Research Article

Gross and Cytological Evaluation of Canine Spontaneous Mammary Neoplasms and Its Correlation with Histopathology and Morphometric Analysis

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ABSTRACT

Incidence of cancer is increasing day by day in canine population. Cytological analysis is a quick, inexpensive and promising diagnostic technique to differentiate the benign and malignant cells. Previous reports have shown very good correlation of its outcome with histopathological findings. Despite this, its potential is still in its infancy in veterinary practice. Considering these facts, we have attempted to enhance the accuracy of the diagnostic outcome with the concomitant application of cytology, histopathology, gross morphology and morphometric analysis in spontaneous mammary gland tumors. In this study, we examined 15 cases of spontaneous canine mammary tumors, wherein 13.3% (2) and 86.6% (13) were found to be benign and malignant tumors, respectively. Amongst these, cytological analysis corroborated very well with histopathological findings in 84.61% (13) and 15.38% (2) incorrect diagnosis. In addition, gross morphology and morphometric analysis of the tumors were also conducted to gain additional insights in the outcome.

Key words: Canine, Cytology, Gross, Histopathology, Mammary gland tumors, Morphometry

INTRODUCTION

Neoplasms in canines are of special interest since their occurrence is prevalent in heterogeneous out bred canine population (Zatloukal *et al.,* 2005). And also they are very similar to human cancers (Douglas and Steven, 2009). Hence Canine is considered as natural spontaneous preclinical animal model for human breast cancer (Rivera and von Euler, 2011). Mammary gland tumor is a common finding in older female dogs and increase as that are not spayed, but occurrence and pathogenesis is depends in variable factors like breed, age, spaying status, number of parturition in dogs (Brodey *et al*., 1983; Kuldip *et al*., 2012) and affect the pet owners as deeply as those of human beings. In addition, the canine females have thrice the incidence of mammary tumor rate as observed for human females (Cullen *et al.*, 2002). In female dogs, the mammary tumors are the second most common tumors after skin tumors (Rezia *et al.,* 2009). Incidence of mammary gland tumor in female dogs represent up to 42% of all the tumors (Misdorp, 2002). Near about 40 to 50 percent of mammary tumors in dogs were found to be malignant (Sorenmo, 2003; Brunelle, 2006). Behavior of mammary tumor is varies with the nature of tumor and

metastasis to regional lymph nodes is decide the fate and prognosis of animals (Kuldip *et al*., 2012). Most of the malignant tumor spread through lymphatic and blood vessels (Marian and Constantin, 2012) this tumor burden and metastasis resulted in anorexia, weight loss, weakness and generally resulted as death in affected animals (Wang *et al*., 2002). Consequently, mortality percentage is increasing day by day. Hence diagnoses of tumors have become extremely important at an early stage. Early and accurate diagnosis plays a vital role to make the treatment successful (Simon *et al.,* 2009). Histology has been principle mode of diagnosis till now but the possibility of complication during surgery and time taken for diagnosis has reduced its importance. Therefore peoples look for the test which can give quick, inexpensive techniques for tumor diagnosis. Cytological diagnosis is important growing filed in veterinary medicine (Sorenmo, 2003; Radmehr *et al*., 2013). It has been utilized in veterinary practice for diagnosis of different lesions in dogs (Masserdotti *et al*., 2005). The on-site evaluation of patients can be useful in ensuring adequacy, and the triage of specimens for secondary studies, if necessary, can also be aided by rapid clinical decision. Morphometric analysis of cytological tumor cell is one of the methods used to

decide the prognosis in human practices for breast cancer. But its application is still infancy in veterinary practices (Simeonov, 2008). In addition to this gross morphology of tumor was described by many researchers (Dolores *et al.*, 2004; Peyman *et al.,* 2013; Radmehr *et al.*, 2013) but its efficacy in diagnosis is neither executed nor compare with cytology and histopathology. Hence, considering all these factors more emphases have been given on cytology, gross morphology and morphometric analysis for diagnosis and further correlation with histopathology. So that in feature we may able to diagnose the nature of tumor for its further treatment and prognosis.

MATERIALS AND METHODS

The current research was performed on 15 cases of spontaneous mammary gland tumors in female dogs accepted for examination and treatment in Surgery and medical OPD of Bai Sarkabai Hospital, Bombay Veterinary College as well as cases from other privet clinics in Mumbai. Details regarding age, sex, breed and location of tumor were documented. The blood samples were collected for hematological analysis using fully automatic CBC analyzer (Abacus, Diatron, Hungary). Differential leukocyte count was conducted by manual method using light microscope (Primo Star, Zeiss, Germany). The tumors were examined for gross morphological appearance, size, shape, cut surface and consistency. Samples for cytological study were collected by fine needle aspiration and fixed in methanol. The staining of cytological smears were performed using Leishman and Wright stain (Vasudevan *et al.*, 2004). The remaining part of the tumor samples were fixed in 10% neutral buffer formalin and processed by routine paraffin embedded block method. Tissue sections (3-5 µm) were stained with H&E stain and histopahtologically evaluated. The mammary gland tumors were cytologically classified as benign or malignant type considering the crucial parameters depicted in Table 1 (Simeonov and Stoikov, 2006).

Morphometric analysis were also performed on the similar tissue slides using light microscope aided with video camera system and computer equipped with the Image Pro express analysis system. The morphometric measurements of the obtained images were conducted considering 25 cells for cytological slides and 50 cells for histopathological slides. Cells exhibiting sharp nuclear and cytoplasmic margin were considered for analysis. Cell nucleus and cytoplasm were contoured by tracing their margins with the aid of a mouse. The following parameters viz area and length of nucleus followed by area and length of cytoplasm in microns were determined using Image Pro express software. The area of cytoplasm was calculated by deducting area of nucleus from area of whole cell. Further, the nucleus to cytoplasm ratio was also calculated.

RESULTS

General observations

In the present study, 15 cases of spontaneous mammary gland tumors bearing female canine were examined. The study revealed 13.3% (2) occurrence of

Table 1: Cytoprognostic parameters

Parameters	Morphology	Benign	Malignant
Cells	Well differentiated cells in cluster	Well differentiated cells in cluster	
	Individual isolated cells		Individual isolated cells
	Anisocytosis		Anisocytosis
	Hypercellularity	Hypercellularity	Hypercellularity
Nuclei	Anisokaryosis		Anisokaryosis
	Macrokaryosis		Macrokaryosis
	Angular nucleoli		Angular nucleoli
	Number of nucleoli	Not more than 2	More than 3
	Mitosis		>6
Cytoplasm	Nucleus to cytoplasm ratio	More than 2	Less than 2
	Basophilia		Basophilia

benign tumor and 86.6% (13) of malignant tumors. The age of the tumor bearing canines ranged from 6 - 18 years with a mean average of 12. Amongst these, the highest incidences of tumor was found to be in the age group of 7-12 years (53.3%) followed by 13-18 years (46.7%), respectively. Least incidence of tumors was found in the group of 1-6 years (6.6%). Considering the anatomical locations, the highest incidences were observed in caudal mammary glands compared to cranial. Out of the total examined cases, nearly 80% (12) of tumor growth were evident in caudal mammary gland, 13.33% (2) in cranial mammary gland and 6.66% (1) were observed at both the locations (Figure1). Pomeranian was found to be the most susceptible breed amongst the clinically examined cases followed by Non-descript canine (Table 2).

Hematological observations

The hematological examination showed that there was significant decrease in hemoglobin count in metastatic tumor bearing canines. However, no statistically significant difference was evident in hemoglobin count for malignant and benign cases. Total leukocyte count was increased significantly in metastatic tumor bearing canines compared to benign and malignant (Table 3). Amongst the differential count profile, an increased number of neutrophil counts were observed in the entire tumor bearing groups. However, statistically significant increment was evident in metastatic tumor bearing canines.

Gross observation of tumors, cytological and histopathological analysis

Cytological and histopathological diagnosis comparison was given in (Table 6). And Correlation between gross morphological, Cytological and histopathological diagnosis in canine mammary tumor was given in (Table 5).

Benign mammary gland tumor

The benign mammary gland tumors were found to be of an average size of 1.6 cm × 4.5 cm. The tumors were white to light brown in color, round to oval in shape and found to be hard in consistency. Multiple cystic cavities filled with slimy amber coloured fluid were observed in the cross section view of the tumor. Cytologically,

Table 2: Summary of Breed wise incidence of tumor

Animal breed	Number of cases	Percent (%)
Pomeranian	8	53.30
German shepherd	1	6.60
Labrador	1	6.60
Non descript	5	33.30
Total	15	100.00

Table 3: Summary of mean hematological values of different growths of dogs. Data is expressed as mean ± SD. Statistical analysis was performed using ANOVA test where the significance level is *P<0.05.

Parameters	Benign	Malignant	Metastatic
Hb	10.05±0.98	10.23±0.62	9.8±0.84
PCV	33.62±2.36	34.46±2.41	31.88±3.10
RBC	5.19±0.38	5.26±0.36	4.94±0.48
TLC	13787.14± 1808.94	14912.00± 2955.57	25944.00± 6283.99
Differential counts			
N	63.00±4.75	74.95±1.37*	80.40±2.28b*
L	30.28±4.03	19.81±1.58*	22.64±3.40*
M	4.42±2.84	3.22±0.46	3.14±0.62
E	1.57±0.41	2.45±0.73	3.28±1.10
Platelets	8.53±4.37	10.73±5.17	10.66±5.78

Table 4: Summary for N/C ratio in cytological and histo-pathological slides

Type of tumor	N/C ratio	
	Cytology Mean±SE	Histopathology Mean±SE
Adenocarcinoma (6 cases)	1.09±0.12	1.05±0.17
Mixed mammary gland tumor (5)	0.81±0.06	0.65±0.04
Cystic adenoma (2)	2.21±0.11	2.32±0.10
Solid duct carcinoma (1)	1.59±0.13	1.61±0.15
Mammary gland Fibrosarcoma (1)	0.93±0.11	1.33±0.05

Table 5: Correlation between Cytological and histopathological diagnosis in canine mammary tumor

Type of tumor	No. of cases	Correct diagnosis	Partially correct	Incorrect diagnosis	Use of gross morphology
Benign					
Cystic adenoma	1	1			
Mixed adenoma	1		1		1
Total	2*	50.00%	50.00%		50.00%
Malignant					
Adenocarcinoma	5	6			
Mixed type	5		5		5
Scirrhous type	1	1			
Duct carcinoma	1			1	
Fibrosarcoma	1			1	
Total	13*	53.84%			38.46%
Total case	15	84.61%	15.38%		

Table: 6: Comparisons of cytological findings with histo-pathological diagnosis

Cytological diagnosis	Histopathological Diagnosis
Adenocarcinoma	Adenocarcinoma
Adenocarcinoma	Mixed Malignant mammary gland tumor
Adenocarcinoma	Mixed cystic Papillary Adenocarcinoma
Inconclusive	Solid duct infiltrating carcinoma
Benign tumor	Benign Cystic Adenoma
Adenocarcinoma	Mixed Adenocarcinoma
Adenocarcinoma	Poorly differentiated scirrhous type of adenocarcinoma
Adenocarcinoma	Mixed cystic Papillary Adenocarcinoma
Adenocarcinoma	Cystic adenocarcinoma
Benign tumor	Benign Mixed cystic adenoma
Adenocarcinoma	Solid Adenocarcinoma
Adenocarcinoma	Mixed cystic Adenocarcinoma
Adenocarcinoma	Solid Adenocarcinoma
Inconclusive	Fibrosarcoma of mammary gland tumor
Adenocarcinoma	Solid Adenocarcinoma

clusters of round, uniform differentiating epithelial cells along with few inflammatory cells were observed. Histopathology of tumor revealed a moderate stroma with cystic dilatation of acini. The acini were filled with acidophilic pink coloured homogenous material. The proliferating acinar cells were also distinctly evident. The tumor was diagnosed as cyst adenoma. Similar cytological features were evident in benign mixed adenoma. Histopathological observation showed cuboidal epithelium of acini with papillary projections towards the lumen of duct. Focal area of myxomatous tissue was seen, suggesting the tumor to be a benign mixed adenoma (Figure 6).

Malignant adenocarcinoma of mammary gland tumor

The average size of adenocarcinoma was found to be in the range of 4.5 cm × 3 cm to 20 cm × 7.5 cm. The tumors were round to oval or multinodular in shape and were of hard consistency. On cut section tissue surface was white colored with few cystic cavities. In two cases distance metastasis leads to focal hemorrhages and ulceration in neighboring gland or subcutaneous area. Cytological features of revealed exfoliation of cells in clusters. Individual epithelial cell contained a round nucleus and a moderate amount of basophilic cytoplasm. Marked anisocytosis and anisokaryosis with multinucleated cells were observed, nucleus to cytoplasm ratio was increased. The cytoplasm contained amorphous basophilic secretory product or vacuoles. On histopathological investigation of growths, simple

adenocarcinomas were composed of malignant epithelial cells. Proliferation of acinar cells showed increased pleomorphism, hyperchromatic nuclei, numerous mitotic figures, stromal invasion and epithelial stratifications in papillary fashion. The lumens were completely or partially occluded with epithelial cells. Fibrous connective tissue divided proliferating cell and formed lobes in one case. In other case, a productive fibrous connective tissue stroma was present, along with papillary projection in duct. Myoepithelial cells proliferation was also observed (Figure 4).

Mixed mammary gland tumor

In mixed malignant mammary gland tumor the average size of tumor was 4.5 cm × 1.5cm.The tumors were round to oval shape and were hard consistency. On cut section diffuse white colored surfaces with few cystic cavities filled with amber colored fluid and white cartilaginous area was seen. Some tumors were hard to cut and white in coloured. Cytological observation showed exfoliation of round to oval shape cells epithelial with eccentric nucleus contained 7-8 nucleoli and basophilic cytoplasm with marked anisocytosis and anisokaryosis. In malignant mixed tumors, histopathology showed coexistence of glandular epithelial components along with heterogeneous stromal elements like myxoid tissue and
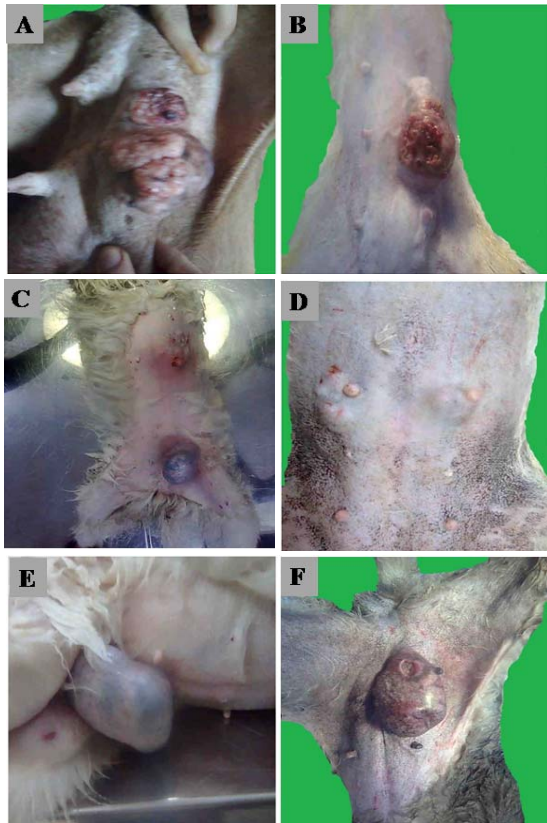
Fig. 1: Photographs of anatomical location of mammary gland tumors: (A) Inguinal (5th); (B) Cranial abdominal (3rd); (C) Inguinal (5th) & Cranial thoracic (1st); (D) Caudal thoracic (2nd); (E) Inguinal (5th); (F) Inguinal (5th).

cartilage. Along with this, cystic dilatation and papillary projection with fibrous connective tissue and myoepithelial cell proliferation was noticed. The myxoid tissues were seen in multifocal area (Figure 2 and 3).

Solid duct carcinoma

One ase of solid infiltrative duct carcinoma was recorded. The size of tumor ranged from 7cm x 3cm and was round shape. Cystic cavities with variable diameter were observed on cut section. The tumor was hard in consistency. Exfoliation of oval, spindle shape cells with moderate basophilic cytoplasm contained 5-6 nucleoli along with fine chromatin. The tumor was not able to diagnose. Histopathologically proliferation of epithelial cells within the duct supported by strong stock fibrous connective tissue was seen. Acinar cells showed malignancy. There was partial or complete occluded lumen with malignant cells and productive fibrous connective tissue (Figure 5).

Scirrhous type of adenocarcinoma:

One case of scirrhous type of adenocarcinoma was observed. The average size of tumor was 7.5cm x 4.6cm. Grossly tumor was oval, nodular in shape & ulcerated. It was hard to cut and form cystic changes with harder consistency because more amount of fibrous connective tissue was present, which impart creamy white coloured to the tissue on cut section. Cytologically cluster of oval to round basophilic cells with marked anisocytosis and
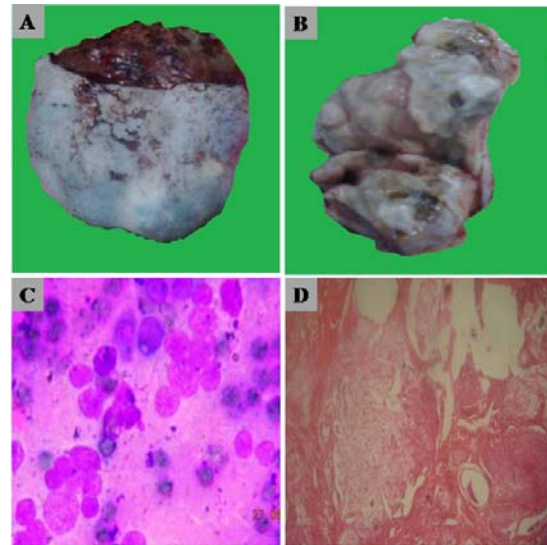
Fig. 2: Photographs of Mixed cystic Adenocarcinoma (A) Gross photograph of round nodular growth; (B) cut section, white gritty tissue with few cystic cavity; (C) round to oval cell showed anisocytosis & binucleated cell; (D) multifocal area of myxomatous tissue with cystic cavities and diffuse proliferation of acinar cell.
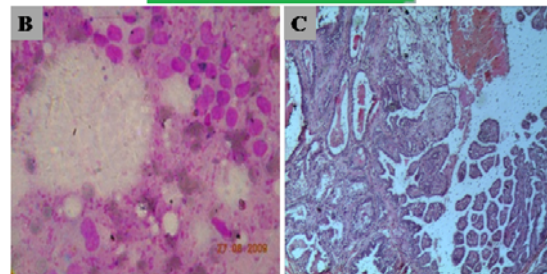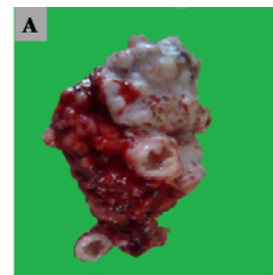

Fig. 3: Photographs of Cystic Papillary Adenocarcinoma (A) Gross photograph of elongated oval multinodular cystic growth; (B) cluster o round binucleated cells; (C) Multiple cystic cavities with finger like papillary projections in the lumen.

anisokaryosis was observed. Histologically multiple cystic cavities filled with pink coloured material. Productive fibrous connective stroma along with proliferation of epithelial cell was observed. Proliferating cells invaded the basement membrane (Figure 7).

Fibrosarcoma of mammary gland

The size of tumor ranged from 4.5cm x 2.5cm and was round shape. On cut section multifocal hemorrhages were seen. The tumor was hard in consistency and pale yellow. Cytologically large number of inflammatory cells mainly neutrophils and RBCs were present. Histopathological
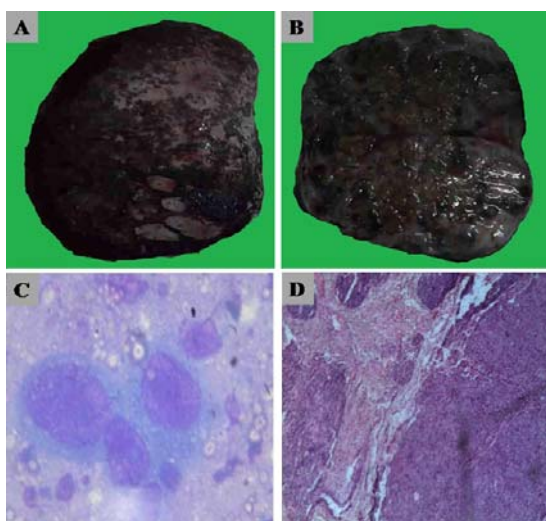
Fig. 4: Photographs of Solid Adenocarcinoma (A) Gross photograph of oval ulcerated growth; (B) Multiple cystic cavities filled with amber coloure jelly like fluid; (C) exfoliated cells with basophilic cytoplasm with anisocytosis and anisokaryosis; (D) Diffuse proliferation of acinar cells with invading the basement membrane.
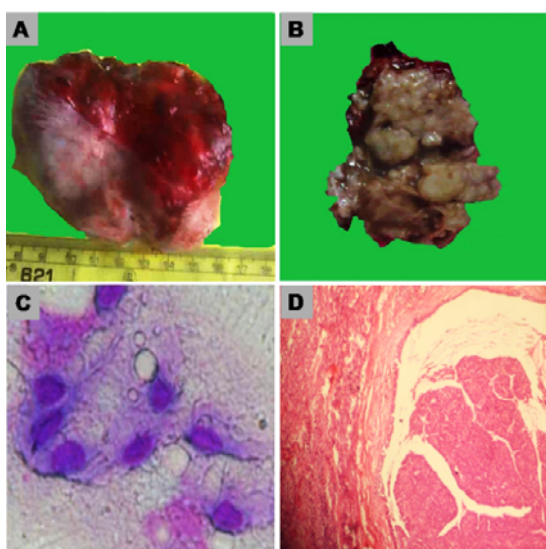

Fig. 5: Photographs of Solid Duct Carcinoma (A) Gross photograph of round to oval growth; (B) Multinodular cystic tumor contained amber coloured fluid; (C) cluster of oval basophilic cells of duct; (D) Proliferation of epithelial cells within the duct supported by strong stock fibrous connective tissue.

observations showed large area of necrosis with infiltration of inflammatory cells and multifocal hemorrhages. Proliferation of fibrous connective tissue showed variable mitotic figures and pleomorphism. The cells were proliferated in different directions and formed whorls.

Morphometric analysis

Nucleus to cytoplasm ration was evaluated in both cytologically and histopahtologically examined tissue (Table 4). The nucleus to cytoplasm ratio was increased in malignant tumor compared to benign tumor.

DISCUSSION

The prevalence of mammary tumor has shown an upsurge in canine population remarkably from last few years. Early diagnosis of cancer plays a pivotal role in reducing the mortality rate of canines. So far, the application of histopathology proved to be the first line of diagnostic tool. However concomitant application of cytological analysis, gross morphological observation and morphometric evaluation along with histopathology could enhance the accuracy and confer deeper insights in diagnosis. In this investigation, 15 cases of spontaneous canine mammary gland tumors were examined for cytology, gross morphological changes, histopathology and morphometric analysis and an attempt was made to correlate their outcome from the diagnosis perspective. Amongst the examined canines, the affected belonged to wide age group of 6-18 years. The highest incidence of tumors was observed in the age group of 7-12 years (53.3%) and 13-18 years (46.7%), respectively. These findings were in agreement with previous reports (Kumar et al., 2011; Radmehr et al., 2013). The rising incidence with age could be attributed to persistent accumulation of somatic mutations over a period of time which could have resulted in the development of cancer (Vegad, 2007). Considering the breed amongst the examined cases, pomerian was found to be the most predisposing breed followed by the non-descript one. It increased in pure breed population this could be attributed to total number of dogs in each breed in an geographic area finding was in concordance with the outcome of Agneta et al., 2005. In the study we revealed that, anatomical location of mammary glands also seems to be an important factor, as the tumors were frequently found at inguinal (5th) mammary gland (80%). Similar finding was also reported by Radmehr et al., 2013, which could be ascribed to greater proliferative changes in inguinal mammary glands in response to estrogen (Kumar et al., 2011). Similar type of results were also observed by Rutteman et al., 2000, which are explained by the fact that, most caudal pairs of glands comprised majority of mammary tissue and are predisposed to mechanical trauma. Health status of the animals accessed hematological profile. There was slight reduction of hemoglobin value in metastatic tumor which indicates development of anemia and could be attributed to decreased erythropoiesis and malignancy through different mechanism (Mellanby, 2004). Significant elevated neutrophil counts indicate the acute inflammatory reaction and possible bacterial infection in the tumor affected animals. Similar finding was observed by Losco, 1986. In contrast to these Dolores et al., 2004 observed inflammatory mammary carcinoma with ulceration in cat and considered it was associated with aggressive behaviour and poor prognosis. Cytological evaluation of cellular and nuclear characteristics details of exfoliated tumor cell were utilized for rapid diagnosis. It helps in identification of origin, type and nature of the cells which facilitates rapid diagnosis of tumor. Fine needle aspiration cytology (FNAC) has been greatly explored for routine diagnosis of breast tumor in humans (Cyrta et al., 2012). However, its application has not yet been well-established in the
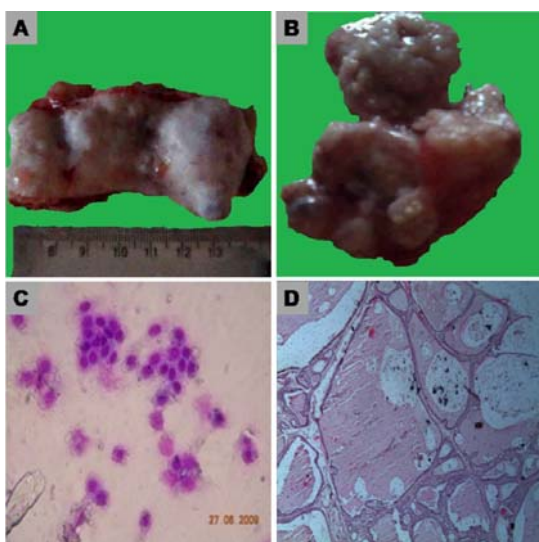
Fig. 6: Photographs of Mixed Cystic Adenoma (A) Gross photograph of oval nodular growth; (B) Nodular cystic tumor with round white hard tissue; (C) cluster of round uniform differentiating epithelia cells; (D)Cystic dilatation of acini, filled with acidophilic pink coloured homogenous material and proliferating acinar cells.
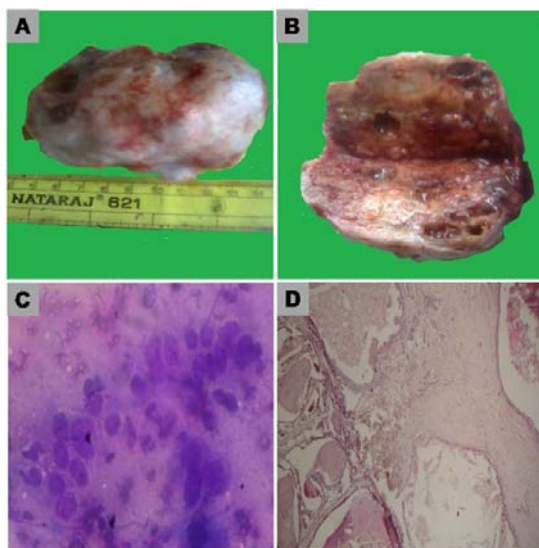

Fig. 7: Photographs of Scirrhous type of adenocarcinoma (A) Oval shape cystic nodular growth; (B) cystic creamy white diffuse tissue with white cartilaginous tissue; (C) cluster of oval to round basophilic cells with anisocytosis and anisokaryosis; (D) cystic osteiod tissue with excessive fibrous connective tissue stroma.

veterinary practice. Mammary gland tumor classified in to three different type's namely epithelial, mixed & mesenchymal cell types cytologically (Alleman and Bain, 2000). In the present study correlation between cytological & histopathological diagnosis for benign tumor was 100% and malignant tumor 84.61% where as incorrect diagnosis was 15.38%. Cytology was not representing the true type of histological type in mixed mammary gland tumor hence gross morphology was utilized to enhance the accuracy of cytological diagnosis (Table 5). In our study, we noticed that cytological

analysis was able to diagnose malignant and benign type of tumors more easily, which is due to easy exfoliation of epithelial cells by FNAC method. Similar explanation was given by Vasudevan *et al*., 2004. However, the diagnosis of mixed-type and mesenchymal tumors were more difficult, as mesenchymal and mixed tissue were difficult to exfoliate. Similar difficulties were also reported by other authors (Zuccari *et al*., 2001). This study clearly indicated that cytological analysis proves to be a promising tool to diagnoses benign and malignant tumor, but incompetent to sub-classify the mammary gland tumors.

Gross morphological observation of the tumors did not help to clearly differentiate between benign and malignant tumors. Nevertheless, it could help to identify mixed type tumors, as they appears lobular or radiating in shape and often consist pathognomonic feature like bone or cartilage which imparts rigid consistency to the tumor. Similar findings were observed in earlier studies (Geovanni *et al., 2012*). Additionally, gross morphological features observed for adenocarcinoma and cystic papillary adenocarcinoma were in line with one reported by Radmehr *et al*., 2013. These features could give probable impression for histopathological type. These observations were further confirmed by conducting histopathological analysis of the same tissue, suggesting cytological and gross morphological outcome are in good agreement with histopathological results.

Morphometric analysis for both cytological and histopathological tissues showed no significant variations in nucleus to cytoplasm ratio, indicating similar morphometric outcome. However, there was an increase in nucleus to cytoplasm ratio in malignant tumors compared to benign. These results are in line with earlier report (Simeonov and Stoikov, 2006).

In case of mixed gland tumors with malignancy, histopathology showed coexistence of glandular epithelial components along with heterogeneous stromal elements like myxoid tissue and cartilage. On morphometry nucleus to cytoplasm ratio of cytology and histopathology was increase. But differentiation from mixed type of tumor was not possible these findings in line with Zuccari *et al*., 2001. This might be due to less exfoliation ability of connective tissue. Therefore we focused on gross morphology of the tumors. Gross morphology of the tumor was useful along with cytology to differentiate mixed type of tumor. Histopathology of tumor confirmed the diagnosis which revealed classical picture of adenoma, adenocarcinoma and mixed tumor.

Conclusion

Cytological diagnosis is useful, quick tool for identification nature of tumor. In addition to this gross morphology provide additional insight for identification of sub classification of mammary tumor. But further confirmation can be achieved by histopathology. Morphometric analysis could help in classification of cell in to benign or malignant nature, this could support the cytological observation. A Combination of three methods could increase to accuracy of diagnosis.

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