



## Cytomorphological, histopathological and immunohistochemical observations on the histiocytic origin of canine transmissible venereal tumour

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### Abstract

The cytogenic origin of canine transmissible venereal tumor (CTVT) still remains unknown. Resulting from paucity of information on the histiocytic phenotypic features and behaviours of CTVT, this study was undertaken to show some uncommon cytomorphological features of CTVT and its immunoreactivity with S-100 protein. Nine cases of CTVT were investigated (6 females and 3 males) using nine fine needle aspirates for cytology and four biopsy samples for histopathology and immunohistochemistry. Cytology revealed CTVT cells with pale basophilic, fine granular cytoplasm and distinct intra-nuclear (3/9) and intra-cytoplasmic (9/9) vacuolations. Erythrophagocytosis by a giant binucleated CTVT cell in one dog and nuclear budding in 2 dogs were observed. Histopathology showed loose sheets and cords of uniformly round to ovoid cells with slight indistinct eosinophilic cytoplasm and a tendency towards glandular as well as syncytial formation. Nuclei were large and round with a single centrally placed nucleolus. The mitotic index was high. Immunohistochemistry of the four biopsy samples revealed negative immunoreactivity to pan-cytokeratin, actin and desmin but positive immunoreactivity with vimentin and S-100 proteins. This is the first report in which CTVT showed erythrophagocytosis, nuclear budding and positive immunoreactivity to S-100 protein. Based on these cytomorphological and immunohistochemical features, we conclude that CTVT is of histiocytic/dendritic origin.

**Keywords:** Cytology, histopathology, Histiocytic origin, immunohistochemistry, Transmissible Venereal Tumour

### Introduction

Canine transmissible venereal tumor (CTVT) is a sexually transmitted disease by physical transplantation rather than infectious means (Goldschmidt & Hendrick, 2002). It is a naturally occurring contagious round cell tumour that affects

both external genitalia and extra-genital sites (Ajayi *et al.*, 2009; Mascarenhas *et al.*, 2014). Previous reports have shown that CTVT arises from allogeneic cellular transplants and abnormal neoplastic cells (Dingli & Nowak, 2006; Murgia *et al.*, 2006). The

disease is most common in tropical and subtropical regions, occurring in free roaming and sexually active dogs (Das & Das, 2000; Mello *et al.*, 2005). There is no age, sex or breed susceptibility and the mucous membrane of the external genitalia of either sex is the predilection site (Das & Das, 2000). Loss of mucosa and skin integrity during coitus, licking of the external genitalia, fighting, biting or other types of contact; sniffing and scratching (Gupta & Sood, 2012) are the means of tumor implantation. Canine transmissible venereal tumor rarely metastasizes and less than 5-17% of metastatic cases have been documented (Gurel *et al.*, 2002; Ajadi *et al.*, 2004; Ajayi *et al.*, 2009) but when metastases occurred, it was usually observed in extra-genital regions (Mascarenhas *et al.*, 2014). The growth rate and metastasis of the tumor depends on the age, sex (mostly occurs in males) and immune status of dogs (Bastan *et al.*, 2008). The initial gross appearance of the tumour is usually a small hyperemic papule which later progresses to nodular, papillary, multilobated, and cauliflower-like pedunculated mass (Eze *et al.*, 2007).

Various studies on the cytomorphological appearance of CTVT have affirmed the usefulness of cytology as a good confirmatory diagnostic tool (Amaral *et al.*, 2007). Based on the morphological characteristics, the tumor has been classified as lymphocytoid (with centrally placed nucleus) and plasmacytoid (with peripherally placed nucleus) (Amaral *et al.*, 2007, Flórez *et al.*, 2012). The plasmacytoid form has been shown to be more aggressive than the lymphocytoid form (Amaral *et al.*, 2007). But the diagnostic usefulness of this cytomorphological classification with regard to the cytogenetic origin of the tumour is unknown.

Previous studies have suggested histiocytic origin of CTVT (Marchal *et al.*, 1997; Mukaratirwa & Gruys, 2004) while other workers have expressed contrary opinions about its origin (Mascarenhas *et al.*, 2014). Transmissible venereal tumor has been shown to be immunopositive to histiocytic markers such as lysozyme, ACM1 and alpha-anti-trypsin (AAT) but negative to cytokeratin, S-100, CD3, neuro-specific enolase and p63 (Mozos *et al.*, 1996). Over the years, S-100 protein and other histiocytic markers have played a significant role in the determination of histiocytic/dendritic tumours in humans (Zeid & Muller, 1993) and in canine histiocytic sarcomas (Thongtharb *et al.*, 2016). Despite positive immunoreactivity of CTVT with other histiocytic tumour markers, its cytogenetic origin and immunoreactivity with S-100 protein still remain to

be established (Mozos *et al.*, 1996). Therefore, this study was undertaken to show some uncommon cytomorphologic features of the CTVT and its immunoreactivity with S-100 protein and possibly correlate these findings with its histiocytic origin.

## Materials and Methods

### *Collection of samples*

Records of gross, cytomorphological and histopathological findings in nine dogs (6 females, 3 males) of different breeds and ages diagnosed with CTVTs at the two Veterinary Teaching Hospitals and Department of Veterinary Pathology, Federal University of Agriculture, Abeokuta and University of Ibadan, Southwestern Nigeria were used in this study. Cytological slides and tissue biopsies submitted between 2012 and 2014 were reviewed. Fine needle aspirates were collected from all nine cases while tissue biopsies were collected from four of the nine cases. All samples were taken before commencement of vincristine therapy. Complete regression of the tumour masses was observed after vincristine sulphate treatment.

### *Cytological evaluations*

Cytological specimens were collected using fine needle aspiration, biopsy and touch imprint. Smears were air-dried, fixed with methanol and stained with Giemsa stain according to the Wright-Giemsa method (Rakich & Latimer, 2011). The cytological preparations were analysed and grouped into three as previously described (Amaral *et al.*, 2007). Briefly, the group with lymphocytoid appearance (LPT) was made up of TVT cells having a 60% lymphocytic appearance while the group with plasmacytoid appearance was made up of TVT cells having 60% plasmacytic appearance (PCT) and a group having mixed cellularity between lymphocytic and plasmacytic appearances (MLP) in which none exceeded 59%.

### *Histopathology and Immunohistochemistry*

The four tumour biopsy samples were fixed in 10% formalin and processed for histopathology. The tissues were dehydrated in graded levels of alcohol, cleared in xylene and embedded in paraffin wax. Paraffin sections (5µm) were cut using a semi-motorized microtome (Leica, RM 2125 Model) and stained with haematoxylin and eosin. Replicate sections of the tissues were stained with toluidine blue to rule out the possibility of mast cell tumor. Diagnosis was made based on the characteristic

cytological and histopathological features of CTVT (Amaral *et al.*, 2007).

For immunohistochemistry studies, the replicate sections were deparaffinized in 4 changes of xylene for 3 minutes each, rehydrated in a graded levels of alcohol, and finally washed with deionised water. Antigens were unmasked with 0.01M sodium citrate buffer (pH 6.0) for 30 minutes at 650W using a microwave oven. This was cooled to room temperature for 20 minutes, followed by 5 minutes of washing in 0.05M Phosphate Buffer Saline-Tween 20 ( TPBS) (pH 7.6). All other steps were performed at room temperature. The sections were incubated in 3% hydrogen peroxide for 15 minutes to inhibit endogenous peroxide. After a brief wash with TPBS, non-specific binding was eliminated by bathing in normal goat serum for 10 minutes. The tissues were then incubated overnight at 4<sup>0</sup>C with specific antibodies (Table 1). After a final wash in TPBS, the slides were incubated with streptavidin-biotin-horseradish peroxidase (Histomark, Goat anti-Rabbit IgG (H+L) KLP, Gaithersburg U.S.A) for 15 minutes. The slides were then rinsed with distilled water and incubated with 3, 3'-diaminobenzidine (DAB) for 10 minutes, rinsed with distilled water and counterstained with Mayer haematoxylin, rinsed and mounted with glycerol for microscopic evaluation. Pancytokeratin, vimetin, actin and desmin monoclonal antibodies were used to exclude the possible differential diagnosis of poorly differentiated carcinoma and mesenchymal tumours.

The staining index of immunohistochemical expression of all the markers was obtained as previously stated by multiplying the staining distribution and intensity scores (Heller *et al.*, 2005). The staining distribution was scored from 0 to 4, with 0 = 0%, 1 = < 10%, 2 = 10–30%, 3 = 31–60%, and 4 = > 61% of cells staining positive. The staining intensity was defined as the strength of the signals

for the positive-staining tumours, with – = no signal, + = weak signal, ++ = moderate signal, and +++ = strong signal.

## Results

Results showed that the gross appearance of the tumors varied in sizes and shapes within the genital tracts and extra-genital sites. Some of the tumor masses were papillary or nodular having a cauliflower-like, pinkish to reddish appearance protruding from the surface of the penis, vagina and vulva. The masses were soft with tendency to bleed and some metastasized to the skin (2/9). The tumours on the skin were raised, circular and ulcerated. The tumor was observed on the skin and the lips in one female Boerboel without genital involvement (Table 2).

### Cytological findings

The cytomorphological features of the nine dogs are depicted in Table 2. Fine needle biopsy aspirates and imprints of the tumors from both genital and extra-genital sites were cellular and similar with no cellular difference between the two sites. The tumor cells occurred discretely and showed anisocytosis with small to large round or oval nuclei (7/9). The nuclei were centrally or eccentrically placed and contained coarse aggregates of chromatin and prominent nucleoli which were usually 1-2 in number. The nuclear:cytoplasmic ratio was approximately 1:1. Numerous mitotic figures were present in all the nine cytological preparations (2-3/HPF). The cytoplasm was pale basophilic and finely granular. The cytology showed distinct and clear intra-nuclear (3/9) and intra-cytoplasmic (9/9) vacuoles of varying sizes. In one of the cases from a female mongrel, without extra-genital metastasis, erythrophagocytosis was observed from a giant binucleated neoplastic cell (Plate I). Nuclear budding (Plates II) was also observed in two dogs in which

**Table 1:** Primary antibodies and the conditions in the immunohistochemical procedure

S/N	Antibody	Type/Clone	Dilution	Incubation	Treatment	Source
1	Anti- Vimentin	Monoclonal mouse, V9	1:50	30 minutes	Microwave	Dako A/S Denmark
2	Anti-desmin	Monoclonal mouse, D33	1:50	30 minutes	Microwave	Dako A/S Denmark
3	Anti-pan Cytokeratin	Monoclonal mouse, PCK-26	1: 200	30 minutes	Microwave	Abcam, USA
4	Anti-actin	Monoclonal mouse, 1A4	1:200	30 minutes	Microwave	Dako A/S Denmark
5	Anti-S100	Polyclonal rabbit, Z0311	1: 300	30 minutes	Microwave	Dako A/S Denmark

**Table 2:** Risk factors and cytomorphologic changes in canine transmissible venereal tumors

S/N	Breed	Sex	Age (years)	Primary site	Extra-genital site	Cytomorphological observations								
						TD	NS	NL	SV	EP	LPT	PCT	MLP	BK
1.	Alsatian	F	3	Vulva	-	mitosis	RD/OV	Central	CYT	-	+	-	-	Haemorrhagic
2.	Mongrel	F	1.5	Penis	-	mitosis	RD/OV	Central/peripheral	CYT	-	-	-	+	Haemorrhagic
3.	Mongrel	F	1.8	Vulva	-	mitosis	RD/OV	Peripheral	CYT/NUC	+	-	+	-	Haemorrhagic/ Neutrophilic
4.	Mongrel	F	3	Vulva	-	mitosis /budding	RD/OV	Central	CYT/NUC	-	-	-	+	Neutrophilic
5.	Mongrel	F	6	Vulva	-	mitosis	RD	Central/peripheral	CYT	-	-	-	+	Neutrophilic
6.	Boerboel	F	2.5	Skin	Lips	mitosis	RD/OV	Central	CYT	-	-	+	-	Haemorrhagic
7.	Boerboel	M	4	Penis	Skin	mitosis	RD	Central/peripheral	CYT	-	-	-	+	Haemorrhagic
8.	Mongrel	M	8	Vagina	-	mitosis /budding	RD/OV	Peripheral	CYT/NUC	-	-	+	-	Neutrophilic
9	Alsatian	M	3	Penis	Skin	mitosis	RD/OV	Central	CYT	-	+	-	-	Haemorrhagic/ Neutrophilic

Note: TD =Type of cellular division, NL= Nuclear location, SV= site of vacuolations, CYT = cytoplasm, NUC = Nucleus, EP= Erythrophagocytosis, LPT=Lymphocytic type, PCT= Plasmacytic type, MLP= mixed lymphocytic plasmacytic, BK=Background, NS=Nuclear shape. RD/OV =Round to oval, +=present, -= Absent

**Table 3:** Histopathological and immunohistochemical changes in canine transmissible venereal tumor

S/N	Cellular infiltrate	Histopathological changes							Immunohistochemical changes							
		GC	MF	GP	MS	CYA	NA	HGE	Pan-cytokeratin	Anti-Vimentin (ImD)	S-100 (ImD)	Anti-desmin	Anti-Actin	Site of immuno-reactivity		
1	NQ LY MQ	+	13-19/HPF	+	+	indistinct	O/C	+	-	+	(2)	+	(8)	-	-	cytoplasmic
2	NQ, LY	-	16-17/HPF	-	-	indistinct	O/C	+	-	+	(2)	+	(12)	-	-	cytoplasmic
3	NQ MQ	-	13-14/HPF	-	+	indistinct	O	+	-	+	(2)	+	(8)	-	-	cytoplasmic
4	NQ, LY, PC	-	10-11/HPF	-	-	indistinct	O/C	+	-	+	(2)	+	(8)	-	-	cytoplasmic

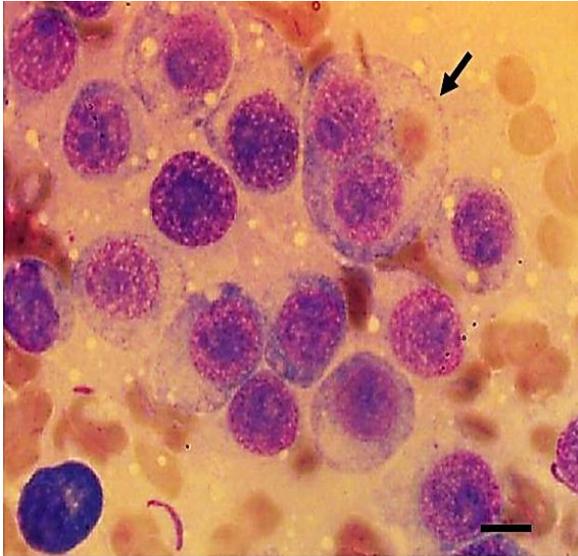
Notes; +=positive/present, -=negative, ImD =Immunohistochemical index, NQ= neutrophils, LY= lymphocytes, MQ= macrophages PC= plasma cells, /HPF= per high power field, CYA= cytoplasmic appearance, NA= Nuclear appearance, O/C= open/close, GC=Giant cell, MF=Mitotic figure, GP=Glandular Pattern, MS =Metastases, HGE = Haemorrhage

small nucleus was seen protruding from the surface of a large nucleus and in some having 2 to 3 nuclei within a slightly basophilic and vacuolated cytoplasm. Both nuclear budding and mitotic figures were observed simultaneously in one of the dogs (Plate III). Two of the 9 cytological smears showed lymphocytoid appearance and 3 showed plasmacytoid morphology, while the remaining 4 smears displayed mixed lymphocytoid/plasmacytoid appearances. A few inflammatory cells such as neutrophils, lymphocytes and macrophages were observed on cytology. A haemorrhagic background was observed in 4 smears while 3 were neutrophilic and 2 were both haemorrhagic and neutrophilic.

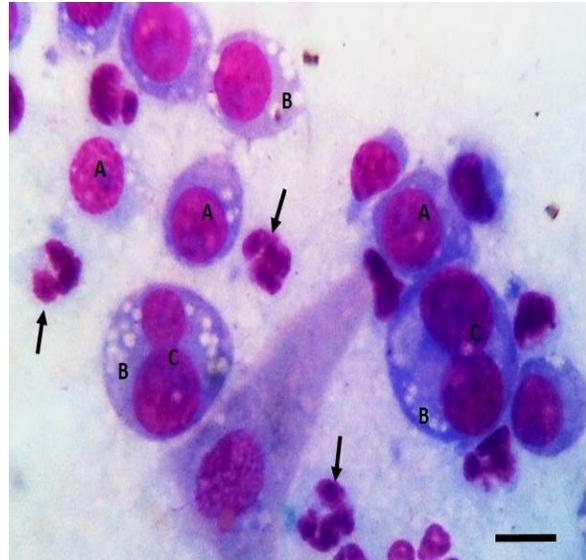
Based on these cytological features, diagnoses of CTVT were made in all the 9 cases.

#### Histopathologic and Immunohistochemical findings

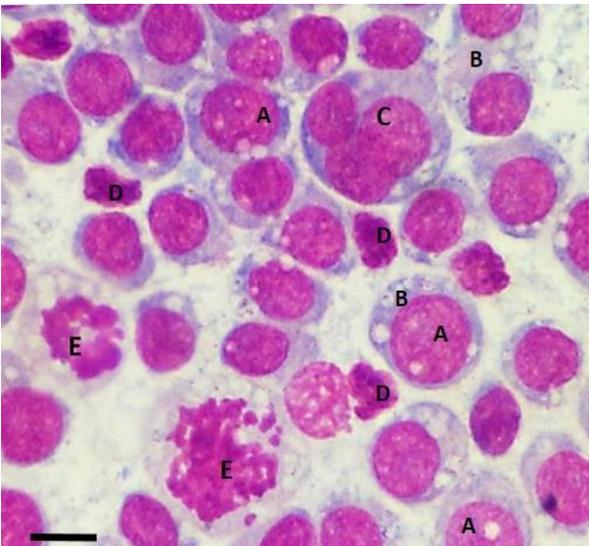
The summary of the histopathological studies are shown in Table 3. The tumor cells were composed of loose sheets and cords of uniformly round to ovoid cells interlaced with fibrous stroma. Nuclei were large and round, with a single centrally placed nucleolus surrounded by marginated chromatin. The nuclei were surrounded by a narrow rim of slightly eosinophilic cytoplasm and indistinct margins with tendency of the tumour cells towards glandular and



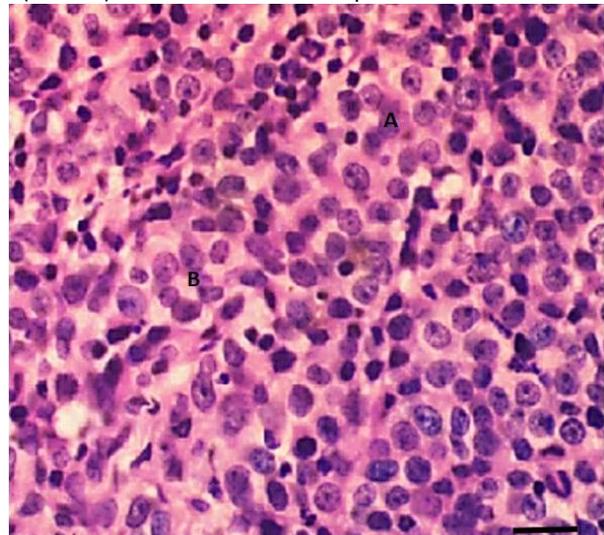
**Plate I:** Fine needle aspirate of plasmacytoidTVT cell showing a binucleated cell undergoing erythrophagocytosis (arrow) with a haemorrhagic background. Giemsa Stain. Bar=40µm



**Plate II:** Fine needle aspirate of transmissible venereal tumour showing intra-nuclear (A) and intra-cytoplasmic (B) vacuolations, two neoplastic cells undergoing nuclear budding (C) and few neutrophils (arrows). Giemsa Stain. Bar=40µm



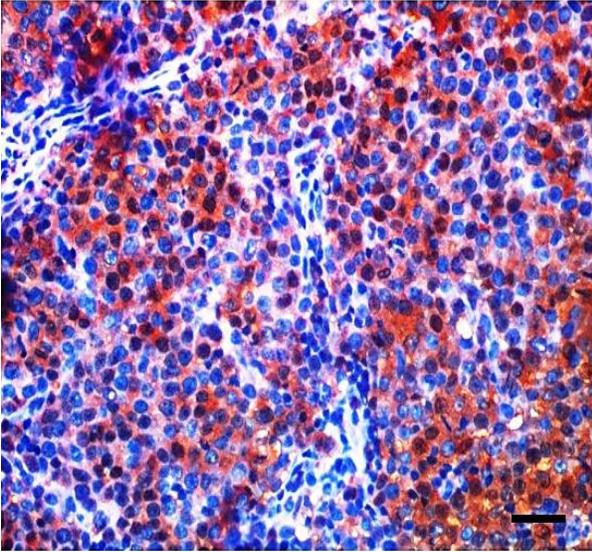
**Plate III:** Fine needle aspirate of transmissible venereal tumour showing intra-nuclear (A), intra-cytoplasmic (B) vacuolations and a three nuclear-budded giant cell (C) and few neutrophils (D) with two mitotic figures (E). Giemsa Stain. Bar=40µm



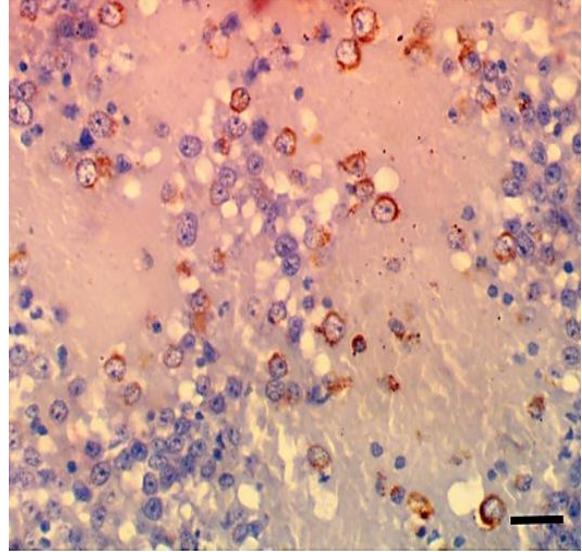
**Plate IV:** Photomicrograph of transmissible venereal tumour showing loose sheets of neoplastic cells with a few giant-like cells (A), acinar formation (B) and numerous mitotic figures. H&E Stain. Bar=30µm

syncytial formation in one of the sections (Plate IV). The mitotic index was high. Tumors were infiltrated with variable numbers of neutrophils, lymphocytes, and macrophages (4/4). Red blood cells were interspersed among the neoplastic cells. The toluidine blue stained slides were negative for mast

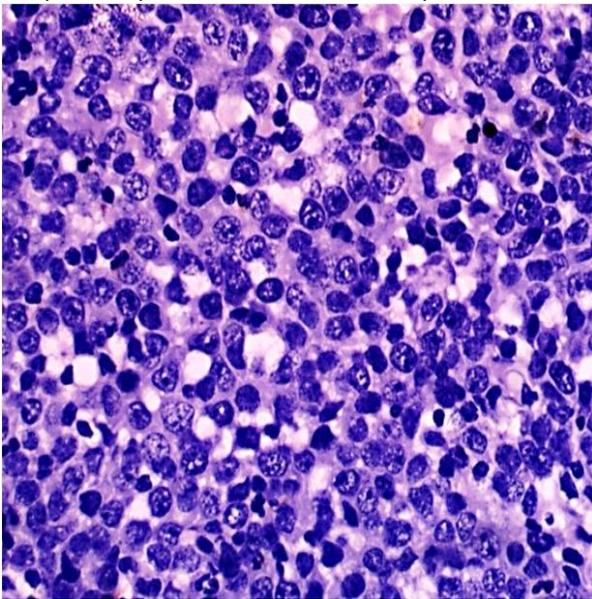
cell tumors due to the absence of cytoplasmic metachromatic granules within the tumor cells. The results of immunohistochemical staining and their respective indices in the 4 cases are shown in Table 3. Immunohistochemical staining revealed moderate cytoplasmic reactivity to S-100 in about



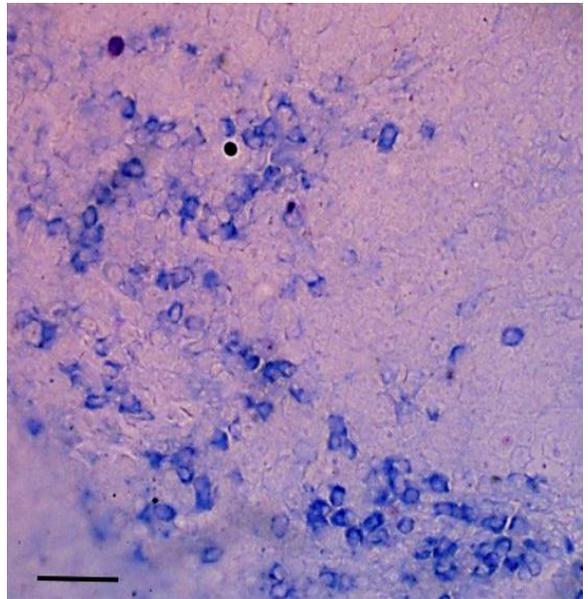
**Plate V:** Photomicrograph of transmissible venereal tumour, vagina, dog. Numerous neoplastic cells showing moderate cytoplasmic immunoreactivity to S-100 protein in most of the neoplastic cells. Biotin Streptavidin peroxidase, DAB. Bar = 35 µm



**Plate VI:** Photomicrograph of transmissible venereal tumour, vagina, showing a few cells with mild cytoplasmic expression of vimentin protein. Biotin Streptavidin peroxidase, DAB. Bar = 35 µm



**Plate VII:** Photomicrograph of transmissible venereal tumour, penis, dog. Numerous neoplastic cells showing non-immunoreactivity to pan-cytokeratin protein. Biotin Streptavidin peroxidase, DAB. Bar = 30 µm



**Plate VIII:** Photomicrograph of transmissible venereal tumour, penis, dog. Numerous neoplastic cells showing non-immunoreactivity to anti-desmin protein. Biotin Streptavidin peroxidase, DAB. Bar = 30 µm

80% cells in three cases (Plates V) whereas this reactivity was mild in a few cells in the fourth case. There was weak cytoplasmic immunoreactivity to vimentin protein in two cases while the remaining two cases expressed moderate cytoplasmic reactivity (Plate VI). There was no immunoreactivity

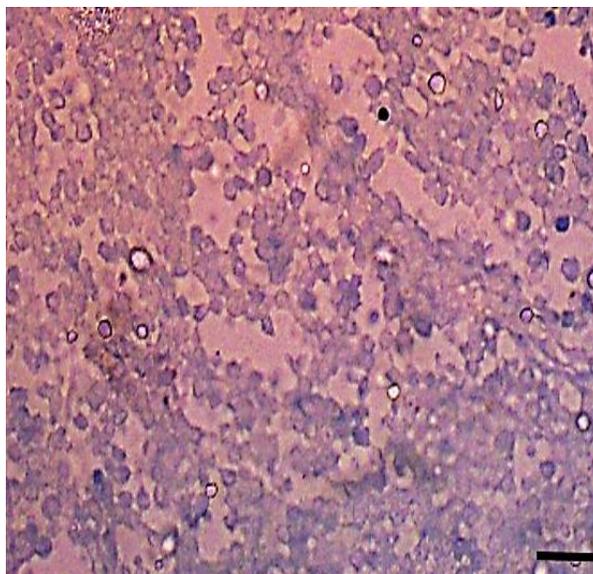
to pan-cytokeratin, actin, and desmin in all the four cases (Plates VII-IX).

#### Discussion

Canine transmissible venereal tumor is one of the most frequently diagnosed neoplasms of the external genitalia and occasionally the internal

genitalia of dogs (Das & Das, 2000). Canine transmissible venereal tumor was reported to occur in sexually mature dogs, although no breed or age susceptibility has been documented (Rogers, 1997). In this study, Nigerian indigenous breeds were mostly affected. This might be due to their large number, uncontrolled breeding and unrestricted movement of unneutered dogs in the study area. The sexually matured dogs above 1 year were mostly affected and this is not different from earlier reports (Das & Das, 2000). Canine transmissible venereal tumor also occurred more in female compared to male dogs in this study which might be due to the fact that one infected male often mates numerous females both in kennels and under free range. The primary cutaneous TVT (though observed only in one dog) without genital involvement is in agreement with previous report (Marcos *et al.*, 2006) which suggested that this might have been due to co-habitation or fighting with infected dogs.

Cytology plays a significant role in the diagnosis of CTVT because of the fact that it is time and cost effective. It also displays distinct nuclear and cytoplasmic features. The cytomorphological features expressed by the nine CTVT cases in this study were not different from previous reports (Amaral *et al.*, 2007) but the phenomenon of erythrophagocytosis, intra-nuclear vacuolation and nuclear budding are rare findings. The phenomenon of erythrophagocytosis in this study further confirmed previous reports in which *Leishmania* amastigotes were found within the cytoplasm of TVT cells (Albanese *et al.*, 2002; Catone *et al.*, 2003). This finding suggests that CTVT cells possess phagocytic ability and hence, histiocytic in nature. Nuclear budding is uncommonly reported in CTVT as a means of cellular proliferation or nuclear abnormality but this phenomenon was observed in two dogs, which might suggest another mode of cellular proliferation or expression of nuclear anomaly in CTVT. Although, the aetiology of the nuclear budding is unknown, previous report affirmed that CTVT cells with plasmacytoid morphology present higher frequency of nuclear abnormalities associated with greater expression of glycoprotein-P (Amaral *et al.*, 2007). This is in agreement with this study in which the two cases with nuclear budding displayed plasmacytoid and mixed lymphocytoid- plasmacytoid appearance. To the best of our knowledge, this is the first documented report on CTVT exhibiting erythrophagocytosis, nuclear vacuolations and budding. Despite the fact that nuclear budding is not



**Plate IX:** Photomicrograph of Transmissible venereal tumour, penis, dog. Numerous neoplastic cells showing non-immunoreactivity to anti- smooth muscle actin protein. Biotin Streptavidin peroxidase, DAB. Bar = 30  $\mu$ m

a known phenomenon in the proliferation of histiocytic cell lines (except in chronic myelomonocytic leukemia) (Padron *et al.*, 2014), the processes of multiple nucleation and erythrophagocytosis by the CTVT cells in this study are suggestive of histiocytic behavior.

Canine transmissible venereal tumor has been immunohistochemically shown to express Proliferating cell nuclear antigen (PCNA) and Ki-67 antigen which have been associated with DNA replication and repair (Amaral *et al.*, 2007). Although immunohistochemical studies of these two proteins were not performed in this study, the presence of numerous mitotic figures and nuclear budding might be as a result of these two proteins.

On the type of cellular infiltrate associated with CTVT, this study observed that the type of leukocytic exudate associated with TVT might be unconnected with the origin of the tumour, contrary to the opinion of some workers (Bloom *et al.*, 1951), but this might depend on the secondary infectious agent, the duration of the neoplasm, and the host immune response (Pai *et al.*, 2011). In early progressive growth phase of the tumor, bacterial contamination of the masses usually occurs with possible neutrophilic and haemorrhagic exudation. This might progress to a subacute (regressive growth) phase in which more of the mononuclear cells (lymphocytes, macrophages and plasma cells)

predominate and to the chronic phase in which these mononuclear cells and fibroplasia are found (Tella *et al.*, 2004). This is in agreement with previous studies (Hsiao *et al.*, 2002; Pai *et al.*, 2011) in which tumor infiltrating lymphocytes were associated with regressive growth phase of the tumor than the progressive growth phase. This lymphocytic infiltration has been shown to confer both cellular and humoral immunity on affected dogs (Vural *et al.*, 2015).

The diagnosis of CTVT by cytology has been preferred over histopathology (Ganguly *et al.*, 2016). This is due to the fact that histopathology of CTVT is very difficult to differentiate from other round cell tumors such as lymphoma, histiocytoma, mast cell tumor (Das *et al.*, 1990; Ganguly *et al.*, 2016). The histopathological changes observed from the four cases were not different from those from previous reports (Boscos *et al.*, 1999; Mukaratirwa & Gruys, 2004; Park *et al.*, 2006; Gupta & Sood, 2012). The tendency towards glandular and syncytial formation in one dog, evidenced by the indistinct cytoplasmic borders of adjoined neoplastic cells (resembling Langerhans's giant cell) is suggestive of histiocytic behavior. The negative immunoreactivity to pancytokeratin in this study further gave credence to the fact that the tumor is of histiocytic and not epithelial origin.

All the four cases examined immunohistochemically gave negative immunoreactivity to actin, desmin and pancytokeratin with positive immunoreactivity to vimetin. Thus, excluding the possible differential diagnosis of poorly differentiated carcinoma and mesenchymal tumours. This is in agreement with previous studies (Park *et al.*, 2006).

Previous studies on the cytogenic origin of TVT have demonstrated positive immunoreactive expression of CTVT cells to histiocytic makers such as anti-lysozyme, anti-macrophages and AAT, suggesting histiocytic origin (Zeid & Muller 1993; Park *et al.*, 2006). However, some workers observed negative immunoreactivity to these proteins (Mascarenhas *et al.*, 2014). Before now, there is paucity of information on the positive immunoreactivity of S-100 protein to CTVT (Zeid & Muller, 1993; Ferreira *et al.*, 2000; Park *et al.*, 2006). In this study, all the four cases were positive for S-100 and vimetin.

Over the years, negative immunoreactivity of S-100 protein to CTVT has been documented by various workers (Zeid & Muller, 1993; Park *et al.*, 2006; Mascarenhas *et al.*, 2014). This has been used to differentiate amelanotic melanomas from CTVT but

its positive immunoreactivity with histiocytic/dendritic tumors in humans and dogs is seldom discussed. In humans, S-100 protein subunits alpha, beta and gamma have been used to confirm histiocytic sarcoma of dendritic and macrophage origin (Takahashi *et al.*, 1984). Histiocytes of dendritic origin are usually positive to S-100 protein subunit alpha but negative to those of macrophage origin while S-100 protein subunits beta and gamma were positive to tumors of macrophage but not to dendritic type.

Also, in human and canine histiocytic sarcoma, vimetin and S-100 proteins have demonstrated strong positive cytoplasmic immunoreactivity to tumor cells (Tomaszewski & Lupton, 1998; Rakich & Latimer, 2011). Moreover, the comparative immunophenotypic features of canine histiocytoma and CTVT showed no significant difference after both tumors expressed positive immunoreactivity to lysozyme, AAT and vimetin (Zeid & Muller, 1993). These workers suggested that the differential diagnosis of the two tumors should be based on clinical and histopathological features. The reason for negative immunoreactivity of CTVT to S-100 protein in previous studies is unknown, but it is possible to speculate that this might have been due to; 1) wrong dilution factor of S-100 protein, 2) the use of wrong or different S-100 protein subunits and 3) poorly differentiated TVT cells in which antigen of S-100 protein had been lost due to poor differentiation of the tumor during the progressive growth phase (Moore & Rosin 1986; Zeid & Muller 1993; Ramos-Vara *et al.*, 2008). In this study, polyclonal rabbit S-100 ( $\alpha$ ,  $\beta$  and  $\gamma$  subunits) protein was used at a dilution factor of 1:300 which gave a strong positive cytoplasmic immunoreactivity by the TVT cells.

In conclusion, the cytological and histopathological features in all the cases examined in this study were similar to those described earlier in genital and extra-genital CTVT (Das & Das, 2000; Mukaratirwa & Gruys, 2004; Park *et al.*, 2006; Bastan *et al.*, 2008; Ajayi *et al.*, 2009), but the expression of features such as erythrophagocytosis, nuclear budding, intranuclear vacuolations and the positive immunoreactivity with S-100 proteins are rare findings and suggestive of histiocytic behavior. This study also revealed that the process of nuclear budding is actively involved in the proliferation of CTVT cells during the progressive growth phase of the tumor. The negative reaction for anti-pancytokeratin, anti-actin, and anti-desmin in the

four cases rule out the possibility of undifferentiated carcinoma and mesenchymal tumours, while the positive immunoreactivity to S-100 showed that this protein should not only be used as a differential marker between amelanotic melanoma and CTVT, but as a biomarker to demonstrate histiocytic origin of CTVT. To the best of our knowledge, this is the first study in which CTVT showed erythrophagocytosis, nuclear budding, and positive immunoreactivity to S-100 protein. Based on these cytomorphological, histopathological and immunohistochemical features, we conclude that CTVT is of histiocytic/dendritic in origin.

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