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Light and Electron Microscopic Study on a Haemogregarine Species Infecting the Viper *Cerastes cerastes gasperitti* from Saudi Arabia

Saleh Al-Farraj
Faculty of Education, King Saud University, Riyadh, Saudi Arabia

Abstract: A *Haemogregarine* sp. infecting the Viper *Cerastes cerastes gasperitti* (35%) from K.S.A. was recorded for the 1st time. Parasites were recorded exclusively in the red blood cells of the Vipers. Erythrocytic parasites measured 17.5×3 μm with a nucleus of 8.1×2.5 μm. Mature Schizonts of 30×2.5 μm and producing 18-30 merozoites by ectomerogony were reported in the endothelial cells of blood capillaries of different internal organs (liver, spleen and lung). The merozoites measured 13×3.5 μm and these were similar to the stages in the red blood cells. The motile stages of the parasite recorded in the present study showed all the general architectures of the Apicomplexa with some pecularities such as presence of micronemes and rhoptries at both sides of the nucleus and the occurance of large number of subpellicular microtubules (80-90) extending to the posterior end of the parasite.

Key words: Haemogregarine, Apicomplexa, parasites, vipers, *Cerastes*

INTRODUCTION

Haemogregarines are intraerythrocytic apicomplexan parasites that had been described from all groups of tetrapod vertebrates (Levine, 1988; Sidall, 1995; Smith, 1996; Al-Sadoon *et al*., 1999; Shazly, 2000). Moreover, reptiles are of special interest to our subject because they are very old in the evolutionary scale and their parasites seem to become stabilized allover the world (Ball, 1967; Bashtar *et al*., 1984; Ahmad, 1998; Al-Sadoon *et al*., 1999; Shazly, 2000). On the other hand, intensive efforts had been oriented to survey parasitic infection of wild animals especially reptiles (Bashtar *et al*., 1984a, b; Abdel-Ghaffar, 1985; Ahmad, 1998; Shazly, 2000, 2003; Abdel-Ghaffar *et al*., 1994; Lainson *et al*., 2003). Furthermore, few studies were carried out on adeleorin blood sporozoan parasites infecting ectothermic Arabian animals (Al-Sadoon and El-Bahrawy, 1998; Al-Sadoon *et al*., 1999; Shazly, 2003), at the same time these studies were investigated only on the; evel of light microscopy.

The present study deals with the prevalence of haemogregarine infection and to describe the blood stages and mergony of these parasites in the Viper Cerastes cerastes gasperetti by Light and electron microscopy and to elucidate the ultrastructural characteristics of these parasite stages.

MATERIALS AND METHODS

A total of 18 adult male and female vipers *Cerastes cerastes gasperetti* (Vipridae, lacertilia, Gasperetti, 1988) were collected from Al-Zulfy city and Jizan city in Saudi Arabia during 2007. They were kept in glass cages with sand in an animal room. The animals were fed once a month with mice.

Animals were examined for blood parasites by preparing thin blood films from each viper by cutting its tail tip. Blood films were air-dried, fixed in absolute methanol for 5 min and stained with 3% Giemsa solution in phosphate buffer (pH 7.3) for 30-45 min. Finally, they were examined microscopically.

From highly infected animals, small pieces of liver, lung, heart, spleen and kidney were excised and fixed in 5% glutaraldehyde buffered in 0.1 M sodium cacodylate buffer (pH 7.4) for at least 4 h at 4°C. The specimens were washed 4-5 times in the buffer and post-fixed in 2% (w/v) OsO_4 in the previous buffer for 2 h, followed by 4-5 washing in the same buffer. Samples then treated with 50% ethanol, then with 1% (w/v) mixture of uranyl acetate-phosphatungestic acid in 70% ethanol for 2 h at 40°C and were left in 70% ethanol overnight.

The specimens were dehydrated in graded ethanol, transferred to propylene oxide and finally embedded in Araldite. Semi and ultrathin sections were cut on a Reichert-Jung Ultracut ultra microtome. Semithin sections were stained with methylene blue and Azur A mixture. Ultrathin sections were stained with alcoholic uranylacetate (20 min) and alkaline lead citrate (25 min) and finally examined with JEM-100 CX-Joel transmission electron microscopy.

RESULTS

Prevalence of the infection of the 18 *Cerastes* vipers under investigation, 7 (35%) were found to be infected

with haemogregarines on initial investigation. The parasitaemia (number of haemogregarine infection/1000 erythrocytes) ranged between 200-400/1000 erythrocytes (Fig. 1). The 3 vipers with high level of parasitemia were chosen for further microscopic preparation, while the rest of vipers were returned again to Al-Zulfy.

Blood stages of the parasite observed in the preipherel blood films were mostly large and intracellular (Fig. 1). However in some cases extracellular parasites were also seen. The parasites were usually infecting exclusively the red blood cells of their host, but none of the leucocytes. Single as well as double infection were common (Fig. 1). All parasitic stages are enclosed in a parasitophorous vacuoles. The average measurements of the erythrocytic stage of the parasite are 17.5×3 μm, while the nucleus measured 8.1×2.5 μm. Parasitized erythrocytes were elongated and hypertrophied (21.8×12.3 μm), whereas normal cells measured 17.9×9 μm. Electron microscopy showed that each intraerythrocytic parasite was covered by a typical coccidian pellicle of two membranes (Fig. 6-13), each 7-9 μm thick and are separated by a space of 10-12 μm (Fig. 13). The inner

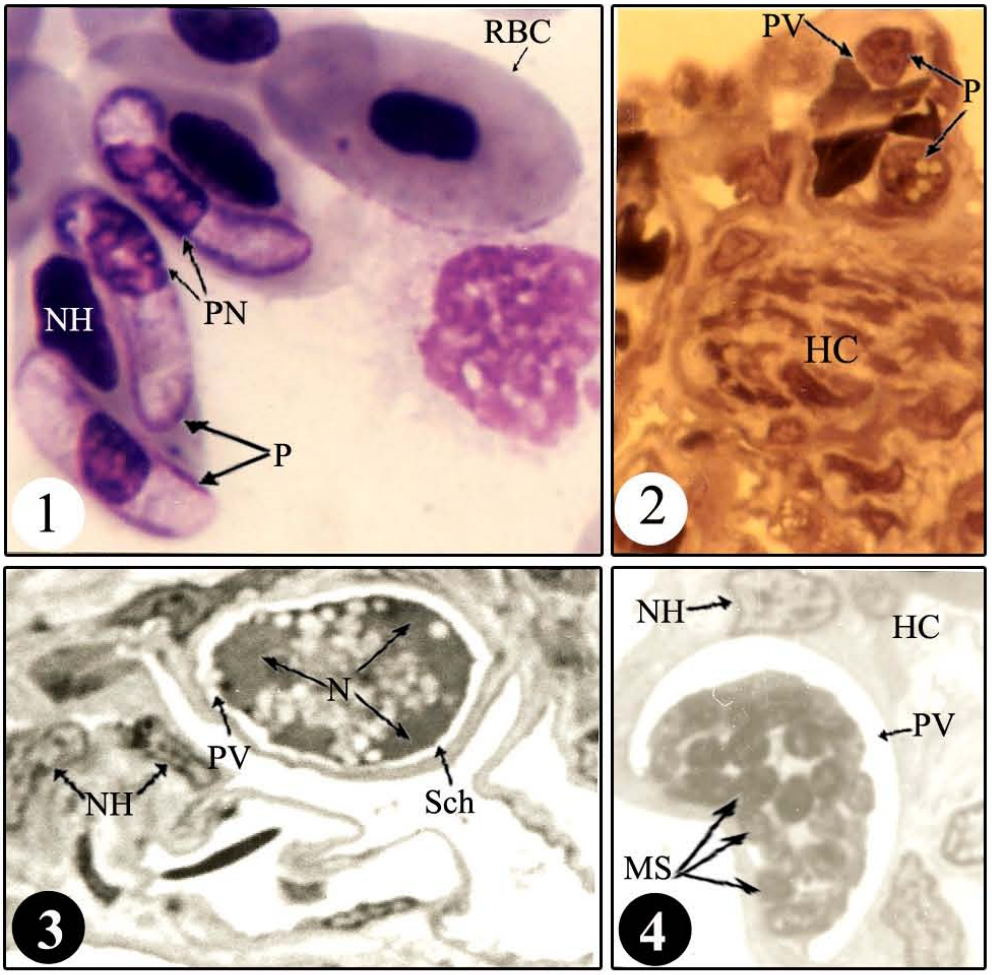

Fig. 1-4 : Light micrographs of some vertebrate stages of the parasite

Fig. 1 : Light micrograph of Giemsa stained vertebrate stages of the parasite (P) showing the infection of red blood cells (RBC) with parasites (P) and the large parasite nucleus (PN) and Nucleus of the host (NH) X 2200

Fig. 2 : Light micrograph showing a parasite (P) in the endothelial cells within parasitophorous vacuole (PV) in lung and Host cells (HC) X 2100

Fig. 3 : Photomicrograph of a developing schizont (Sch) enclosed in a parasitophorous vacuole (PV), Nucleus (N) and Nucleus of the host (NH) in lung X 2000

Fig. 4 : Photomicrograph of a mature schizont (Sch) in a parasitophorous vacuole (PV), containing mature merozoites (MS) X 2350

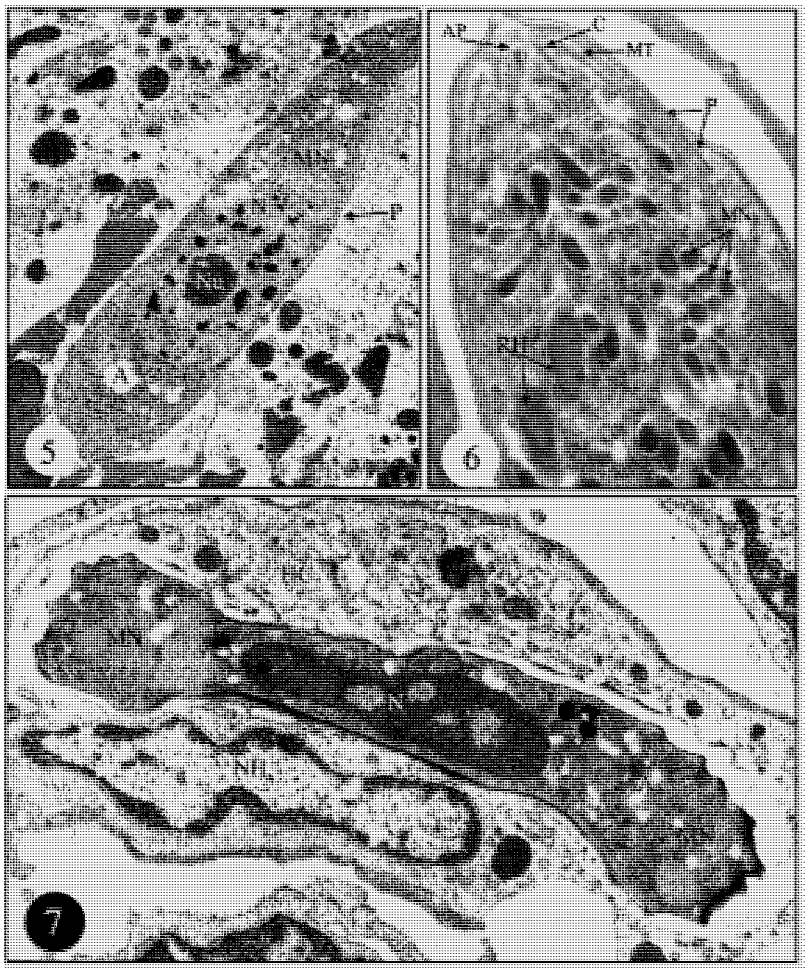

Fig. 5-13 : Transmission electron microscopy of blood stages and merogony
Fig. 5 : Electron micrograph of a longitudinal section of the parasite (P) showing micronemes (MN), Nucleus (N), nucleolus (Nu) and amylopectin (A) X 11500
Fig. 6 : A magnified part of the apical region of the parasite (P) showing, anterior polar ring (AP), Conoid (C), rhoptries (RH), micronemes (MN) and subpellicular microtubules (MT). X 21000
Fig. 7 : Electron micrograph of a parasite. just infecting a host cell (HC) and enclosed by a parasitophorous vacuole (PV). Nucleus of the host (NH), micronemes (MN), Nucleus (N) and amylopectin (A) X 17000

membrane of the pellicle was interrupted at the anterior polar ring and at the micropore (Fig. 6-13). At the apical pole of the parasite, a conoid of 0.32 μm long, covered with a canopy-like structure was present (Fig. 6). Rhoptries (8-10) and micronemes (up to 160) are found on both sides of the nucleus (Fig. 5-7), both have dark osmiophilic homogenous contents (Fig. 5-7 and 12-13). Beneath the pellicle, there are 80-90 subpellicular microtubules running from the anterior pole (polar ring) to the posterior region of the parasite. The parasite has a large nucleus and a prominent nucleus is often seen (Fig. 5, 12).

Merogony and merozoites: Schizonts in different developmental phases were observed in the endothelial cells of blood capillaries in different organs (spleen, lung, liver) as well as in the lumen of the lung and liver capillaries (Fig. 2-12). Schizogony began when the parasite left the infected erythrocytes in the capillaries and grew distinctly in size through accumulation of large amounts of the reserve food materials as well as the normal cell organoids and became a trophozoite (Fig. 2 and 8). These trophozoites usually lodged in the lumen of the capillaries or invade the endothelial and parenchyma cells developing into schizonts, meanwhile

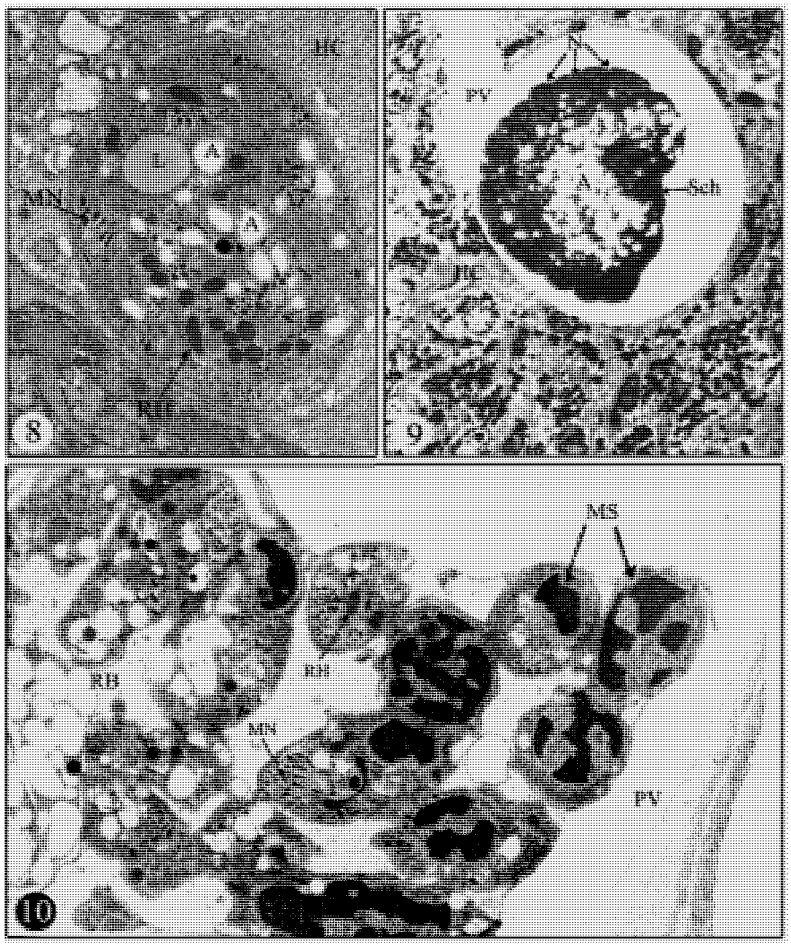

Fig. 5-13 : Transmission electron microscopy of blood stages and merogony
Fig. 8 : A cross sectioned young meront or merozoite in a host cell (HC), showing rhoptries (RH) and micronemes (MN), Lipids (L) and amylopectin (A) X 16500
Fig. 9 : A cross-sectioned developing schizont (Sch) in a parasitophorous vacuole (PV) peripheral nuclei of the schizont (N), host cell (HC) and amylopectin (A) X 12000
Fig. 10 : Electron micrograph of cross-sectioned mature schizont (Sch) with merozoites (MS) showing apical complex structures (RH, MN, …) in a parasitophorous vacuole (PV) with a residual body (RB) X 18000

they were enclosed within parasitophorous vacuoles (Fig. 3-9). As gradual development of schizonts proceeded, most characteristics of the apical complex structures disappeared (Fig. 9). Moreover, rapid nuclear divisions took place and the resulting nuclei migrated to the preiphary of the schizonts underneath the outer boundary, where daughter merozoites were developed as finger-like outgrowths from the surface of the meront, a process known as ectomerogony (Fig. 11-12). Merozoite formation began with the development of osmiophilic thickenings, along the limiting membrane of the meront in areas overlying the nuclei. These areas began to elevate into conical protrusions and the merozoite bud continued to elongate and protrude into the parasitophorous vacuole. The inner membrane complex of this young merozoite extended posteriorly during this time, incorporating a nucleus while other apical complex structures appear in the merozoite. As development of the merozoite completed, it detached off from the residual cytoplasm (Fig. 4-10, 12). Mature schizonts of 30×25 μm giving rise to 18-30 merozoites, each of 13×3.5 μm with a nucleus of 5.3×2.5 μm (Fig. 4, 10, 12). All details of the ultrastructural characteristics of the merozoites described here with are typical to those described before for the blood stages.

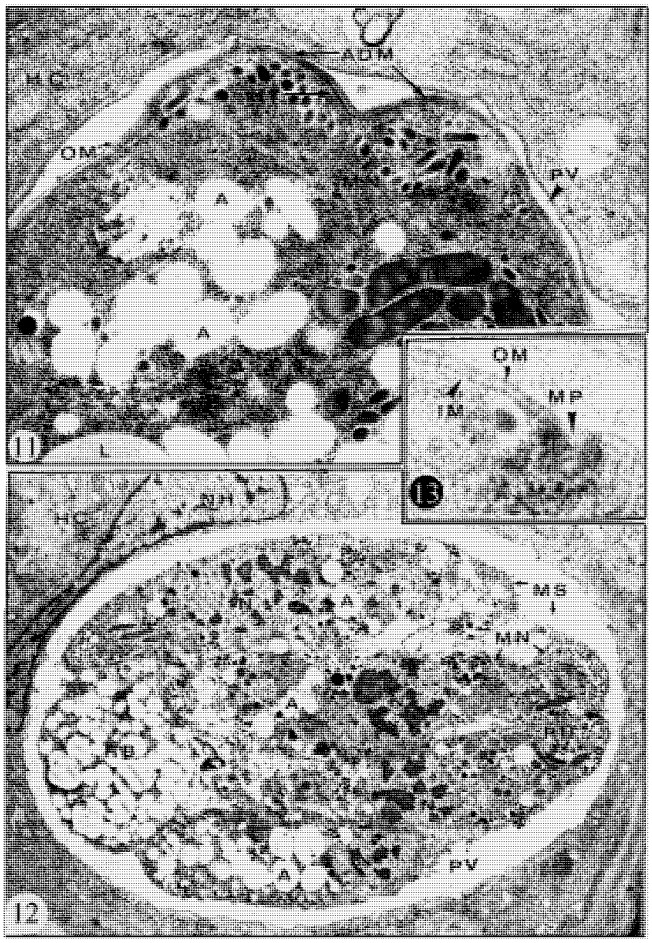

Fig. 5-13: Transmission electron microscopy of blood stages and merogony

Fig. 11 : Showed the ectomergonous development of merozoites at (ADM) anlage of developing merozoite in the parasitophorous vacuole (PV). outer membrane (OM) amylopectin (A), rhoptries (RH), micronemes (MN), lipids (L) subpellicular microtubules (MT) and Host cells (HC) X 22000

Fig. 12 : Electron micrograph of mature schizont (Sch) with merozoites (Ms) and a residual body (RB), amylopectin (A) micronemes (MN), Nucleus (N), in a parasitophorous vacuole (PV) X 19500

Fig. 13 : Electron micrograph of a micropore (MP), showing the pellicle of the parasite of two membranes (OM and IM) X 30.000

DISCUSSION

The present haemogregarine parasite described here with was the first adeleid protozoan parasite to be recorded in the viper *Cerastes cerastes gasperetti*. Moreover, Haemogregarines belong to a group of blood parazoa which are yet poorly studied, Classification of a certain haemogregarine species depending upon many criteria as site of schizogony and mode of transmission after the specific sporogonic process within its haematophagus vector as well as its host range (Bashtar *et al*., 1984a; Ahmed, 1998; Shazly, 2000). In the present investigation only stages of the parasite within the vertebrate host were available and no further identification is possible, so it is usually termed as a haemogregarine species.

The recorded rate of infection in the vipers represented a considerably moderate rate of natural infection among wild animals. However, vipers are seldom suffer massive mortality or epizootics because of the normal dispersal and territorialism of the species (Schmidt and Roberts, 1981; Ahmed, 1998). Although red blood corpuscles were recorded as the common site of infection with the present haemogregarine as well as other

haemogregarines (Bashtar *et al*., 1984a, 1987; Shazly, 2000, 2003; Abdel-Ghaffar *et al*., 1991; Lainson *et al*., 2003), leucocytes were recorded as site of infection with haemogregarines in some animals (Killick-Kendrick, 1984). Moreover, the present study revealed that infected red cells were hypertrophied as it recorded before (Bashtar *et al*., 1984a, 1991; Shazly, 2003; Lainson *et al*., 2003).

One of the most striking features of the present parasite was the overall ultrastructural similarity with other haemogregarines and all coccidian species (Bashtar *et al*., 1984a; Bashtar and AbdelGhaffar, 1987; Bashtar, 1990; Abdel-Ghaffar *et al*., 1994; Ahmed, 1998; Al-Sadoon *et al*., 1999; Shazly, 2000, 2003; Kamler *et al*., 2002; Jakes *et al*., 2003; Lainson *et al*., 2003).

These similarities included the fine structure such as pellicle, subpellicular microtubules, micronemes, rhoptries, micropore, conoid and the apical polar ring. However, many characteristic pecularities of haemogregarines were reported, such as, the presence of rhoptries and micronemes on both sides of the nucleus and the occurance of 80-90 subpellicular microtubules. Furthermore, stages of the parasite inside erythrocytes and the merozoites are bounded by a pellicle of two membranes, which is similar to other haemogregarines (Bashtar *et al*., 1984b; Bashtar, 1988, 1990; Abdel-Ghaffar *et al*., 1994, Ahmed, 1998; Mehlhorn, 2001; Diniz *et al*., 2002; Jakes *et al*., 2003). In contrast to the previous results some authors claimed that haemogregarines have three-layered pellicle (Paterson *et al*., 1988; El-Wasila, 1989; Shazly *et al*., 1992; Desser *et al*., 1995). Furthermore, (Vivier *et al*., 1970) announced that a pellicular complex of a triple layer was characteristic for all motile and infectious stages of sporozoa and that the description of only two unit membranes may be due to imperfect fixation or insufficient enlargement, which might occurred in the present study.

The merogonic stages of the presented parasite herein were found to develop in the parenchyma cells of liver and spleen as well as in the pulmonary endothelial cells of infected vipers. Similar observations were previously reported for other haemogregarines (Bashtar, 1988, 1990; Bashtar and Abdel-Ghaffar, 1987; Bashtar *et al*., 1991, 1994; Shazly, 2000, 2003). However, the testis has been reported to be the site of merogonic development for a haemogregarine species infecting the lizard *Lacerta saxicola* (Beyer *et al*., 1983). Also, nodular lesions in the heart, adipose tissues, diaphragm, mesentery and tongue of *Martes melapus* were found to contain haemogregarine schizonts (Yanai *et al*., 1995).

Following liberation of the parasites from erythrocytes and invading of the host cells, they change considerably in size, shape and structure into a new functional stationary immotile stage (trophozoiteschizont). This was evidenced by the loss of most of the inner membrane complex and the apical complex constituents (Bashtar, 1988; Bashtar *et al*., 1991; Bashtar and Abdel-Ghaffar, 1987; Shazly, 2000; Mehlhorn, 2001; Jakes *et al*., 2003). Nevertheless, the cyst forming coccidians are known to maintain their 3-layered pellicle during schizogony (Scholtyseck, 1973). The nucleus of the schizont eventually underwent many repeated divisions and the resulting daughter nuclei migrated to the periphery of the schizont directly underneath the outer boundary. The first indication of merozoite formation was a thickening and protrusion of the schizont boundary at sites where a nucleus was close to this membrane and this structure is termed merozoite anlage. Similar observations were reported on the coccidia as well as the subsequent development and formation of merozoites, where such process was described as ectomerogony (Sohltyseck, 1973; Bashtar *et al*., 1984a, b, 1991, 1994; Shazly, 2000, 2003; Jakes *et al*., 2003; Lainson, 2003). The fine structure of the free merozoites was similar to that described for the erythrocytic stages of the parasite, except for the presence of more amylopectin granules in the merozoites. This was observed before in many coccidian merozoites (Bashtar *et al*., 1984; Bashtar, 1988, 1990; Ahmed, 1998; Shazly, 2000; Mehlhorn, 2001; Jakes *et al*., 2003). Although the red blood cells occupied by parasites were hypertrophied in most cases, there is no apparent pathological effect on the infected vipers, contrary to fish haemogregarines which were reported to be pathogenic and sometimes fatal to their infected hosts (Kirmse, 1980; Khan and Newman, 1982).

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