Veterinary Pathology, Faculdade de Ciências Agrárias e Veterinárias of Universidade Estadual Paulista (UNESP/Jaboticabal) and from the Laboratory of Experimental Pathology of Campinas, São Paulo, Brazil. Tissues used, animal source and number of cases are summarized in **Table 1**. The cases and species included in the present study corresponded either to those which presented diagnostic problems (more frequently), or to those which served as tests for future research projects.

Immunohistochemistry

Serial sections were placed on silanized slides and dewaxed. Antigen retrieval was performed after hydration and endogenous peroxidase blocking with 3% H_2O_2 in a steamer (T-Fal®, France) at 90° C for 30 minutes. The slides were then incubated overnight (18 hours) at 4° C with the primary antibodies specified in **Table 2**. The detection systems for each primary antibody were used according to the suppliers' instructions (two polymer-based detection systems, EnVision [K1491; Dakocytomation, Carpenteria, CA, USA] and EnVision Plus [K4001, Dako], and a catalyzed signal amplification system, CSA [K1500; Dako], were used, as also listed in Table 2). Labeling was visualized with 3,3'-diaminobenzidine (D-5637; Sigma, St. Luis, MO, USA) added to H_2O_2 3% in phosphate-buffered saline (pH 7.6). Positive controls consisted of human tissues known to be reactive for each marker. Negative controls were performed by replacing the primary antibody by bovine serum albumin (BSA 1% in phosphate-buffered saline, pH 7.6) in the animal samples. Immunolabeling was evaluated microscopically.

Results

The description of immunolabeling for each marker is described in **Table 3** and some examples are shown in **Figures 1** to **8**.

Tabela 2 **Specification of antibodies used in the present study**

Antibody	Supplier	Clone	Dilution	Amplification system	Tissue	Animal
T-cell, CD3	Dako	PC3/188A	1:50	EnVision	Lymph node	Dog
T-cell, CD3	Novocastra	PS1	1:50	EnVision	Lymph node	Dog, bovine
T-cell, CD8	Novocastra	1A5	1:40	EnVision	Lymph node	Monkey
T-cell, CD8	Novocastra	1A5	1:800	CSA	Lymph node	Monkey
T-cell, CD8	Dako	C8/144B	1:50	EnVision	Lymph node	Monkey
B-cell, BLA-36	Novocastra	A2742	1:50	CSA/Envision	Lymph node	Dog, bovine
B-cell, CD79a	Dako	HM57	1:50	CSA/EnVision	Lymph node	Dog
CD117, c-kit	Dako	Polyclonal	1:50	EnVision	Mastocytoma	Dog
CD31	Dako	JC70A	1:10	EnVison	Hemangiosar-coma	Dog
Lysozyme	Dako	Polyclonal	1:400	EnVision	Cutaneous histiocytoma	Dog
Myeloperoxidase	Dako	Polyclonal	1:400	Envision	Cutaneous histiocytoma	Dog
Pancytokeratin, AE1/AE3	Dako	Polyclonal	1:50	EnVision	Skin	Dog
NSE	Dako	BBS/NC/VI H14	1:100	EnVision	Neuroendocrine tumor	Dog
Sinaptofisin	Dako	Polyclonal	1:50	EnVision	Neuroendocrine tumor	Dog
S-100 protein	Dako	Polyclonal	1:800	EnVision	Melanoma	Dog
Melanocytic antigen, HMB45	Dako	HMB35	1:10	EnVision	Melanoma	Dog
Vimentin	Dako	V9	1:50	EnVision	Melanoma, mesothelioma	Dog, bear
PLAP	Dako	8A9	1:50	EnVision	Seminoma	Dog
TTF-1	Dako	8G7G3/1	1:50	EnVision Plus	Lung	Dog
p-53 protein	Novocastra	CM-1, polyclonal	1:50	EnVision	Lymphoma	Dog
c-myc protein	Dako	9E11	1:50	EnVision Plus	TVT	Dog
Proliferation antigen, Ki-67	Immuno-tech	MIB-1	1:50	EnVision Plus	TVT	Dog
Proliferation antigen, Ki-S-5	Dako	Ki-S-5	1:50	EnVision Plus	TVT	Dog
Proliferation antigen, Ki-67	Novocastra	MM-1	1:50	EnVision Plus	TVT	Dog
PCNA	Dako	PC10	1:100	EnVision	Mastocytoma	Dog
PCNA	Novocastra	PC10	1:100	EnVision	Mastocytoma	Dog

NSE: neuron specific enolase; PLAP: placental alkaline phosphatase; TTF-1: thyroid transcription factor; TVT: transmissible venereal tumor; PCNA: proliferating cell nuclear antigen.

Tabela 3 Specific immunolabeling for each marker used in this study

Antibody	Supplier	Number of positive cases	Immunolabeling
T-cell, CD3	Dako	5/5	Intense, membrane of small lymphocytes in paracortical areas
T-cell, CD3	Novocastra	5/5	Intense, membrane of small lymphocytes in paracortical areas
T-cell, CD8	Novocastra	8/8	Weak when the Envision detections system was used. Intense with CSA detection system, membrane of scattered small lymphocytes in follicles and paracortical areas
T-cell, CD8	Dako	0/8	Not reactive
B-cell, BLA-36	Novocastra	10/10	Moderate, membrane of small lymphocytes and blasts in follicles and scattered lymphoid cells in paracortical areas
B-cell, CD79a	Dako	10/10	Intense, transmembrane of small lymphocytes and blasts in follicles and scattered lymphoid cells in paracortical areas
CD117, c-kit	Dako	9/9	Variable, cytoplasm of neoplastic mast cells, Golgi pattern
CD31	Dako	1/1	Moderate, cytoplasm of hemangiosarcoma cells
Lysozyme	Dako	10/10	Intense, cytoplasm of histiocytes and granulocytes
Myeloperoxidase	Dako	10/10	Intense, cytoplasm of granulocytes in a granular pattern
Pancytokeratin, AE1/AE3	Dako	4/4	Intense, cytoplasm of epithelial cells
NSE	Dako	4/4	Moderate, cytoplasm of neuroendocrine cells
Sinaptofisin	Dako	3/3	Moderate, cytoplasm of neuroendocrine cells
S-100 protein	Dako	5/5	Intense, cytoplasm of mesenchymal cells
Melanocytic antigen, HMB45	Dako	0/4	Not reactive
Vimentin	Dako	5/5	Intense, cytoplasm of mesenchymal cells
PLAPH	Dako	1/1	Moderate, cytoplasm of seminoma cells
TTF-1	Dako	1/1	Intense, nuclei of pneumocytes
p-53 protein	Novocastra	8/8	Intense, nuclei of neoplastic lymphoid cells
c-myc	Dako	2/2	Variable; nuclei of TVT cells
Proliferation antigen, Ki-67	Immunotech	10/10	Intense, nuclei of TVT cells
Proliferation antigen, Ki-S-5	Dako	10/10	Intense; nuclei of TVT cells
Proliferation antigen, Ki-67	Novocastra	10/10	Intense; nuclei of TVT cells
PCNA	Dako	10/10	Variable, nuclei of lymphocytes and mast cells
PCNA	Novocastra	10/10	Variable, nuclei of lymphocytes and mast cells

NSE: neuron specific enolase; PLAPH: placental alkaline phosphatase; TTF-1: thyroid transcription factor; TVT: transmissible venereal tumor; PCNA: proliferating cell nuclear antigen.

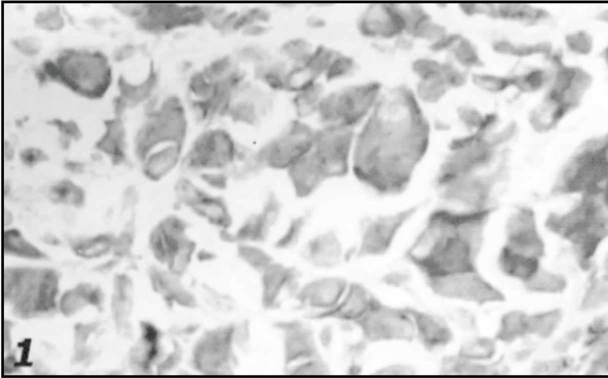

Figure 1 – *Mesothelioma, anti-vimentin, cytoplasm stainig (x 1.000)*

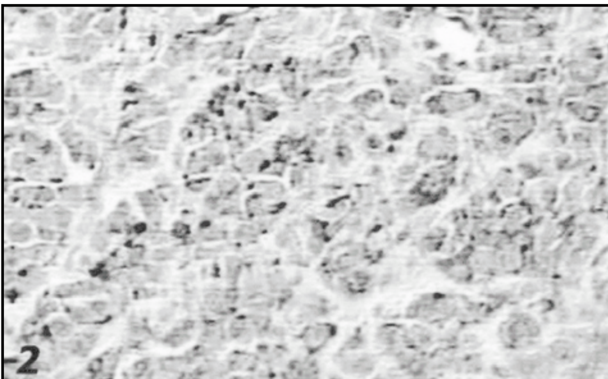

Figure 2 – *Neuroendocrine carcinoma, anti-neuron specific enolase (NSE), cytoplasm staining (x 800)*

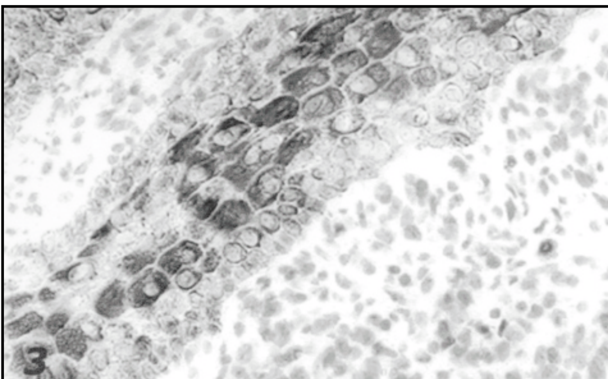

Figure 3 – *Skin, anti-cytokeratin AE1/AE3, cytoplasm staining (x 800)*

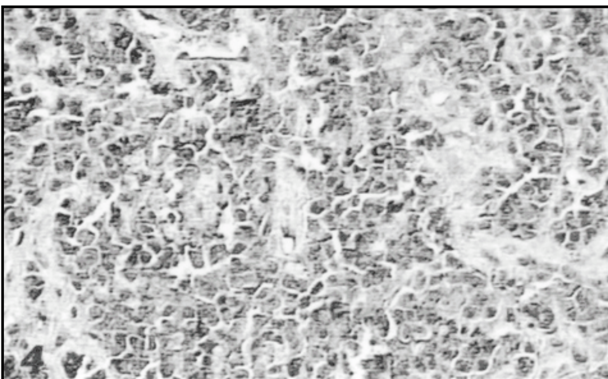

Figure 4 – *Melanoma, anti-S100 protein, cytoplasm staining, (x 400)*

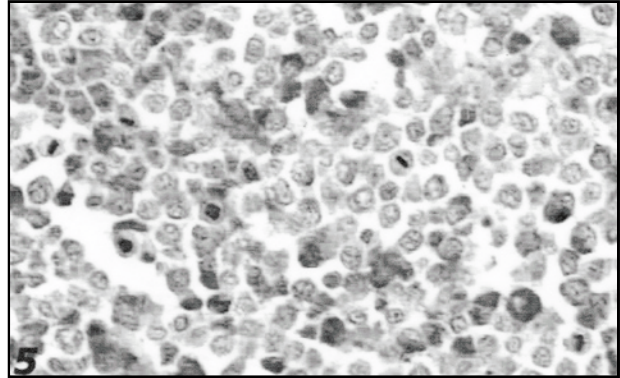

Figure 5 – *Seminoma, anti-placental alkaline phosphatase (PLAP), cytoplasm staining (x 800)*

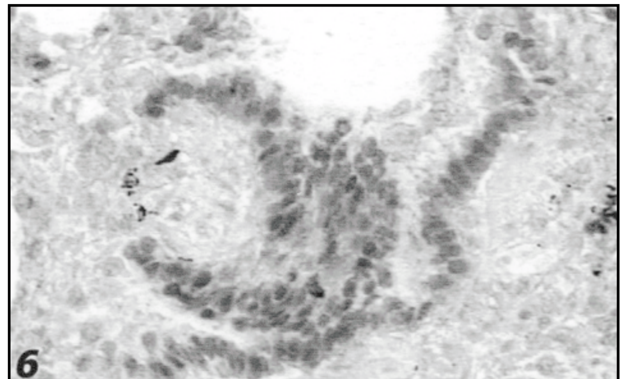

Figure 6 – *Lung (pneumocytes), anti-thyroid transcription factor-1 (TTF1), nuclear staining (x 800)*

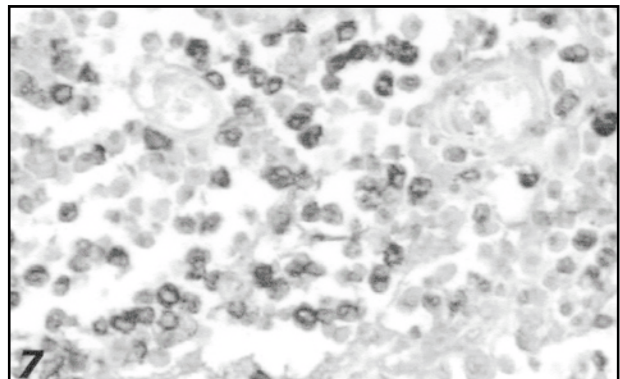

Figure 7 – *Cutaneous histiocytoma, anti-lysozyme, cytoplasm staining (x 800)*

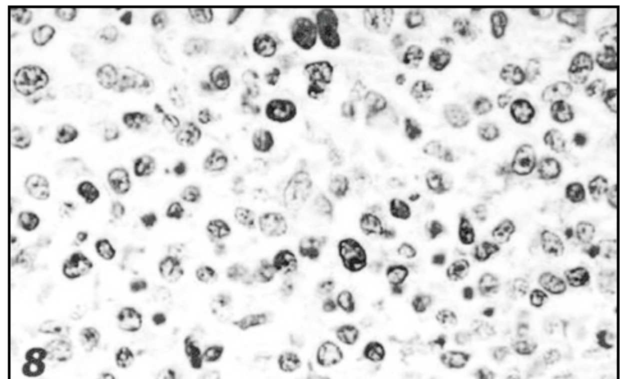

Figure 8 – *Mastocytoma, anti-proliferation marker Ki-67, nuclear staining (x 1.000)*

Discussion

Most antibodies developed for animals are suitable for fresh tissue and have a limited application in routine veterinary histopathology. The most frequently used immunomarkers for animals in the routine diagnosis are polyclonal, as they contain antibodies to a wider range of epitopes, increasing sensitivity of the method, sometimes in detriment of specificity. Antigen retrieval techniques, especially the heat-induced ones, have been widely used in human pathology since the 80's, allowing reliable immunodetection of epitopes distorted by formalin fixation[4, 17, 21]. These techniques have proven efficiency in routine veterinary pathology, permitting the utilization of a wider spectrum of markers, including monoclonal and those intended for human use, as shown by our results.

The polymer-based detection systems (e. g., EnVision and EnVision Plus) have increased sensitivity of immunoreactions and reduced one of its steps[19]. In our study, this system has shown good results for most antibodies. However, in some cases, a signal amplification was needed (e. g., for the pan-B marker CD79a, as previously demonstrated by Sueiro *et al.*)[22]. The catalyzed signal amplification system is very sensitive[19], but may result in unacceptable background staining. To overcome this adverse effect, thorough washing between the steps of the reaction and, sometimes, the over dilution of the primary antibody are necessary.

Antibodies to pancytokeratin AE1/AE3, neuron specific enolase (NSE), synaptofisin (SNF), S-100 protein and vimentin were previously described as showing cross reactivity in different animal species[17, 20, 31]. Antibody to AE1/AE3 showed specificity in epithelial cells in our cases. The use of heat-induced epitope retrieval (HIER) and the polymer-based detection system were efficient, allowing a higher dilution of primary antibody and replacing the more imprecise method of enzymatic digestion, as previously reported[20].

The melanocytic antigen (Dako, clone HMB45) did not react in any test performed, using the same procedures described by Sulaimon *et al.*[27].

The antibodies to NSE and SNF also showed specific labeling, but in lesser intensity than the positive controls. This reinforces the need of testing different conditions of dilutions and reactions to adapt the use of antibodies for use in different animal species. Antibody to S-100 proteins showed intense cytoplasmic reactivity, accompanied by scattered nuclear positivity[24, 26]. Strong labeling for vimentin in a case of mesotheliona of bruin (*Ursus arctos middendorffi*) demonstrates the widespread preservation of this antigen in various species.

Lymphoid markers are largely used in human pathology and have been shown to cross react in various animal species[8, 9, 14, 22, 29, 30, 32]. Nevertheless, the list of antibodies for use in veterinary pathology is still small. In 1993, Jones *et al.*[7] demonstrated cross reactivity of the antibody to CD3 in frozen sections of various domestic species, such as horses, bovines, pigs and chicken. This was later confirmed in formalin-fixed, paraffin-embedded material, using different detection and amplification methods[8]. In our cases, CD3 was expressed in strong intensity in cell membrane of T lymphocytes of the species tested. Even the use of HIER and the polymer-based detection system did not allow higher dilution of the primary antibody, but no background unspecific reactivity was seen. Antibody to CD8 for use in animals is available only for frozen tissue[15]. The anti-human CD8 showed satisfactory immunolabeling in a hyperplastic monkey (*sagüi una*) lymph node. Another clone used (Dako, clone C8/144B) did not react, what stresses the need to test different suppliers and clones when the use in other species is intended. The cross reactivity of the pan-B antibodies to CD79a and BLA-36 has already been demonstrated[8]. In our cases, reactivity for CD79a was more intense than for BLA-36, but a more sensitive detection system (CSA) was necessary for the first. A faint positivity in the muscular layer of blood vessels was also seen in specimens stained for CD79a, in accordance with previous reports[2].

Antibodies to lysozyme and myeloperoxidase labeled intensely histiocytes and myeloid cells of canine lymph nodes. The usefulness of these markers to differentiate histiocytic and myeloid neoplasms from lymphoma and other small cell neoplasia was demonstrated elsewhere[22].

The thyroid transcription factor (TTF-1) had already been shown to cross react with normal and neoplastic canine thyroid[16]. The placental alkaline phosphatase (PLAPH) was not previously described in animal tumors. In our study, both were demonstrated in strong intensity in canine tissue, as compared with the positive human controls.

Reactivity of CD117/c-kit in cell membrane and cytoplasm was intense in our cases of canine mastocytoma, as previously described[10, 18, 25], confirming its utility in the identification of difficult cases. The polyclonal antibody to the p53 protein (Novocastra, clone CM1) showed strong nuclear reactivity, confirming the applicability of this marker in research and diagnostic veterinary pathology[5,

[6, 10, 11, 18, 22, 23]. Immunoexpression of c-myc was strong in the nuclei of the case of canine transmissible venereal tumor (TVT) tested in this study, corroborating previous reports[1, 35]. This protein has also been demonstrated in canine breast tumors[6].

Amino acid sequences of proliferating cell nuclear antigen (PCNA) present high homology among mammalians, and also in superior vegetal[28]. In our cases, nuclear reactivity for PCNA was strong, with faint cytoplasmic staining in mitotic cells[12, 34]. In humans and animals, detection of the antigen Ki-67 has proven superior to PCNA in evaluation of proliferation index[3, 13, 33]. In our experience, the best clone for application in canine pathology was MIB-1, as compared with MM-1 and Ki-S-5.

Conclusion

In the present study it was confirmed that many of the antibodies produced for use in human histopathology might be applied in veterinary pathology. Further studies are needed to increase the list of applicability of these antibodies to different animal species. It must be stressed that in this type of study, some variables, such as clone of antibody, dilution, antigen retrieval method, and detection system, have to be evaluated.

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