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Comparative Hematology, Morphology and Ultrastructure of Blood Cells in Monocellate Cobra (*Naja kaouthia*), Siamese Spitting Cobra (*Naja siamensis*) and Golden Spitting Cobra (*Naja sumatrana*)

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ABSTRACT

Blood samples of 17 monocellate cobras (*Naja kaouthia*), 12 Siamese spitting cobras (*Naja siamensis*) and 6 golden spitting cobras (*Naja sumatrana*) in the Queen Saovabha Memorial Institute were collected from ventral caudal vein for basic hematology, light microscopic, scanning and transmission electron microscopic features of blood cells. There was no hematozoa detected in all monocellate cobras. Five Siamese spitting cobras (42%) and four golden spitting cobras (74%) were positive for *Hepatozoon* sp. Lymphocytes were the most commonly observed leukocytes in all cobras and average 6-8 μm in diameter. Azurophils were the second most commonly observed leukocytes, averaging 10-17 μm in diameter. Azurophils in golden spitting cobra contained more number of azurophilic granules and ribosomes than the other cobras. Heterophils were the largest leukocytes, averaging 16-19 μm in diameter. Eosinophils in monocellate cobras revealed large bulging granules and usually were medium-sized cells, averaging 10-14 μm in diameter. Eosinophils in Siamese spitting cobra were larger than those in monocellate cobra but contained smaller granules. Eosinophils in golden spitting cobra showed bulging granules but smaller than those in monocellate cobra. Basophils in all kind of cobras were similar and as small as lymphocyte but revealed many small granules polarity. Scanning electron microscopic examination revealed the surface of thrombocytes, erythrocytes, lymphocyte, azurophil and heterophil in monocellate cobra. Transmission electron microscopic examination showed organelles within azurophil and thrombocyte of Siamese spitting cobra and azurophis and gamonts of *Hepatozoon* sp. in erythrocytes of golden spitting cobra.

Key words: cobra, hematology, *Hepatozoon*, *Naja*, morphology, ultrastructure

INTRODUCTION

The cobras are in the genus *Naja*, subfamily Bungarinae which includes king cobra (*Ophiophagus*) and krait (*Bungarus*). The cobras in Thailand compose of monocellate or monocled cobras (*Naja kaouthia*), Siamese or Indo-Chinese spitting cobras (*Naja siamensis*) and golden or equatorial spitting cobras (*Naja sumatrana*), (Cox *et al*., 1998). The Queen Saovabha Memorial Institute (QSMI) has initiated a captive-breeding program since 1994 to supply healthy snakes for venom and antivenom production. These venomous snakes prey on mice and occasionally on puff-faced watersnakes. Most venomous snakes were highly infected with *Hepatozoon* sp. (Salakij *et al*., 2001).

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Morphologic characteristics of reptile blood cells are heterogeneous. Variations in cell characteristics and cell populations existed between species within the order Squamata (Alleman *et al*., 1999). Hematologic examination would be useful for evaluating the health of the snakes, however, basic hematologic values and morphology have not been described in these species. The purpose of this study was to obtain the hematologic data and morphologic characterization of blood cells in three species of cobras.

MATERIALS AND METHODS

Blood samples of 17 monocellate cobras, 12 Siamese spitting cobras and 6 golden spitting cobras were collected from ventral caudal vein during December 2000 to June 2001. The monocellate cobra was captive-breed in QSMI while the Siamese spitting cobras and the golden spitting cobras were wild-caught and reared in QSMI for 3 years. Blood smears were prepared immediately, then air-dried and stained by Wright's (W) and Wright's-Giemsa (WG) stains. The whole blood samples were kept in EDTA, stored at 4°C and processed within 2 hours. The complete hematology was performed as previous described by Salakij *et al*., 2002a.

Blood smears were fixed in methanol and stained by WG stain (Benjamin, 1978) for determination of differential leukocyte count, identification of hematozoa infection and morphological evaluation of all blood cells. *Hepatozoon* sp. infection was graded by quantifying the number of infected erythrocytes (RBCs), as described by Salakij *et al*. (2001). At least 200 WBCs were counted for differential WBC determination. For comparison of pattern of staining, blood smear from 2 samples of each cobras were stained with 1-step Wright's staining method that did not require methanol fixation prior to staining (Benjamin, 1978).

For each parameter obtained, data from each group of cobra were calculated for means, variances and standard error (SE) using SPSS for window™ (Norusis, 1993). Significant difference among means were determined using an independent sample T-test model.

For scanning electron microscopy (SEM), a drop of blood from two monocellate cobra were fixed using 1.5% glutaraldehyde (GA) in 0.1 M phosphate buffer pH 7.3 (PB) at 4°C overnight. Specimens were dehydrated through a graded acetone series, coated with gold and viewed under Jeol JSM-5600 LV scanning electron microscopy. For transmission electron microscopy (TEM), buffy coats from 2 Siamese spitting cobra and 2 golden spitting cobras were fixed in 2.5% GA and PB for 24 hours, postfixed with 1% osmium tetroxide and embedded in Spurr's epoxy resin. Ultrathin sections stained with uranyl acetate and lead citrate, were examined using Jeol 1200Ex TEM. Identification of blood cells by TEM was based on the relative number, size, shape and distribution of granules and on nuclear appearance.

RESULTS

There was no hematozoa detected in all monocellate cobras. Five Siamese spitting cobras (42%) and four golden spitting cobras (74%) were positive for *Hepatozoon* sp. (Table 1). There were no significant differences of all hematologic values among the cobras except the MCHC, absolute number of heterophils, eosinophils, plasma protein, fibrinogen and reticulocyte count (Table 2).

The *Hepaozoon* sp. gamonts found in Siamese spitting cobras were small (Figure 1a) while in golden spitting cobras the gamonts were very large (Figure 1b). The gamonts were in the cytoplasm of enlarged RBCs (Figure 1a, 1b). Some gamonts were free from RBCs but still within their parasitophorous vacuole membrane (PVM, Figure 1c). Ultrastructurally, intra-RBC *Hepaozoon* gamont was in PVM and displaced the nucleus (Figure 7c and d). Hook-like formation of PVM was seldom detected (Figure 7c). There was an

Table 1 Numbers and percentages of *Hepatozoon*-negative and positive in the cobras subgrouped according to sex.

Groups	*Hepatozoon* negative		*Hepatozoon* positive		Total
	Male	Female	Male	Female	
Monocellate cobras	15	2	0	0	17
Siamese spitting cobras	5	2	5	0	12
Golden spitting cobras	1	1	3	1	6
Total	21	5	8	1	35
%	60	14	23	3	100

Table 2 Comparative hematology (mean ± SE) among the cobras.

	Monocellate cobras	Siamese spitting cobras	Golden spitting cobras
Number	17	12	6
PCV (%)	21.2 ± 1.2	21.3 ± 1.8	18.8 ± 2.4
Hb (g/dl)	6.5 ± 0.4	6.9 ± 0.6	4.8 ± 0.7
RBC ($x10^6/\mu l$)	0.616 ± 0.052	0.576 ± 0.042	0.657 ± 0.086
MCV (fl)	362.7 ± 18.9	371.6 ± 24.9	289.3 ± 9.6
MCH (pg)	110.1 ± 5.90	120.3 ± 9.62	71.2 ± 3.1
MCHC (g/dl)	30.5 ± 0.7^a	32.3 ± 1.5^b	24.8 ± 1.7^c
WBC ($x10^3/\mu l$)	14.316 ± 1.265	12.025 ± 0.880	9.816 ± 1.046
Azurophils ($x10^3/\mu l$)	3.993 ± 0.785	3.089 ± 0.512	2.632 ± 0.623
Heterophils ($x10^3/\mu l$)	0.664 ± 0.170^a	0.226 ± 0.069^b	0.473 ± 0.146^{ab}
Eosinophils ($x10^3/\mu l$)	0.197 ± 0.061^a	0.011 ± 0.011^b	0 ± 0^{bc}
Basophils ($x10^3/\mu l$)	0 ± 0	0 ± 0	0.015 ± 0.015
Lymphocytes ($x10^3/\mu l$)	9.259 ± 0.903	8.562 ± 0.709	6.695 ± 0.590
Monocytes ($x10^3/\mu l$)	0 ± 0	0 ± 0	0.03 ± 0.2
Azurophils (%)	26.1 ± 3.7	25.2 ± 3.5	26.0 ± 4.5
Heterophils (%)	4.4 ± 1.0	1.9 ± 0.5	4.7 ± 1.3
Eosinophils (%)	1.1 ± 0.08	1.4 ± 0.3	0 ± 0
Basophils (%)	0 ± 0	0 ± 0	0.2 ± 0.2
Lymphocytes (%)	66.9 ± 4.4	71.8 ± 3.5	69.2 ± 4.1
Monocytes (%)	1.2 ± 0.4	1.0 ± 0.5	0 ± 0
PP (g/dl)	5.56 ± 0.43^a	6.84 ± 0.63^b	9.6 ± 0.4^c
Fibrinogen (mg/dl)	28.6 ± 28.6^a	0 ± 0^a	200.0 ± 44.7^c
Agg. Reticulocytes (%)	1.6 ± 0.6^{ac}	4.2 ± 0.8^b	1.5 ± 0.3^c
Punct. Reticulocytes (%)	6.8 ± 1.0^{ac}	32.7 ± 6.9^b	13.8 ± 4.5^c

The figures on the same row with the same letter are not significantly different ($p > 0.05$).

electron-lucent space between the *Hepaozoon* gamont PVM and RBC cytoplasm (Figure 7b). The gamonts possessed a nucleus, many micronemes (Figure 7b and 7c), dense granules (Figure 7c) and vacuoles.

Erythrocytes

Erythrocytes (RBCs) from all cobras were homogeneous in color but moderately anisocytosis (Figure 1). Cytoplasmic vacuoles were found in less than 1% of RBCs (Figure 1d). The young RBCs were seldom shown (Figure 1d). *Hepatozoon*–infected RBCs were larger than those non-infected ones (Figure 1a and b) and hypochromic (Figure 1a). The mitotic figure of RBCs in golden spitting cobra rarely appeared (Figure 1e). By SEM, RBCs were ellipsoid, lacking a dome appearance at the site of the nucleus (Figure 5a and b). The width of RBCs in monocellate cobra were significantly less than in Siamese spitting cobra and golden spitting cobra whist the length of RBCs in Siamese spitting cobra were significantly less than in golden spitting cobra (Table 3).

Thrombocytes

Thrombocytes were elongate and approximately half the size of mature RBCs (Figure 1f). Their aggregation turned into round cells (Figure 1f and 5a). However, they could be differentiated from lymphocytes by the characteristic perinuclear and cytoplasmic vacuolation (Figure 1f). Ultrastructurally, thrombocytes contained a nucleus, mitochondria, vacuoles and dense core granules (Figure 7a).

Leukocytes

Leukocytes (WBCs) of the cobra were categorized into 6 groups; azurophil, heterophil, eosinophil, basophil, lymphocyte and monocyte. For comparison, the blood smears stained with one step Wright's stain provided staining quality for identification of all blood cell type but in Wright's stain the RBCs stained more basophilic (Figure 1g and h).

Lymphocytes in cobras were the most prevalent circulating cells (Table 2). They were small, well differentiated averaging 6-8 μm in diameter (Figure 2e, 3e, 4e, Table 3). There were no morphologic difference among lymphocytes in three kinds of cobras. Some vacuoles were present in the cytoplasm of lymphocytes in monocellate cobras (Figure 2e) and Siamese spitting cobras (Figure 3e).

Azurophils were the second most commonly

Table 3 Comparative blood cell diameters in micrometer (mean ± SE) among the cobras.

	Monocellate cobras	Siamese spitting cobras	Golden spitting cobras
Number of cells	40	40	40
RBC (width)	9.45 ± 0.11^{a}	10.10 ± 0.12^{b}	9.90 ± 0.09^{b}
RBC (length)	16.65 ± 0.12^{a}	16.25 ± 0.18^{ab}	16.70 ± 0.16^{ac}
Azurophils	12.95 ± 0.22^{a}	11.38 ± 0.30^{b}	13.23 ± 0.27^{a}
Heterophils	16.05 ± 0.19^{a}	15.13 ± 0.25^{b}	14.95 ± 0.26^{b}
Eosinophils*	13.23 ± 0.39^{a}	15.65 ± 0.34^{b}	14.70 ± 0.50^{b}
Basophils*	8.87 ± 0.17^{a}	8.90 ± 0.19^{a}	10.05 ± 0.34^{b}
Lymphocytes	7.48 ± 0.23^{ab}	6.63 ± 0.19^{b}	7.53 ± 0.18^{a}
Monocytes*	15.87 ± 0.37^{a}	15.40 ± 0.53^{ab}	15.00 ± 0.30^{b}

The figures on the same row with the same letter are not significantly different ($p > 0.05$).
*Only 20 cells from each group were measured.

observed leukocytes, which contained fine indistinct azurophilic granules. They were round and 11-14 μm in diameter (Table 3). The nuclei were round to irregular with clump chromatin locating centrally to eccentric (Figure 2a, 3a, 4a). The cytoplasm of azurophils in golden spitting cobra were stained more azuroplilic than those of the other two cobras. Ultrastucturally, they contained numerous membrane-bound granules, some mitochondria, rough endoplasmic reticulum and many ribosomes (Figure 6a, 6b). The organelles in azurophils of Siamese spitting cobra were less than those of golden spitting cobra (Figure 6c, 6d). Monocytes were not frequently observed and their characters were similar to mammalian monocytes (Figure 2f, 3f, 4f).

Heterophils were the largest of the WBCs averaging 14-18 μm in diameter (Table 3). They

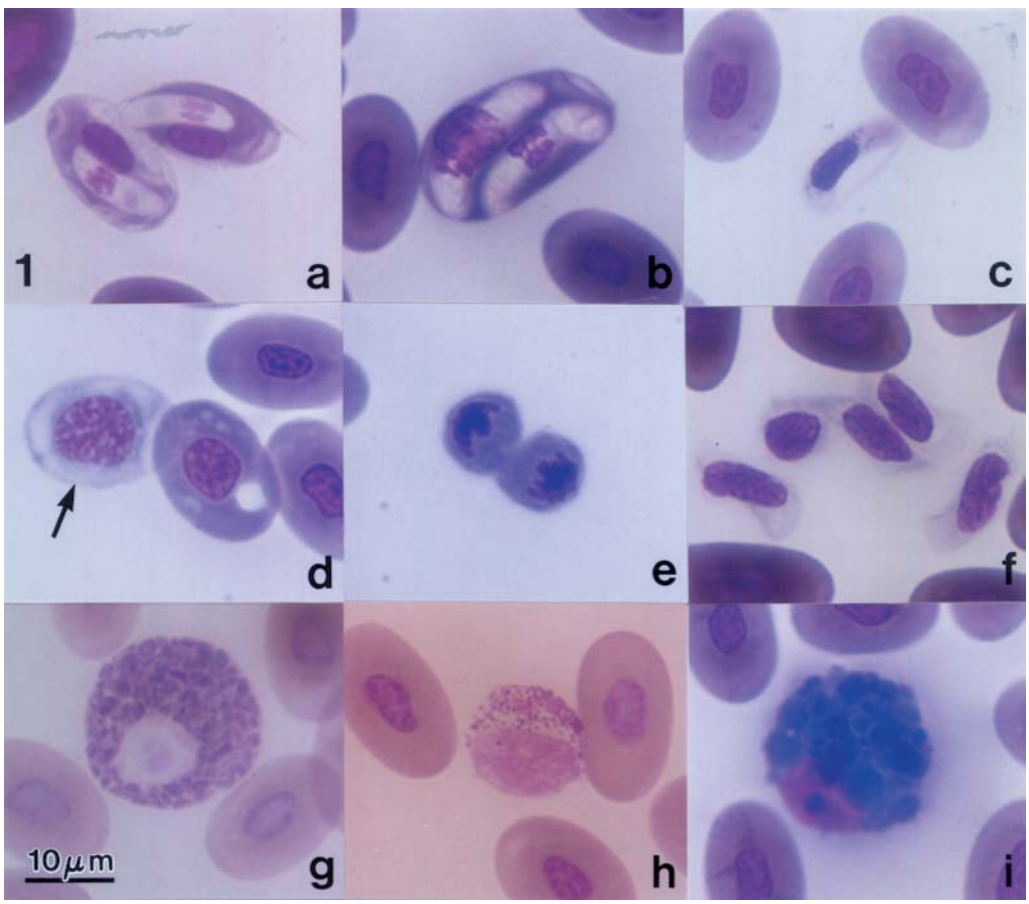

Figure 1 (a) Two gamonts of *Hepatozoon* sp. in two erythrocytes (RBCs) of Siamese spitting cobra. Note the hypochromic RBCs. (b) Two large gamonts of *Hepatozoon* sp. in one RBC of golden spitting cobra. Note the larger size than RBCs in (a). (c) Free *Hepatozoon* sp. in golden spitting cobra. (d) Young erythrocyte (arrow) and vacuole in RBC of golden spitting cobra. (e) Two daughter cells of RBCs in golden spitting cobra. (f) Five thrombocytes in monocellate cobra. Wright's-Giemsa stain (WG). (g) Hetrophil in golden spitting cobra. Wright's stain (W). (h) Basophil in golden spitting cobra. W stain. (i) The large eosinophil with 18 μm in diameter in golden spitting cobra. Note the variation in size of granules and eccentric nucleus. WG stain.

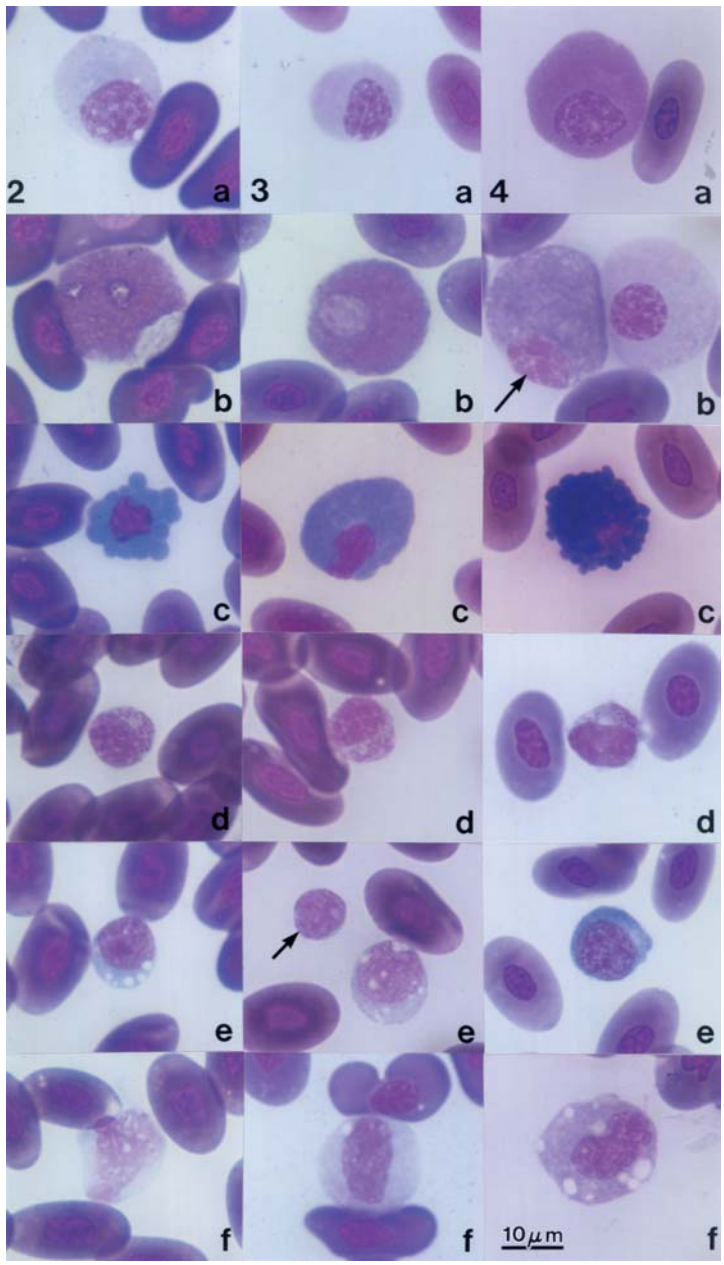

Figure 2 WBCs in the monocellate cobra. (a) An 16 μm azurophil. (b) A 19 μm heterophil. (c) An eosinophil with bulging large granules. (d) A 9 μm basophil. (e) A 10 μm lymphocyte with vacuolated cytoplasm. (f) A 17 μm monocyte. WG stain.

Figure 3 WBCs in the Siamese spitting cobra. (a) A 13 μm azurophil. (b) A 18 μm heterophil. (c) An eosinophil with many large granules packed in the cytoplasm. (d) A 10 μm basophil. (e) A 8 μm (arrow) and a vacuolated 10 μm lymphocytes. (f) A 14 μm monocyte. WG stain.

Figure 4 WBCs in the golden spitting cobra. (a) An 16 μm azurophil with dark margenta cytoplasm. (b) A 19 μm heterophil (arrow) and an azurophil. (c) An eosinophil. (d) A 10 μm basophil. (e) A 11 μm lymphocyte. (f) A 16 μm monocyte. WG stain.

contained large numbers of irregular shape, dull eosinophilic granules (Figure 2b, 3b, 4b). By Wright's stain, the heterophil granules were easily seen by reddish-orange bright color (Figure 1g). There were no morphologic difference among heterophils in three kinds of cobras. By SEM, their granule contour was bulging showing the custard apple-liked appearance (Figure 5d).

Eosinophils in monocellate cobras revealed very large bulging granules (Figure 2c) and usually were medium-sized cells, averaging 11-15 μm in diameter (Table 3). Eosinophils in Siamese spitting cobra were larger than those in monocellate cobra but contained smaller granules (Figure 3c). Eosinophils in golden spitting cobra revealed bulging granules but smaller than those in monocellate cobra (Figure 4c) and in some occasion very large cells were also detected (Figure 1i). By Wright's stain, eosinophil granules were stained dark blue the same as by WG stain. The eosinophil numbers were very low in all kinds of cobra (Table 2).

Basophils were very small, averaging 9-11 μm in diameter (Table 3) and slightly smaller than eosinophils (Figure 2d, 2c, 3d). They contained small dark purple staining metachromatic granules polarized opposite to the round eccentric nucleus. By Wright's stain, basophil granules stained darker than those in WG stain (Figure 1h).

DISCUSSION

The incidence of hematozoa infection in the Siamese spitting cobras and golden spitting cobras

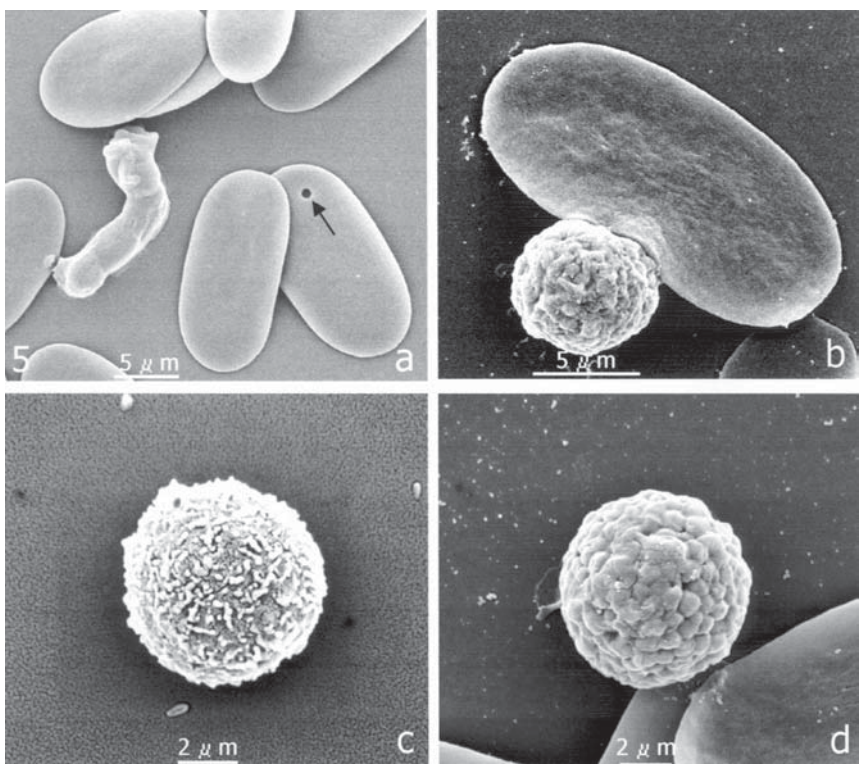

Figure 5 Scanning electron micrographs of blood cells in monocellate cobra. (a) Four aggregated thrombocytes (left) and RBC with small cytoplasmic vacuole (arrow). (b) Lymphocyte with irregular surface membrane. (c) Azurophil, showing villi-like surface. (d) Heterophil with custard apple-like appearance of the granule contour and some micropores.

were as high as the other snakes in Queen Saovabha Memorial Institute (Salakij *et al*., 2001). Some hematological values were different from the normal hematologic parameters for reptile (Mader, 2000) such as the PCV was lower whist the total WBC and the plasma protein was higher than the reference (Mader, 2000). This study also revealed that hematozoa parasitism of Siamese spitting cobras and golden spitting cobras RBCs had no effect on anemia since there were no significant differences of all RBC parameters except the MCHC. These results supported the finding of no clinical disease demonstrated in infected snakes (Campbell, 1986).

Lymphocytes in the cobras were the most prevalent circulating cells like those in King cobra (Salakij *et al*., 2002a), puff-faced watersnakes (Salakij *et al*., 2002b) and the other snakes (Mader, 2000). Some researchers characterize azurophils as monocytes with azurophilic granules (Campbell, 1986).

It was quite difficult to differentiate eosinophils from basophils in WG stained smears

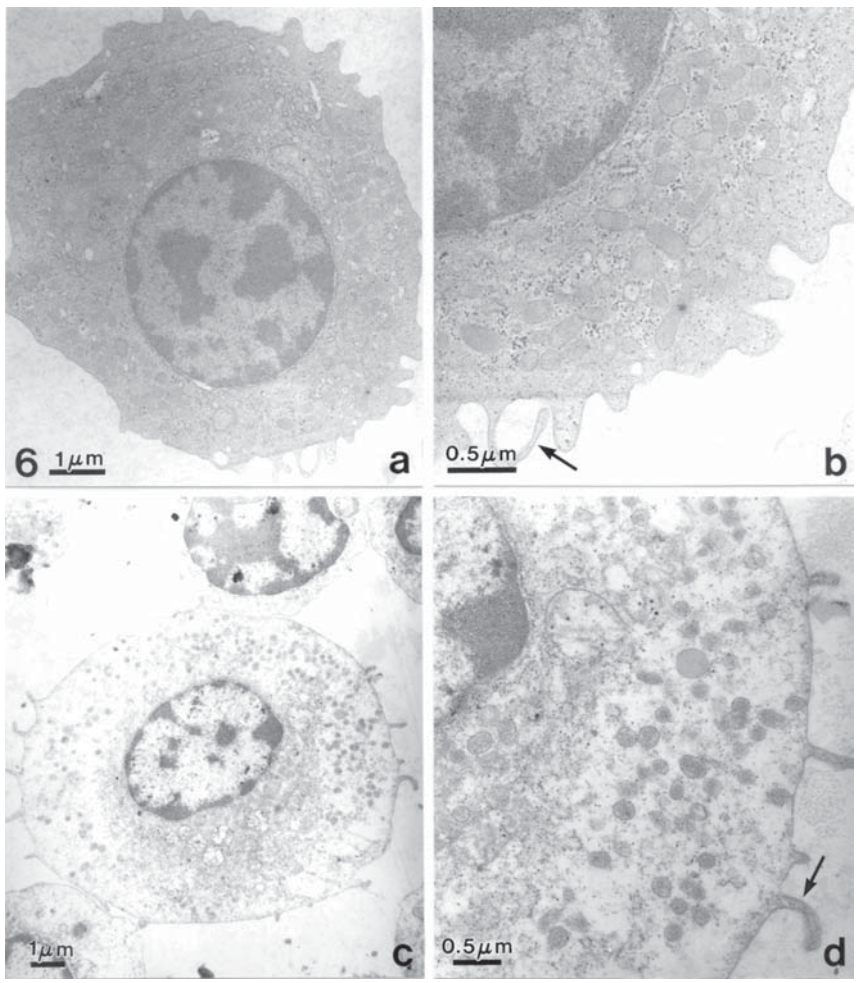

Figure 6 Transmission electron photomicrographs of (a) Azurophil from golden spitting cobra. (b) Higher magnification of the azurophil in (a) showing many granules, ribosomes and villi-like surface (arrow). (c) Azurophil from Siamese spitting cobra. (d) Higher magnification of the azurophil in (c). Note the cytoplasmic process (arrow).

because of the bluish coloration of their granules. They were identifed more easily on Wright's stained preparation. The eosinophil granule characteristic in the cobras was similar to those of iguanas and psittacines (Hawkey and Dannett, 1989) which contained dark purple staining metachromatic granules obscuring the unlobed nucleus. The large-sized eosinophils found in golden spitting cobra should be the characteristic of eosinophil in snakes which were larger than those of the other reptiles (Mader, 2000). The large bulging granules of eosinophils in monocellate cobra and golden spitting cobra were similar to some eosinophils in king cobra (Salakij *et al*., 2002a). But the granules of eosinophil in Siamese spitting cobra were not bulged out the surface but seemed to be packed in the

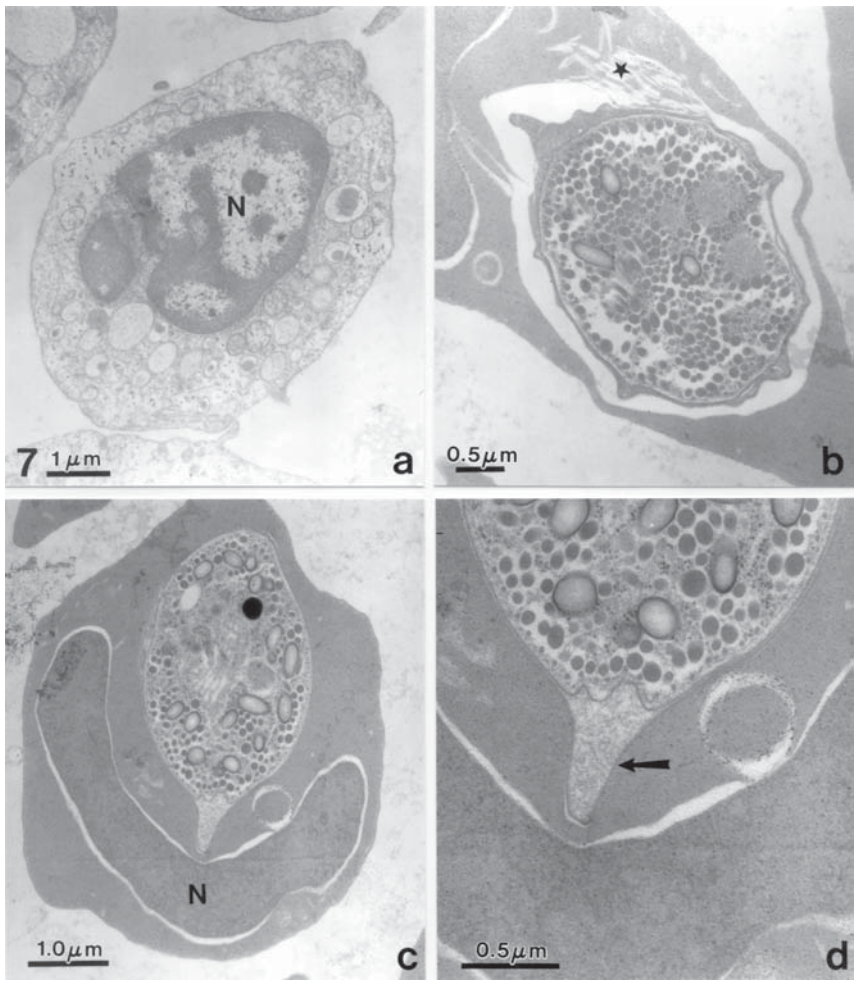

Figure 7 Transmission electron photomicrographs of (a) Thrombocyte of Siamese spitting cobra containing nucleus (N), mitochondria, vacuoles and dense core granules. (b) Cross-section of *Hepatozoon* sp. in RBC of golden spitting cobra. The hemoglobin around the protozoa was denatured (*). (c) Cross section of RBC in golden spitting cobra containing *Hepatozoon*. Note the displacement of the host nucleus (N). The *Hepatozoon* gamont contains Golgi apparatus, one dense granule and may micronemes. (d) Higher magnification of a *Hepatozoon* in (c). Note hook-appearance of the parasitophorous vacuole membrane (arrow).

cytoplasm.

The low number of eosinophils in the cobras (Table 2) and their bluish granules might make it misidentified as basophils. The finding of eosinophils in all kinds of cobras supported the existence of these leukocytes in snakes eventhough they were not identified in eastern diamondback rattlesnakes (Alleman *et al*., 1999).

The small gamonts of *Hepatozoon* sp. found in Siamese spitting cobras were similar to those found in the banded krait (*Bangarus fasciatus*) of the QSMI (Salakij *et al*., 2001). The large gamonts found in golden spitting cobras were referred as the large gamonts found in mangrove snakes and mangrove pit vipers of the QSMI (Salakij *et al*., 2001). The other hematozoas; *Haemogregarina* sp. and trypanosomes found in puff-faced watersnakes of the QSMI (Salakij *et al*., 2002b) were not detected in the cobras.

These results provided comparative hematologic data and a guide for identification of blood cells in nonocled, Siamese spitting and golden spitting cobras. *Hepatozoon* infection was relatively common in wild-caught Siamese spitting and golden spitting cobras but not in captive-bred monocellate cobras. The study may be beneficial for further study and related research.

ACKNOWLEDGMENTS

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