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Original Article
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Detection of occult metastases of mammary carcinomas in lymph nodes of dogs by immunohistochemistry combined with histochemistry techniques[1]

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ABSTRACT.- Barraza V.C.T., Buttelli P.H.S., Vargas I., Goldani M.A., Medeiros A.M., Sangoi F.A., Flores M.M. & Kommers G.D. 2024. **Detection of occult metastases of mammary carcinomas in lymph nodes of dogs by immunohistochemistry combined with histochemistry techniques**. *Pesquisa Veterinária Brasileira 44:*e07450, *2024*. Laboratório de Patologia Veterinária, Departamento de Patologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Av. Roraima 1000, Cidade Universitária, Bairro Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: glaukommers@yahoo.com

Lymph node status is considered an important clinical prognostic factor in canine mammary carcinomas and women's breast neoplasms. However, occult isolated tumor cells (ITCs) can be missed during hematoxylin and eosin (HE) analyses. Immunohistochemistry (IHC) for cytokeratin can be used to detect carcinomatous occult ITCs in mammary drainage lymph nodes. However, brown pigments, such as hemosiderin and ceroid in lymph nodes, may hinder the search for occult metastases by IHC utilizing DAB (3,3'-diaminobenzidine) as the chromogen. The aim of this study was to identify ITCs in canine lymph nodes of cases in which it was not detectable by routine HE evaluation through IHC for cytokeratin (AE1/AE3) combined with histochemistry techniques, such as Perls' Prussian blue and periodic acid-Schiff (PAS), to improve the detection of occult metastases when hemosiderin and ceroid were present in these lymph nodes. For this, 25 tubulopapillary mammary carcinomas with their respective submitted 29 regional lymph nodes, previously given as free of tumor cells by HE analyses, were selected. Mammary tumors were graduated, and vascular invasion was investigated in these tumors. The submitted lymph nodes were reevaluated in HE, looking for occult metastases. IHC for cytokeratin (AE1/AE3) was used to detect occult metastases in mammary lymph nodes. Subsequently, a combined technique of IHC with Perl's Prussian blue (for hemosiderin) or PAS (for ceroid) was performed to optimize the detection of ITCs by IHC, distinguishing them from pigments. Occult metastases were classified by their microanatomical location in subcapsular, cortical and medullary. Hemosiderin and ceroid were searched in lymph nodes and quantified as low, moderate, or high. The amount of pigments with a percentage of ITCs was also compared. Isolated tumor cells were found in 24.1% (7/29) of mammary lymph nodes. These ITCs were located mainly in subcapsular sinuses (4/7; 57.1%), followed by cortical (2/7; 28.5%) and medullary sinuses (1/7; 14.3%). There were concomitant lymph nodes with ITCs in 33.4% (2/6) of cases with vascular invasion. Hemosiderin and ceroid were present in about 90% of the 29 lymph nodes analyzed. In 42.8% (3/7) of lymph nodes with ITCs, hemosiderin and/or ceroid were in the same location as

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ITCs. It was found that lymph nodes in which ITCs were detected also present high amounts of hemosiderin (3/7; 42.9%) and low amounts of ceroid (5/7; 71.4%). In this study, IHC for cytokeratin (AE1/AE3) was an efficient method to detect occult tumor cells. IHC combined with Perls' Prussian blue or with PAS proved to be a helpful way to investigate the presence of occult metastases in the lymph nodes of mammary canine tumors. It allowed distinguishing hemosiderin and ceroid, respectively, from ITCs in the same slide of IHC (immunostained by DAB), favoring a more accurate analysis by pathologists, which can be useful for the oncological staging of these patients.

INDEX TERMS: Immunohistochemistry, isolated tumor cells, cytokeratin, lymph nodes, mammary tumors.

RESUMO.- [**Detecção de metástases ocultas de carcinomas mamários em linfonodos de cães por imuno-histoquímica combinada com técnicas histoquímicas.**] O status nodal é considerado um importante fator prognóstico clínico em cães com carcinomas mamários e em mulheres com neoplasmas mamários. No entanto, durante a análise histopatológica convencional (coloração de hematoxilina e eosina - HE), células neoplásicas podem permanecer ocultas no linfonodo. Imuno-histoquímica (IHQ) para citoqueratina pode ser utilizada para detectar células carcinomatosas isoladas ocultas em linfonodos de drenagem mamária. Entretanto, a presença de pigmentos marrons, como a hemossiderina e o ceroide em linfonodos, pode dificultar a procura por metástases ocultas durante a análise por IHQ utilizando o DAB (3,3′-diaminobenzidina) como cromógeno. O objetivo deste estudo foi identificar células tumorais isoladas (CTIs), não detectadas pela avaliação de rotina (HE), em linfonodos de cães, através de IHQ para citoqueratina (AE1/AE3) combinada com técnicas histoquímicas, como azul da Prússia e ácido periódico Schiff (PAS), para otimizar a detecção de metástases ocultas, quando os pigmentos hemossiderina e ceroide estavam presentes nos linfonodos. Para esta finalidade, 25 carcinomas mamários túbulo-papilares com seus respectivos 29 linfonodos regionais remetidos, previamente diagnosticados como livres de células neoplásicas pelo HE foram selecionados. Os tumores mamários foram graduados e a presença de invasão vascular foi investigada nestes tumores. Os linfonodos submetidos foram reavaliados histologicamente no HE, à procura de metástases ocultas. IHQ para citoqueratina (AE1/A3) foi utilizada para detectar metástases ocultas nos linfonodos mamários. Subsequentemente, técnicas combinadas de IHQ com azul da Prússia (para hemossiderina) ou PAS (para ceroide) foram realizadas para otimizar a detecção de CTIs através da IHQ, distinguindo-as dos pigmentos. As metástases ocultas foram classificadas pela sua região microanatômica nodal em subcapsular, cortical e medular. Hemossiderina e ceroide foram identificados no linfonodo e classificados de acordo com sua quantidade em baixa, moderada e alta. Também foram comparadas a quantidade de pigmentos com a porcentagem de CTIs. Foram encontradas CTIs em 24,1% (7/29) dos linfonodos mamários. Estas CTIs estavam localizadas principalmente em seios subcapsulares (4/7; 57,1%), seguido pela cortical (2/7; 28,5%) e seios medulares (1/7; 14,3%). Em 33,4% (2/6) dos casos com invasão vascular havia, concomitantemente, linfonodos de drenagem com CTIs. Hemossiderina e ceroide estavam presentes em cerca de 90% dos 29 linfonodos analisados. Em 42,8% (3/7) dos linfonodos com CTIs, hemossiderina e/ou ceroide estavam na mesma localização das CTIs. Os linfonodos que apresentaram CTIs também apresentavam grande quantidade de hemossiderina (3/7; 42,9%) e pequena quantidade de ceroide (5/7; 71,4%). Neste estudo, IHQ para citoqueratina (AE1/AE3) foi um método eficiente para detecção de células tumorais ocultas. A técnica combinada de IHQ com azul da Prússia ou com o ácido periódico de Schiff (PAS) mostrou ser uma maneira útil de investigar a presença de metástases ocultas em linfonodos de cães com tumores mamários caninos, distinguindo a hemossiderina e o ceroide, respectivamente, de CTIs na mesma lâmina de IHQ (imunomarcadas por DAB), favorecendo uma análise mais acurada pelos patologistas, o que pode ser útil no estadiamento oncológico destes pacientes.

TERMOS DE INDEXAÇÃO: Imuno-histoquímica, células tumorais isoladas, citoqueratina, linfonodos, tumores mamários.

INTRODUCTION

Mammary neoplasms are common tumors in areas where ovariohysterectomy is unusual and can represent 50-70% of all neoplasms in intact bitches (Goldschmidt et al. 2016). Lymph node status is one of the most important clinical prognostic factors in canine mammary tumors (Sorenmo et al. 2011, De Araújo et al. 2015) and breast cancer in women (Ahmed et al. 2014). The modified staging system used for mammary carcinomas in dogs classified animals with cytological or histological evidence of metastases in the lymph node as stage IV (Sorenmo et al. 2012). However, this system does not consider the presence of occult metastases detected by immunohistochemistry (Matos et al. 2006).

A new proposal for a histological staging system of mammary canine carcinoma was made and considered these occult metastases, detected by immunohistochemistry (IHC), as positive nodal stage (Chocteau et al. 2019). Occult metastases are defined as clusters of neoplastic cells detected by IHC in lymph nodes previously negative on the HE slides examination (Matos et al. 2006). According to the 7th edition of the Cancer Staging Handbook from the American Joint Committee on Cancer (AJCC) (Edge et al. 2010), isolated tumor cells (ITCs) were defined as metastases ≤0.2mm or less than 200 tumor cells, micrometastases are deposits between >0.2 and ≤2mm and macrometastases are deposits >2mm (Edge et al. 2010, Ahmed et al. 2014).

Studies about occult metastases in mammary canine lymph nodes are scarce (Matos et al. 2006, Szczubial & Lopuszynski 2011, De Araújo et al. 2015, Coleto et al. 2018). Additionally, considering the frequent observation of brown pigments in macrophages, such as hemosiderin and/or ceroid in mammary drainage lymph nodes, this raises the concern that these pigments may mask the presence of true immunostained ITCs when using the DAB (3,3′-diaminobenzidine) chromogen. DAB is a brown-colored chromogen often used for IHC staining in

veterinary pathology laboratories, particularly in developing countries where access to other colored chromogens may be more limited.

The aim of this study was to identify ITCs in canine lymph nodes in a case series in which it was not detectable by routine hematoxylin and eosin (HE) evaluation through IHC for cytokeratin (AE1/AE3) combined with histochemistry techniques, such as Perls' Prussian blue (for hemosiderin) and Periodic acid-Schiff (PAS; for ceroid), as a tool to detect occult metastases in lymph nodes of dogs with canine mammary tumors, allowing visual distinguishing between hemosiderin or ceroid and true metastatic immunomarked cells on the same slide.

MATERIALS AND METHODS

Ethical approval. In this study, we did not perform any animal experiments. All the data were obtained from the archives of biopsy reports. All the samples (paraffinized tissues) used in this study came from the archives of the diagnostic routine of the Veterinary Pathology Laboratory.

Samples. A retrospective investigation of biopsy cases of canine mammary tumors submitted to and archived at a veterinary pathology service linked to a veterinary teaching hospital was performed in 12 years (2010-2021) to select samples for this study. Only mammary tumors with tubulopapillary histological subtype and cases in which regional lymph node was referred to as "free of tumor cells" in the pathology report were selected. Only one case of metastasis was referred as "suggestive" in the report. There were some cases in which more than one lymph node in the same dog was analyzed. The lymph nodes were inguinal and/or axillary. Information on the location of the mammary tumor and the affected lymph nodes was taken from the biopsy reports.

Histopathology. New histological sections of mammary tumors and lymph nodes were prepared for evaluation. Samples were sectioned at 3μm and stained with hematoxylin and eosin (HE). Lymph nodes were sectioned semi-serially for three histological sections; two were reserved for IHC, and another was stained with HE. Microscopically, the mammary tumors were classified into Grades I, II, and III, according to Peña et al. (2013). Also, the presence or absence of vascular invasion was evaluated in these tumors. The presence or absence of isolated tumor cells (ITCs) was investigated for regional lymph nodes. For ITCs, it was considered histologically less than 200 cells, according to Edge et al. (2010). The presence of brown pigments was also observed in the lymph nodes.

Immunohistochemistry (IHC). Silanized slides with 3μm histological sections of each lymph node were used. The presence of ITCs was evaluated by IHC. The epithelial cells were identified with anti-cytokeratin mouse monoclonal antibody (Clones AE1/AE3; Dako; 1:200 dilution). Sections were dewaxed and rehydrated. Endogenous peroxidase was blocked with hydrogen peroxide (3%) for 2x 10 min. Antigen retrieval was achieved by microwaving (10 min at full power) in TRIS-EDTA pH 9.0. Blocking of nonspecific reactions was performed with a protein blocker (EP-12-20532, EasyPath) at room temperature (RT) for 10 min. Sections were incubated with the primary antibodies diluted in PBST for 1h at 37°C. A polymer-HRP system (EasyLinkOne-HRP, EP-12-20502, EasyPath) was used (at RT for 30 min), followed by substrate development with 3,3'-diaminobenzidine (DAB; EasyPath). After 5 min, the reaction was stopped in water. After that, the slides were counterstained with Harris hematoxylin. As a positive control for cytokeratin AE1/AE3, a lymph node sample with confirmed mammary gland carcinoma metastasis was used. A section of the analyzed tissue was used as a negative control for each case, incubated only with the antibody diluent (PBST). It was considered positive tumor cells by IHC, individual or groups of cells with polygonal format, stained in strong golden brown and with central nuclei.

IHC combined with Perl's Prussian blue or periodic acid-Schiff (PAS). For the purpose of differentiating macrophages containing hemosiderin (staining in strong blue to blue-green with Perl's Prussian blue) from epithelial neoplastic cells (staining in brown), the staining of Perl's Prussian blue (Histokit Perls; EP- 11-20015; EasyPath) was combined to another round of IHC (Clones AE1/AE3), as follows. After the reaction of DAB was stopped in tap water, ferrocyanide and hydrochloric acid in the same proportions were mixed and added to the slides for 30 min. The slides were washed in distilled water and counterstained with Harris hematoxylin. On the other hand, for the purpose of differentiating macrophages containing ceroid (staining in strong pink/magenta with PAS) from epithelial neoplastic cells (staining in brown), the staining of PAS (Histokit Periodic Acid-Schiff; EP- 12-20014; EasyPath) was combined with IHC as follows. After the reaction of DAB was stopped in tap water, a periodic acid solution was added to the slides for 10 min. The slides were washed in tap water for 3 min and distilled water for 1 min. Schiff's solution was added to the slides for 15 min in a dark chamber and then the slides were washed in tap water for 3 min and counterstained with Carazzi's hematoxylin. The positive and negative controls and the interpretation of neoplastic cells were the same as described in the IHC section. For hemosiderin, it was considered present when stained in strong blue to blue-green. Ceroid was considered present when stained in pink or magenta. After the IHC, when tumor cells were found, the HE sections of lymph nodes were reevaluated, searching for ITCs. Regarding pigments, hemosiderin and ceroid were searched in lymph nodes and classified in low, moderate, or high amounts. The microanatomical location (subcapsular, cortical, and medullary) of pigments in the lymph nodes was also evaluated. The amount of pigments with a percentage of ITCs was also compared.

RESULTS

Samples

Among the 25 canine mammary tumors collected, the most common location was the inguinal mammary gland (8/25, 32%). Out of 29 lymph nodes analyzed, most were from the inguinal region (18/29, 62.1%); the remaining were axillary (5/29, 17.2%), and location not specified (6/29, 20.7%).

Histopathology

Out of 25 canine mammary tumors, 11 were Grade I (44%), 12 were Grade II (48%), and two were Grade III (8%). There was evidence of vascular invasion in 20% (5/25) of the neoplasms. Of 29 lymph nodes analyzed, only one axillary lymph node was suspected of metastasis; the remaining 28 lymph nodes were interpreted as free of tumor cells. Golden brown pigment (hemosiderin) and opaque brown pigment (ceroid) were observed in the drainage lymph nodes. As described below, their identification and quantification (as hemosiderin or ceroid) were helped with special stains.

IHC and IHC combined with Perl's Prussian blue or PAS

Isolated tumor cells (ITC). ITCs from mammary carcinomas were showed by IHC in seven of 29 lymph nodes (24.1%), originally considered free of tumor cells by histopathological analyses (HE) (Fig.1). These ITCs were located mainly in subcapsular sinuses

(4/7, 57.1%), followed by cortical (2/7, 28.5%) and medullary sinuses (1/7, 14.3%). All lymph nodes that present ITCs were from dogs with Grade II mammary carcinomas. Two out of six lymph nodes (33.4%) removed from dogs showing vascular invasion in the primary tumor had ITCs (Table 1).

Pigments. Three out of seven (42.8%) of the lymph nodes with ITCs had neoplastic cells and pigments in the same location (Fig.2). In two of three cases (66.6%), there was agreement at localization of neoplastic cells and hemosiderin, and in one of three cases (33.4%), the same occurred with ceroid.

Hemosiderin. Perl's Prussian blue stain evidenced the presence of hemosiderin in 93.1% (27/29) of the lymph nodes. This pigment was most present in medullary sinuses (15/27, 55.6%) and in the cortical (10/27, 37.0%), less present in subcapsular sinuses (2/27, 7.4%), and it was present mostly in high amounts (11/27, 40.8%) (Table 2).

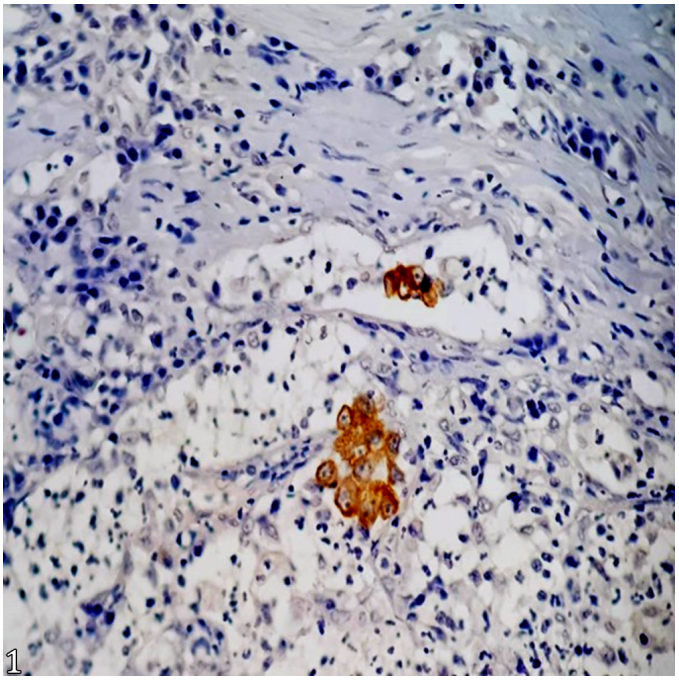

Fig.1. Lymph node of canine with mammary carcinoma subtype tubulopapillary. Isolated tumor cells are intensely immunostained for cytokeratin (AE1/AE3), located in the cortical region. Polymer method-HRP (IHC), obj.20x.

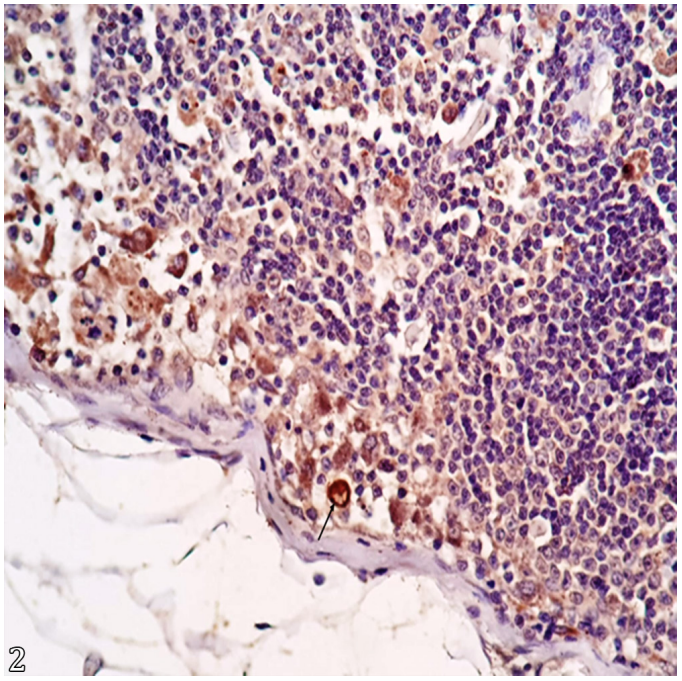

Fig.2. Lymph node of canine with mammary carcinoma subtype tubulopapillary. Isolated tumor cells (ITCs; arrow) are intensely immunostained for cytokeratin (AE1/AE3). However, pigments such as hemosiderin and ceroid are all colored brown and can mask the immunostained ITCs. Polymer method-HRP (IHC), obj.20x.

Table 1. Frequency of isolated tumor cells in lymph nodes (LN) of a female dog with mammary carcinoma detected by immunohistochemistry

	Primary tumor (n=25)	Number of analyzed LN n=29 (%)	Absence of nodal metastasis n=22 (%)	Isolated tumor cells n=7 (%)
Grade	I	11 (37.9)	11 (100.0)	0 (0.0)
	II	16 (55.1)	9 (56.2)	7 (43.8)
	III	2 (7.0)	2 (100.0)	0 (0.0)
Vascular invasion	No	23 (79.3)	18 (78.2)	5 (21.8)
	Yes	6 (20.7)	4 (66.6)	2 (33.4)

Table 2. Microanatomical location and quantification of hemosiderin in mammary lymph nodes with isolated tumor cells

		Number of lymph nodes n=27 (%)	Absence of metastasis n= 20 (%)	Isolated tumour cells n=7 (%)
Microanatomical location*	Subcapsular Sinuses	2 (7.4)	2 (100.0)	0 (0.0)
	Cortical	-	-	-
	Medullary sinuses	10 (37.0)	7 (70.0)	3 (30.0)
		15 (55.6)	11 (73.3)	4 (26.7)
Amount*	Low	10 (37.0)	8 (80.0)	2 (28.6)
	Moderate	6 (22.2)	4 (66.6)	2 (28.6)
	High	11(40.8)	8 (72.7)	3 (42.9)

* Analyzed in slides of immunohistochemistry combined with Perl's Prussian blue.

Three out of seven (42.9%) lymph nodes that present ITCs also had high amounts of hemosiderin (Fig.3).

Ceroid. PAS stain evidenced the presence of ceroid in 96.5% (28/29) of lymph nodes in different locations and amounts. This pigment was present in cortical (15/28; 53.6%) and medullary sinuses (13/28; 46.4%); it was not present in subcapsular sinuses and mostly appeared in low amounts (21/28; 75.0%) (Table 3). Five out of seven (71.4%) lymph nodes with ITCs also had low amounts of ceroid; 28.6% of the lymph nodes with ITCs also had high amounts of ceroid (Fig.4).

DISCUSSION

In this study, it was successfully combined the techniques of IHC (for cytokeratin) and histochemistry with Perls' Prussian blue (for hemosiderin) and IHC (for cytokeratin) with PAS (for ceroid), in order to differentiate immunostained neoplastic cells (ITCs) from macrophages containing these brown pigments. Furthermore, occult metastases (with IHC and histochemistry) were demonstrated in 24.1% of lymph nodes previously negative on HE staining.

Regarding occult metastasis, studies on the occurrence of ITCs and micrometastasis in canine mammary tumors are scarce (Matos et al. 2006, Szczubial & Lopuszynski 2011, Coelho 2014, De Araújo et al. 2015, Coleto et al. 2018) if compared with data available in humans (Galea et al. 1991, McGuckin et al. 1996, Millis et al. 2002, Reed et al. 2004, Tan et al. 2008, Van Deurzen et al. 2008, Loya et al. 2009, Ahmed et al. 2014, Liikanen et al. 2018, Wang et al. 2021). In this study, the use of IHC combined with histochemistry techniques (Perls' Prussian blue and PAS)

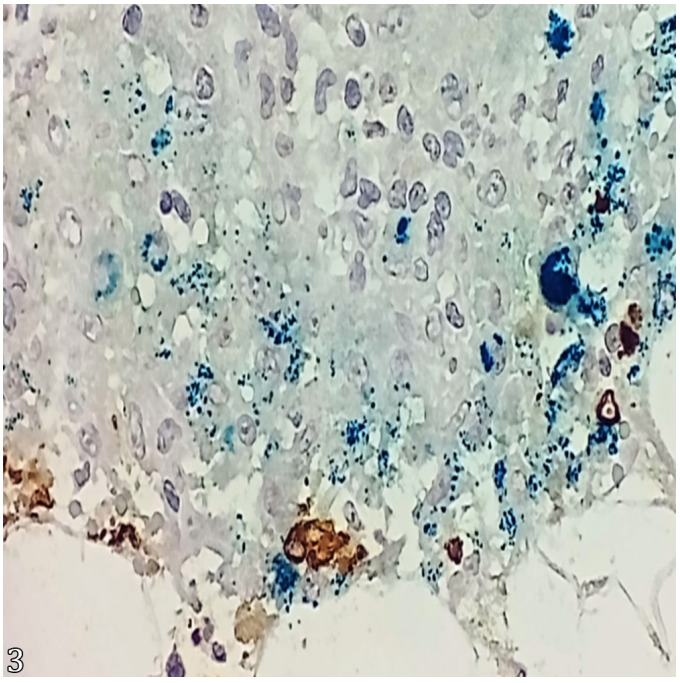

Fig.3. Lymph node of canine with mammary carcinoma subtype tubulopapillary. Isolated tumor cells are intensely immunostained for cytokeratin (AE1/AE3) and located in subcapsular sinuses. A high amount of hemosiderin is also observed in the subcapsular sinuses, and it is strongly stained in blue, allowing easily differentiated neoplastic cells from hemosiderin to be found in the same slide. Polymer method-HRP (IHC) combined with Perls' Prussian blue, obj.40x.

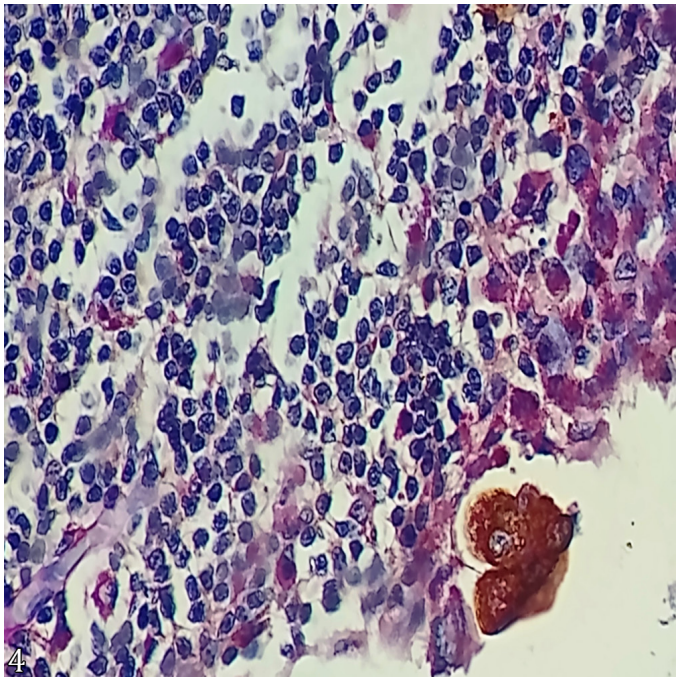

Fig.4. Lymph node of canine with mammary carcinoma subtype tubulopapillary. Isolated tumor cell is intensely immunostained for cytokeratin (AE1/AE3), located in cortical region. A high amount of ceroid is also observed in the cortical region, and it is strongly stained in pink/magenta, allowing easily differentiated neoplastic cells from ceroid to be found in the same slide. Polymer method-HRP (IHC) combined with PAS, obj.40x.

Table 3. Microanatomical location and quantification of ceroid in mammary lymph nodes with isolated tumor cells

		Number of lymph nodes n=28 (%)	Absence of metastasis n= 21 (%)	Isolated tumour cells n=7 (%)
Microanatomical location*	Subcapsular sinuses	0 (0.0)	0 (0.0)	0 (0.0)
	Cortical	-	-	-
	Medullary sinuses	15 (53.6)	12 (80.0)	3 (20.0)
		13 (46.4)	9 (69.2)	4 (30.7)
Amount*	Low	21 (75.0)	16 (76.1)	5 (71.4)
	Moderate	3 (10.7)	2 (66.6)	1 (14.3)
	High	4(14.3)	3 (75.0)	1 (14.3)

* Analyzed in slides of immunohistochemistry combined with periodic acid-Schiff (PAS).

increased by 24.1% the detection of ITCs in canine mammary lymph nodes previously considered negative for metastasis. In veterinary research focused on the presence of ITCs, the detection was increased by 35.2% (Coleto et al. 2018), and for studies focused on micrometastasis, the detection was increased by 6.8% (Coelho 2014) and 9.2% (Matos et al. 2006).

In human studies, ITCs were identified in 2.1% (Ahmed et al. 2014) and 12.5% (Van Deurzen et al. 2008) and micrometastasis in 5% (Ahmed et al. 2014), and 23% (Van Deurzen et al. 2008) of breast cancer sentinel lymph nodes. The overlook of these occult metastases may be explained by the presence of scarce individual cells, small clusters or hemorrhage in the lymph nodes that could have masked these cells. The other possibility is that cancer cells were not present in the original sections stained with HE and were present in adjacent sections stained with IHC (Matos et al. 2006). However, in the cases analyzed here, the new sections of lymph nodes stained with HE remained apparently free of metastases.

The subcapsular sinuses were the microanatomical location more frequently affected by ITCs in lymph nodes in this study. In another study on dogs, more ITCs were found in the medullary sinuses (Coleto et al. 2018). In humans, an association between microanatomical location and prognostic survival was search, and the presence of tumor cells in capsular lymphatics, subcapsular or intraparenchymal sinuses of sentinel lymph nodes was associated with high incidence of non-sentinel lymph nodes metastases (Ahmed et al. 2014). When restricted to subcapsular sinuses, it had a significant unfavorable effect on overall survival, similar to those with larger metastases (Reed et al. 2004). However, other studies in human breast cancer, found a correlation between subcapsular location and less non-sentinel lymph node metastases (Van Deurzen et al. 2008, Fink et al. 2011), based on the concept that tumor cells follow an order, arriving in subcapsular sinuses, later present subcapsular outgrowth of cancer cells into the parenchyma, and finally extend to medullary sinuses (Van Deurzen et al. 2008).

Most lymph nodes submitted in the analyzed case series were from the inguinal region, as reported by other authors (De Araújo et al. 2015, Coleto et al. 2018). This could be related to the surgical approach for mammary tumors, which is, in most cases, a unilateral chain mastectomy with the removal of the regional inguinal lymph node (Fossum 2018). ITCs were present in 33.4% of lymph nodes excised from cases with tumor vascular invasion. In dogs, lymphovascular invasion (LVI) was demonstrated to be a sensitive (85.6%) and specific (73%) indicator of lymph node metastasis; however, the lymph node of drainage is not always submitted for histopathological analyses. For this reason, LVI was added in the nodal status in a new proposal for histological staging system in mammary carcinomas in dogs (Chocteau et al. 2019). In humans, LVI also is associated with an increased incidence of sentinel lymph node metastases (Mittendorf et al. 2008, Ahmed et al. 2014). Also, the presence of ITCs was particularly associated with LVI; this information is strong evidence that ITCs are true metastases (Mittendorf et al. 2008).

In this study, all lymph nodes in which it was detected isolated tumor cells by IHC were excised from Grade II mammary tumors. It has been reported that Grades II and III tumors invade the lymphatic system and metastasize more frequently in dogs (Rasotto et al. 2012, Coleto et al. 2018). In women, high-grade tumors were associated with an increased incidence of sentinel lymph node metastases (Ahmed et al. 2014). In the present study, it was selected only simple tubulopapillary carcinomas; this approach was made in order to standardize the parameters evaluated (presence of occult ITCs and targeted pigments, hemosiderin and ceroid) and because this histological subtype is the most prevalent in this laboratory routine (Oliveira Filho et al. 2010).

Regarding prognostic value, for dogs, it was found that the presence of ITCs in lymph nodes was associated with lower overall survival (De Araújo et al. 2015). However, in other studies, there were no significant difference in overall survival between female dogs with lymph nodes showing occult ITCs and lymph nodes without ITCs (Coleto et al. 2018), and only metastases greater than 2mm demonstrated to be essential for post-surgical prognosis (Szczubial & Lopuszynski 2011). In women, it was found that the presence of ITCs in sentinel lymph node is associated with an increased risk of distant metastases (Liikanen et al. 2018). In one study, it was reported that 12.5% of metastases (one macrometastase and two micrometastases) in non-sentinel lymph nodes when ITCs were found in the sentinel lymph node; this demonstrated the potential metastatic spread from ITCs and suggested that patients with these cells may benefit for adjuvant systemic treatments (Liikanen et al. 2018). These literature findings reinforce the importance of searching for more sensitive techniques for diagnosing possible metastatic cells in lymph nodes, including attempts to combine approaches, such as those in this study.

In previous case reports in the veterinary field, one case of carcinoma in a blue and gold macaw (Anjos et al. 2012) and two dogs with cutaneous squamous cell carcinoma with mucinous metaplasia (Santos et al. 2021), it was briefly mentioned one type of combined IHC and histochemistry technique, in which IHC for cytokeratin (AE1/AE3), followed by Alcian blue, were performed in order to allow visualization of neoplastic epithelial cells (immunomarked with cytokeratin) and ventricular secretory products (koilin) or mucin, respectively, stained with Alcian blue in the same slide of IHC.

In this study, brown pigments were observed in the histiocytes of mammary drainage lymph nodes. These pigments were hemosiderin and ceroid. Hemosiderin is a golden brown pigment derived from hemoglobin during hemolysis (Wang et al. 2010). Its presence in mammary lymph nodes may be associated with erythrophagocytosis secondary to trauma during the biopsy of mammary nodules or to previous trauma to the mammary tissue (Listinsky 1988). Ceroid is a granular opaque dark brown pigment, a complex lipid compound resulting from hyperoxidation of unsaturated fatty acids (Nakayama et al. 1993). It was possible to observe that hemosiderin (seen in 93.1%), because it is golden brown, resembles more the immunostaining by the DAB (3,3′-diaminobenzidine) chromogen, and the simultaneous use of the Pearls' Prussian blue stain was very useful to distinguish them. Although ceroid was observed in 96.5% of the lymph nodes in low amounts, this pigment is opaque brown, representing less of a challenge than hemosiderin in the interpretation of the IHC results. Despite this, the simultaneous use of IHC and PAS staining helped in doubtful cases.

CONCLUSION

Combined techniques of immunohistochemistry (IHC) for cytokeratin (AE1/AE3) with Perls' Prussian blue or with Periodic acid-Schiff (PAS) can be very helpful in investigating isolated tumor cells (ITCs) in mammary regional lymph nodes containing hemosiderin and ceroid, allowing differentiate true immunomarked cells from these pigments during the IHC evaluation. This finding is especially important in developing countries where DAB is one of the most common chromogens used for IHC, and access to other colored chromogens may be more limited. This study also ratifies the importance of utilizing IHC for cytokeratin (AE1/AE3) to identify ITCs in canine lymph nodes of cases that were not detectable by routine hematoxylin and eosin (HE) evaluation.

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