Braz J Vet Pathol, 2026, vol. 19, e019003
DOI: https://doi.org/10.24070/bjvp.1983-0246.019003

BJVP
Brazilian Journal of Veterinary Pathology
ISSN 1983-0246

Original Full Paper

Fine-needle aspiration cytology and cell block technique for grading canine mammary tumors: diagnostic feasibility and prognostic utility

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Submitted: September 25th, 2025. Accepted: December 22nd, 2025.

Abstract
Fine-needle aspiration cytology (FNAC) is an essential tool for evaluating canine mammary tumors (CMTs), yet its accuracy for grading requires validation. This study aimed to evaluate the diagnostic accuracy, malignancy grading, and architectural patterns using FNAC and agarose cell block (CBA) compared to histopathology in 30 CMTs obtained from surgical specimens. Additionally, the correlation between cytological grading and sentinel lymph node metastasis was investigated. Diagnostic efficacy for malignancy was 90% for FNAC and 97% for CBA. Regarding malignancy grading, concordance with histopathology was 65% for FNAC and 95% for CBA. Moreover, CBA allowed for morphological classification, showing moderate agreement (60%; k=0.50) with histopathological subtypes. A significant positive correlation (p=0.016) was observed between FNAC malignancy grade and inguinal lymph node metastasis. In conclusion, CBA proves to be a promising tool for tumor grading and architectural assessment, while the proposed cytological grading system serves as a feasible prognostic indicator for metastatic risk, highlighting the need for future studies to validate these findings in clinical practice.

Keywords: agarose cell block, canine mammary tumors, cytopathology, fine-needle aspiration, grading, veterinary oncology.

Introduction

Canine mammary tumors (CMTs) are the most common neoplasms in intact and older female dogs (26), and patient survival is strongly correlated with the timeliness and quality of the diagnosis, which has evolved steadily to provide greater specificity in tumor type classification (7, 16, 26). Although histopathology remains the gold standard for diagnosing CMTs (13), fine-needle aspiration cytology (FNAC) has gained prominence. This technique involves aspirating cells from an anatomical site, providing a representative sample of the tumor mass with minimal invasiveness and low cost (1, 3, 17). Consequently, cytology has become an essential tool for the initial evaluation of CMTs (3), distinguishing between malignant and benign processes and aiding in malignancy grading, which plays a significant role in treatment planning and prognosis (11, 17).

To enhance diagnostic capabilities, the agarose cell block (CBA) technique has been implemented (28). This method offers advantages such as increased cellular concentration in low-cellularity samples, improved evaluation of architectural patterns, and better preservation of nuclear and cytoplasmic details (28, 29). In human medicine, cell blocks are routinely used alongside FNAC for the assessment of breast tumors (18).

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Regarding prognosis, malignant mammary tumors most commonly metastasize via the lymphatic system (23). The lymph nodes draining the mammary glands function as sentinel nodes and are the first indicators of metastatic spread (23). Identifying neoplastic involvement in these nodes is crucial for tumor staging and therapeutic planning (23). However, a direct correlation between cytological findings and the metastatic potential of tumors has yet to be fully established. Given the clinical and epidemiological similarities between CMTs and human breast cancer, research on these diagnostic methodologies and prognostic factors contributes significantly to comparative oncology (7).

Therefore, the aim of this study was to evaluate the diagnostic accuracy and malignancy grading of CMTs using FNAC and CBA compared to histopathology. Additionally, this study aimed to assess the architectural patterns identified by CBA and to investigate the correlation between cytological malignancy grades and the presence of metastases in sentinel lymph nodes.

Material and Methods

Samples

The procedures and methods used in this project were submitted for evaluation and approved by the Ethics Committee on Animal Use (CEUA, FOA - UNESP, Araçatuba, SP, Brazil, protocol No. 512/2023).

In this prospective study, conducted over a three-month period, samples were initially collected from 42 mammary tumors obtained from unilateral mastectomies performed at the Veterinary Hospital Luiz Quintiliano de Oliveira of the Faculty of Veterinary Medicine of Araçatuba (FMVA – UNESP). All specimens were sent for histopathological analysis at the Veterinary Pathology Sector (SPV) of the institution.

To ensure diagnostic reliability, strict exclusion criteria were applied. Samples were excluded if they were deemed unsuitable for cytological or CBA analysis due to low cellularity (scarcity of content), extensive necrosis, severe blood contamination (hemodilution), or if paired FNAC and CBA collection was not possible. Consequently, a total of 30 tumors met the inclusion criteria and were selected for the final analysis.

These 30 tumors were identified in 26 mammary chains (two chains had two tumors each, and one chain presented three tumors). In 21 of the mammary chains, the inguinal lymph node (ILN) was present, collected, and sent for histopathological processing. All samples were properly identified to allow correlation between cytology, CBA, and histopathological analyses

Fine Needle Aspiration Cytology (FNAC)

At the SPV, after macroscopic evaluation of the mammary chains, FNAC of the tumors was performed using 22 to 24-gauge needles attached to 10 mL syringes. The syringe plunger was retracted to create a negative pressure of 6 to 8 mL while the needle was moved in a 'fan-like' motion through different areas of the tumors. Soft regions suggestive of necrosis or ulcerated areas were not aspirated.

From each tumor's FNAC, 3 to 5 smears were prepared using the squash technique (slide-over-slide). The smears were air-dried and immediately stained with a Romanowsky-type quick stain (Panótico Rápido®, Laborclin, Brazil). For microscopic analysis, the smear with the highest cellularity and the least blood and necrosis contamination was selected, as recommended by Layfield (18).

There is a limited number of publications in the literature regarding the most appropriate method for cytological analysis of mammary tumor smears, and there is no consensus among authors. Pierini et al. (22) and Layfield (18) suggest that cytological evaluation should be performed by analyzing 10 fields at 40× magnification. Therefore, we established our own cytological evaluation protocol, based on these authors, for smear assessment. Each smear was divided into three areas: two peripheral regions (A and C) and one central region (B). Each area was evaluated in three fields (peripheral and central), first at 10× magnification and then at 40× magnification, following a vertical scanning direction. Additionally, a random field evaluation was performed, completing a total of 10 fields for diagnostic conclusion (Figure 1).

The diagnostic classification in cytology was based on benign or malignant processes. For malignant cases, a grade was assigned based on a score obtained following the criteria proposed by Kuppusamy et al. (17) (Table 1).

The smears were diagnosed and graded by two experienced veterinary pathologists at different times, and in cases where there was disagreement between the evaluators' analyses, a joint reassessment was performed.

Agarose Cell Block (CBA)

In two syringes containing FNAC samples from each tumor, 90% ethanol was added for fixation over a 24-hour period, during which the samples were kept refrigerated (4°C). After this step, the material was transferred to 10 mL Falcon tubes and centrifuged in an analog centrifuge at 1500 rpm for 3 minutes (29). The supernatant was discarded, and 3% liquid agarose (at approximately 45-50°C) was added to the sediment at the bottom of the tube.

The material was then centrifuged again at 1500 rpm for 3 minutes to form a compact pellet. At the end of the process, the solid pellet (Figure 2) was removed, sectioned into transverse slices, and placed in a cassette. The samples were then fixed in 10% neutral buffered formalin for 24 hours, followed by routine paraffin-embedding and histological processing. Sections of 3 µm thickness were cut and stained with Hematoxylin and Eosin (H&E).

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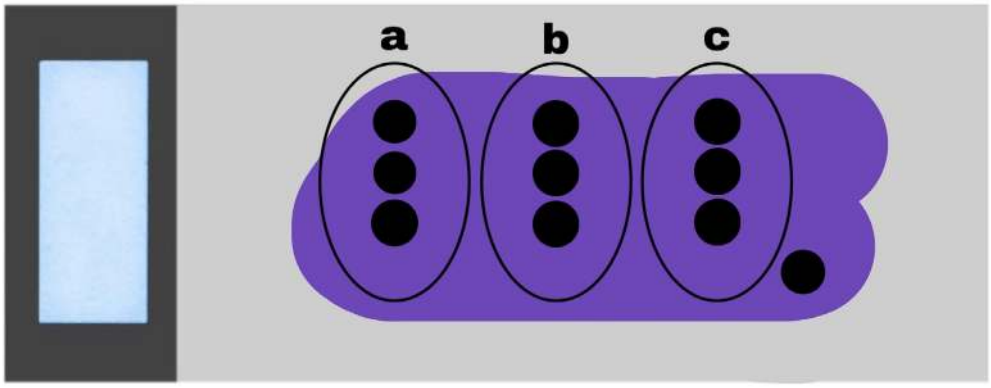

Figure 1. Protocol for evaluating TMCs smears. The cytological sample was divided into three areas: two peripheral areas (a and c) and one central area (b). A random field evaluation (black circle) was performed completing 10 evaluation fields.

Table 1. Score card for grading canine mammary tumors adapted from Kappusamy et al. (17).

Cytological features	Score 1	Score 2	Score 3
		10×	
Cell dissociation	Mostly in clusters	Clusters and single cells	Single cells
Syncytia Formation	1-2	2-4	More than 5
Necrosis	Mild	Moderate	Marked
Tubule formation	Marked	Moderate	Mild/absent
		40×	
Cellularity	10-20 cells	20-50 cells	>50 cells
Cell size	1-2x red cell size	3-4x red cell size	>5x red cell size
Cell uniformity	Mild pleomorphism	Moderate pleomorphism	Marked pleomorphism
Nuclear margin	Smooth	Irreguar	Budding/clefts
Nuclear size	<3x red cell size	3-5x red cell size	>5x red cell size
Nuclear pleomorphism	Absent	Mild to moderate	Marked
Chromatin	Fine	Moderate granular	Coarse
Nucleoli	Indistinct	Noticeable	Prominent
Mitotic count	Absent	1-2	More than 3
Naked tumor nuclei	<3x red cell size	3-5x red cell size	>5x red cell size
Inflammatory cells	<4 cells	5-10 cells	>10 cells

A total score of 15 was considered benign. Grade 1: score 16 – 25; Grade 2: score 26 – 35; Grade 3: score 36 – 45.

The CBA slides were classified as either benign or malignant processes, and for malignant cases, histological grading was assigned according to the system proposed by Elston & Ellis (12) (Table 2). Additionally, the proliferation of epithelial or mesenchymal components was identified either in isolation or in association; the latter was classified as mixed tumors. The morphological pattern of the epithelial component was also analyzed and, when possible, classified as tubular, papillary, or solid (4, 6, 19).

Histopathology

The macroscopic analysis involved visual inspection and palpation of all mammary glands present in the surgical specimen for the identification and description of tumor formations. After performing FNAC for the preparation of cytopathology and CBA samples, 1.5 cm × 1.5 cm fragments were collected from the neoplastic lesion and from the tumor border with healthy tissue. The number of fragments collected was proportional to tumor size: one fragment for tumors up to 3 cm, three to five fragments for tumors between 3 and 5 cm, and a minimum of five fragments for tumors larger than 5 cm, for histopathological analysis.

Additionally, an effort was made to identify the presence of the ILN by dissecting the fibroadipose tissue surrounding the inguinal mammary gland. When present, the lymph node was separated from the chain. ILN fragments were fixed in 10% buffered formalin for 48 hours. After this period, tissue sectioning was performed, and histopathological slides were prepared and stained with hematoxylin and eosin (H&E).

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The histopathological diagnosis and malignancy grading of the mammary tumors, as well as the evaluation of the ILN, were also based on the classification by Goldschmidt et al. (13).

Statistical analysis

For statistical analysis, the diagnostic and malignancy grading results from FNAC and CBA were compared with the diagnosis and malignancy grading from histopathology, the latter considered the gold standard test. The correlation between the diagnostic and grading methods was determined after assessing the normal distribution of the results using the Shapiro-Wilk test, followed by application of Cohen's Kappa test (k) as follows: values <0 suggest no concordance; values between 0-0.20 suggest a slight concordance; 0.21-0.40 suggest reasonable concordance; 0.41-0.60 moderate concordance; 0.61-0.80 substantial concordance; 0.81-1 nearly perfect concordance. The correlation between FNAC grading and metastatic involvement of the ILN was evaluated using Pearson's correlation coefficient (r). The significance level was set at $p<0.05$. Analyses were performed using Jamovi software, version 2.3 (The Jamovi Project, 2023).

Results

Epidemiological data

Regarding the breed of dogs with CMTs included in this study, mixed-breed dogs were the majority, represented by 15 (57.7%) animals, followed by 4 (15.4%) Shih Tzus and 2 (7.7%) Poodles. The Boxer, Labrador, Fox Paulistinha, Golden Retriever, and Yorkshire Terrier breeds were each represented by only 1 (3.8%). The age range of the dogs with CMTs varied from 7 to 16 years, with a mean age of 10.4 years.

The mammary chain in female dogs is divided into the cranial thoracic gland (M1), caudal thoracic gland (M2), cranial abdominal gland (M3), caudal abdominal gland (M4), and inguinal gland (M5). In our study, the most frequently affected gland by CMTs was M5 (53%), followed by M4 (33%), while M3 (7%) and M2 (7%) were the least affected.

Histopathological evaluation

For the evaluation and grading of CMTs, histopathology was considered the gold standard diagnostic method. Among the 30 mammary tumors, 2 (6.7%) were benign neoplasms, 27 (90%) were malignant neoplasms, and only 1 (3.3%) was a non-neoplastic lesion. The benign tumors were classified as benign adenomyoepithelioma and benign mixed tumor. The non-neoplastic lesion was characterized as a fibrocystic process. Among the malignant tumors, the most prevalent type was carcinoma in a mixed tumor, with 14 samples, followed by tubular carcinoma with 7 samples, 3 papillary carcinomas (2 invasive papillary carcinomas and 1 solid papillary carcinoma), and 1 basaloid carcinoma. In the other 2 samples, the diagnosis was fibrosarcoma and carcinosarcoma. Regarding the malignancy grade assigned in the

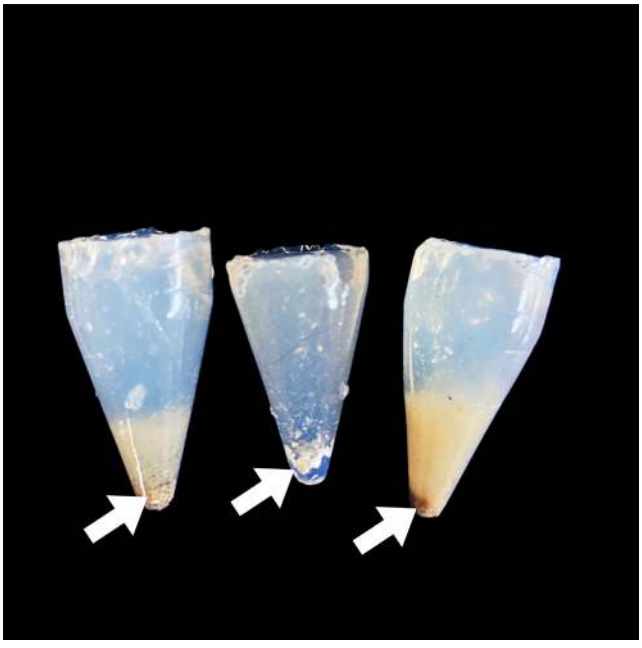

Figure 2. CBA technique. Solid pellet with concentrated vacuumed contents (arrow) ready for sectioning.

Table 2. Score card for grading canine mammary tumors from Elston and Ellis (12).

Feature	Score
Tubule formation	
Most tumors (>75%)	1
Moderate degree (10-75%)	2
Little or none (<10%)	3
Nuclear pleomorphism	
Small	1
Moderate increase in size and variability	2
Marked variation	3
Mitotic counts	
0-7	1
8-16	2
>17	3
Grade	
Total score 3 – 5	1
Total score 6 – 7	2
Total score 8 – 9	3

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histopathological evaluation, 6 samples were classified as Grade 1, 6 samples as Grade 2, and 8 as Grade 3. The tumors classified as benign neoplasms, the sarcomas (carcinosarcoma and fibrosarcoma), and 5 carcinomas in a mixed tumor that did not contain at least 10 foci of basement membrane invasion (4, 21) were not graded.

Cytological evaluation

Based on the cellular and nuclear criteria observed at low (10x) and high (40x) magnification, the samples were graded as Grade 1 (Figure 3A), Grade 2 (Figure 3B), and Grade 3 (Figure 3C). In the cytopathological evaluation, all 30 samples were diagnosed as malignant neoplasms. Regarding the malignancy grade, 17 samples were classified as Grade 2, 9 samples as Grade 1, and only 4 samples as Grade 3.

The concordance between benign and malignant diagnoses in the 30 samples, comparing cytological and histopathological evaluations, was 90% (k=0.0 due to the total number of malignant diagnoses in cytology). For the correlation between malignancy grading, benign tumors (3 samples), non-graded carcinomas in a mixed tumors (5 samples), and sarcomas (2 samples) was excluded, as the grading system is primarily intended for epithelial neoplastic processes. The concordance between cytological and histopathological malignancy grading of malignant tumors was 65% (k=0.49). The false-positive diagnoses in cytology were classified as Grade 1 malignant. The greatest discrepancy between cytological and histopathological grades occurred in Grade 3 tumors, which were underestimated by the cytological method. The comparison between the diagnoses and malignancy grades reported in the cytological and histopathological reports is presented in Table 3.

CBA evaluation

The samples of CMTs processed using the CBA technique were classified as benign or malignant, and a grade was assigned to the malignant specimens. In the CBA evaluation, out of the 30 samples, 2 were classified as benign neoplasms and 28 as malignant neoplasms. Regarding the malignancy grade (Figure 4), 8 samples were classified as Grade 1, 11 samples as Grade 2, and 7 samples as Grade 3. One sample was classified as a mixed malignant proliferation (with both epithelial and mesenchymal components), and one as a sarcoma. When comparing the CBA method with histopathology in the 30 samples, the concordance between benign and malignant diagnoses was 97% (k=0.78). The concordance between malignancy grading in the CBA and histopathology for the 20 malignant tumors was 95% (k=0.92). Only one sample, diagnosed as carcinoma in mixed tumor by histopathology, was classified as Grade 3 malignancy, while in the CBA it was graded as Grade 2.

The comparison between the diagnoses and malignancy grades in the CBA and histopathology is presented in Table 4. In the CBA, samples with epithelial cell proliferation forming well-defined and diffuse patterns across the slide were classified as tubular, papillary, solid, mixed (when both epithelial and mesenchymal proliferation were present), or sarcoma. The histopathological diagnoses (histotype) were compared to the cellular patterns identified in the CBA (Table 5). Between the methods used to classify tumor architecture, a moderate agreement was observed (60%; k=0.50). The results of the evaluation using different methods demonstrated that the CBA technique allowed for grading

Table 3. Benign tumors (BG) and grading of malignant tumors (G1, G2 and G3) assigned in cytological (CT) in relation to histopathological (HP) diagnoses in 23 samples.

CT	HP				Total
	BG	G1	G2	G3	
BG	0	0	0	0	0
G1	3	5	1	0	9
G2	0	1	5	5	11
G3	0	0	0	3	3
Total	3	6	6	8	23

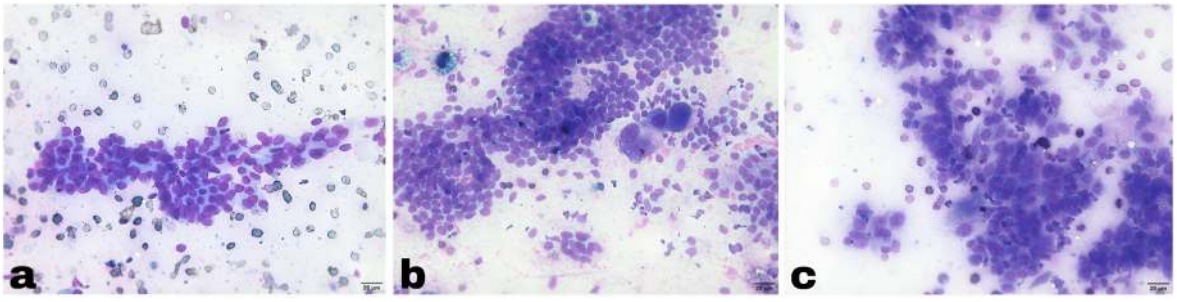

Figure 3. Photomicrograph of cytology of TMCs. (a). Grade 1 malignancy presenting small cells (<2 red blood cells) with mild cellular pleomorphism, fine chromatin, and absence of nucleoli. Diff-Quick. (b). Grade 2 malignancy presenting medium-sized cells with moderate pleomorphism, sometimes evident nucleoli, and irregular margins. Diff-Quick. (c). Grade 3 malignancy presenting cells with marked cellular pleomorphism, evident nucleoli, and irregular chromatin. Diff-Quick.

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the malignancy of the CMTs and successfully identifying tissue morphology, which is limited in conventional smear cytology (Figure 5).

ILN evaluation

Of the 26 mammary chains received at the SPV, 21 included the inguinal lymph node (ILN). In 5 (23.8%) ILNs the presence of neoplastic cells was observed in the subcapsular sinus. Regarding the location of the mammary gland with nodal metastatic tumor, 2 (9.5%) ILNs belonged to the mammary chain with involvement of M4, and 3 (14.3%) ILNs were from the chain with M5 involvement, with one of these lymph nodes coming from a chain where both M4 and M5 were affected.

After grading the malignancy of the CMTs according to the Kuppusamy et al. (17) cytological grading system, we observed that 4 of these nodal metastases came from Grade 2 tumors and 1 from Grade 3. No ILN metastasis was found in any sample classified as Grade 1. While in the histopathological diagnosis, 1 was Grade 2 and 4 was Grade 3.

To assess the relationship between cytological malignancy and metastatic potential, a Pearson correlation analysis was performed. The variables analyzed were the total cytological grade assigned to each tumor (ranging from 1 to 3) and the status of metastasis in the ILN (coded as 1 = absent; 2 = present). This analysis was restricted to caudal mammary glands (M4 and M5), as these primarily drain to the ILN (23). The results demonstrated a significant positive correlation ($r = 0.494$; $p = 0.016$), indicating that higher cytological grade are associated with the presence of nodal metastasis.

Discussion

In our study, mixed-breed dogs, ranging from middle-aged to elderly, were the most affected by CMTs, consistent with epidemiological patterns widely reported in the literature (10, 24, 26). It is well established that the

Table 4. Benign tumors (BG) and grading of malignant tumors (G1, G2 and G3) assigned in agarose cell block (CBA) in relation to histopathological (HP) diagnoses in 23 samples.

CBA	HP				Total
	BG	G1	G2	G3	
BG	2	0	0	0	2
G1	1	6	0	0	7
G2	0	0	6	1	7
G3	0	0	0	7	7
Total	3	6	6	8	23

Table 5. Tumor type in histopathological diagnoses and the architecture presented in the CBA of each sample of the 30 CMTs.

Tumor type	CBA diagnoses
Benign mixed tumor	Benign epithelial tumor
Benign adenomyoepithelioma	Benign mixed tumor
Fibrocystic process	Tubular carcinoma
Carcinoma in a mixed tumor (14 samples)	Carcinoma in a mixed tumor (3 samples)
	Tubular carcinoma (2 samples)
	Solid carcinoma
	Carcinoma (8 samples)
Papillary carcinoma (3 samples)	Papillary carcinoma (3 samples)
Tubular carcinoma (7 samples)	Tubular carcinoma (7 samples)
Basaloid carcinoma	Solid carcinoma
Carcinosarcoma	Carcinosarcoma
Fibrosarcoma	Sarcoma

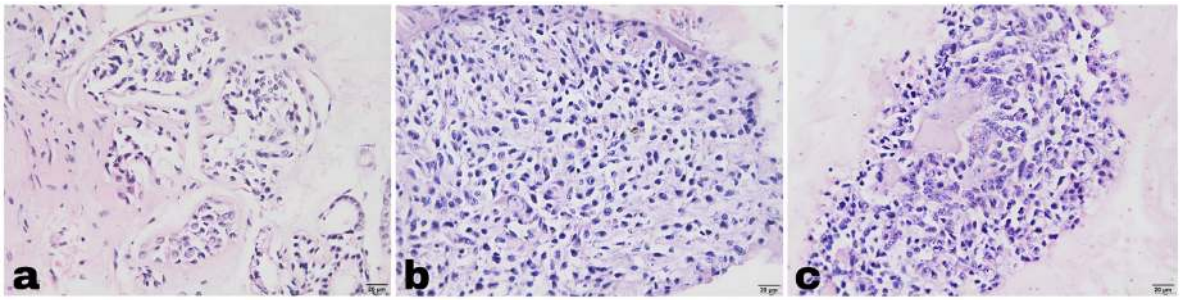

Figure 4. Photomicrograph of CBA of TMCs. (a). Note the marked tubular architecture, uniform nuclei, and absence of mitosis. Hematoxylin and eosin. (b). Grade 2. Discrete tubular formation, nuclei with moderate pleomorphism, and presence of mitotic figures. Hematoxylin and eosin. (c). Grade 3. Absence of tubular formation, marked pleomorphism, and mitotic figures. Hematoxylin and eosin.

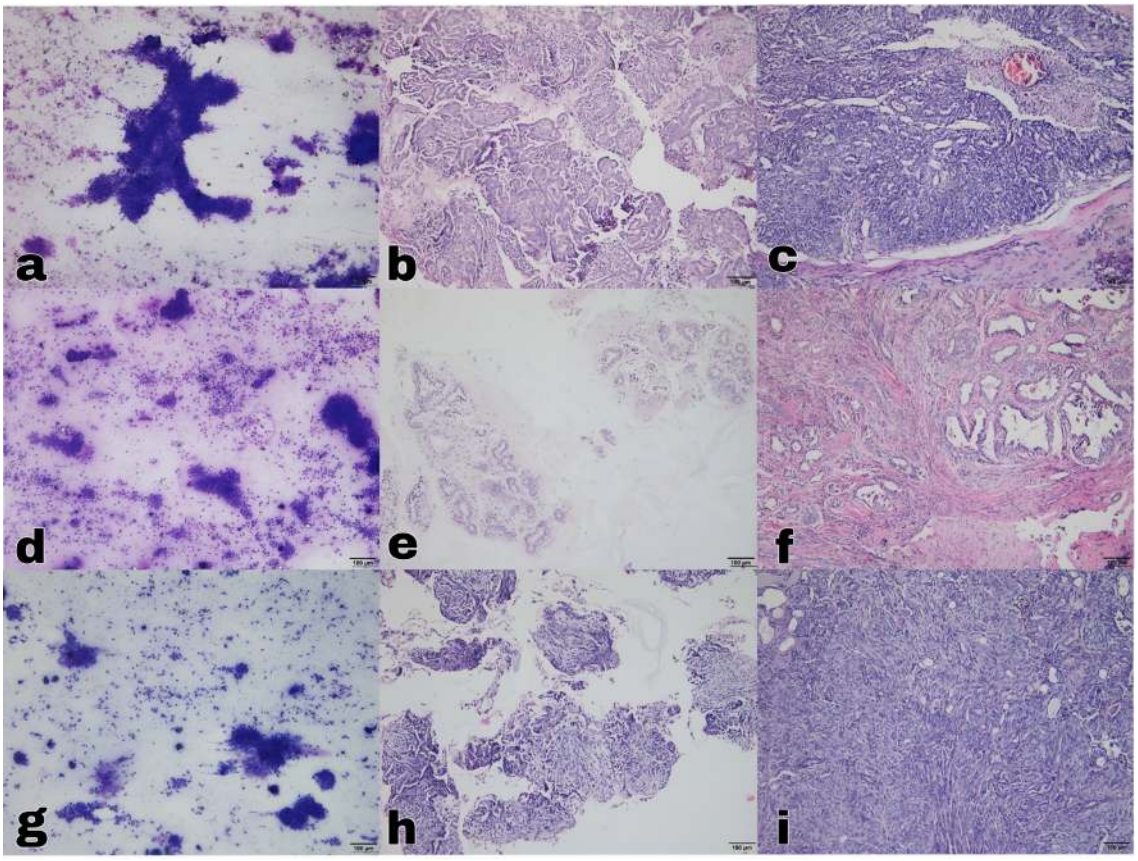

Figure 5. Correlation of FNAC, CBA, and Histopathology Analysis. (a) Photomicrograph of FNAC characterized as a Grade 2 malignant sample. Diff-Quick. (b) Photomicrograph of CBA showing malignant epithelial proliferation in papillary arrangements, the same tumor as in a. Hematoxylin and eosin. (c) Photomicrograph of histopathology of solid papillary carcinoma, Grade 2, the same tumor as in a and b. Hematoxylin and eosin. (d) Photomicrograph of FNAC characterized as a Grade 2 malignant sample. Diff-Quick. (e) Photomicrograph of CBA showing malignant epithelial proliferation in tubular arrangements, the same tumor as in d. Hematoxylin and eosin. (f) Photomicrograph of histopathology of carcinoma in a mixed tumor, Grade 2, the same tumor as in d and e. Hematoxylin and eosin. (g) Photomicrograph of FNAC characterized as a Grade 3 malignant sample. Diff-Quick. (h) Photomicrograph of CBA showing malignant epithelial and mesenchymal proliferation, the same tumor as in g. Hematoxylin and eosin. (i) Photomicrograph of histopathology of carcinosarcoma, the same tumor as in g and h. Hematoxylin and eosin.

occurrence of CMTs increases with life expectancy, as they are predominantly found in middle-aged to older female dog populations and are rare in animals under 5 years of age, with a higher occurrence of benign CMTs in younger dogs (11, 24, 26).

The caudal abdominal (M4) and inguinal (M5) mammary glands are generally the most commonly affected by tumors due to their larger amount of mammary tissue (10, 27). In agreement with these anatomical predispositions, our study observed the highest prevalence of tumor involvement in M4 and M5.

Toríbio et al. (26) evaluated 132 female dogs undergoing mastectomy and found that 90.9% of tumors were malignant, predominantly carcinomas in mixed tumors. Similarly, in the present study, regardless of the diagnostic method used, most CMTs were diagnosed as malignant, and histopathological classification confirmed a predominance of carcinoma in mixed tumors, reinforcing findings reported by other authors (5, 8, 10).

Cuellar et al. (10) observed a 70% concordance between benign and malignant diagnoses assigned by cytology and histopathology in 50 CMTs. Our study demonstrated a superior concordance of 90%. The authors of the cited study emphasize that obtaining clinical information and properly applying the cytological sampling technique directly influence diagnostic quality, which may have been a differentiating factor in our study, although cytology was unable to identify any benign tumor.

Dolka et al. (11), in a pioneering study on cytological grading of CMTs, reported a higher occurrence of Grade 1 and Grade 3 tumors and a lower occurrence of Grade 2 in cytology. They also noted a higher number of false positives among Grade 1 malignant tumors, attributed to nuclear changes in benign CMTs that mimic malignancy (3), a phenomenon also observed

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in our analysis. Indeed, cytology has proven to be a diagnostic method with good sensitivity but presents analytical challenges regarding specificity due to the mimicry of benign cellular populations (11). In our study, the concordance rate between cytological and histopathological grading was 65%, which is slightly lower than that reported by Dolka et al. (11) (72.1%).

Consistent with our results, Taniguchi et al. (25) performed cytological grading of mammary tumors in women and found the highest levels of discordance in Grade 2 and Grade 3 tumors. This is likely due to the high prevalence of high-grade (Grades 2 and 3) carcinomas in mixed tumors in our sample set, which present heterogeneous intratumoral cell populations that, when sampled by FNAC, may result in a non-representative sample (11).

In the cytology of CMTs, the evaluated samples showed moderate to marked cellularity. We prioritized collecting samples representative of the tumor mass, avoiding necrosis and hemorrhage. When FNAC is performed properly, proliferating epithelial cells are easily retrieved, providing high cellularity (15, 18). regarding cytological findings, the observation of multinucleated giant (syncytial) cells was rare, except for one sample classified as Grade 3 in histopathology. This is consistent with Yildirim and Gurel (27), who reported syncytial cells in high-grade tumors, and Bonzanini et al. (2), who identified them in poorly differentiated carcinomas.

CBA samples frequently exhibit tissue architectural characteristics and can help elucidate the relationship between distinct cellular populations (28). In our study, the CBA method proved suitable for identifying cellular patterns. Zanoni et al. (29) reported a diagnostic concordance of 81% between CBA and histopathology in CMTs, whereas our study achieved a higher concordance of 97%. To our knowledge, there are no prior reports applying malignancy grading in CBA for CMTs. We observed a 95% concordance in malignancy grading with CBA, surpassing FNAC. However, CBA analysis does not provide information about tumor invasion areas, which remains a limitation exclusive to the histopathological method.

The superiority of CBA over conventional cytology in preserving architectural features has been well documented (20, 29). Moreover, clinical interest often lies in performing additional tests like immunocytochemistry. In this context, CBA stands out for enabling lifetime sample storage and expanding diagnostic options (14).

Lymphadenectomy combined with mastectomy is recommended for mammary tumors larger than 3 cm or with evidence of malignancy. However, lymph node removal may not occur due to inadequate intraoperative identification (23). This may explain the absence of the ILN in 5 of the 26 mammary chains evaluated in this study. Regarding lymphatic drainage, our findings align with anatomical descriptions (9), where M4 and M5 drain primarily into the ILN.

In malignancy grading by FNAC, a significant positive correlation was found between higher cytological grades and lymphatic invasion. To the best of our knowledge, the present study is the first to relate this grading system to this variable. Therefore, this finding suggests that the evaluated morphological criteria may indicate a likelihood of nodal metastasis. This supports observations by Dolka et al. (11), where higher grades correlated with metastasis and reduced survival in CMTs.

This study has limitations that must be acknowledged. First, the relatively small sample size (n=30) limits the generalization of the findings, characterizing this research as a pilot study. Future investigations with larger cohorts are necessary to validate these results robustly. Second, the samples were collected *ex vivo* (immediately after mastectomy), which eliminates hemodynamic artifacts typically seen in live patients, potentially overestimating the quality of cytological samples compared to clinical routine. Finally, a selection bias exists, as only tumors with surgical indications (mastectomy) were included, which likely contributed to the overrepresentation of malignant cases and high-grade tumors in our sample set.

Conclusion

This study confirms that both Fine-Needle Aspiration Cytology (FNAC) and Agarose Cell Block (CBA) are reliable techniques for the initial diagnosis of canine mammary tumors. However, the CBA technique demonstrates superior performance in malignancy grading and architectural evaluation, achieving high concordance with histopathology. Regarding the proposed cytological grading system, although less accurate in predicting the specific histological grade, it proved to be a valuable prognostic indicator, as high cytological scores were significantly associated with metastatic involvement in sentinel lymph nodes. Therefore, the combined use of these methods refines the preoperative assessment. Future studies with larger sample sizes and *in vivo* validation are encouraged to further consolidate these findings as routine prognostic tools in veterinary oncology.

Conflict of Interest

The authors declare no competing interests.

Acknowledgments

We gratefully acknowledge the scientific initiation scholarship granted by the São Paulo Research Foundation (FAPESP) for supporting this work.

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