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Poxvirus infection in Nile crocodiles (*Crocodylus niloticus*)

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An outbreak was encountered of numerous yellowish cutaneous nodules in one- to two-year-old farmed Nile crocodiles (*Crocodylus niloticus*) in Kasaba Bay Crocodile Farm at Lake Tanganyika in Zambia during 1988. Out of 4000 crocodiles of different age groups, 300 yearlings were affected and 82 of those affected died. The lesions were prominent on the head, especially around the eyelids, the nostrils, both sides of the mouth, ventral neck, ventral pale belly, limbs and the root of the tail. Histologically, the epidermal lesions revealed large focal areas of marked acanthosis accompanied by hyperkeratosis and parakeratosis. In the prickle cells, there were multiple small neutrophilic granular inclusions and large eosinophilic homogeneous cytoplasmic inclusions. Electron microscopically, there were numerous poxvirus particles and matrices in the cytoplasm of many prickle cells. Dark blue homogeneous cytoplasmic inclusions in toluidine blue sections consisted of an electron opaque matrix with mature viral particles (160 × 200 × 230 nm). Furthermore, there were clumps of granular matrix containing immature viral particles (200 × 399 nm) in the cytoplasm. Various features of viral developing processes were observed.

RECENTLY commercial crocodile farming in Zambia is receiving much attention as it can produce valuable skin and meat to earn foreign exchange. But the great problem in crocodile farming is the presence of infectious diseases (Foggin 1985).

Reports of viral diseases in reptiles are very few (Jacobson 1980). In crocodiles and related species, an adenovirus-like infection in Nile crocodiles (*Crocodylus niloticus*) (Jacobson et al 1984), skin lesions associated with pox-like virus in juvenile captive spectacled caimans (*Caiman sclerops*) (Jacobson et al 1979) and poxvirus infection in Nile crocodiles (*C niloticus*) (Foggin 1985, Horner 1988) have been described.

An outbreak of poxvirus infection with high mortality was encountered among crocodiles on one farm. The purpose of the present report is to describe briefly the gross and microscopic pathology of Nile crocodiles infected with poxvirus with special reference to the ultrastructure of cytoplasmic inclusions.

Materials and methods

During 1988, farmed Nile crocodiles, which were reared in Kasaba Bay Crocodile Farm, on Lake Tanganyika in Zambia, formed the basis of the study. Out of 4000 crocodiles of different age groups ranging from hatchlings to five year olds only the 300 yearlings were affected. Out of these 300 affected, 82 died (27·3 per cent) with nodular lesions and complications of lesions. Of the six crocodiles showing lesions, four were dead and two were alive when received. All were subjected to detailed necropsy and laboratory investigation. One of these was examined by electron microscope.

For histological observations, skin samples were fixed in 10 per cent neutral formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin (H&E).

Formalin-fixed specimens corresponding to the histological lesions containing cytoplasmic inclusions were refixed in 1 per cent Millonig's osmium tetroxide and embedded in Epon 812. Thin sections were stained with toluidine blue and examined under light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Hitachi H 600L electron microscope.

Results

Gross pathological findings

Yellowish or occasionally brownish nodular lesions were observed on the skin. The lesions varied from unraised spots to raised plaque-like nodules which occasionally had shallow ulcers. The size of the nodules was usually 2 to 3 mm but sometimes up to 6 mm in diameter. The lesions occurred most prominently on the head, especially around the eyelids, the nostrils and both sides of the mouth. Some lesions were also seen inside the mouth as

FIG 1: Raised nodular skin lesions on the ventral belly of an affected crocodile

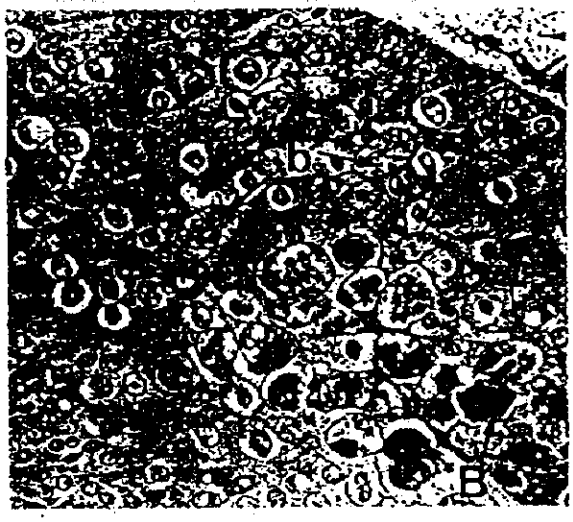

FIG 2: Ballooning of epithelial cells having small granular cytoplasmic inclusions (b) and large homogeneous cytoplasmic inclusions (B). H&E ×300

irregular plaques, and these lesions sometimes coalesced and tended to be larger. Lesions were also seen on the ventral neck, ventral pale belly (Fig 1), the limbs and the root of the tail. The skin lesions often showed hyperkeratinisation with a crust. Internal organs did not have any significant gross pathological changes.

Microscopic findings

The epidermal lesions consisted of large focal areas of marked acanthosis accompanied by hyperkeratosis and parakeratosis. The prickle cells were enlarged and showed ballooning. There were multiple small neutrophilic granular inclusions and large eosinophilic homogeneous cytoplasmic inclusions when stained with H&E (Figs 2 and 3). The latter were also seen in the horny cells. There was brown pigmentation in the subepidermal areas and sometimes vascularisation and perivascular infiltration of heterophils and lymphocytes, and proliferation of fibrocytes in the dermis.

The nucleus showed various changes, such as swelling, loss of chromatin, stippling formation, clumping, shrinkage and margination. There were no characteristic lesions in the visceral organs.

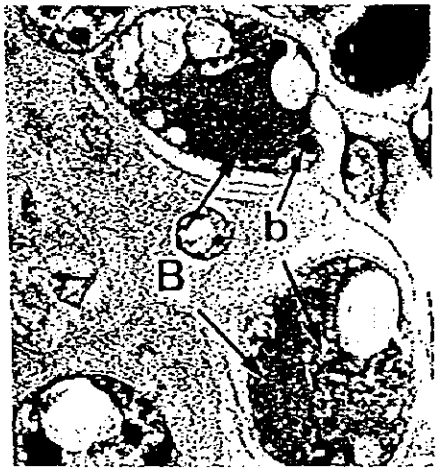

FIG 3: Epithelial cells showing granular cytoplasmic inclusions (b) and homogeneous cytoplasmic inclusions (B). H&E ×600

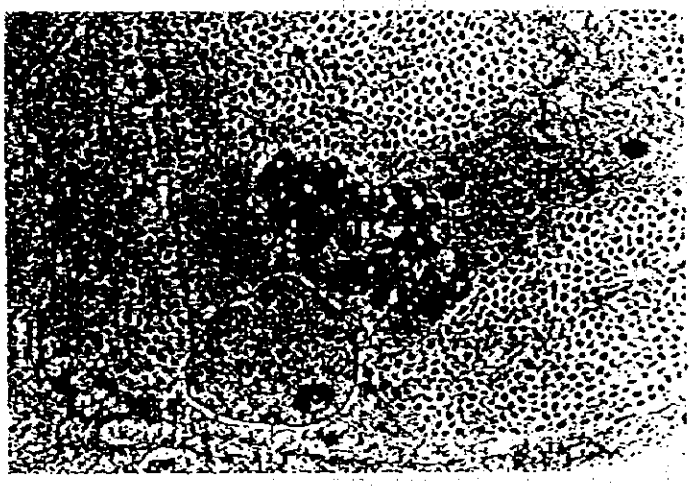

FIG 4: Numerous viral particles and dark matrix in the cytoplasm of an epithelial cell. The cytoplasm contains aggregations of degenerated mitochondria (m). ×5000

Electron microscopic findings

There were numerous viral particles and a dark matrix in the cytoplasm of many prickle cells. The cytoplasm contained aggregations of degenerated mitochondria and endoplasmic reticulum (Fig 4). Dark blue homogeneous cytoplasmic inclusions in toluidine blue sections consisted of an electron opaque matrix with mature viral particles. Furthermore, there were clumps of granular matrix containing immature viral particles in the cytoplasm of prickle cells, which were considered to represent the light blue granular inclusions in the toluidine blue sections.

Viral factories contained dense viroplasm, immature viral particles and brick-shaped mature virions. Membranous structures (shell) were developing around the viroplasm, some with micelles (Figs 5 and 6). The viroplasm was electron opaque and surrounded by a granular structure.

Micelles were approximately 15 nm in diameter and were detected adjacent to the opening of immature viral particles (Figs 7 to 9). Immature viral particles were numerous within the cytoplasmic inclusions. A developing process from dense viroplasm to fine granules with shell and to mature viral particles was observed in several cells.

Immature viral particles were ovoid, about 200 × 300 nm and were composed of a shell and an inner core. The shell consisted of two thin membranes. Each shell was about 9 to 12 nm in width, and a core with open spaces surrounded by a unit membrane was formed within the dense immature particles. Spaced radial projections measuring about 22 nm in length were regularly seen on the exterior of the outermost layers of these particles. Occasionally two cores enclosed by an electron opaque thick membranous structure were wrapped together by common double membranes (Fig 6).

Mature viral particles were brick-shaped, and measured about 160 × 200 × 230 nm. The central core was brick-shaped and depressed in the centre, appearing dumb-bell-shaped in some sections, and the outer envelope contained an electronlucent line (Fig 10).

The apparent maturation process of the poxvirus is represented in Fig 11. At first a viroplasm matrix with granular structure and micelles was formed, followed by the appearance of a shell, immature viral particles, particles with radial projections and finally mature viral particles. The morphogenesis of this virus probably is similar to other poxviruses.

The nuclei showed chromatin margination and contained both filamentous structures and aggregations of coarse and fine granules (Fig 12).

Discussion

Foggin (1985) and Horner (1988) described poxvirus infection in crocodiles and Jacobson et al (1979) reported pox-like skin lesions in captive caimans. These are the only reports associated with poxvirus in reptiles. The light microscopic findings of our study were similar to those reported by Foggin (1985) and Horner (1988). Electron microscopic studies made by previous authors described only the morphology and size of viral particles (Jacobson et al 1979, Horner 1988).

In the present study, various features of the viral development process were observed electron microscopically. Virus-induced alterations were mainly associated with the viroplasm zones in the cytoplasm, but nuclear changes, such as filamentous structures were also seen. Recent biochemical studies (Hruby et

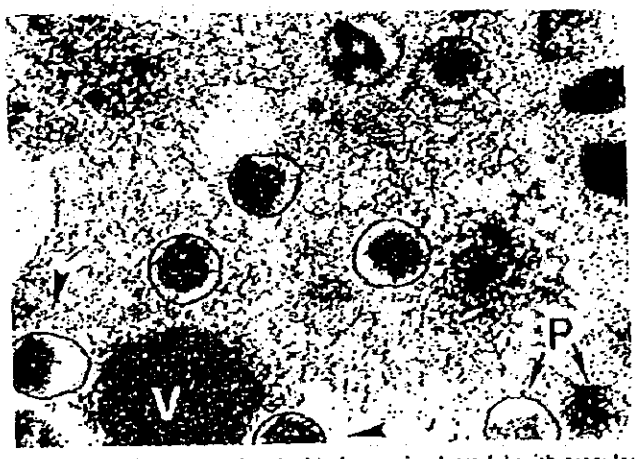

FIG 5: Viral factory associated with dense viroplasm (v) with granular structure and immature viral particles (arrowed). Particles (P) are being processed to immature viruses. × 38,000

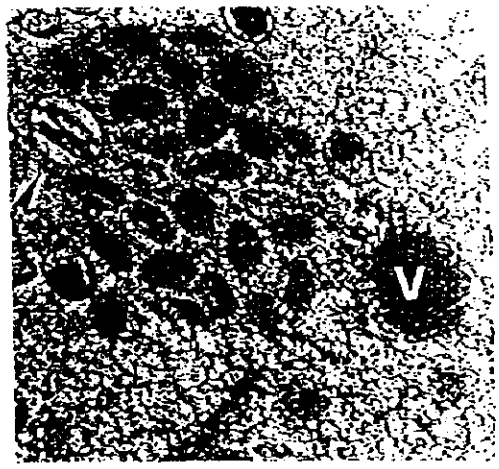

FIG 6: Viral factory showing twin particles enclosed in a common membrane (arrowed). × 29,000

FIG 7: Early development from micelles to shell in the cytoplasm (arrowed). × 35,000

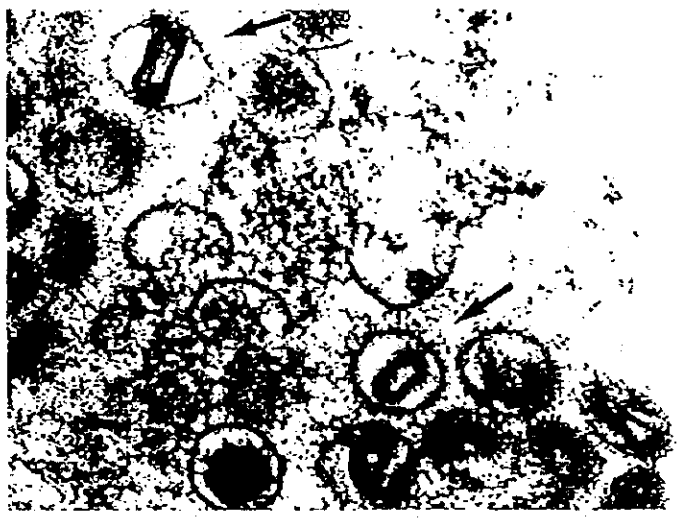

FIG 8: Intermediate development showing micelles, shell, immature particles and particles with radial projections on the surface (arrowed). × 50,000

al 1979a,b) confirmed that the host cell nucleus was necessary for poxvirus replication, and a significant fraction of vaccinia virus DNA synthesised was found in the nucleus (La Colla and Weissbach 1975, Hruby et al 1979a,b). But another report by Silver et al (1979) suggests that vaccinia virus replication may not require the host nuclear transcription. In the present study, morphological changes of the nucleus such as filamentous structures and other granular structures were seen, but their relevance to poxvirus infection and viral replication are still obscure. Similar nuclear morphological changes were also reported in other pox group virus infections (Conroy and Meyer 1971, Wilson et al 1972, Okada and Fujimoto 1975, Kim et al 1977, Moriguchi 1977, Pospischil and Bachmann 1980, Okada and Fujimoto 1984).

When the membrane of immature viral particles was closed, dense material started to accumulate inside it. Dales (1963) showed that the nucleoid of the vaccinia virus was composed of filaments measuring 15 to 40 nm width and suggested that the filaments represented the viral DNA or a DNA protein complex. Nagington and Horne (1962) also recognised such structures. Such filaments were not observed within the maturing particles in the present material, perhaps because the dense material lacks a regular orientation. An increase in density of the immature particles reported in this study may be related to the synthesis

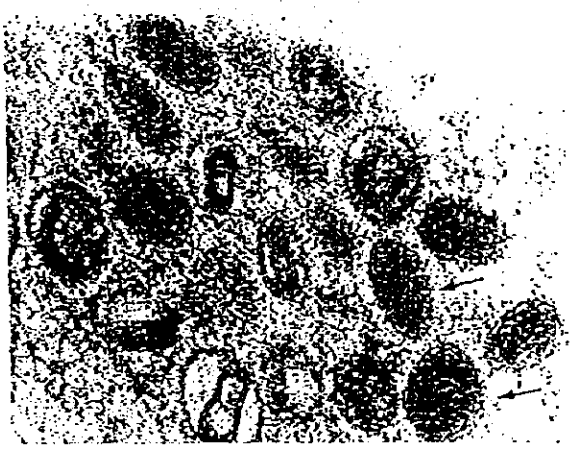

FIG 9: Late development showing mature particles (arrowed). × 50,000

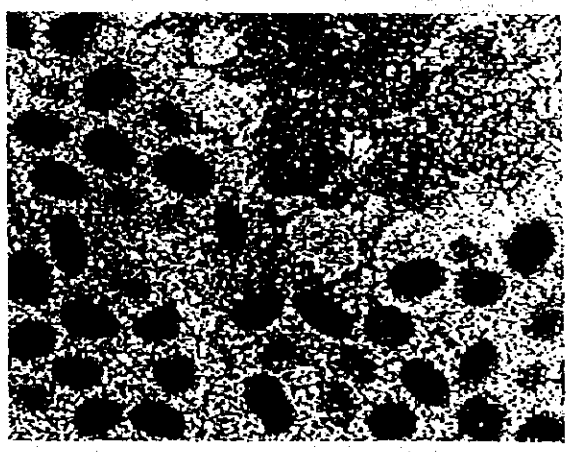

FIG 10: Magnification of Fig 4. There are numerous mature particles and degenerated mitochondria (m). × 28,000

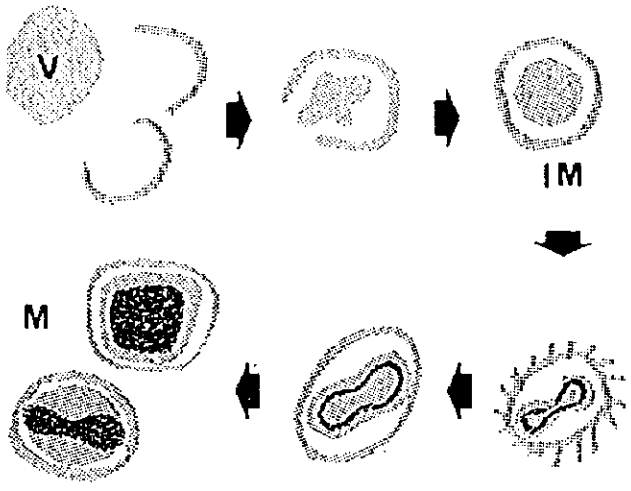

FIG 11: Diagram of presumed maturation process. V viroplasm, IM immature particle, M mature particles

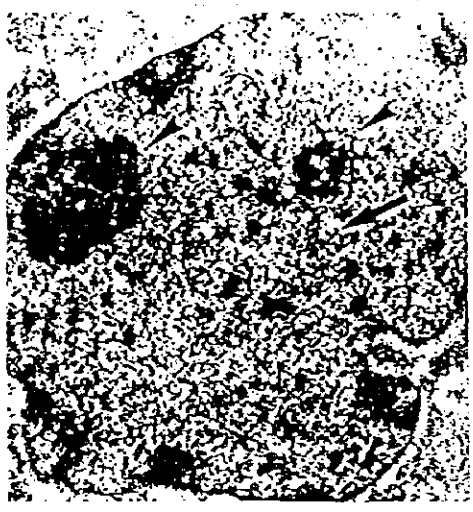

FIG 12: A mass of fine and coarse granules (arrowed) in the nucleus. × 17,000

of viral DNA (De Harven and Yohn 1966).

The radial projections observed on the viral surface were the same as in previously reported cases (De Harven and Yohn 1966, Patrizi and Middelkamp 1967, Kajioka et al 1984). The projections appeared in the transitional stage from immature to mature and seemed to vanish late, in the final stage of development. It is suggested that the assembly of these subunits plays a role in viral membranogenesis in the initial phase and that these accumulated subunits are available for the construction of future viral membranes. Alternatively they may be synthesised in excess as a result of some imbalance or asynchrony in the various synthetic processes triggered by the presence of infecting viral DNA in factory areas (De Harven and Yohn 1966).

The final stage of the maturation process included the formation of a dumb-bell-shaped core of the poxvirus.

Occasionally two viral particles enclosed in a common membrane were observed. Similar findings have been reported previously in swine pox (Kim et al 1977) and vaccinia (Tsutsui 1983) virus infected cells.

There are no previous reports on developmental forms of poxvirus in crocodiles and further studies will be needed for the precise identification of the causal agent of the disease.

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PATHOLOGICAL STUDY ON SWINE LYMPHOSARCOMA
– MORPHOLOGY, LINEAGE AND IMMUNOPHENOTYPE OF TUMOUR CELLS AND MALIGNANCY GRADE –

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Based in part on a thesis submitted by the author to the graduate school of Azabu University, in partial fulfillment of the requirement for Ph.D. degree, 1990.
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Abstract: Thirteen cases of swine lymphosarcoma were examined to clarify the relationships among morphology, lineage and immunophenotype of tumour cells and malignancy grade. One of the 2 localized cases was a B-cell neoplasm despite its thymic location. All cases showed diffuse manner of proliferation; 12 cases were medium-sized cell type and 1 case was large cell type. Starry-sky appearance was not characteristic of one particular type. Immunostaining confirmed that most cases were B-cell tumours. Six were of μ and 4 were of γ immunophenotype. T-cell tumour was not diagnosed and 2 were negative for both B- and T-cell markers. Ultrastructures of the 2 negative cases suggested B-cell lineage. Morphologically, 13 cases of this series included 9 high and 4 low grade malignancies. According to the findings of the argyrophilic nucleolar organizer regions associated proteins method and applying similar criteria as for human non-Hodgkin's lymphomas, they were classified 10 high grade and 3 low grade malignancies; thus showing a high degree of agreement with ordinary malignancy grading. Morphometry failed to show any significant differences except that round shaped nuclei were more predominant in neoplastic cells than in normal lymphocytes. Immunophenotype of tumour cells may not be predictive of histology and malignancy in swine lymphosarcoma.

Key words: swine, lymphosarcoma, immunophenotype, AgNOR method, malignancy grade.

Introduction

Swine lymphosarcoma (SLSA) is one of the commonest tumours in the pig and often seen in young animals without sex or breed predisposition. It is believed to be sporadic, while familial cases have been described [24, 31]. Leukemic changes may appear in the terminal stage [13]. Most SLSAs are considered to be B-cell origin [15-17, 26], but not much is known about the immunophenotype or malignancy grade, nor has there been any report on morphometry in SLSA. In human non-Hodgkin's lymphoma (NHL), immunophenotype has been shown to be sufficiently predictive of histology and malignancy grade [29]. Morphometeric difference has been shown between subclasses of human NHL [37].

The aim of the present study is to clarify the relationships among histopathology, lineage and immunophenotype and malignancy grade in SLSAs. Morphological study was extended to morphometry of nuclear configuration of tumour cells. Argyrophilic nucleolar organizer regions-associated proteins (AgNOR) method [3, 7, 10] was employed to asses malignancy grade objectively.

豚のリンパ肉腫の病理学的研究－腫瘍細胞の形態，由来，免疫学的表現型とその悪性度分類－
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Materials and Methods

Thirteen SLSAs were investigated. Touch smears of neoplastic lesions were studied with Giemsa's stain. Several touch smears of each case were set aside for AgNOR staining. Small pieces of fresh material were frozen in liquid nitrogen and stored at −80°C for cryosectioning. Tissue samples were fixed in 10% phosphate buffered fromalin and embedded in paraffin wax. Four μm serial sections were cut and stained with haematoxylin and eosin, periodic acid Schiff and Watanabe's silver stains for histopathology.

SLSAs were grossly divided into systemic and loclized types and diagnosed according to the classification proposed by lymphoma study group in Japan [9, 33]. Malignancy was graded based on the form and distribution pattern of nuclear chromatin, nucleolar features and frequency of mitotic figures [19, 34].

Indirect immunoperoxidase and a two-round reaction cycle of indirect Immunostaining [21] and protein A gold-silver stain [8] were employed for cytoplasmic immunoglobulin (CIg) staining using serial paraffin sections and the following antibodies; rabbit anti-swine IgG (Cappel, PA, USA and Nordic, The Netherlands), rabbit anti-swine IgM (Nordic, The Netherlands) and peroxidase conjugated $F(ab')^2$ fragment goat anti-rabbit IgG (Cappel, PA, USA).

Frozen sections were fixed in either acetone alone or acetone followed by periodate-lysine-paraformaldehyde. Avidin-biotin peroxidase method was employed to detect T-cell antigen in frozen sections using a monoclonal antibody 8/1 against swine Pan T antigen (mouse hybridoma ascites) reported by Saalmüller et al. [30] and supplied by Dr. G. Wittmann, Federal Research Center for Virus Diseases of Animals, Germany and biotinylated horse anti-mouse IgG and horseradish peroxidase avidin D (Vector, CA, USA).

Serial paraffin sections and smears fixed in ethanol for 5 minutes were stained by the improved one-step AgNOR stain [3, 7, 10] for the evaluation of malignancy grade.

Tiny tissue blocks fixed in 2.5% glutaraldehyde or 10% phosphate buffered formalin were immersed in 1% OsO_4 and embedded in Epon 812 (Taab, Berkshire, UK.). One μm thick plastic sections stained by Toluidine blue were used to study nuclear morphometry employing Olympus XL500 image analyzing system (Olympus Co., Japan). In each high power dry field, 25 to 35 nuclei were outlined to obtain areas and perimeters and calculate form factor (FF) and nuclear contour index (NCI) according to the following formulae:

$FF=4\pi\ Area/(Perimeter)^2$ [5],

$NCI=Perimeter/\sqrt{Area}$ [36, 37].

FF, NCI and their standard errors of mean (SEM) were used for statistical comparison of normal lymph nodes and SLSAs both low and high AgNOR grades by Wilcoxon's test.

Ultrathin sections of negative immunostaining cases were stained with uranyl acetate and lead citrate and examined on Hitachi H-300 electron-microscope at 75 kV.

Results

1. Tumour morphology:

1) Anatomical form:

According to the distribution of gross lesions, 11 cases were classified as systemic form and 2 cases as localized form (Table 1). One (K2) of the 2 localized cases had lesions restricted to the small intestine. The other localized case (T2) mainly involved the thymus area.

2) Manner of proliferation and cell type:

All 13 cases showed the diffuse manner of proliferation. Comparing with benign macrophage nucleus, 13 SLSAs were classified into 12 cases of medium-sized cell type and 1 large cell type. Starry-sky appearances were present in both medium and large cell types of SLSA (Table 2).

2. Cell lineage and immunophenotype:

1) Immunostaining results:

Table 1. Biodata and distribution of gross neoplastic lesions.

	H1	H2	H3	H4	H5	H6	K1	K2	T1	T2	T3	T4	T5
Sex	CM	CM	F	F	CM	CM	F	F	F	F	F	CM	CM
Body weight (Kg)	110	100	100	70	110	60	200	100	?	?	?	?	?
Age	6m	6m	6m	5m	6m	6m	3y	6m	6m	?	adult	6m	6m
Clinical signs	?	+	?	+	?	?	+	?	?	+	+	+	?
– Body lymph nodes –													
Sub-mandibular lnn. L	2+	–	–	–	–	–	–	–	–	–	–	–	2+
R	2+	–	–	–	–	–	–	–	–	–	3+	–	2+
Superficial cervical lnn.													
L	+	–	–	–	+	2+	3+	–	2+	–	–	–	2+
R	+	–	–	–	–	2+	3+	–	2+	–	–	–	2+
Deep cervical lnn. L	3+	–	–	–	–	–	–	–	–	–	–	–	–
R	3+	–	–	–	–	–	–	–	–	–	–	–	–
Axillary lnn. L	–	–	–	3+	–	–	–	–	–	–	–	–	2+
R	–	–	–	3+	–	–	–	–	–	–	–	–	2+
Mediastinal lnn. cranial	2+	–	–	2+	–	2+	–	–	–	–	–	–	–
caudal	2+	–	–	2+	–	2+	–	–	–	–	–	–	–
Sub-iliac lnn. L	–	–	–	–	–	–	3+	–	–	–	–	–	2+
R	–	–	–	–	–	–	3+	–	–	–	–		2+
Internal iliac lnn. L	–	–	2+	2+	2+	3+	3+	–	2+	–	3+	3+	2+
R	–	–	2+	2+	2+	3+	3+	–	2+	–	3+	3+	2+
Popliteal lnn. L	–	–	–	–	2+	2+	2+	–	3+	–	–	–	–
R	–	–	–	–	+	+	2+	–	3+	–	–	–	–
Other body lymph nodes	–	–	–	–	–	2+	–	–	–	–	–	–	–
– Organs and organ-associated lymph nodes –													
Spleen	–	(*)	+	–	2+	(2+)	–	–	(*)	–	(*)	*	*
Splenic lnn.	–	–	–	–	–	(2+)	–	–	–	–	(2+)	–	–
Liver	2+	3+	+	+	3+	(3+)	*	–	2+	*	(*)	2+	+
Hepato-gastric lnn.	2+	(+)	(3+)	–	(3+)	(3+)	(3+)	*	(3+)	–	(3+)	(3+)	(2+)
Pancreatic lnn.	–	–	–	–	–	–	–	–	–	–	(2+)	–	–
Small intestine	–	–	–	–	–	–	–	3+	–	–	–	–	–
Colonic lnn.	–	(2+)	–	–	–	–	–	–	–	–	–	–	–
Ano-rectal lnn.	–	(+)	(3+)	–	–	–	–	–	–	–	–	–	–
Mesenteric lnn.	–	–	–	–	–	–	–	(2+)	–	–	(3+)	–	–
Kidneys	*	2+	–	+	+	+	–	–	–	–	*	+*	–
Renal lnn. L	–	–	(3+)	–	–	(2+)	(3+)	–	(2+)	–	*	(2+)	–
R	–	–	(3+)	–	–	(2+)	(3+)	–	(3+)	–	–	(2+)	(3+)
Aortic lnn.	–	–	–	–	–	–	–	–	–	(+)	–	–	–
Vertebral lnn.	–	–	–	–	–	–	–	–	(3+)	–	–	–	–
Tracheo-bronchial lnn.													
cranial	(+)	–	(2+)	(2+)	–	(2+)	–	–	(2+)	–	–	–	(2+)
caudal	(+)	–	(2+)	(2+)	–	(2+)	–	–	(2+)	–	–	–	(2+)
Other organs	–	+*	2+	–	–	–	2+	–	–	3+	2+	–	–
Anatomical type	Sys	Sys	Sys	Sys	Sys	Sys	Sys	Loc	Sys	Loc	Sys	Sys	Sys

Key: CM; castrated male, F; female, m; age in months, y; age in years, ?; unknown or without record, lnn; lymph node, –; no gross neoplastic lesion, +; slight, 2+; moderate, and 3+; marked neoplastic change, ( ); organ swelling, *; with necrosis and haemorrhage, Sys; systemic, Loc; localized.

Table 2. Cyto-histological findings, LSG classification and malignancy grade

	H1	H2	H3	H4	H5	H6	K1	K2	T1	T2	T3	T4	T5
– CYTOLOGY –													
Cytoplasm	S	W	S	S	W	S	S	S	S		S	S	S
Nucleus													
size	Me	La	Me	Me	Me	Me	Me	Me	Me		Me	Me	Me
configuration	Ir	Ir	Ir	Ir	Ir	S/I	Ir	Ir	Ir		S/I	S/I	S/I
chromatin	F	F	C	C	C	F	F	F	C		F	C	C
Nucleolus; increase													
in size & number	2+	3+	2+	2+	2+	3+	3+	4+	3+		4+	3+	3+
eccentric location	2+	3+	2+	2+	2+	3+	3+	3+	2+		3+	3+	2+
aggregation	–	+	–	–	–	+	–	–	–		–	–	–
– HISTOPATHOLOGY –													
Manner of proliferation	D	D	D	D	D	D	D	D	D	D	D	D	D
Cell size	Me	La	Me	Me	Me	Me	Me	Me	Me	Me	Me	Me	Me
Mitotic figures	2+	2+	2+	2+	+	2+	2+	+	+	+	3+	2+	2+
Necrosis	–	–	–	–	–	–	2+	+	–	+	–	–	–
Histopathological malignancy grade	L	H	L	L	L	H	H	H	H	H	H	H	H
Classification according to "LSG"	MED	LAR	MED	MED	MED	MED	MED	MED	MED	MED	MED	MED	MED

Key: S; scanty, W; wide, Me; medium in size, La; large, Ir; irregular and indebted, S/I; smooth and slightly indented, F; fine granular, C; coarse granular and dispersed, D; diffuse, L; low, H; high, MED; medium-sized cell type, LAR; large cell type. Imprint smear was not available for T2 case.

Table 3. Immunohistochemical reactivities and immunophenotypes

	H1	H2	H3	H4	H5	H6	K1	K2	T1	T2	T3	T4	T5
IgG (H+L)													
frozen	–	2+	–	+	–	NE	#	–	+	+	–	+	2+
paraffin	–	3+	–	+	+	NE	#	–	2+	–	+	2+	+
IgG (Fc)													
frozen	–	NE	–	NE	–	+	#	–	NE	NE	NE	NE	NE
paraffin	–	3+	–	+	+	+	#	–	2+	–	–	2+	+
IgM (Fc)													
frozen	–	–	2+	3+	+	2+	#	+	+	2+	2+	+	+
paraffin	–	–	–	–	–	–	#	–	–	–	–	–	–
Pan T antigen	–	–	–	–	–	–	#	–	–	–	–	–	–
Immunophenotype	?	γ	μ	μ	?	μ	#	μ	γ	μ	μ	γ	γ

Key: NE; not examined, #; difficult to determine the reactivities because of severe necrosis. +; weak positive reaction, 2+; moderate positive reaction, 3+; marked positive reaction.

Ten out of 13 SLSAs stained positive for CIg, none for Pan T-cell antigen and 2 were negative for both CIg and Pan T-cell antigen. Immunostaining result in case (K1) could not be interpreted because of high necrotic changes. Of the 10 CIg-positive cases, 6 had μ chain and 4 had γ chain. Among the positive cases, CIg staining intensity varied from weak to strong and positive-staining cells showed patchy to diffuse distribution (Table 3).

2)Ultrastructures of negative immunostaining cases:

H-1: Tumour cells were characterized by rounded nuclei, prominent mitochondria and a scattered distribution of short rough endoplasmic reticula, many of which showed dilatation. Many tumour cells had desmosome-like structures.

H-5: Tumour cells had roundish-oval nuclei and were rich in mitochondria. Mitochondria were swollen, vacuolated and lacked discernible cristae, but short rough endoplasmic reticula and desmosome-like structures were observed.

3. Malignancy grade

1) Morphological malignancy grading:

Nine cases were histologically classified as high grade malignancy and 4 cases as low grade malignancy.

2) AgNOR malignancy grading:

Ten cases with marked increase in size and numbers of AgNOR-positive granules in their nuclei were classified as high grade malignancy. Three cases with moderate increase in sizes and to some extent in numbers of AgNORs were diagnosed as low grade malignancy (Table 4).

Of the 10 AgNOR high grade malignancy cases

Table 4. AgNOR staining results

	Cont.	H1*	H2	H3	H4*	H5	H6	K1	K2	T1	T2*	T3	T4	T5
Imprint smear	+	NE	NE	+	3+	+	3+	NE	NE	NE	NE	2+	3+	3+
Section	+	2+	2+	+	3+	+	3+	2+	2+	2+	+	2+	2+	2+
Grade	L	H	H	L	H	L	H	H	H	H	L	H	H	H

Key: Cont.; control, NE; not examined, because of no unstained imprint smears, *; no imprint smears were available for T2 case, +; small number of AgNORs, 2+; moderate increase in AgNORs, 3+; marked increase in AgNORs, L; low, H; high.

Table 5. Summary of results

	H1*	H2	H3	H4*	H5	H6	K1	K2	T1	T2*	T3	T4	T5
Anatomical type	Sys	Sys	Sys	Sys	Sys	Sys	Sys	Loc#1	Sys	Loc#2	Sys	Sys	Sys
Manner of proliferation	D	D	D	D	D	D	D	D	D	D	D	D	D
Classification according to "LSG"	MED	LAR	MED	MED	MED	MED	MED	MED	MED	MED	MED	MED	MED
Immunophenotype	(B)	γ	μ	μ	(B)	μ	NE	μ	γ	μ	μ	γ	γ
Histopathological malignancy grade	L	H	L	L	L	H	H	H	H	H	H	H	H
AgNOR malignancy grade	H	H	L	H	L	H	H	H	H	L	H	H	H

Key: *; discrepancy between two malignancy grading methods, Sys; systemic, Loc; localized, #1; located in mesenteric lymph node, #2; located in anterior mediastinum, D; diffuse, MED; medium-sized cell type, LAR; large cell type, (B); ultrastructure suggesting B-cell nature, NE; not examined, L; low, H; high.

Table 6. Nuclear morphometry results

Group		AgNOR malignancy grading	
Parameter	Control	Low grade	High grade
NCI (x)	4.104±0.094	3.968±0.02	3.974±0.091
NCI (sem)	0.036±0.005	0.028±0.008	0.026±0.008
FF (x)	0.761±0.031	0.835±0.053	0.805±0.034
FF (sem)	0.011±0.001	0.008±0.001	0.008±0.002

Key: Control; normal lymph node, NCI; nuclear contour index, FF; form factor, x; mean value, sem; standard error of mean

Table 7. Results of Wilcoxon's analysis

Parameter	Groups compared	p level
NCI (x)	Control vs. Low*	p>0.05
	Control vs. High*	p>0.05
	Low vs. High*	p>0.05
NCI (sem)	Control = Low	
	Control = High	
	Low = High	
FF (x)	Control vs. Low*	p>0.05
	Control = High	
	Low = High	
FF (sem)	Control = Low	
	Control vs. High*	p>0.05
	Low = High	

Key: Control; normal lymph node, NCI; nuclear contour index, FF; form factor, x; mean value, sem; standard error of mean, *; significant difference

8 SLSAs were cyto-histologically evaluated as high grade malignancy. Two cases (H1, H4) which were histologically assigned low grade malignancy were assessed high grade malignancies by AgNOR method. One case (T2) histologically graded as high grade malignancy was assigned low grade malignancy following AgNOR method (Tables 5).

4. Morphometry:

Using SEM of NCI, there was no significant difference among normal lymphocytes, AgNOR low and AgNOR high grade cases about nuclear indentation. Both neoplastic and normal lymphocytes had a predominance of indented nuclei as indicated by their high mean NCI values, although mean NCI values of neoplastic cells were somewhat lower than that of normal lymphocytes. SEM of FF values was significantly lower in neoplastic cells than in normal lymphocytes, thus implying that round-shaped nuclei were predominant in tumour cells than in normal lymphocytes. There was no significant difference between AgNOR low grade malignancy cases and AgNOR high grade malignancy cases concerning nuclear shape (Tables 6 & 7).

Discussion

SLSA was investigated from multiple viewpoints to clarify the relationships among tumour morphology, cell lineage and immunophenotyupe and malignancy grade. For anatomical types, "systemic or localized" preferred to "multi-centric, alimentary and thymic" in the previous paper [14] to emphasize the nature of each tumour.

One of the 2 localized cases was a B-cell tumour in spite of its thymic location in accord with Nakajima et al. [26]. Some thymic type SLSAs are probably B-cell tumours derived from lymph nodes in the anterior mediastinum, involving the thymic area. Mediastinal lymphomas of B-cell origin have been re-

ported in man [32]. These facts will highlight the need for immunophenotyping when one is examining thymic type lymphosarcoma in animals.

From the present study and a few available papers [9, 26], medium sized cell type is supposed to be quite common in SLSAs. Contrary to Hayashi et al. [9], starry-sky appearance was not characteristic of particular type in agreement with Ashley's observation that it was not specific to or pathognomonic of Burkitt's lymphoma [1].

Surface immunoglobulin (Ig) and/or CIg are the best single marker for B-cell lineage [25, 28]. Present study could not determine the monoclonicity of CIg positive cells, but there were little confusion due to infiltrative or residual lymphoid cells because they were easily interpreted in serially cut paraffin sections. Immunostaining showed varied staining intensities and distributions of CIg-positive cells as seen in human NHL [22, 27]. This variety may reflect different stages in development or cell cycle [22]. Immunohistochemical study confirmed the reported observation that most SLSAs are B-cell origin and display μ or γ chain immunophenotype and that SLSA derived from T-cell is rare.

Electron-microscopy of the 2 negative immunostaining cases indicated ultrastructures of B-cell lineage [16]. The negative immunostaining could be due to incomplete functional maturation, failure to express Ig genes or loss of Ig genes as a result of genetic instability in proliferating tumour cells. This loss of Ig can occur in later stages of lymphoma development [27]. At certain stages of follicular B-cell maturation in man, Ig is not expressed normally [11, 27]. Lymphoma derived from such non-expressing cells are invariably Ig negative. Null-cell lymphoma is rare in man [18, 27] and has not been reported in pigs though Binns claimed to have encountered such cases [2].

B-cell lymphomas can exhibit nodular (or follicular) and/or diffuse manner of proliferation [15, 26]. Nodular growth tends to become diffuse in advanced long-standing cases; such lymphomas however may retain their former cytological characteristics [16]. Some diffuse SLSAs may be evolved from nodular SLSA with passage of time.

NORs are demonstrable by virtue of the argyrophilia of their associated non-histone proteins [7, 10]. In some human tumours, AgNOR positive granules have been shown to increase with cellular deterioration and malignancy grade [6], while it has not been proven in animal tumours. On the basis of AgNOR method, the results of SLSA malignancy grade were similar to those reported for NHL of man with 5 NOR-bearing chromosomes [7, 12]. If the increase in AgNOR positive granules with malignancy is related to a rise in chromosomal segregation [7], it is very interesting to note that swine with only 2 NOR-bearing chromosomes [20] showed similar results as for humans where there are 5 NOR-bearing chromosomes [7, 12]. The number of NOR-bearing chromosomes seemed not to influence the applicability of AgNOR method [3, 7] for grading lymphosarcoma in domestic animals.

In this study, AgNOR malignancy grading showed high correlation with cytological evaluation. However, there were some discrepancies between the grades of some cases given by the 2 methods. Such problems justify the need for an objective approach based on quantifiable parameters in the evaluation of malignancy. AgNOR method will offer a potential for objective malignancy grading in SLSAs.

Morphometric differences have been shown to exist between subclasses of large and small cell type NHL [37]. This study failed to show any significant difference between large cell type and medium-sized cell type SLSAs concerning the degree of indentation configuration of tumour cell nuclei. In both types of SLSAs, indented but round-shaped nuclei with high NCI and low FF values predominated among tumour cells in agreement with previous report on NHL [35]. Round-shaped nuclei were significantly more predominant in SLSA cells than in normal lymphocytes. In man, nuclei of high grade NHL are reported to have more rounded configuration than their low grade counterparts or lymphocytes

in follicular hyperplasia [5], but in this study, there was no significant difference between AgNOR high and low grade SLSAs regarding nuclear shape.

Present results seemed to suggest that immunophenotype of tumour cells did not correlate with histology in SLSAs, whereas it has been shown to be predictive of the tumour histology and malignancy grade in NHL [4, 29]. In canine lymphosarcoma [23], clinical behaviour and response to cancer therapy have been shown to be related to tumour cell immunophenotype as in NHL [29]. Considering malignancy grade as the basis for clinical behaviour, immunophenotype of constituent tumour cells may not be predictive of malignancy grade and clinical behaviour in SLSAs.

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要　約

豚の好発腫瘍の一つである豚のリンパ肉腫(SLSA)は，しばしば若齢豚にみられるが，品種/系統あるいは性素因の報告はない。イギリスと日本で家族性に発生したSLSAが報告されたが，発生は散発的で，末期に白血化することもある。SLSAの大部分は，B細胞性腫瘍と考えられているが，免疫学的表現型や悪性度分類についての知見は乏しく，SLSA細胞の形態計測に関する報告もない。

本研究の目的は，核の形態計測を含む腫瘍の形態学的所見，腫瘍細胞の由来と免疫学的表現型ならびに悪性度の間に何らかの関係があるかどうかを明らかにすることであった。

13例のSLSAを検索の対照として細胞学的，組織学的，免疫組織化学的並びに電子顕微鏡的検索を行い，悪性度分類のために核小体形成域（NOR）関連蛋白のワンステップ銀染色（AgNOR）法を用いた。核の形態計測には準超薄切片を作ってトルイジン青染色を施し，核周長と核の面積を画像解析装置で計測して，NCI (nuclear contour index) と FF (form

factor）を算出し，Wilcoxon 検定を行った。

11例が全身型，2例が限局型 SLSA で，細胞学的には12例の中細胞型と1例の大細胞型に分類されたが，増殖様式は13例すべてが濾胞型であった。"starry sky 像" は，特定の細胞型に特徴的ではなかった。広範な壊死で1例の免疫染色結果は判定不能。10例が細胞質内免疫グロブリン陽性でB細胞腫瘍に分類されたが，T細胞腫瘍は無かった。免疫学的マーカー陰性の2例は，電顕検索でB細胞由来が示唆された。B細胞腫瘍10例中，免疫学的表現型は6例が μ，4例が γ であった。

形態学的には，9例が高悪性度，4例が低悪性度に分類された。AgNOR 染色所見をヒトの NHL と同様の基準で評価すると，10例が高悪性度，3例が低悪性度となり，純形態学的悪性度分類と AgNOR 法による悪性度分類は高い一致率を示した。

核の形態計測では腫瘍細胞と正常リンパ球の核膜の切れ込みの程度には有意差が無かったが，腫瘍細胞では円みのある核をもつ細胞が優勢であった。AgNOR 染色低悪性度症例と高悪性度症例の間には，核の形状に関しては有意差がなかった。

以上の成績から，SLSA 腫瘍細胞の免疫学的表現型からは，腫瘍の組織像や悪性度を予測出来ないように思われる。

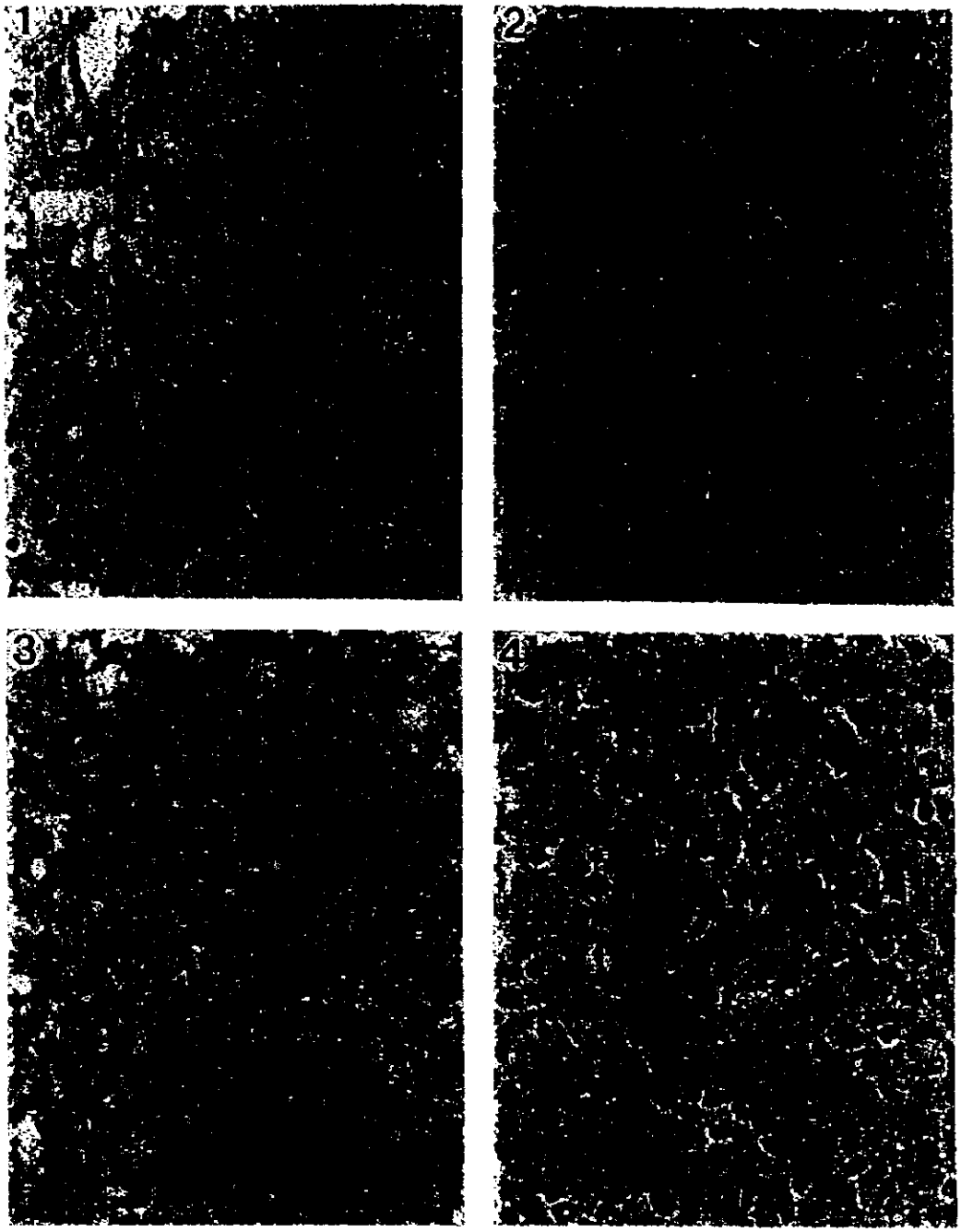

Fig. 1. Case No. H-2. Cytoplasmic immunoglobulin G seen in neoplastic cells proliferating in bone marrow. ×300. Two-round reaction cycle of indirect immunoperoxidase method using paraffin section.

Fig. 2. Case No. H-4. Cytoplasmic immunoglobulin M. Frozen section of neoplastic lymph node. ×300. Two-round reaction cycle of indirect immunoperoxidase method.

Fig. 3. Case No. T-2. Cytoplasmic immunoglobulin M. Paraffin section of tumour mass. ×300. Two round reaction cycle of indirect immunoperoxidase method.

Fig. 4. Case No. T-1. Cytoplasmic immunoglobulin G seen in neoplastic cells proliferating in the liver. ×300. Immunostaining by Fujimori's Protein A Gold-silver method using paraffin section.

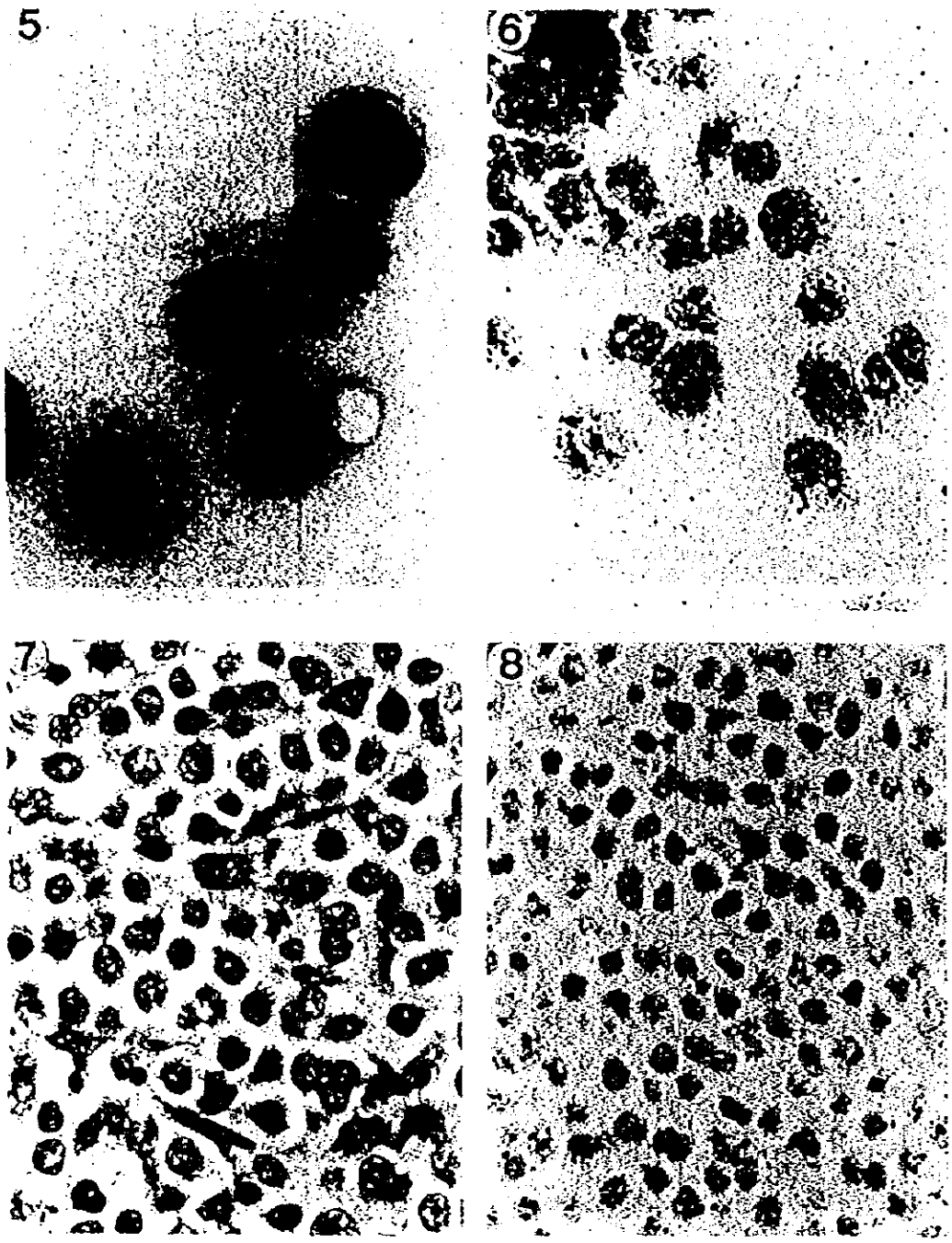

Fig. 5. Case No. H-6. Medium-sized tumour cells with scanty cytoplasm and round nucleus containing eccentric nucleoli. Imprint smear of lymph node. ×900. Giemsa's stain.

Fig. 6. Case No. H-6. AgNOR high grade malignant tumour showing marked increase in AgNOR positive granules. Imprint smear of lymph node. ×600. One-step AgNOR stain.

Fig. 7. Case No. H-6. Medium-sized cell type, diffuse lymphoma. Two mitotic figures are present in this field. Paraffin section of lymph node. ×600. HE stain.

Fig. 8. Case No. H-6. AgNOR high grade malignancy showing marked increse in AgNOR positive granules. Paraffin section of lymph node. ×600. One-step AgNOR stain.

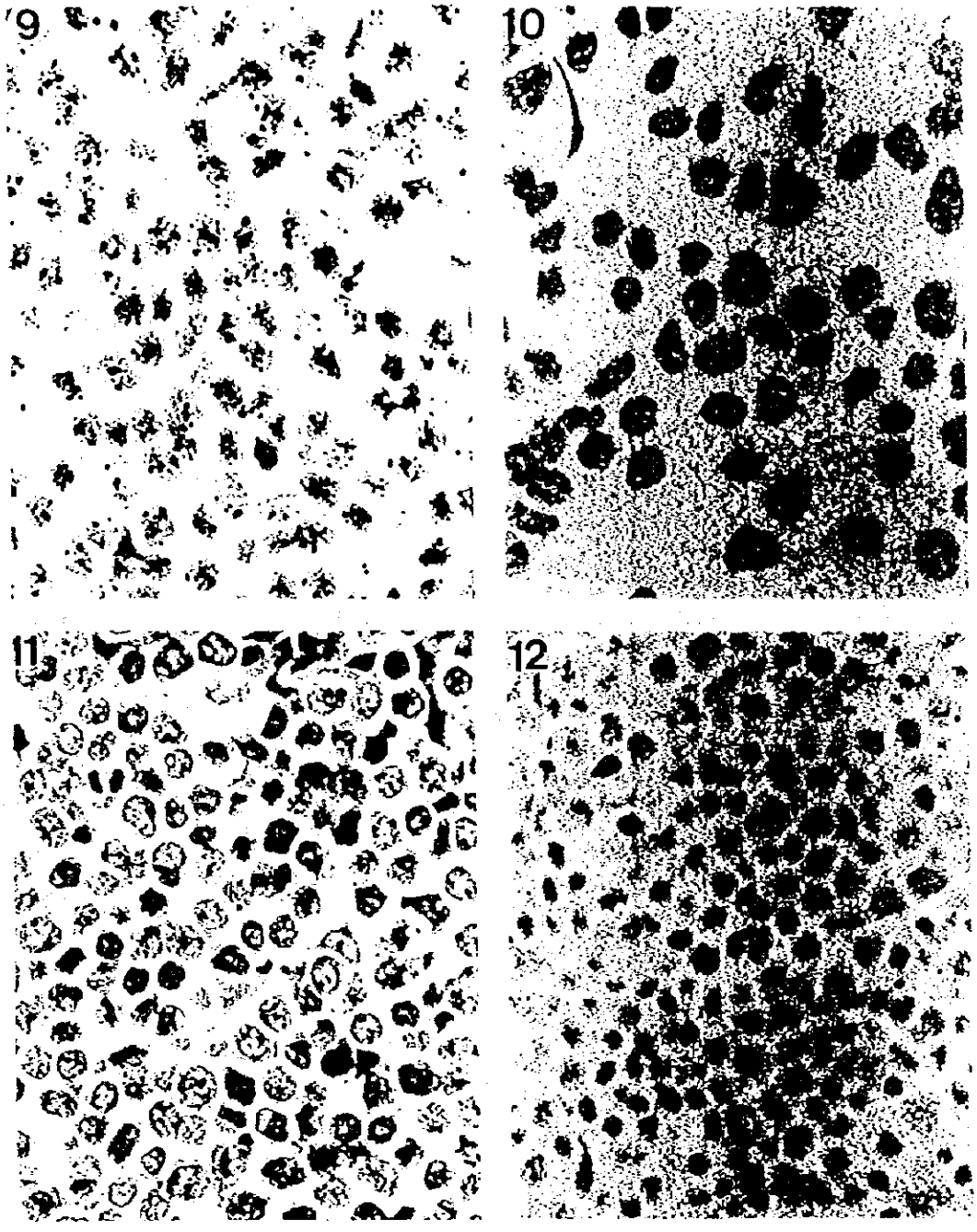

Fig. 9. Case No. H-4. Increased in AgNORs seen in a AgNOR high grade malignancy that was once diagnosed low grade malignancy by routine histopathology. Paraffin section of lymph node. ×600. One-step AgNOR stain.

Fig. 10. Case No. H-5. AgNOR low grade malignancy showing no conspicuous increase in AgNORs. Imprint smear of lymph node. ×600. One-step AgNOR stain.

Fig. 11. Case No. H-5. Medium-sized cell type, diffuse lymphoma according to the classification proposed by lymphoma study group in Japan. ×600. HE stain.

Fig. 12. Case No. H-5. AgNOR low grade malignancy showing no conspicuous increase in AgNOR positive granules. Paraffin section of lymph node. ×600. One-step AgNOR stain.

Hydranencephaly in Newborn Calves in Zambia

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ABSTRACT. Hydranencephaly without arthrogryposis was observed in three newborn Holstein-Friesian calves, born during one week in a 400-cow dairy herd in Zambia. The affected calves were blind and exhibited nystagmus and depressed behavior. Akabane virus infection was suspected based on the pathological findings and etiological data, however, we could not determine the exact causative agent.—KEY WORDS: hydranencephaly, Zambia.

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Hydranencephaly is defined as a complete or almost complete absence of cerebral hemispheres, leaving only membranous sacs filled with cerebrospinal fluid. It occurs in many domestic animals, but is most common in calves, either sporadically or as minor epizootics [12]. As hydranencephaly in calves has a high incidence in specific geographic locations in certain years only, viral infections are considered as possible etiologic factors [5]. The viruses that are well established as potential causes of hydranencephaly in calves are Akabane [8], bovine virus diarrhea-mucosal disease (BVD-MD) [2], bluetongue (BT) [9], Wesselsbron (WD) [4], and Chuzan virus [10]. The present paper describes an occurrence of hydranencephaly in Holstein-Friesian calves in Zambia, and discusses possible etiologies.

Three Holstein-Friesian calves aged 27 to 34 days exhibited clinical symptoms including blindness, nystagmus, and depressed behavior soon after birth. All three calves were born within one week during September 1989 from dams that calved for the first time, in a 400-cow dairy farm located 30 kilometers northeast of Lusaka, Zambia. The same dams gave birth to normal calves after the subsequent pregnancies. On the same farm, three calves born between June and July 1987, were reported to have manifested hydranencephaly. Moreover, on the neighboring farm with two stud herds of 250 purebred Boran cows and 200 purebred Hereford cows, four arthrogrypotic calves have been found between August and November 1987 (M. Schneebeli, unpublished data).

The calves were sacrificed by bleeding under anesthesia and samples for histological examination were fixed in 10% formalin and processed routinely, sectioned, and stained with hematoxylin and eosin (H.E.) in case 1 and 2. Serum antibodies against Akabane, BVD-MD, BT, WD, and Rift Valley fever (RVF) viruses were titrated in the serums of the three calves, collected after colostrum ingestion. The dams of three calves were vaccinated against RVF approximately three months before insemination.

Macroscopical findings were mainly observed in the central nervous system. Case 1 showed hydranencephaly and porencephaly of the cerebellum, case 2 and 3 had hydranencephaly. The basal ganglia, hippocampus, thalamus, and midbrain were slightly reduced in size and were exposed due to the almost complete loss of the cerebral hemispheres (Fig. 1). Mild to moderate decubitus was a common finding in all cases. Case 1 showed enlargement of lymph nodes and case 2 showed lobar pneumonia of the right middle lobe, respectively. However, none of the calves had arthrogryposis.

Microscopically, the remaining cerebrum, interbrain, and midbrain were hypoplastic (hypoplasia of the neural components of the cerebral hemispheres was most severe) and dilatation of the third ventricle and the mesencephalic aqueduct were apparent, but no inflammatory lesions were observed. The thalamus contained some small foci of mineralization. In the cerebellum, focal cerebellar cortical dysplasias and aplasias in some folia, especially near the cerebellar peduncles, were common findings in both cases. In case 1, there were rarefaction of nerve fibers and cavitations that lacked organized cellular margins in the medullary corpus and the folial white matter (Fig. 2). No

Fig. 1. Hydranencephalic brain in the cranium. Remnants of thin cerebral tissue and exposed basal ganglia, hippocampus, thalamus, and midbrain. Proencephaly of the cerebellum.

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Fig. 2. Low power view of the lesion. Focal cerebellar cortical aplasia. Cavitation in the folial white matter. H.E. × 46.

significant changes were found in the pons, the medulla oblongata, and the spinal cord.

In the serological study, antibodies to Akabane virus in two calves and Wesselsbron virus in one calf were demonstrated, respectively (Table 1). Further serological survey performed on 100 dairy cows from the same farm that we got present three cases and 90 cows from the neighboring farm with Boran purebred cows showed a positive result for Akabane virus in 43% and 30%, respectively (S. Inoue, unpublished data).

In cattle, toxic plants, nutritional deficiencies, and infectious diseases are considered as possible causative factors for hydranencephaly, however, genetic predisposition to hydranencephaly has no evidence in farm animals, including cattle [13]. The occurrence of encephalic malformation and the failure of dams to produce more than a single abnormal calf were in keeping with recognized criterion for viral teratogens [1]. The well-established potential viral causes of hydranencephaly include Akabane [8], BVD-MD [2], BT [9], WD [4], and Chuzan virus [10]. We examined serum antibody titers for the above mentioned four viruses except Chuzan virus. However, the three calves had already ingested colostrum before serum samples were collected. Therefore, we could not clarify the relationship between the present malformation and the Akabane virus infection, serologically.

In bovine hydranencephaly cases, the remaining cerebrum has little diagnostic value, because of the resemblance of the macroscopical and microscopical findings in each viral infection. While, the lesions of the cerebellum associated with hydranencephaly have some value for differential diagnosis.

Table 1. Serological findings with hydranencephaly[a]

	Akabane[b]	BVD-MD[c]	WD[d]	BT[e]
Case 1	1:8	Neg	Neg	Neg
Case 2	1:8	Neg	1:40	Neg
Case 3	Neg	Neg	Neg	Neg

a) Serum test against RVF is all positive for above three samples because of vaccination.
b) Virus Neutralization Test.
c) Indirect Immonofluorescent Antibody Test.
d) Hemagglutination Inhibition Test.
e) Agar Gel Diffusion Test.

There are two types of cerebellar lesions associated with bovine virus induced hydranencephaly: 1) normal cerebellum or slightly small-sized cerebellum (hypoplasia) with or without only rare minor microscopic changes and 2) hypoplasia of the cerebellum with severe microscopic changes. The former includes Akabane [8], WD [4], and BT virus infection [9], and the latter includes BVD-MD [2] and Chuzan virus infection [10]. In Akabane disease, the cerebellum was sometimes reduced its size, but never less than two-thirds of the diameter of the normal cerebellar hemisphere [8]. If the cerebellum had some lesions, it contained only rare minimal microscopic changes [14]. However, there is a controversial description in the cerebellar lesion of the Akabane virus infection [6]. Hartley *et al.* [6] grouped the lesions suspected Akabane virus infections into five groups. Group 5 included the calves born with hydranencephaly and cerebellar cavitation. The pathological findings of the present cases in Zambia resembled those of the Akabane disease epizootic which Hartley *et al.* [6] had observed in Australia.

In southern African countries, the main vector for Akabane virus transmission, the zoophilic species of *Culicoides*, is distributed in Zambia [7], suspected Akabane virus infection cases have been reported in lambs in Rhodesia [11], and the Akabane virus has been isolated from *Culicoides milnei* and *Culicoides imicola* in Zimbabwe [3]. We also found a high percentage of Akabane virus antibody in cattle in Zambia as mentioned above. There is a possibility that our present cases were Akabane virus infection, however, we could not determine the exact causative agent. Further study will be necessary for etiological agents of hydranencephaly in calves in Zambia.

ACKNOWLEDGEMENTS. We thank Dr. H. H. Booker, Veterinary Research Institute, Onderstepoort, for the serology of bluetongue, Wesselsbron, and Rift Valley fever viruses and Messrs. P. Chama, M. Silumbwe, and C. Sakala, for technical assistance.

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ORCHIEPIDIDYMITIS DUE TO *BRUCELLA* IN A KAFUE LECHWE

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INTRODUCTION

Bacteria of the genus *Brucella* are gram-negative, non-motile, non-sporeforming small rods. They are intracellular parasites transmissible to wide range of animal species including man, in which they generally cause nonlethal infections. Early bacteremia is followed by localisation of the infection in the genital organs and monocyte-macrophages.

Several species of wildlife (African buffalo, hippopotamus, zebra, eland and impala) have tested serologically positive for brucellosis[1]. Brucellosis was reported to be widespread in Zambia [2].

Suzuki demonstrated close contact of Kafue lechwe with cattle in the Lochinvar National Park [4], and detected serologically *Brucella* positive Kafue lechwe [5]. In order to find out pathological condition in testis of Kafue lechwe, testes from seven lechwe from Lochinvar National Park, Zambia were examined. The results are presented here.

MATERIALS AND METHODS

During August 1994, seven pairs of testes were collected from Kafue lechwe culled at the Lochinvar National Park, Zambia. The measurements of the testes were recorded. The pieces of testes were fixed in buffered 10% formalin, dehydrated, embedded in wax, thin-sectioned and stained with HE as routine.

RESULTS AND DISCUSSION

The testicular measurements are shown in Table I. The left testis of case 1 was enlarged(8.0 x 7.0 x 5.0 cm) than the average of left testes (6.8 x 4.3 x 4.0). It had thick and hard testicular capsule and the cut surface revealed irregularly scattered caseous foci in the parenchyma.

Histologically the focal lesions were built of necrotic tubules and intertubular interstitial inflammation. The tubules were embedded in granulomatous ground solitarily or coalescently and occluded with thick pus-like detritus. Basal layer of cells were damaged in some tubules. The interlobular interstitial tissue was infiltrated with many lymphomonocytes and neutrophils (Fig. 2,3,4). Vascular changes were marked by hyperemia, hemorrhage and occasional thrombosis. Tunica albuginea was thickened with fibro-granulomatous tissue and small amount of fibrinous exudate was on the serosa. Seminiferous tubules outside the lesions were atrophied and showed changes of aspermatogenesis. The lining cells were edematous and swollen. Sertoli cells and occasionally degenerated germ cells and sperms were observed in the lumen. The right testes had an average size (7.0 x 4.5 x 5.0 cm). Histologically, all testes except the left side of case no. 1 showed mild regressive spermatogenesis(Fig. 1).

In the epididymis, while some tubules were empty or contained small amount of sperms, the others were necrotic and contained some detritus as in the necrotic seminiferous tubules. The interstitial tissue showed fibrosis and infiltration with lympho-monocytic cells.

Orchitis may be interstitial (interlobular), intralobular or necrotizing. Ease of separation of these types varies with the stage of the lesion. Most often it arises by hematogenous infection[3]. In the present case, the lesions were focal granulomatous interstitial orchitis with necrotic and suppurative tubular degeneration. It suggests that lesions were initiated at the interstitium hematogenously and the lesions were spreading through tubules. Since sequestration for necrosis was not yet built and circulatory disturbances were present, the lesion was regarded as subacute phase.

Histological finding of brucellosis is not specific but characteristic. However, the characteristic histological lesions in left tests of one lechwe and the circumstantial information are suggestive of *Brucella* species infection. The bovine brucellosis is prevalent in Zambia, and seropositive *Brucella* cases were detected in the Kafue lechwe by Suzuki [5]. It is more likely that lechwe got infected from domestic cattle which have close contacts with them.

The most of bovine *Brucella*-orchitis occurs unilaterally, but, even so, the affected animals are sterile because of the admixing of

inflammatory products with semen and thermal degeneration [3]. The present affected Kafue lechwe must have been sterile, and since the lesion was progressive, it might have been a potential source of spread of *Brucella* infection to other lechwe in contact in the Lochinvar National Park. It needs further study to find and the extent of *Brucella* infection in Kafue lechwe and other ruminant animals in the Park.

Table 1. Measurements of testes of Kafue lechwe (cm)

Case No.	Right side	Left side
1	7.0 x 4.5 x 5.0	8.0 x 7.0 x 5.0*
2	7.0 x 4.5 x 4.0	7.5 x 4.5 x 4.0
3	7.0 x 4.5 x 4.0	6.0 x 4.5 x 4.0
4	7.0 x 4.5 x 4.0	7.0 x 4.0 x 5.0
5	5.5 x 4.0 x 3.5	6.0 x 3.8 x 3.0
6	7.0 x 4.5 x 4.0	7.0 x 4.5 x 4.0
7	7.2 x 4.7 x 4.0	7.0 x 4.5 x 4.2
Average	6.8 x 4.5 x 4.1	6.8 x 4.3 x 4.0

*: Not included in calculation of average.

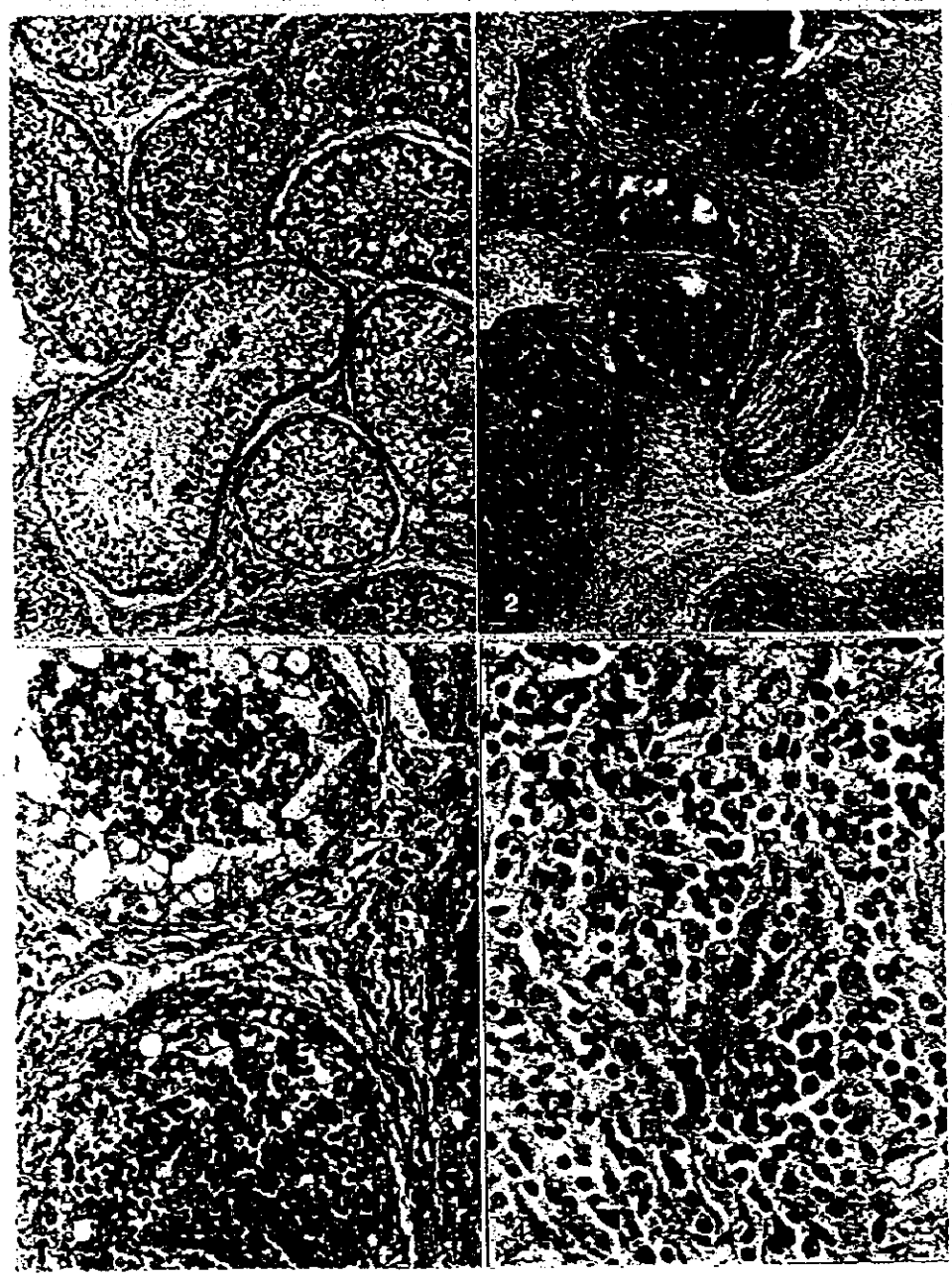

Fig. 1. Mild regressive spermatogenesis in the testis of case No. 3 (x 150).

Fig. 2. Testicular focal lesion. Seminiferous tubules are filled with detritus. A thrombosis is located centrally (x 60).

Fig. 3. Lining cells are still reserved in the upper seminiferous tubule but disappeared in the bottom one (x 300).

Fig. 4. Fibroblasts and inflammatory cells are proliferating interstitially in the testicular lesion (x 600).

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ACUTE FATAL THEILERIOSIS IN A 4- MONTH-OLD ELAND IN ZAMBIA

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INTRODUCTION

The most important species of *Theileria* affecting cattle in southern Africa is *Theileria parva*, and it has now been tentatively divided into three subspecies: *T. p. parva* (the cause of classical East Coast Fever); *T. p. lawrencei* (the cause of corridor disease); and *T. p. bovis* (the cause of Zimbabwe theileriosis)[6]. However, the theileriosis in Zambia has not been classified clearly. *Theileria taurotragi* affects not only eland, but also cattle mildly, and it has been known collectively as *T. mutans*.

Theileriosis in eland has been described [1, 2, 6, 7, 8]. Theileriosis in a young eland, introduced to Zambia from Zimbabwe, was diagnosed. Pathological findings are reported in this communication.

HISTORY OF THE CASE

The eland was male, 4-month-old, born at a game farm in the Kabwe District, in Zambia and weighed 90 kg. The eland herd had originally been introduced from Zimbabwe 2 years ago. The present size of herd was 32 heads. The other elands in the herd were apparently healthy.

The animal was found lying down in the morning 14th February 1995. It showed dyspnea with short rasping breath and discharged fluid from the eyes and mucous from the mouth. Tetracycline was given but proved ineffective, and it died at 11.00 a.m. The animal was slightly dehydrated, otherwise looked in good nutrition. 15 ticks infested on the body.

MATERIALS AND METHODS

The animal was autopsied at the Veterinary Laboratory, School of

Veterinary Medicine, the University of Zambia. During autopsy, smear samples were taken from the blood and spleen and stained with Giemsa stain.

Specimens were taken from liver, spleen, lung, heart, kidney adrenal, bone marrow from femur and rib, thymus and thyroid, fixed in buffered 10% formalin, dehydrated, embedded in wax, thin-sectioned and stained with hematoxylin and eosin.

RESULTS

Autopsy findings: 1. Anemia, 2. Hyperplastic splenomegaly, 3. Hyperplasia of lymph nodes, 4. Cloudy discoloration of the liver, 5. Dark red discoloration of the renal parenchyma, 6. Hyperplasia of the adrenal cortex, 7. Subendocardial petechiae, 8. Frothy discharge in the airduct, 9. Mild jaundice, and 10. Gelatinous degeneration of the femoral bone marrow.

In the blood smear, 87% of erythrocytes contained single to several (maximum nine) dot-, comma- or thread-like piroplasmas and 33% of lymphocytic cells schizonts (Fig. 1).

Histopathologically, most conspicuous finding was presence of amorphous syncytial multinuclear giant cells with schizonts (maximum 350 x 150 μm in size) in the portal veins and sinusoids in the liver (fig. 2), in the pulmonary arteries in the lung, in the cortical and medullar sinuses in bronchial, mediastinal, cervical, hepatic and pancreatic, lymph nodes (fig. 3), and in the bone marrow of the femur and rib (fig. 4). Intralobular focal necrosis in the liver and haemorrhages in the lymph nodes and bone marrow were observed. The spleen was congested and the white pulp was hyperplastic and crowded with lympho-monocytic cells but hemosiderosis was scarce. In the kidney, most of Bowman's capsule contained protein-aceous substance. In the thyroid gland, follicles became small and varied in size and the colloid substance was depleted. The femur bone marrow was hypoplastic and edematous. The thymus was regressive.

The digestive canal and central nervous system revealed no remarkable finding.

DISCUSSION

Martin and Brocklesby (1960) found a protozoan parasite in an eland and it was designated as *Cytauxzoon taurotragi* [7]. In the animal the superficial lymph nodes were enlarged and thin blood film showed a very heavy infection (over 90%) of the erythrocytes. Histologically the liver, lymphnodes and lung showed many schizonts of the *Cytauxyzoon* type. The schizonts varied in size from 10 x 10 μm to 175 x 68 μm, with an average of 58 x 34 μm.

In the present case, the parasites were found in 87 % of the erythrocytes. Amorphous multinuclear giant cells with large schizonts were found not only in the liver, lymph nodes and lung, but also in the bone marrows. The size of schizonts was bigger and their presence in many tissues were extensive than described by Martin and Brocklesby (1960).

The type and location of exoerythrocytic schizogony is used as the basis for dividing the family *Theileriidae* into two genera: *Theileria*, with small or medium-sized exoerythrocytic schizonts in lymphocytes, and *Cytauxyzoon*, with large exoerythrocytic schizonts in phagocyte cells [5]. Stagg et al. succeeded to cultivate *Cytauxyzoon* from a sick eland and demonstrated the schizonts in lymphoblastoid cells and in monocytic macrophage-like cells [8]. Now *Cytauxyzoon taurotragi* is called *Theileria taurotragi*.

A stabilate of *Theileria sp. (T. taurotragi)* appeared to have low pathogenicity for eland and cattle although one eland died on challenge, showing a 50 % piroplasm parasitemia [8]. *T. taurotragi* from eland and *Theileria sp.* (INDOBOGO) from cattle are same species which are adapted to different hosts [3,4]. The present case was diagnosed as fatal *Theileria taurotragi* infection, but there was no gastrointestinal lesion as reported by Grootenhus et al. [1]. We also observed large collections of schizonts in massive multinucleated cell even in the bone marrow, not reported earlier in eland.

The infected parasite would have been introduced with eland from Zimbabwe or one from cattle kept nearby. The reason for why only one eland out of 32 was affected seriously might depend on individual disposition.

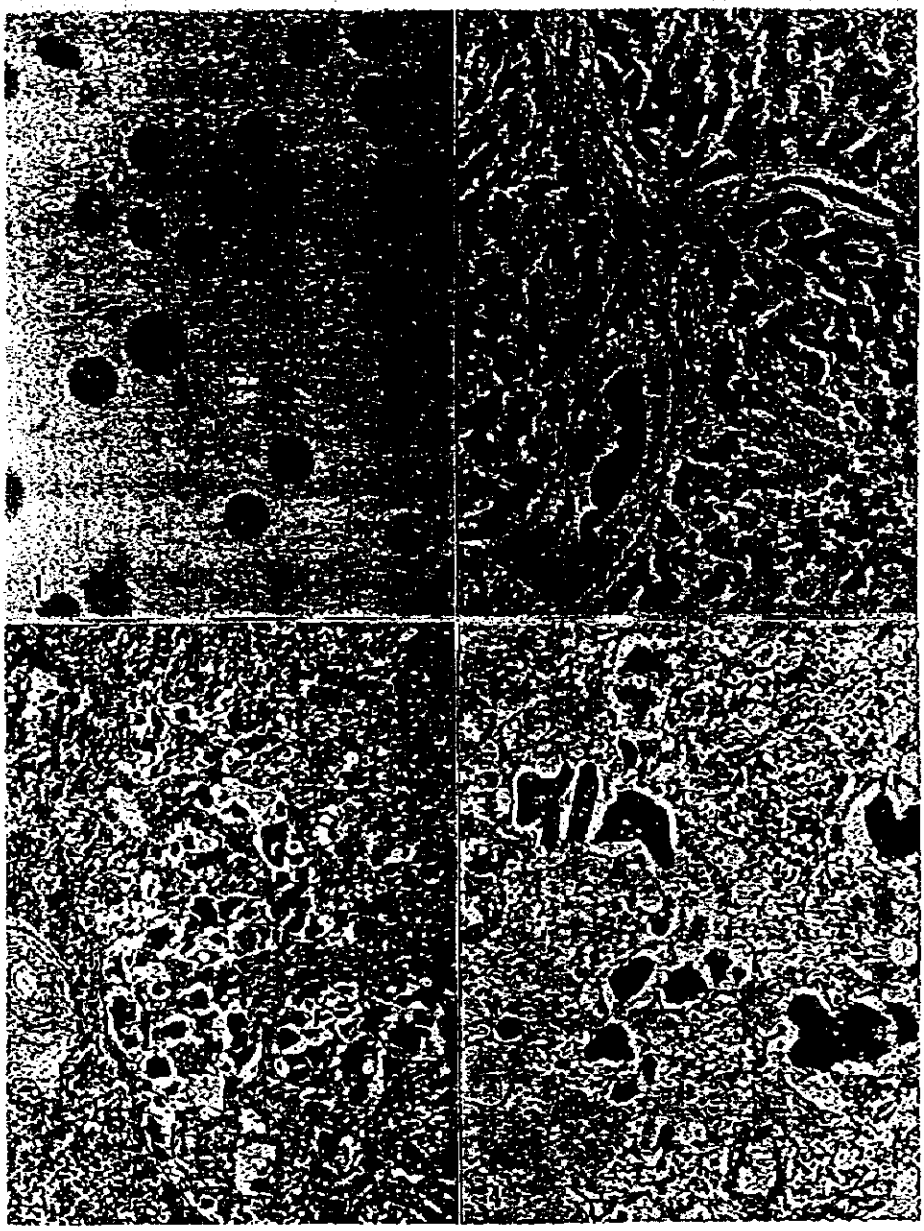

Fig. 1. Blood smear. Plural small-sized piroplasmas in almost all erythrocytes (x 1500).

Fig. 2. Schizonts-laden multinuclear giant cells in portal vein and sinusoid (x 150).

Fig. 3. Numerous schizonts-laden cells in sinus of the bronchial lymph node (x 60).

Fig. 4. Schizonts-laden multinuclear giant cells in the femoral bone marrow (x 60).

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GLOBOID CELL LEUKODYSTROPHY IN A MALTESE DOG

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Globoid cell leucodystrophy (GCL), Krabbe's disease, is an inherited (autosomal recessive) disorder caused by deficiency of the enzyme β galactocerebrosidase in oligodendrocytes and Schwann cells. Deficiency of the enzyme results in an accumulation in both types of cell of galactocerebroside or its precursor psychosin with resultant damage. The degenerated cells are taken up by macrophages, globoid cells, and the disease is characterised by demyelination, axonal loss and astrogliosis throughout the central and peripheral nervous systems. In the dog, GCL has been recognised in the Basset hound, Beagle, Blue tick hound, Cairn terrier, Dalmatian, Miniature Poodle, Pomeranian and West Highland White terrier. The authors report here the first case of GCL in a Maltese dog. The case is a four month old male non-pedigree Maltese dog born in Lusaka. The animal had hind leg paralysis and urinary incontinence unresponsive to treatment. Its two litter mates had been affected with similar clinical signs previously. The dog was autopsied on May 14, 1990. Histologically, many macrophages or multinuclear giant cells containing a PAS-positive substance (globoid cells) were observed in the white matter of the cerebrum, brain stem, cerebellum and spinal cord. This lesion was bilaterally symmetrical. The symptoms are difficult to differentiate clinically from other disorders of the central nervous system. The lineage of GCL may be present in Zambia. Small animal practitioners should keep this disease in mind.

SMALL RUMINANTS AND COMPANION ANIMALS

OVINE VERMINOUS ENCEPHALOMYELITIS: SETARIOSIS?

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Five Dorper lambs raised on a farm near Lusaka suffered from posterior paralysis in February 1995. One of them was autopsied at the School of Veterinary Medicine 1 hour after death. The carcass maintained a high body temperature even after death. Acute pulmonary congestive oedema was the most marked necropsy finding. Histologically, acute or subacute focal lesions were found in the spinal cord and brain. Since these foci had apparently arisen from mechanical destruction, and were accompanied by eosinophil infiltration, it was assumed that these lesions resulted from parasitic migration, although the worm itself was not detected. The most probable parasite must be *Setaria* spp. Ovine paralysis due to *Setaria digitata* occurs often in Asia and the pathogenesis has been studied well. The proper host of *S. digitata* is cattle, and aberrant migration to the central nervous system of sheep occurs in the rainy season after microfilaria infestation through mosquito transmission. *S. digitata* has not been detected in Africa but *S. labiatopapillosa* is found commonly in cattle in Zambia. Besides, many species have been observed in Zambian wild ruminants: *S. hornbyi, labiatopapillosa, boulengeri, pillersi, africana and yorkei*. We suspect that ovine paralysis caused by *Setaria* spp. may be in existence and overlooked. We would like to detect the larva, identify the species and clarify the pathogenesis of this disease in collaboration with practitioners.

THE FIRST CASE OF CANINE VISCERAL LEISHMANIASIS IN ZAMBIA: PATHOLOGICAL FINDINGS

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The first case of canine visceral leishmaniasis in Zambia was discovered in a 12 year old male Australian cattle dog (Australian heeler) in September 1994. At necropsy, splenomegaly, fatty degeneration of the liver and nodular lesions in the renal cortex were observed. A prominent histopathological finding was proliferation and spreading out of amastegote-laden macrophages (ALM). The ALM were distributed throughout the spleen. There was severe fatty degeneration in the liver and ALM were scattered diffusely or as aggregates accompanied with monocytic infiltration. ALM were scattered in the alveolar capillaries of the lung with little cellular reaction. ALM free within blood vessels were observed in the liver and lung. There were focal lesions caused by ALM and released amastegotes in the adrenal cortex. The nodular renal lesions were diagnosed as degenerative interstitial nephritis, which was caused probably by amastegotes released from the macrophages. It seemed that the renal cortical interstitium was not a favourable environment for development of *Leishmania*. Since the dog had been kept in Lusaka and was obviously parasitaemic, based on the histological findings, the animal would have been a carrier of leishmaniasis. The first human case of visceral leishmaniasis in Zambia was reported from the Southern Province in 1973. Sand flies are known to be vectors of leishmaniasis, however, their ecology in Zambia is obscure. It is known that *Leishmania* can be transmitted by direct contact with the lesion or mediated by some blood-sucking arthropods. The prevalence of *Leishmania* is high in HIV-infected patients. Infection of leishmaniasis is related to immunosuppression, and so visceral leishmaniasis could well be a serious problem in Zambia.

Onderstepoort Journal of Veterinary Research, 64:77–79 (1997)

RESEARCH COMMUNICATION

Canine visceral leishmaniosis: first case in Zambia

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Visceral leishmaniosis was discovered in a male 12-year-old Australian cattle dog in September 1994. Canine leishmaniosis has not previously been reported in Zambia. At necropsy, splenomegaly, fatty degeneration of the liver and focal lesions in the renal cortex were observed. Histopathologically, focal diffuse proliferation of amastigote-laden macrophages (ALM) were found in the spleen and liver. Amastigotes were diffusely distributed in the pulmonary alveolar wall, and formed minute lesions in the adrenal cortex. Focal degenerative interstitial nephritis and myocarditis were observed, but ALM were hardly found in these lesions. The animal had lived in Lusaka for the previous 2 years at least, where it was likely to have had a *Leishmania* parasitaemia. Although the ecology of sandflies in Zambia is still veiled, the present case of canine leishmaniosis could be an indication of widespread leishmaniosis in this coutry, not only in dogs, but also in humans, particularly as human immunodeficiency virus (HIV) infection is prevalent in the country.

The subject was a male 12-year-old Australian cattle dog brought to the Veterinary Teaching Clinic at the University of Zambia on 5 September 1994, with a 5-d history of anorexia, polydipsia and longstanding ocular discharge. The dog was suspected to have been poisoned with horticultural chemicals. The animal had been kept in Lusaka for at least the previous 2 years, but no previous history was available.

Necropsy was performed within 2 h of death. Specimens from the spleen, liver, kidney, heart, lung, small intestine, pancreas, urinary bladder, prostate, testis, adrenal gland, thyroid and brain were taken and fixed in 10% buffered formalin, thin-sectioned after routine processing, and stained with haematoxylin and eosin (H & E).

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Physical examination of the dog revealed tachycardia, weak pulse, icterus and splenomegaly. Haematological data were as follows:

Packed-cell volume	29 %
Red-blood cells	$3{,}7 \times 10^6/\mu\ell$
White-blood cells	$14{,}6 \times 10^3/\mu\ell$
Neutrophils	13,090/µℓ
Lymphocytes	1,095/µℓ
Eosinophils	131 /µℓ
Monocytes	263 /µℓ
Basophils	29 /µℓ
Haemoglobin	9,2 g/dℓ
Total protein	8,0 g/dℓ
Serum protein	6,5 g/dℓ
Aspartate aminotransferase (AST)	39,7 [normal value (n.v.) quoted by Bistner & Ford 1995] 5–80] IU/ℓ
Alanine aminotransferase (ALT)	43,1 (n.v. 5–25) IU/ℓ
Alkaline phosphatase (ALP)	379,5 (n.v. 20–120) IU/ℓ
Gamma-glutamyltransferase (GGT)	13,9 (n.v. 1,4–11,5) IU/ℓ
Blood-urea nitrogen (BUN)	55,7 (n.v. 10–22) mg/dℓ
Creatinine	5,1 (n.v. 0,4–1,5) mg/dℓ

Body mass was 20 kg. Mild jaundice was observed. The spleen was swollen (30 x 5 x 2 cm) and the parenchyma was pulpy-hyperplastic and unevenly congested. The liver had a mass of 900 g and was light yellowish brown. Many irregular, whitish focal lesions were present in the kidney cortex. The heart was dilated and round in shape. The aorta had several hollowed scars in the intima caused by migration of *Spirocerca lupi*. The right testicle was atrophied with a thick capsule, while the left showed nodular hyperplasia.

The spleen was congested unevenly and ALM were scattered diffusely or as aggregates throughout the parenchyma (Fig. 1). The ALM were ovoidly swollen, and contained about 20 or more amastigotes. The average amastigote size was 3,0 x 2,4 µm, and had an eccentrically located, ovoid nucleus and a structure corresponding with that of a kinetoplast. Walls of some central arterioles were thickened with hyalin. ALM of various sizes were scattered diffusely or

as aggregates throughout the liver, accompanied by slight lymphocytic infiltration. In the heart, necrotizing inflammations with marked infiltration of lymphocytes and monocytes were observed. Some intramyocardial arterioles were thickened with hyalin. In the lung, amastigotes were spread diffusely or within macrophages in the alveolar walls (Fig. 2). In the kidney, the lesions observed macroscopically, were focal aggregates of degenerated monocytic cells in the interstitium, whereas ALM were hardly found. In the adrenal gland, minute degenerating lesions were found in the zona glomerulosa, accompanied by ALM and free amastigotes released from ruptured ALM. A few lymphocytes infiltrated into the leptomeninx. Fibrous thickening of arterial walls and demyelination were noted in the cerebellar white matter. The testes were atrophied and aspermatogenetic.

The amastigotes were round or ovoid in shape, and the size, 3,0 x 2,4 µm, ranged between the previously reported 2,5–5,0 x 1,5–2,0 µm (Slappendel 1988) or 2,4–3,1 x 1,5–2,6 µm (George, Nielsen, Shively, Hopek & Mroz 1976). The nucleus was round and located eccentrically, and a kinetoplast was observed in the cytoplasm. While the ALM in the present case contained about 20 or more amastigotes in the section (Fig. 1), George *et al.* (1976) reported that 25–30 amastigotes were found in macrophages, and Simpson, Harvey & French (1982) reported their finding of at least ten amastigotes per macrophage in the bone marrow.

Visceral leishmaniosis in the Old World is caused by parasites of *L. donovani* complex (*L. donovani*, *L. infantum* and *L. chagasi*) (WHO 1990). In Africa, endemic areas of visceral leishmaniosis are located in northern and eastern Africa (WHO 1984, 1990). In Zambia, only two cases of human leishmaniosis have been reported; the first was in Southern Province, by Hira, Naike & Egere (1973), and the other in 1976 (WHO 1990). The disease has been found in adjoining countries such as Tanzania, Namibia, Malawi, Zaire and Zimbabwe (WHO 1990). In South Africa, cutaneous leishmaniosis was diagnosed in sheep in 1989 and 1990, and the visceral form was diagnosed in two dogs, one in Durban, in 1964 (of which the life history was uncertain), and the other (which had never left the country) in the Orange Free State, in 1987 (Van der Lugt & Stewart 1994).

Because a full history was not available for the present case, it is not known where the animal had become infected. Even if the dog had been infected in another country, it had been kept in Zambia for at least 2 years, where it could have been a parasitaemic carrier. The acute onset of disease in this case was suspected to have been caused by poisoning which resulted in hepatic fatty degeneration. Ferrer (1992) mentioned that elevation of ALT and AST could occur in canine visceral leishmaniosis. Elevations of AST, ALT, ALP and GGT in the present case, were probably related to both poisoning and hepatic *Leishmania* infection. Increases of BUN and creatinine were apparently related to the renal lesions (Ferrer 1992).

In the present case, the dog was 12 years old, and in the histological findings there were apparently senile changes such as testicular atrophy and fibrous thickening of the arterial walls in the brain. Hyaline degeneration in the spleen and heart might be amyloid degeneration, as described by George *et al.*

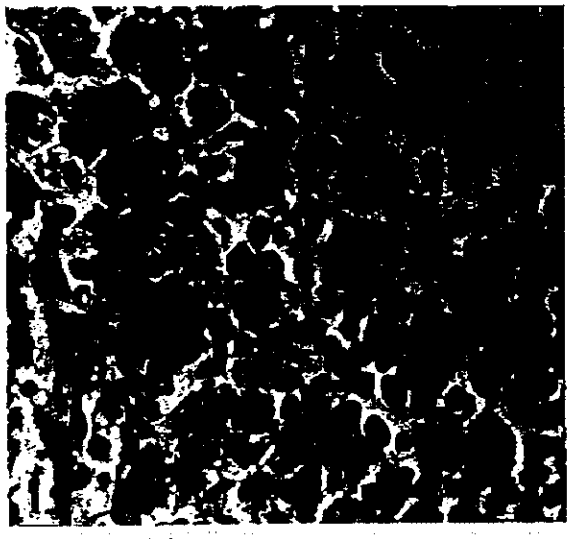

FIG. 1 Amastigote-laden macrophages are gathered in the red pulp of the spleen

x 600, H & E stain

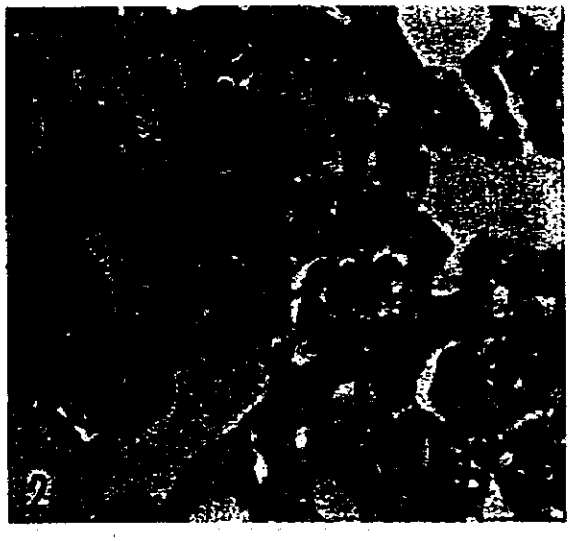

FIG. 2 Amastigotes are observed in macrophage (arrow) or diffusely (around star marks) in the pulmonary alveolar walls

x 600, H & E stain

(1976), regarding visceral leishmaniosis. Mild lymphocytic infiltration in the leptomeninx might be related to generalized leishmaniosis, while the demyelination in the cerebellar white substance would be a senile change.

Ecology of sandflies, vectors of leishmaniosis in Zambia, is not described in the textbook of Ashford & Bettini (1987) nor by Lewis & Ward (1987). *Leishmania* is transmissible by other blood-sucking arthropods or by direct contact (Longstaffe, Jefferies, Kelly, Bedford, Herrtage & Darke 1983; Huss & Ettinger 1992; Slappendel 1988). Leishmaniosis is known to be an opportunistic infection, and is prevalent among HIV-infected patients with acquired immune deficiency syndrome (AIDS) (Dedet, Lambert & Pratlong 1995; Baily & Nandy 1994). Numazaki, Luo & Suzuki (1994, 1995, 1996) reported that the HIV-positive rate in Zambia was high:

Patients in the University Teaching Hospital (UTH)	54.0 %
Mothers of children in the urban community of Lusaka	22.3 %
Children admitted to UTH	43.6 %

The occurrence of canine visceral leishmaniosis could indicate a risk of canine and human leishmaniosis spreading in Zambia.

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SIGNIFICANCE OF OCCURRENCE OF CANINE SALMONELLOSIS IN LUSAKA

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Infections with organisms of the *Salmonella* spp. is of importance from two distinct aspects: food poisoning in man and disease in domestic animals. In dogs, signs of salmonellosis may be mild or severe gastro-enteritis, septicaemia and death. We report the occurrence of salmonellosis in three companion dogs (case No. 1: 7 years old; case Nos. 2 and 3: both 2 months old) in Lusaka, Zambia. Clinically, all the cases exhibited nervous signs and had a history of diarrhoea. Besides, case No. 1 had a persistent vaginal discharge and case No. 3 belonged to a litter of six puppies which had an episode of diarrhoea. *Salmonella enteritidis* was isolated from the vaginal discharge and liver of case Nos. 1 and 2 respectively, while *S. typhimurium* was isolated from various organs of case No. 3. These species of *Salmonella* are also pathogenic to man. Predominant histopathological findings included colitis and meningitis in case No. 1. Case No. 2 was associated with canine distemper characterised by a demyelinating encephalitis and bronchopneumonia, and there was meningo-encephalitis in case No. 3. These dogs were apparently shedding the organisms in the faeces or discharges while raised in circumstances close to people. Thus, salmonellosis in companion dogs may be a source of infection for humans. The neurological signs manifested by the present cases may confound the diagnosis of salmonellosis and may be mistaken for rabies, canine distemper or other causes of encephalitis. Veterinarians in Zambia should therefore bear this in mind.

An outbreak of aflatoxicosis in swine: Clinical, clinical pathological

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Abstract

An outbreak is reported in a pig herd at an agricultural training institute, which was fed mouldy maize. Clinical signs observed included, vomiting, recumbency, paddling, trembling, unsteady gait, weight loss and death. A total of 18 pigs (60%) died within two weeks from a herd of 30. There was a significant increase in the liver enzymes, GOT and GPT. Blood urea nitrogen was also markedly elevated. Necropsy of two pigs revealed congested livers and petechiae haemorrhages in the *Longisimus dorsi* muscles. Analysis of the poorly stored mouldy grain and livers using thin layer chromatography revealed aflatoxin B. Although information on the prevalence of mycotoxicoses in Zambia is limited, conditions for their widespread occurrence exist throughout the country especially during the rainy season.

Poultry Disease

ASPECTS OF NEWCASTLE DISEASE IN ZAMBIA

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In 1952 Newcastle Disease(ND) was first reported in Zambia. We started virological investigations into ND in 1988 and isolated Newcastle Disease Virus(NDV) from chickens at the end of 1988. Since then NDV has been isolated in 6 cases and therefore present situation and countermeasures are described.

PRESENT SITUATION

Although our ND investigations are recent, NDV has already been isolated in 6 cases(Table 1). In most cases there was hemorrhagic ulceration in the digestive tract and a relatively high mortality. Further serological investigation using the Hemagglutination Inhibition(HI) test revealed high percentage antibody positive (74 %) in the whole country. This finding suggests that NDV infection exists throughout Zambia.

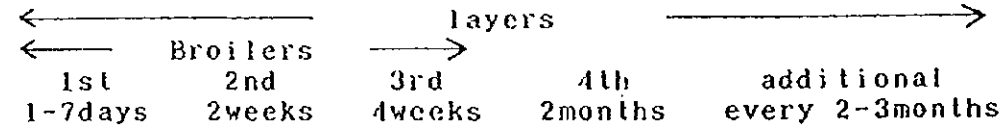
Table 1 NDV isolated field cases

No	Date	Place	Breed(Species)	Age	Mortality	Type of farm
1	12/88	Mazabuka	avian	-	-	-
2	6/89	Mazabuka	broiler	6 weeks	97%	school
3	7/89	Mazabuka	local	-	90%	compound
			guinea fowl	-	-	
4	7/89	Mongu	local	-	40%	-
5	12/89	Lusaka	layer	32 weeks	30%	commercial
6	2/90	Lusaka	layer	10 weeks	70%	commercial

Note:Mortality includes estimates

PREVENTION

Vaccination is the most important and useful preventative action against ND and ND live vaccine(B1 strain) is available in Zambia. As infection in Zambia is widespread the following vaccination program is recommended:



ESTABLISHMENT OF DIAGNOSTIC PROCEDURE

Correct diagnosis is the first step in the prevention of any diseases including ND. However virus isolation requires sophisticated facilities and equipment. Therefore serological examination, especially HI test which requires only simple equipment, is recommended at present. In order to establish this test in the country, we will endeavour to supply HI antigen in the future.

Avian diseases diagnosed at the School of Veterinary Medicine

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This article reports on the findings of a retrospective study of avian diseases diagnosed at the Veterinary School during the years 1989 and 1990. The findings for both broilers and layers are given in tables 1 and 2. There were five major significant findings:

1. Omphalitis was the most frequently diagnosed disease in broiler chickens (20.8% of broiler cases).
2. In layers, salmonellosis was the most often diagnosed disease (21.3% of cases in layers).
3. Vitamin E deficiency was found to be a serious problem in broilers (17.8% of broiler cases).
4. Gumboro disease was the second most common disease in layers (9.8%).
5. Coccidiosis was a significant problem in both layers and broilers, being 6.6% and 5.9% respectively.

These results can be used to indicate disease problems in commercial flocks only as the greater majority of avian cases presented at our school come from commercial flocks. They indicate that there is much that can be done from a husbandry point of view to increase the performance of commercial poultry flocks.

Table 1: Avian diseases diagnosed in broiler chickens during 1989 and 1990

Disease	No. of cases		Percentage	
	1989	1990	Total	(%)
Orephalitis	7	14	21	20.8
Vitamin E deficiency	14	4	18	18.2
Coli septicaemia	8	6	14	13.9
Mycoplasmosis	4	4	8	7.9
Staphylococcal infection	4	4	8	7.9
Salmonellosis	5	2	7	6.9
Coccidiosis	5	2	7	6.9
Gumboro disease	3	2	5	5.0
Mismanagement	1	3	4	4.0
Nutritional deficiency	1	2	3	3.0
Heat stroke	0	2	2	2.0
Streptococcal infection	2	0	2	2.0
Aspergillosis	0	1	1	1.0
Marek's disease	1	0	1	1.0
Vitamin A deficiency	1	0	1	1.0
Total	52	49	101	100.0

Table 2: Avian disease diagnosed in layer chickens during 1989 and 1990

Diseases	No. of cases 1989	1990	Percentage Total	(%)
Salmonellosis	1	12	13	21.3
Gumboro	1	5	6	9.8
Infectious coryza	3	3	6	9.8
Coccidiosis	0	4	4	6.6
Coli septicaemia	4	0	4	6.6
Mycoplasmosis	1	3	4	6.6
Mismanagement	0	3	3	4.9
Nutritional deficiency	0	3	3	4.9
Omphalitis	2	1	3	4.9
Vitamin A deficiency	0	3	3	4.9
Aspergillosis	2	0	2	3.3
Newcastle disease	1	1	2	3.3
Avian leucosis	0	1	1	1.6
Fowl pox	1	0	1	1.6
Marek's disease	1	0	1	1.6
Poisoning	1	0	1	1.6
Pseudomonad infection	1	0	1	1.6
Staphylococcal infection	1	0	1	1.6
Streptococcal infection	1	0	1	1.6
Vitamin E deficiency	0	1	1	1.6
Total	23	38	61	100.0

An Occurrence of Vitamin A Deficiency in Chickens in Zambia

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KEY WORDS: chicken, vitamin A deficiency, Zambia.

Recently, commercial poultry farms have been developed rapidly in Zambia and productivity of these farms is deeply dependent on the quality of feeds. Vitamin A is one of the most important nutrients in poultry diets; it is essential for optical vision, growth and integrity of mucous membranes [3]. Vitamin A deficiency, which causes a high mortality, retarded growth, and low egg production in poultry [5], is therefore responsible for economic loss of the farms. Although it is sometimes difficult to be distinguished from other respiratory diseases [3], it is very important to diagnose the disease accurately to avoid severe economic loss. Vitamin A deficiency in poultry in Africa has been reported in Uganda [1] and Nigeria [6]. The present report is the first description of the disease in Zambia.

The flocks concerned were housed on a poultry farm in Kabwe, 100 km north of Lusaka. There were 4 layer flocks and 1 broiler flock. Day old chicks were bought from a nearby hatchery and the initial numbers in the flocks are given in Table 1. They were fed with broiler-starter until 3 weeks old in broilers and layers, broiler-finisher until 8 weeks old in broilers and until 20 weeks old in layers. Layers were given layers-mash after 20 weeks of age. These poultry feeds were produced by a company in Kabwe and the composition was not indicated.

Autopsy of five birds from each flock was carried out at the School of Veterinary Medicine, University of Zambia. For histological examination, organs including the mucous membranes of oral cavity and eyelids from birds in the first and fifth flocks were fixed in 10% formalin. Paraffin sections were cut and stained with hematoxylin and eosin (HE).

The outline of clinical symptoms and the mortality is given in Table 1. Birds in the first and fifth flocks were emaciated. Their eyelids were stuck together and a large amount of caseous exudate was presented in the eye (Fig. 1). White pustule-like lesions of various sizes, from pin point to 3 mm in diameter, were observed on the mucous membrane of the oral cavity, pharynx, esophagus and tongue (Fig. 2). Birds in the second layer flock showed no ovarian activity. However, the birds in the third and fourth layer flock showed no specific lesions.

The main histological lesions were seen on the mucous membranes of the tongue, palate, pharynx and throughout esophagus. The squamous epithelium of those mucous membrane showed marked acanthosis and hyperkeratosis. No inclusion body was seen in the epithelial cells. The mucous glands of those tissues were severely distended by squamous metaplasia and filled with epithelial debris (Fig. 3). The mucous membrane of eyelids showed slight acanthosis and hyperkeratosis. No specific lesions were seen in the other tissues.

All birds in the flocks were given a mixture of 20% of newly produced concentrate with 80% maize meal immediately after the macroscopical diagnosis of vitamin A deficiency. The concentrate was mixed with 5% of poultry premix which contains high level of vitamin A (Table 2). The mortality in the first and fifth flocks decreased rapidly, and egg productivity in the other flocks improved dramatically within a week. However, other 100 birds (25%) from the broiler flock were culled because of severely retarded growth.

The first layer and fifth broiler flocks showed a high mortality and pustule-like lesions in the oral and esophageal mucosa as well as typical clinical signs such as retarded growth and swollen eyes [3–6]. The second, third and forth layer flocks showed only reduced egg productivity which responded to the treatment immediately. As absorption of vitamin A is quite rapid, chickens not in an advanced stage of deficiency should respond promptly [3].

Table 1. Clinical symptoms and the mortality observed in five flocks with vitamin A deficiency

No	Chicken	Age (weeks)	Number in flock	Clinical symptoms	Mortality
1	Layer	5	200	Retarded growth Swollen eyes	50%
2	Layer	30	600	No egg production	<1%
3	Layer	50	600	Reduced egg productivity from 60% to 30%	<1%
4	Layer	70	600	Reduced egg productivity from 60% to 30%	<1%
5	Broiler	6	400	Retarded growth Swollen eyes	50% and 25% culled

Fig. 1. Swollen eyes and a large amount of cheesy material of a chicken from the fifth flock.

Fig. 2. Pustule-like lesions in the pharynx and esophagus of a chicken from the fifth flock.

Fig. 3. Distended mucous glands filled with epithelial debris in the esophagus from a chicken in the fifth flock. HE stain. × 125.

Based on these findings, all flocks concerned were diagnosed as having vitamin A deficiency.

Young chicks given a vitamin A deficient diet may exhibit clinical signs at the end of the first week if the birds are the progeny of hens fed a diet low in vitamin A. On the other hand, if they are a progeny of hens receiving adequate amount of vitamin A, signs and lesions associated with vitamin A deficiency may not appear until the 6th or 7th week of age [3]. In the present case, the chicks of the first and fifth flocks displayed clinical symptoms at 2

Table 2. Contents of premix in each 5 kg

Item	Quantity
Vitamin A	12,000,000 IU
Vitamin D3	2,000,000 IU
Vitamin E	20,000 IU
Vitamin K3	2 g
Vitamin B12	15 mg
Vitamin B6	4 g
Vitamin B2	7 g
Vitamin B1	2 g
Methionine	1,300 g
Biotin	100 mg
Folic Acid	. 1 g
Niacin	30 g
Choline	250 g
Calcium Pantothenate	10 g
Zinc	50 g
Selenium	15 g
Iodine	1 g
Iron	25 g
Manganese	70 g
Copper	5 g

and 3 weeks of age, respectively. Therefore, this suggests that chicks in these two flocks were the progeny of hens receiving an insufficient amount of vitamin A. Insufficient vitamin A during foetal development would have contributed to the high mortality seen in these flocks.

Uche [6] reported vitamin A deficiency in chickens associated with intestinal coccidiosis and Auro [1] reported the disease in association with ascariasis. Infection with intestinal coccidiosis may cause a reduction in plasma carotenoid levels [2] and a reduction in liver stores of vitamin A [3]. Also, failure to convert carotene to vitamin A in the intestinal tract has been known to occur in chronic intestinal parasitism [1]. However, there were no parasites in any flocks in the present case. It is likely that vitamin A deficiency in the present case was caused by feeding of vitamin A deficient feeds.

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Coccidiosis which brings about minor economical loss as long as anticoccidial drugs are used has not been significant in this country. However occasional outbreaks have been reported, possibly due to bad management. Almost all nutritional problems might have resulted from the bad quality of chicken feed available. In many cases farm-made feed produced nutritional problems. Nutritional disorders highlight a need to improve feed quality for chickens. Cases of other diseases such as mismanagement and omphalitis were often submitted from small farms. Although many omphalitis cases must have been related to colibacillosis, the cases which were not checked by culture were included in the category of other diseases.

In conclusion the present figures show the occurrence of many simple bacterial infections and small number of viral disease cases.

Reference

GROSS, W. B. (1984) Colibacillosis. In: Diseases of Poultry. 8th edn. Eds. M. S. Hofstad. H. J. Barnes, B. W. Clarnek, W. M. Reid and H. W. Yoder. Jr. Iowa State University Press.

Table 1. Cases of avian disease at the diagnostic laboratory of the School of Veterinary Medicine at the University of Zambia.

Diseases	1989	1990	1991	1992	1993
Colibacillosis	14	5	4	31	56
Salmonella infections					
Fowl typhoid	0	9	10	13	31
Other serovars	6	5	3	13	8
Other enterobacteriaceae	0	0	4	0	2
Staphylococci	5	4	1	5	5
Streptococci	3	0	2	0	3
Pseudomonas	0	0	2	0	1
Mycoplasma	3	9	12	3	1
Other bacteria	3	3	3	0	1
Fungal infection	2	1	0	0	0
Marek's Disease	1	0	0	0	0
Newcastle Disease	1	1	5	2	1
Fowl Pox	1	0	2	0	0
Gumboro Disease	4	7	5	0	4
Coccidiosis	3	7	6	4	1
Other parasites	0	0	0	2	0
Vitamin E deficiency	14	5	2	10	0
Vitamin A deficiency	3	1	0	0	0
Other nutritional	3	5	10	4	2
Diseases associated with mismanagement & omphalitis	10	58	13	56	26
Total	76	120	84	143	142

The effect of starvation and *Escherichia coli* infection on yolk sac retention in chicks

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Summary Feed starvation and experimental colibacillosis were examined for the effects on yolk sac retention in newly hatched chicks. Forty eight hours starvation resulted in retention of the yolk sac. Experimental colibacillosis also resulted in yolk sac retention. These results suggest that systemic stress enhanced the yolk sac retention in chicks.

Retention of the yolk sac is one of the factors responsible for loss of baby chicks during the first three to four days after hatching, and has been described in relation to omphalitis and mushy chick disease'. In some respects it might be more appropriate to regard yolk sac retention as a manifestation of certain bacterial infections aggravated by poor hygiene on farms and faulty management at the hatchery. The period between hatching and the initiation of feeding in chicks has been considered to affect the size of the yolk sac retained. One-day feed starvation after hat-ching is recommended in some countries. Many farmers may not have been caring for the initiation of feeding by chicks after hatching.

The purpose of the present study is to investigate the effect of starvation after hatching and *Escherichia coli* infection on yolk sac retention in chicks.

Materials and Methods

Fifteen-day old Arbor Acres embryo-nated eggs, were purchased from a local commercial hatchery. The eggs were incubated in the laboratory. Hatched chicks were divided into 6 groups of 8-10 chicks each. The groups consisted of non-treated control, 24 hours starvation, 48 hours starvation, no starvation with *E. coli* inoculation, 24 hours starvation with *E. coli* inoculation, and 48 hours starvation with *E. coli* inoculation. All the chicks were maintained under the same conditions at 25^{0}C in a room with artificial illumination.

The *Escherichia coli* strain used in the present study was isolated in the Diagnostic Laboratory of School of Veterinary Medicine, the University of Zambia, from a chick suffering from colibacillosis, and was typed by Dr. T. Tsukamoto in Osaka Institute of Public Health, Japan as O2:H5. Experimental chicks were inoculated intranasally with 0.5 ml of *E. coli*-cultured broth, which was estimated to have 10^8 colony-forming units of the organism.

Chicks were weighed on hatching day, 7 days and 14 days post hatching. At 7 days after hatching 4 birds from each group were slaughtered, and examined pathologically for the retention of yolk

sac, weight of yolk sac, and the lesions of colibacillosis. At 14 days after hatching the rest of chicks were slaughtered for examination. The liver, heart, spleen and yolk sac were taken from the chicks at necropsy and an attempt was made to culture microorganisms with special reference to *E. coli*.

Data of body weight and weight of retained yolk sac were analysed by Student's t test.

Results

Data of body weight were shown in Table 1. No significant differences were observed among the groups.

Pathological findings including yolk sac retention and results of bacterial culture were described in Table 2. In groups 1 and 2, neither yolk sac retention nor other pathological findings were observed. *E. coli* was not isolated. In group 3, yolk sac retention was found in all of 7-day old chicks necropsied, but no retention in 14-day old chicks was observed. Although in this group there were two birds each positive for *E. coli* culture at both 7 and 14 days after hatching, the isolates were not serotyped. In groups 4, 5, and 6, yolk sac retention was observed in all birds necropsied at 7 days after hatching. The retained yolk sacs in birds of groups 5 and 6 were significantly larger than those in the birds of groups 3 and 4. At 14 days after hatching, however, no yolk sac retention was seen in groups 4, 5 and 6. In these three groups infected with *E. coli* many birds showed the evidences of colibacillosis lesions like perihepatitis, pericarditis and peritonitis at necropsy.

Discussion

Feeding immediately after hatching has been considered as one of the causes of retention of the yolk sac by minimizing utilization of yolk sac energy. In the present study non-starvation without *E. coli* inoculation have taken away yolk sac retention as clearly as 24-hour starvation group, whereas 48-hour starvation resulted in retention of the yolk sac. Chicks must have felt long term starvation to be systemic stress, and therefore they might have failed to dispose of the yolk sac.

It is evident from this study that intranasal inoculation with *E. coli* culture broth reproduce colibacillosis in young chicks. Apparently colibacillosis gave chicks another systemic stress (2). Even if the chicks were starved, *E. coli* infection could have readily caused yolk sac retention in group 4. Concurrent stress with starvation and *E. coli* infection showed some synergistic effects on the size of retained yolk sacs in groups 5 and 6.

In conclusion, yolk sac retention is dependent upon the degree of stress which chicks receive, but it is still to be elucidated whether retention of the yolk sac would affect the severity of other diseases.

Table 1. The effects of different treatments on body weight.

Group	No. of chicks	Starvation (hours)	*E. coli* infection	Body weight (gms) 0 (days old)	7 (days old)	14 (days old)
1	8	0	No	45.6 +/- 2.7	69.6 +/- 1.8	91.9 +/- 3.3
2	10	24	No	47.5 +/- 3.2	70.5 +/- 1.9	90.7 +/- 3.4
3	9	48	No	46.4 +/- 4.2	71.9 +/- 3.5	92.8 +/- 3.2
4	9	0	Yes	45.3 +/- 3.0	70.9 +/- 2.1	88.4 +/- 1.8
5	11	24	Yes	46.4 +/- 2.7	70.6 +/- 1.5	89.5 +/- 2.6
6	10	48	Yes	46.4 +/- 3.4	72.1 +/- 2.1	89.1 +/- 1.0

Mean +/- standard deviation

Table 2. The effects of starvation after hatching and *Escherichia coli* infection on colibacillosis and yolk sac retention in chicks.

Group	Peri-hepatitis	Peri-carditis	Perit-onitis	Yolk sac reten-tion	Size of yolk sac (gm)	Isolation of *E. coli*
1	0/4[a] 0/4[b]	0/4 0/4	0/4 0/4	0/4 0/4		0/4 0/4
2	0/4 0/6	0/4 0/6	0/4 0/6	0/4 0/6		0/4 0/6
3	0/4 0/5	0/4 0/5	0/4 0/5	4/4 0/5	1.3 +/- 0.1[c]	2/4 2/5
4	4/4 5/5	0/4 0/5	4/4 3/5	2/4 0/5	1.6 +/- 0.1	4/4 5/5
5[d]	4/4 5/5	2/4 2/5	4/4 2/5	4/4 0/5	2.1 +/- 0.1	4/4 5/5
6[e]	4/4 5/5	4/4 5/5	4/4 5/5	4/4 0/5	2.5 +/- 0.2	4/4 5/5

a: Fractions in upper row: numerators show number of birds positive for the subject; denominators show the number of birds examined on the 7th day.

b: Fractions in lower row: numerators show number of birds positive for the subject; denominators show the number of birds examined on the 14th day.

c: Mean +/- standard deviation.

d: Two birds were found dead by the 7th day.

e: One bird was found dead by the 7th day.

*: Significantly different from groups 3 & 4 ($p < 0.05$)

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Seminar abstract, (August 25, 1994)

Morphology of antibody production system and mucosal immune system in domestic animals

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The development of immune system and the related cell populations in domestic animals have been studied with a view of effective prevention of infectious diseases. The origin of immunocompetent lymphoid cells has been designated to thymus and bursa of Fabricius in poultry. In the duck spleen, ontogenetical dependent areas of lymphoid tissues on thymus, or bursa of Fabricius, or independent area on both lymphoid organs have been designated by thymectomy and/or bursectomy. Recently they have been further designated immunohistochemically by using anti-thymocyte monoclonal antibodies against CD4 and CD8. In the splenic lymphoid tissue, periarterial lymphoid tissue (PA) is thymus-dependent. Peri-ellipsoidal lymphoid tissue (PE) and germinal centres (GC) are bursa-dependent, whereas perivenous lymphoid tissue (PV) is independent on the two organs. In the process of antibody production by treatment of horseradish peroxidase, GC is one of the sites for proliferation of cells with antibody producing capacity, by contact with the macrophages which bear the antigen-antibody complex on their surface.

Mucosal immune system mediated by the secretory IgA is established on a varietey of local mucous membranes, and has been recognized to function as a first defence barrier for microorganism infection. In the wall of intestine of rabbit, secretory IgA localized in the epithelial cells of the intestinal villi. In the propria, IgA-producing cells are predominant in any intestinal portions except for the caecal Peyer's patch where IgG-producing cells are more frequent. For further approach to the mechanism of mucosal immunity in morphological aspect, relationship of the immuno-competent cells in mucosa should be clarified with immunoelectron microscopy by using monoclonal antibodies raised against lymphocytes and macrophages

Seminar abstract, (August 16, 1994)

Immunohistochemistry applied for diagnostic and research purpose

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Immunohistochemistry comprises the use of an antiserum/monoclonal antibody for *in situ* identification of the corresponding antigen/epitope in a tissue section. There are many methodological variants for visualising antigens *in situ*. In this seminar focus will be on immunofluorescence and avidin/streptavidin biotin complex methods.

The basic principle is based on the use of an immune serum raised in an appropriate animal species (rabbit, sheep, goat) or a monoclonal antibody (of mouse origin) for detection of the corresponding antigen in a section of a fixed, paraffin-embedded specimen or a frozen section. Given that the antigen is preserved and that there are specific antibodies available, there is no limit to the type of antigen that can be identified. The reaction between the antigens and the antibodies has to be made visible to the human eye, typically by use of fluorescing compounds or enzymes incubated with the corresponding substrate/ chromagen solutions. The former is visualised in a fluorescence microscope, the latter in an ordinary light microscope.

The tissue preparation is one of the most important aspects of immunohistochemistry and however refined the immunohistochemical detection method may be, the result of antigen localisation will in the end depend on the handling of the tissue specimen. The fixative is the most important factor and ethanol gives better sensitivity of immunohistochemical methods than formalin fixation. The reasons for the reduced staining sensitivity using formalin fixation, relies on the cross-linking of proteins and subsequent masking of antigens. Several efforts have been made to overcome the masking effect of formalin, and proteolytic treatment of sections is one way to re-expose antigens concealed during fixation. Trypsin or pronase has been extensively used for this purpose with marked beneficial effects and with increased sensitivity as a result.

Evaluations of validity in immunohistochemical techniques should include repeatability (precision), specificity and sensitivity. Evaluation of precision includes standardisation of the preparation of buffers/diluents, incubation time, concentrations of the antibody solutions, and training of personnel. The specificity should be evaluated by two sets of criteria, method specificity and antibody speciality. The former includes the evaluation of any unwanted interactions, while the latter is more difficult to

each other. It is important to understand the disease status of village chickens for the development of the poultry industry in Zambia. Further investigations on village chickens should be carried out in other areas of Zambia.

TABLE 1 : The antibody rates to avian disease agents in Shingoma village.

NO	IBO	*S. pullorum*	*M. gallisepticum*	*M. synoviae*
10/10	17/18	0/18	18/18	18/18
(100.0)	(94.4)	(0.0)	(100.0)	(100.0)

* Number of positive samples / number of samples examined. (percentage)

A five-year summary of diseases of chickens diag-nosed in the School of Veterinary Medicine at the University of Zambia, Lusaka.

M. Hirai[1], E. Baba[2], G. S. Pandey[3]

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Few reports on occurrences of poultry diseases from developing countries are available. The School of Veterinary Medicine in the University of Zambia which was founded in 1985 has been providing diagnostic services to veterinarians and farmers who submit broiler and layer chicken carcases for necropsy. The diagnoses during the last five years (1989-93) have been summarised.

Live or dead chickens of both broiler and layer types were submitted for diagnosis in 1989 to 1993. The diagnosis records were retrieved according to the numbers of outbreaks regardless of the number of chickens submitted at each outbreak (Table 1). Many submissions included two to five chickens as specimens. The presenting history of the poultry farm was recorded, and the chickens were examined pathologically, microbiologically, and parasitologically.

A normal scale poultry farm rears several hundred birds for broilers, breeders or layers in the Lusaka area. Only a small number of big commercial farms keep several thousands of birds. Transportation is not always convenient even for big farms, such that chickens must have been submitted for diagnosis to the Diagnostic Laboratory only when farmers were suffering from severe problems such as high mortality. Many latent cases of small outbreaks might not have been considered.

Colibacillosis has been described as one of the opportunistic infections which cause few large-scale outbreaks, nevertheless an increasing number of colibacillosis cases is a sign that the management and environment of farms in this area might not have been improved. In the present survey, concurrent infection with *E. coli* and *Mycoplasma spp.* was not obvious. Colibacillosis cases did not always accompany respiratory disorders or omphalitis. Since the relationship between serotype and pathogenicity have been described (Gross 1984), an investigation on serotyping *E. coli* isolates is suggested in order to understand the epidemiological situation in this country.

Cases of Salmonella infections have tended to increase in number. In a serological survey of parent stocks in local hatcheries it was noticed that a number were contaminated with *Salmonella gallinarum* (unpublished). *S gallinarum* rather than *S pullorum* was isolated and classified according to its biological properties. Despite the importance of agricultural policy against fowl typhoid the incidence of the disease tends to increase. The initial approach to prevent an outbreak should be education of farmers to understand that economic losses due to fowl typhoid are greater than the expense of disease control. Even in cases where half of introduced chicks were killed by salmonellosis, the local farmers try to treat them with antibiotics to attempt to recover financial losses.

Large outbreaks of Mycoplasma infections in 1991 brought severe disaster to farmers. The serum samples collected were immunologically positive for *M. gallisepticum*. Good management might have minimized the outbreaks.

No severe outbreaks of viral infections were observed during the 5 year period. Almost all commercial farms have been using good vaccination programmes against the important viral diseases. The inconvenience of communicating between farms helps to minimize epidemics of lethal viral diseases.

Other sporadic diseases such as coccidiosis, parasitic diseases, or fungal infections are rarely submitted from farms where low levels of the outbreaks exist.

Coccidiosis which brings about minor economical loss as long as anticoccidial drugs are used has not been significant in this country. However occasional outbreaks have been reported, possibly due to bad management. Almost all nutritional problems might have resulted from the bad quality of chicken feed available. In many cases farm-made feed produced nutritional problems. Nutritional disorders highlight a need to improve feed quality for chickens. Cases of other diseases such as mismanagement and omphalitis were often submitted from small farms. Although many omphalitis cases must have been related to colibacillosis, the cases which were not checked by culture were included in the category of other diseases.

In conclusion the present figures show the occurrence of many simple bacterial infections and small number of viral disease cases.

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Mycoplasma	3	9	12	3	1
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Fungal infection	2	1	0	0	0
Marek's Disease	1	0	0	0	0
Newcastle Disease	1	1	5	2	1
Fowl Pox	1	0	2	0	0
Gumboro Disease	4	7	5	0	4
Coccidiosis	3	7	6	4	1
Other parasites	0	0	0	2	0
Vitamin E deficiency	14	5	2	10	0
Vitamin A deficiency	3	1	0	0	0
Other nutritional	3	5	10	4	2
Diseases associated with mismanagement & omphalitis	10	58	13	56	26
Total	76	120	84	143	142