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ORIGINAL ARTICLE

# Immunostaining of lectin glycoconjugates in primary and metastatic canine mammary neoplasms

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## Abstract

The aim of this study was to investigate glycoconjugate immunostaining of the biotinylated lectins: UEA - *Ulex europaeus* (gorse), PNA - *Arachis hypogaea* (peanut), HPA - *Helix pomatia* (Roman snail), and DBA - *Dolichos biflorus*, in normal mammary glands (control; $n = 7$), mammary neoplasms ($n = 111$), and mammary neoplasm metastasis ($n = 10$). One hundred twenty-eight mammary gland tissue samples were used; 7 samples without history of neoplasms (control). The remaining samples (121) were mammary neoplasms or metastasis. In all cases (UEA, HPA, PNA, and DBA), the binding to normal epithelium was uniform. The immunohistochemical marking was in the membrane and cytoplasm of epithelial cells in most of the cases. Large variations of binding between cell membranes, cytoplasm, and nuclei occurred in the neoplastic and metastatic tissues. Though lacking statistical differences, UEA, PNA, HPA, and DBA lectins revealed different marking patterns between tissues, being metastatic foci less marked in comparison with normal and neoplastic mammary gland tissues. In addition, there were no significant differences in the immunostaining of lectins between tumors where HPA was the most expressed in papillary carcinoma I, PNA in papillary carcinoma II, DBA in carcinosarcoma, and HPA and DBA in mixed carcinoma. It was concluded that the lectins have diagnostic potential and they have been useful for differentiation between normal and neoplastic breast tissue of metastatic foci in mammary tumor in dogs.

**Keywords** Diagnosis of neoplasms · Lectin histochemistry · Tumor · Oncology

## Introduction

Mammary tumors are the most frequent neoplasms in dogs. Approximately 75% of these tumors are malignant, becoming an important problem in veterinary medicine. Metastasis of these tumors preferentially affects the lungs (Cassali et al. 2011). For pathologists, diagnosis of canine mammary neoplasms constitutes a challenge due to the nomenclature and classification of these tumors has not been well defined, changing according the classification adopted (Cassali et al. 2011). The use of immunohistochemical tumor markers aid to predict and prognosticate mammary gland cancer, metastatic

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epithelial cells in sentinel lymph nodes, diagnostic mammary gland lesions, determinate possible origins of metastatic neoplasms, and even study the behavior of tumor stem cells (Magalhães et al. 2017). In this sense, other markers can be included, such as genes related to proliferation, differentiation, cellular invasion, progesterone receptor, and apoptosis (Manuali et al. 2012). Proteins present in some tissues and related to cellular phenomena, mainly recognition and transport of substances, can be also used. Lectins are proteins of non-immune origin that bind hydrophobically and non-covalently to certain carbohydrates. They are considered molecular glyco-code decoders, recognizing sugars responsible for adhesion, migration, and invasion functions and detecting subtle differences between complex carbohydrate structures (Nangia-Makker et al. 2002).

In dogs, studies using lectin immunohistochemistry to identify neoplastic cellular changes have been carried out in different tissues, such as prostate, pancreas, cutaneous endothelia, megakaryocytic lineage cells, lymph nodes, thymus, chondrocytes, spermatogenic cells, adrenal glands, perianal and endometrial endothelium, extracellular connective tissue matrixes, leptomeninges, and renal duct tissue. However, there are few studies in mammary neoplasms (Castagnaro and Canese 1991; Burchell et al. 2001; Cassali et al. 2011).

The aim of this study was to investigate the formation of lectin glycoconjugates of *Ulex europaeus* (UEA), *Arachis hypogaea* (PNA), *Helix pomatia* (HPA), and *Dolichos biflorus* (DBA) in normal mammary glands, mammary neoplasms, and metastases of mammary neoplasms of female canines. The study also aimed to verify the use of these glycoconjugates in the diagnostic of the different canine mammary neoplasm histological types.

## Materials and methods

### Experimental groups

One hundred twenty-eight tissue samples were used: control group—mammary gland without neoplasm of female canines ($n$ = 7/all samples were from the archive of the Department of Veterinary Pathology of the FCAV-UNESP, Jaboticabal/study is retrospective) and 121 mammary neoplasm and metastasis samples, of which 10 were adenoma, 10 mammary carcinoma grade I (solid pattern), 9 mammary carcinoma grade II (solid pattern), 7 mammary carcinoma grade I (papillary pattern), 8 mammary carcinoma grade II (papillary pattern), 7 mammary carcinoma grade III (papillary pattern), 8 mammary carcinoma grade I (tubular pattern), 8 mammary carcinoma grade II (tubular pattern), 10 mammary carcinoma grade III tubular pattern, 9 carcinoma grade I (mixed tumor), 9 carcinoma grade II (mixed tumor), 10 carcinoma grade III (mixed tumor), and 10 metastasis tissues (Table 1). Then, these were embedded in paraffin and processed for histopathology (Castagnaro and Canese 1991).

Neoplasms were classified according to the Mammary Neoplasms Criteria (Cassali et al., 2011). The grade of malignancy was judged from I to III according to Misdorp et al. (1999) associating tubule formations, hyper-chromaticism, mitosis, and nuclear pleomorphism. The experimental groups were composed according to what was described by Misdorp et al. (1999).

### Lectin histochemistry

All samples were fixed in 10% formaldehyde solution, buffered with phosphate solution (pH 7.4), and routinely processed until embedding in paraffin. Serial 4 μm cuts of each sample were performed in microtome (Leica Biosystems, Nussloch, Germany – Jung *autocut* 2055®), stained with hematoxylin and eosin, and then processed for lectin histochemistry. The following biotinylated lectins were used: UEA *Ulex europaeus* (carqueja) (1:200 v/v), PNA *Arachis hypogaea* (peanut) (1:200 v/v), HPA *Helix pomatia* (Roman snail) (1:400 v/v), and DBA *Dolichos biflorus* (Mirim) (1:400 v/v) (Table 2).

Then, tissues were deparaffinized in xylene and rehydrated in alcohol (decreasing dilutions), incubated for 30 min in an 8% hydrogen peroxide solution in absolute methanol to block endogenous peroxidase. Afterwards, heat treatment in a steamer (Philips Walita, Koninklijke, Amsterdam – Walita RI31037®) using a solution of sodium citrate (pH 6.0) was performed. The sections were then treated with nonspecific reaction blocking solution (Dako, Santa Clara, USA – *proteinblockserum-free* - X0909) and the sections covered with biotinylated lectins in optimized dilutions (previously standardized) and incubated at 4 °C for 18 h. Then, samples were incubated for 30 min with streptavidin/peroxidase complex (Dako, Santa Clara, USA – Kit L SAB+ System - K0690) at room temperature.

The reaction was revealed in chromogenic diaminobenzidine (Dako, Santa Clara, USA – DAB - K3466) for 5 min. Samples were washed in distilled water and counterstaining was performed with Harris hematoxylin (1–2 min). In all steps, slides were washed (3×) with phosphate-buffered saline (PBS) at 0.01 M, pH 7.2 for 5 min. Finally, the slides were subjected to an increasing battery of alcohols and xylenes, assembled with Canada balsam, and observed at light microscope (Cassali et al. 2011). As a negative control, the marked lectin solution was replaced by an antibody dilution (Antibody Diluent with Background Reducing Components - S3047) for all histochemical lectin reactions.

**Table 1** Number of samples for normal canine mammary. The canine mammary neoplasms are classified according to histological type, grade, and metastasis

| Group | No. of samples | Histological type |
|---|---|---|
| 1 | 7 | The control group (mammary without neoplasia) |
| 2 | 10 | Adenomas |
| 3 | 10 | Mammary carcinoma solid pattern grade II |
| 4 | 9 | Mammary carcinoma solid pattern grade III |
| 5 | 7 | Mammary carcinoma papillary pattern grade I |
| 6 | 8 | Mammary carcinoma papillary pattern grade II |
| 7 | 7 | Mammary carcinoma papillary pattern grade III |
| 8 | 8 | Mammary carcinoma tubular pattern grade I |
| 9 | 8 | Mammary carcinoma tubular pattern grade II |
| 10 | 10 | Mammary carcinoma tubular pattern grade III |
| 11 | 9 | Carcinoma in mixed tumor grade I |
| 12 | 9 | Carcinoma in mixed tumor grade II |
| 13 | 10 | Carcinoma in mixed tumor grade III |
| 14 | 6 | Carcinosarcomas |
| 15 | 10 | Metastases |

### Evaluation of cellular type, marking distribution, and number of marked cells

Cell evaluations were performed in a binocular light microscope coupled to a digital equipment for photomicrography (Nikon, Tokyo, Japan – Nikon E200). All images were treated with the Automatic Contrast option (removing shadows with the enhancement of the image and mapping of the lighter and darker pixels remaining in the image). Before counting, it was analyzed the cell types marked and the marking distribution (Cassali et al. 2011; Magalhães et al. 2017). To quantify the frequency of marking, four fields were randomly selected per cut and a total of 100 cells were counted between labeled and unlabeled using a 40× objective (Magalhães et al. 2017). Results were expressed as percentage of labeled cells (Cassali et al. 2011).

### Statistical analysis

Comparisons between all of the lectins (UEA, PNA, HPA, and DBA) were performed for each histological type (groups 1 to 15) and between groups. The results were submitted to analysis of variance and comparison of averages was performed using Tukey test ($p < 0.05$) (Snedecor and Cochran 1974).

**Table 2** Biotinylated lectins used in mammary neoplasms, normal breast tissue, and metastases, with their specific binding sites

| Lectins | Binding site | Dilutions | Source |
|---|---|---|---|
| UEA | A-L-fucose | 1:200 | EY lab ref: 28091-7 |
| PNA | Galactosamine- β | 1:100 | EY lab ref: 2882-2 |
| HPA | N-acetylgalactosamine | 1:400 | EY lab ref: 280823-2 |
| DBA | A-N-acetylgalactosamine | 1:400 | Vector ref: 280823-1 |

*UEA*, *Ulex europaeus* (carqueja); *PNA*, *Arachis hypogaea* (peanut); *HPA*, *Helix pomatia* (Roman snail); *DBA*, *Dolichos biflorus* (Mirim); *Dilutions*, Antibody Diluent (S3047); *EY*, EY Experience Lab®; *Vector*, laboratory vector®

## Results

The epithelium of the mammary glands without neoplasms was fairly uniform for all of the lectins used. Marking was observed predominantly in the epithelial cell membranes and in the cytoplasm (Fig. 1a).

Significant variations in the neoplastic tissue occurred in epithelial cell membranes, cytoplasm, nuclei, inflammatory myoepithelial, endothelial cells (Fig. 1b), fibroblasts, and chondrocytes. In the metastatic foci (group 15/Table 1), the marking was predominant in cytoplasm membranes, epithelial cell nuclei, inflammatory cell membranes, and cytoplasm (Fig. 1c).

Adenomas presented greater HPA than PNA lectin marking (Fig. 2a). Solid carcinoma grade II group presented greater DBA lectin marking in comparison with UEA and PNA lectins ($p < 0.05$). In this analysis, it was observed that UEA differed significantly from PNA marking (Fig. 2b). No significant difference was observed between lectins of solid carcinoma grade III.

It was observed that papillary carcinoma grade I group presented greater HPA lectin marking, differing significantly from PNA and UEA marking (Fig. 2c). In grade II papillary carcinomas, it was observed a greater PNA marking, differing significantly from UEA marking (Fig. 2d). In grade III papillary carcinomas, no significant difference was observed.

No significant differences were observed in the grade I tubular carcinomas group. However, in the grade II tubular carcinoma group, it was observed greater PNA and DBA lectin marking, differing significantly from UEA marking (Fig. 2e). In grade III tubular carcinomas, we observed

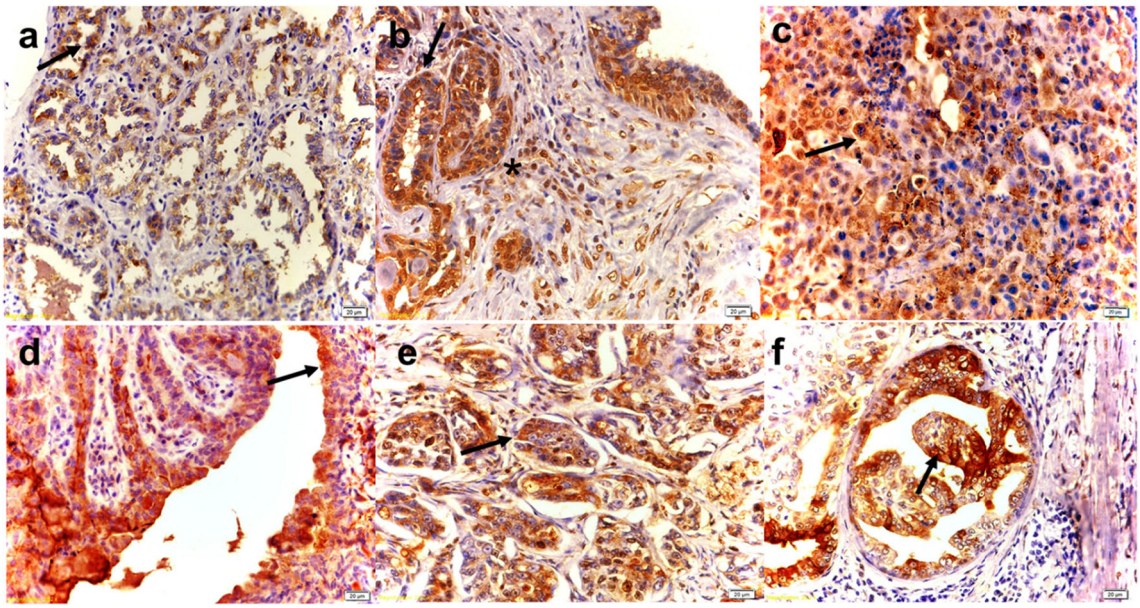


**Fig. 1** Photomicrograph of canine mammary. **a** Non-neoplastic mammary gland in dogs, UEA lectin receptor marking in the epithelial cell cytoplasm (arrow; × 20). **b** Tubular carcinoma grade II in canine female, marking of DBA lectin receptors in the cytoplasm of epithelial cells (arrow), and marking of connective tissue nucleus (star). **c** Canine mammary metastatic neoplasia in lymph node, marking of the UEA lectin receptors in the cytoplasm of epithelial cells (arrow). **d** Carcinoma grade I in mixed tumor, in a feminine canine, receptor marking for the PNA lectins in the cytoplasm of epithelial cells (arrow). **e** Metastatic mammary gland carcinoma in lymph node, markings in HPA lectin receptors in epithelial cell cytoplasm (arrow). **f** Metastatic mammary gland carcinoma in lymph node, markings in HPA lectin receptors in epithelial cell cytoplasm (arrow). DAB chromogen, against Harris's hematoxylin; × 40 objective

greater HPA lectin marking ($p < 0.05$) compared with PNA marking (Fig. 2f).

In the grade I and III mixed tumor carcinoma groups, no significant differences were observed between lectins. However, in the grade II mixed tumor carcinoma group, we observed greater HPA and DBA lectin marking significantly different to PNA marking (Fig. 2g).

The carcinosarcoma groups presented greater DBA lectin marking compared with HPA and PNA marking (Fig. 2h). And in the metastasis group, no significant differences were observed.

In comparative analyses between the histological groups, no statistical differences between any histological types were observed in UEA lectin marking. Few markings were observed using the PNA lectin in the solid carcinoma grade II group, which differed significantly from the groups of papillary carcinoma grade II, tubular carcinomas grades I, II, and III, mixed tumor carcinoma grade I, and the metastasis group (Fig. 1d).

For the HPA lectin, few carcinosarcoma group markings were observed, significantly differing from the other groups: control, solid carcinoma grade II, papillary carcinomas grades I, II, and III, tubular carcinomas grades I and III, and carcinomas in mixed tumors grades I, II, and III (Fig. 1g).

For the DBA lectin, less marking was observed ($p < 0.05$) in the papillary carcinoma grade III group compared with the solid carcinoma groups grades II and III, the papillary carcinoma grade I, tubular carcinomas grades I and II, mixed tumor carcinomas grades I, II and III, carcinosarcoma, and the metastatic group (Fig. 1f).

## Discussion

The lectin receptor immunostaining by lectin immunohistochemistry technique is a useful test to diagnose different types of neoplasms. In the current study, normal tissue (mammary gland without neoplasms) presented no significant difference in the immunostaining of the different lectins. In neoplastic cells, the same lectin binding to **the** membrane, cytoplasm, and nucleus of epithelial cells, inflammatory cells, endothelial cells, fibroblasts, myoepithelial cells, and chondrocytes. This reflects the heterogeneity of the cells, as well as the various grades of differentiation in differing tumors, and within a single tumor. Similar results were found by Augustin et al. (1990) after evaluating the phenotypic endothelial cell characterization with lectin histochemistry.

In both normal and neoplastic tissues, it was observed a pattern of intense and diffusely distributed coloration. Due to the intensity of staining observed, it was opted for a semi-quantitative assessment, through establishing percentages of marked cells. The strengthening of glycoconjugate expression is not necessarily due to elevated biosynthesis, it may also be a reflection of incomplete carbohydrate chain synthesis.

In the metastatic foci, it was observed a smaller amount of marking cells in all the lectins studied, possibly due to

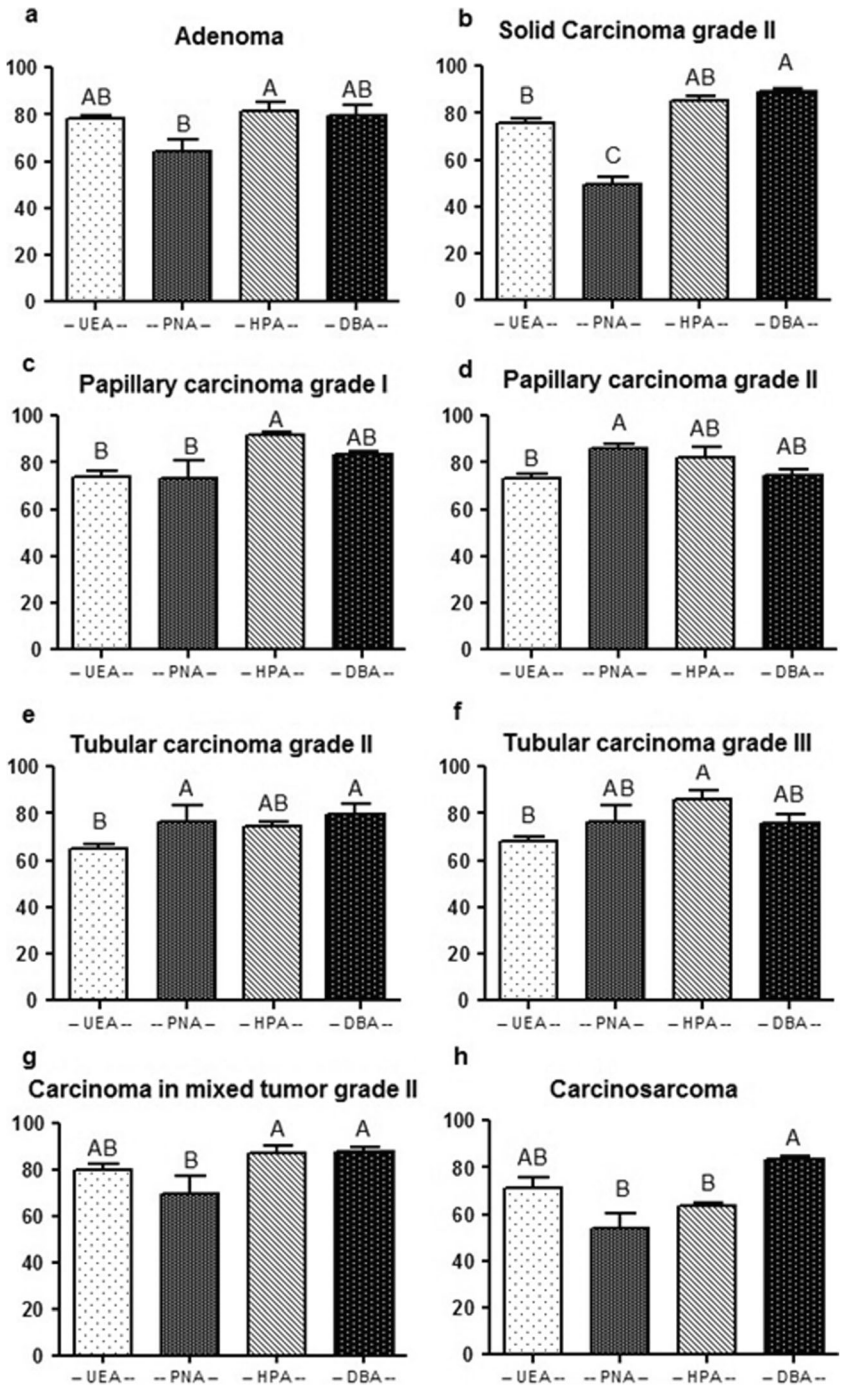


**Fig. 2** Vertical columns represent means of each group in the sampling times. Vertical bars represent the standard deviation of the mean. Columns with letters in common do not differ by the level of 5% by Tukey test by lectin: *Ulex europaeus* (UEA), *Arachis hypogaea* (PNA), *Helix pomatia* (HPA), and *Dolichos biflorus* (DBA) for the groups that presented statistical differences: **a** Adenoma ($n = 10$); **b** solid carcinoma grade II ($n = 10$); **c** papillary carcinoma grade I ($n = 7$); **d** papillary carcinoma grade II ($n = 8$); **e** tubular carcinoma grade II ($n = 8$); **f** tubular carcinoma grade III ($n = 10$); **g** carcinoma in mixed tumor grade II ($n = 9$); **h** carcinosarcoma ($n = 10$)

expression losses in (GalNac) N-acetylgalactosamine (HPA), (α-GalNac) α-N-acetylgalactosamine (DBA), (1-3) GalNa galactose β (PNA), and α fucose (UEA). This may be related to the metastasis process, where the metastatic cells lose adherence to adjacent cells and many of the relations mediated by cell surface glycans and their interactions with carbohydrate-binding proteins (lectins) (Varki 1993; Wells and Hart 2003).

The UEA lectin specifically binds α-L-fucose; however, it is regarded an unspecific marker (Ordofiez and Batsakis 1984). In the current study, no significant difference was observed between the canine mammary tumor types. Castagnaro and Canese (1991) evaluated the correlation between expression of glycoconjugates and squamous cell maturation, using skin from five male dogs with no history of neoplasms, and ninety-five dogs with varied carcinomas observing no UEA marking for any histological type.

In PNA lectin that specifically binds to Gal- β- (1-3 GalNac), it was observed low marking in solid carcinoma grade II group, which differed significantly from the other

histological types. The marking occurred predominantly in the membrane and cytoplasm of epithelial cells. Similar results were found by Castagnaro and Canese (1999), who observed solid epithelial cytoplasmic marking, ranging from moderate to intense in canine mammary carcinomas.

The HPA lectin binds specifically to N-acetylgalactosamine (GalNac), and in the present study, it was observed that it presented less marking in carcinosarcoma group comparing with the other lectins. Burchel et al. (Burchell et al. 2001) observed on the different biological behavior of these cell types and noted that lectin marking characteristics in neoplastic cells, especially in invasive and metastatic strains, were relevant for assessing anaplastic changes. In the DBA lectin, which binds specifically to α-N- acetylgalactosamine (α-GalNac), it was observed less marking in the papillary carcinoma grade III group, which also differed significantly from the other histological types. Sams et al. (1990), assessing abnormalities in lectin markings for familial polyposis and hereditary non-polyposis neoplasm colorectal in humans, observed a significant reduction for DBA marking in neoplastic tissues. The reduction was assigned to failures in the synthesis of long oligosaccharides, remaining only the shorter chains that do not terminate with α-GalNac.

UEA, DBA, HPA, and PNA lectins revealed that marking patterns differed between non-neoplastic canine mammary tissues and mammary neoplasms. However, no significant differences in marking patterns between differing neoplastic histological types were observed; similar results were found by Castagnaro and Canese (1990), who observed the maintenance of glycosylation in different dog epithelial neoplasms immunostaining by the different lectins tested. Comparing between normal and neoplastic mammary gland tissues, there was less marking in the metastatic foci. Among the tumor types, there was a significant difference in the immunostaining of lectins. HPA was the most expressed in papillary carcinoma I, PNA in papillary carcinoma II, DBA in carcinosarcoma, and HPA and DBA in mixed carcinoma.

In dogs, studies using lectin histochemistry to identify neoplastic cell changes have already been carried out in several tissues (Skutelsky et al. 1987; Uchida et al. 2001; Kobayashi et al. 2008). Few studies, however, have been carried out with breast neoplasms (Ejuri et al. 1999) and apparently none so far that compared primary neoplasms and metastases.

Lectins can be used to identify cells that are difficult to differentiate with conventional techniques. In some cases, they replace more complex and costly procedures. They can be used in frozen cuts and samples fixed in formaldehyde and included in paraffin, which allow to perform retrospective studies. They have a higher level of specificity than other coloring techniques used to identify carbohydrates, such as periodic acid Schiff and Alcian blue. It was concluded that the lectins tested have diagnostic potential. They are useful for differentiation between normal and neoplastic breast tissue of metastatic foci in mammary tumor in dogs.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** All samples were included in paraffin and were selected from the archive of the Department of Veterinary Pathology (FCAV-UNESP, Jaboticabal-SP, Brazil), because of this, there is no certificate number for approval of the Ethics Committee.

**Disclaimer** The authors are the only ones responsible for the content and writing of the paper.

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