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# Investigation of Tickborne Pathogens within Naturally Infected Brown Dog Tick (Ixodidae: *Rhipicephalus Sanguineus*) in Egypt by Light and Electron Microscopy

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## ABSTRACT

Tick borne pathogens present a significant health challenge to animals and human because a single tick may transmit multiple pathogens to a mammalian host during feeding. The present study detected tick-borne pathogens from pet dogs. A total of 666 ticks were collected from 144 pet and sheltered dogs in Egypt from April to September 2018. For hemolymph, midgut and salivary gland smears 546 ticks were used as well as 360 egg smears from 120 female tick were examined by light microscope. The infected ticks were prepared for transmission electron microscopy. Ticks were identified; *Rhipicephalus sanguineus*. Light microscopy showed infection rates of 44.69, 68.50 and 15.75%, in hemolymph, midgut and salivary gland, respectively. *Hepatozoon canis* recorded the highest rates in hemolymph and midgut (49.82 and 35.89%, respectively), but *Theileria* spp. was the lowest (0.73 and 2.93%, respectively). In salivary gland smears, *Babesia canis* was detected in 13.55% and *Theileria* spp. in 1.83%. Mixed infection in same tick was recorded in 4.76 and 0.37% in midgut and salivary gland smears, respectively. *Babesia canis* stages were recovered from 15% of egg smears. *R. sanguineus* was naturally infected by *Babesia, Theileria, Hepatozoon* and *Anaplasma phagocytophilum* as well as mixed infections of protozoa accompanied by a complicated sign of diseases and failure in accurate diagnosis.

**Key words:** *Anaplasma, Babesia, Hepatozoon, Rhipicephalus sanguineus*, *Theileria*, TEM.

## INTRODUCTION

*Rhipicephalus sanguineus* is the most widely distributed tick worldwide and has a broad host-range including birds, reptiles, amphibians and mammals. It has both veterinary and zoonotic importance as it causes blood loss in the host and transmits several pathogens such as *Babesia canis* to dogs and rickettsial pathogens to humans (Walker, 2003; Rehman *et al*., 2019; Mahmoud *et al*., 2020). The tick guts were examined by many authors for detection of *Babesia* and *Theileria* spp. to study sexual reproduction and development of ookinete which penetrate salivary gland (Schein *et al*., 1975; 1977; Gough *et al*., 1998). Also, *Hepatozoon spp.* gametogony and sporogony detected in gut (Mathew *et al*., 1998; Baneth *et al*., 2007). *Anaplasma phagocytophilum* multiplication also, was described in tick tissue or on tissue culture derived from tick gut (Blouin and Kocan, 1998; Dyachenko *et al*., 2013). Asexual reproduction of *Babesia* and *Theileria* spp. has been observed in the cells of the salivary gland of their tick vectors, leading to the formation of sporozoites (Mehlhorn *et al*., 1979; Schein *et al*., 1979; Zaman *et al*., 2020; Ali *et al*., 2020). Regarded to close relationship between human and dogs as well as the great importance of *R. sanguineus* as a vector for many pathogens, the present study originated to give spot light on tick borne pathogens in *R. sanguineus* by light microscope and transmission electron microscopy. Since Light microscope only recorded few details of many stages as sporozoites in the salivary glands of the hosts, then TEM was applied to detect more details of these stages.

## MATERIALS AND METHODS

### Sample collection

Firstly, the body of each dog was inspected especially head, ears, axilla, abdomen and inter digital spaces to detect ticks if present. Tick collections (n=666) were performed directly from 144 pet and sheltered dogs from Cairo and Giza Governorates, in collection tubes. Some of ticks preserved in 70% alcohol for morphological identification and others for smear preparation. The collected ticks were identified by using binocular microscope (Zeiss Stemi 2000-C) according to Walker (2003).

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**Preparation of smears**

A Tick's surface was sterilized twice with 75% ethanol and once with phosphate buffered saline (PBS) before preparation of hemolymph smears. Ticks were viewed and dissected under a dissecting microscope (Patton *et al.*, 2012).

A) Tick's surface was sterilized twice with 75% ethanol and once with phosphate buffered saline (PBS) before preparation of hemolymph smears. Ticks were viewed and dissected under a dissecting microscope (Patton *et al.*, 2012).

B) Midgut and salivary gland: out of 666 a total of 546 ticks were dissected (Edwards *et al.*, 2009). After immobilization of tick, the ticks were washed with a drop of PBS to prevent desiccation of the tissues. The scutum was removed with a micro scalpel. At this point, connective tissue and tracheae are apparent and must be removed to observe deeper structures. The anterior salivary glands were appeared as grape like structures at the proximal end of the tick. There are also other sets of salivary glands located near the midgut. The gut appeared as a dark red, spider shaped structure. Smear preparations from tick gut or salivary glands were made by placing the specimen on a glass slide, squashing it with another slide and smearing it out in a single movement. The smears were then air dried, fixed in methanol for 5 min. and stained in a 10% solution of Giemsa stain for 30 min (Qayyum *et al*., 2010), then examined under oil immersion lens (X 1000) by light microscope (Olympus).A total of 360 egg smears were prepared from 120 engorged female ticks eggs for detection of transovarian transmission of *Babesia canis* infection. Ovipossion induced in laboratory according to method described by Okon *et al*. (2011). Each tick was individually incubated at 26-28°C and 85% RH using saturated potassium chloride. Only a mass of eggs obtained from the 4$^{th}$ day till the 6$^{th}$ day of oviposition were squashed against clean glass slides (Nefedova *et al.*, 2004). Slides were fixed in methanol, stained with Giemsa and examined by light microscope with oil immersion lens.

**Transmission electron Microscope (TEM)**

The engorged female ticks were dissected in PBS then midgut and salivary glands were immersed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4). The tick specimens were left in the same fixative for 24 hours at 4°C, then rinsed in 0.1 M cacodylate buffer and post-fixed in 1% Osmium tetroxide for one hour. After rinsing in the buffer, the material was dehydrated in graded ethanol and embedded in Araldite. Semithin sections were cut by ultra-microtome and stained with toluidine blue for examination by light microscope (Nunes *et al.*, 2006). Ultra-thin sections, cut on with a diamond knife then stained on copper grids with uranyl acetate and lead citrate (Abuowarda *et al.*, 2015; 2020). Grids were visualized through electron microscope (JEOL-1200EX), Faculty of Science, Ain Shams University.

## RESULTS

Morphologically ticks recruited from dogs were identified as *R. sanguineus* (Fig. 2). Basis capituli were hexagonal in shape with sharp lateral angles. The mouth and basis capituli were equal in size and palp pedicels were short, eyes were slightly convex (Fig. 2G) and eleven festoons were detected (Fig. 2A). Spiracle plates in both sexes with tails which are narrow, less than the adjacent festoon width (Fig. 2E, F). In male lateral grooves were a distinct and long (extending from eyes to the festoons). Subanal shields were absent and anal opening surrounded by comma shaped adanal plates with large accessory adanal plates. Caudal appendage was broad in fed males (it protrudes as a slight bulge) (Fig. 2B). Female of *R. sanguineus* had a broad U-shaped genital opening (Fig. 1D) and scutum posterior margin with a distinctly concave curve posterior to the eyes (Fig. 2C,H). *R. sanguineus* can attach everywhere on the dog, however ears, axilla inter-digital spaces, back, and inguinal region were their preferred attachment sites (Fig. 3).

The incidence rates of infection with tick borne blood parasites in *R. Sanguineus* were detected by Giemsa stained smears prepared from the tick hemolymh, midgut and salivary gland (Table 1; Fig. 2). Total infection rates were 68.50, 44.69 and 15.75%, in midgut, hemolymph and salivary gland respectively. *Hepatozoon canis* recorded the highest incidence rates in midgut and hemolymph (49.82 and 35.89%, respectively), while *Theileria* spp. was the lowest one (2.93 and 0.73% respectively). The prevalence of *Babesia canis* and *Theileria* spp. in salivary gland smears were recorded to be 13.25 and 1.83% respectively. In contrast, *H. canis* never be detected. The percentage of mixed infection in the same tick was recorded in midgut and salivary gland smears (4.76 and 0.37 % respectively). Moreover, egg smears revealed that, 15% were infected only by *Babesia canis* stages. *Anaplasma phagocytophilum* was not detected in all smears.

The morphological differentiation of some tick borne blood parasites in midgut, hemolymph and salivary gland smears by light microscopic examination revealed presence of different stages of *Hepatozoan canis* (Fig. 4). *H. canis* macrogamete appeared as spherical cells with a large, round and eccentric nucleus (Fig. 4A). *H. canis* zygotes characterized by eccentric and oval shaped nucleus and granulated cytoplasm (Fig. 4B). Mature oocysts were round and filled with sporocysts (Fig. 4D). Oocysts at various stages of development were detected in the same tick, they ranged in size from100-250µm x 100 µm.

From midgut smears, the only stage of *Babesia* that detected was round form with central nucleus which represents early stages of zygote (Fig. 5A-C). The macro and microgametes could not be detected in the present study. *Theileria* stages in midgut were round with peripheral nucleus. They represented macrogametes (Fig. 6A). They were about 3µm in diameter which, increase in size after fertilization to form zygote. The zygote appeared round with pale cytoplasm and nuclear materials located at the cell margin. Its diameter was 5µm then increased up to 10 µm and the nucleus became peripheral again (Fig. 6B-D). Furthermore, motile kinetics were detected in hemolymph smears which appeared as club shaped structure with dark polar cap at the anterior end (Fig. 6E).

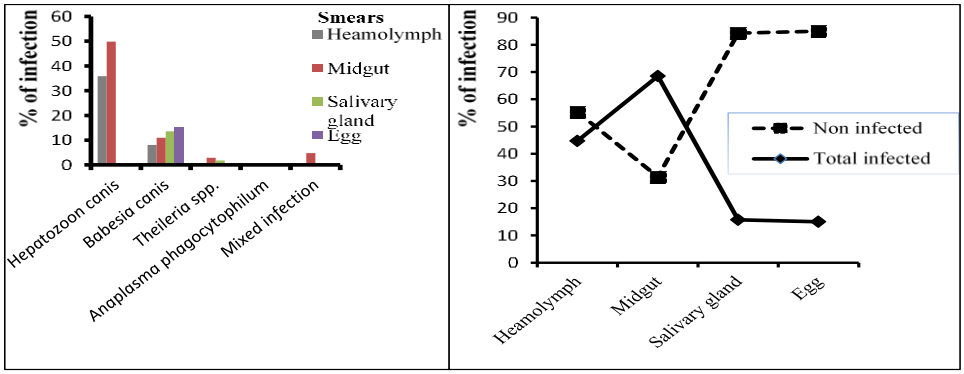


**Fig. 1:** Infection rates of tick-borne protozoan parasites in smears prepared from *R. sanguineus*

**Table 1:** Incidence rates of infection with tick borne blood parasites in smears from brown dog ticks

| Tick borne pathogens | Examined tick smears from total of 546 ticks | | | Three egg smears from each of 120 females |
|---|---|---|---|---|
| | Hemolymph | Midgut | Salivary gland | |
| | Infected No. (%) | Infected No. (%) | Infected No. (%) | Infected No. (%) |
| *Hepatozoon canis* | 196(35.89) | 272(49.82) | 0(0) | 0(0) |
| *Babesia canis* | 44(8.1) | 60(10.99) | 74(13.55) | 54(15) |
| *Theileria* spp. | 4(0.73) | 16(2.93) | 10(1.83) | 0(0) |
| *Anaplasma phagocytophilum* | 0(0) | 0(0) | 0(0) | 0(0) |
| Mixed infection | 0(0) | 26(4.76) | 2(0.37) | 0(0) |
| Non infected tick | 302(55.31) | 172(31.50) | 460(84.25) | 306(85) |
| Total | 244(44.69) | 374(68.50) | 86(15.75) | 54(15) |

Salivary gland smears showed presence of penetrating kinete which rounded up to form sporont which then begins binary fission in case of *Babesia* sporozoites and multiple fission in case of *Theileria* sporozoites. They were morphologically same but differ in size as *Babesia* sporozoites were 3-5 µm (Fig. 5 D) and *Theileria* sporozoites were 1.8-2.5 µm (Fig. 6F). Egg smears showed different stages of *Babesia* as small rode, club and sausage shaped (Fig. 5E, F).

Concerning light microscope examination of semithin histological sections prepared from midgut and salivary gland of *R. sanguineus*, *Anaplasma phagocytophilum* colony detected in gut tissues (Fig. 7C). Early oocysts of *H. canis* after fertilization were detected in semithin sections from gut with eccentric and oval shaped nucleus and granulated cytoplasm (Fig. 7D). On the other hand, salivary gland parasitized cell became filled with the dividing stages of *Babesia canis*. So that, most of the parasitized cells had granular appearance and the host cell nucleuses were pushed toward the margins of the cells or even obscured by sporozoits. In addition, a round structure with centrally located nucleus measuring 5 µm in diameter was detected in salivary acini type I and II (spront). It represented the ookinete after penetrating the salivary gland and rounded up before beginning of division to form infective stage (sporozoites) (Fig. 7A, B).

In the midgut of *R. sanguineus*, the early oocyst of *H. canis* appeared as ovoid structure with large round nucleus, its cytoplasm was granular with few micronemes and rhoptries. Its size was 10 x 7.15µm (Fig. 8A). *H. canis* early oocyst has peripheral nucleus with large nucleolus and more granular cytoplasm with electron dens granules and has few numbers of micronemes. Oocyst cytoplasm filled with amilopectin granules and lipid vacuoles, its wall was undulated and folded. Its size was 12.74 x 8.2µm (Fig. 8B). Sporocysts varied in size from 2.2-3.6x2.13-2.74µm. Early sporocyst after separation from oocyst has many lipid vacuoles which exhausted during further division. Young sporocyst has many granular aggregates (crystalloid bodies) then divided to sporozoites. This sporocyst has some lipid vacuoles and single nucleus with distinct nucleolus (Fig.8C). The nucleus with a thick wall consolidated above the plasmalemma was then divided into multi nucleated sporocyst and the lipid vacuoles were exhausted (Fig.8D). Note worthily, no parasitophorous vacuole surrounding *Hepatozoon* stages were detected.

In addition, *A. phagocytophilum* was observed in midgut tissue as cytoplasmic double membranes vacuoles of different sizes containing mainly polymorphic organisms with size ranged from approximately 0.592-1.49µm (Fig. 8E). These polymorphic organisms appeared highly electron dense which represented infective stage that can infect other cells. Some of those electron dense organisms were observed free in the cytoplasm of infected cells after rupture of the double walled vacuoles (Fig.8F). Electron microscopic examination of salivary gland tissue illustrated the penetrating kinetes (K) of *Babesia canis* which became ovoid or spherical in shape and their pellicles were reduced in many places (Fig. 9A). The cut sections through penetrating kinetes (K) revealed that these kinetes were closely filled with micronemes, double walled organelles which resemble to mitochondria. These kinetes also, have accumulations of the endoplasmic reticulum and situated directly in the cytoplasm of the host cell without surrounding by a parasitophorous vacuole and, no rhoptries were observed. They measured 10.1x8.82µm. Binary fission of this kinete began and the dividing stages became globular parasites their size varied from 2.75-3.61x2.35-2.86µm in diameter. The cytoplasm of the dividing stages was completely free from the organelles mentioned in the kinete. Their cytoplasm appeared relatively electron pale and provided with large vacuole and numerous ribosome-like granules (Fig. 9B, C). The dividing stages transferred to pyrifom sporozoites which had a three-layered pellicle, with 4-6 rhoptries and few micronemes. It measured about 3.62 x2.83 µm (Fig 9D). Furthermore, cross section through the apical pole of the sporozoites showed that the membrane had a few micropores and had some organelles as golgi apparatus, rhoptries and some microneme-like structures (Fig. 9E).

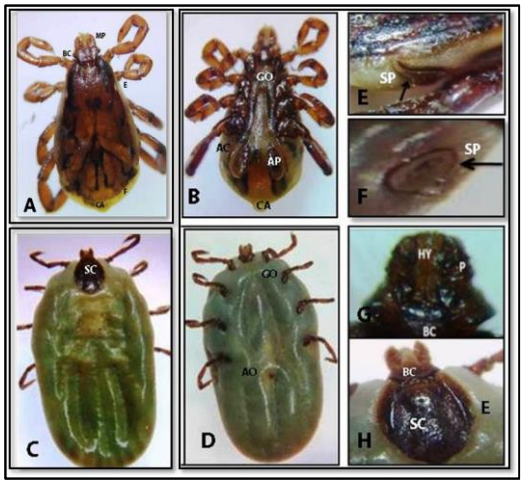


**Fig. 2:** Male and female *R. sanguineus* showed (A) Male dorsal view, (B) Male ventral view, (C)Female dorsal view, (D) Female ventral view, (E) Male spiracular plate, (F) Female spiracular plate, (G) Ventral view of mouth part and basis capituli and (H) Scutum posterior margin in female (X 20): (P: palps, HY: hypostome. Mp : mouth part, BC :basis capituli, E: eye, F: festoons, CA: caudal appendage, GO: genital opening, AO: anal opening, AP: adanal plate, AC: accessory adanal plate SC: scutum, E: Eye).

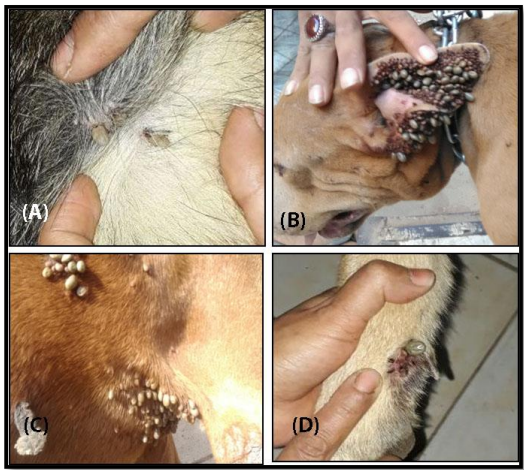


**Fig. 3:** Predilection attachment sites of *R. sanguineus* on infested dogs.

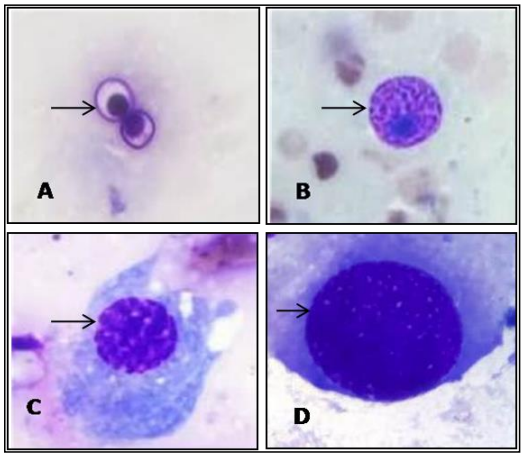


**Fig. 4:** Giemsa stained gut smears of *R. sanguineus* showed developmental stages of *H. canis* (A) Female macrogamete before fertilization, (B) zygote (early oocyst), (C) young oocyst with irregular cytoplasm in early sporogony, (D) mature oocyst filled with ripe sporocysts (X 1000).

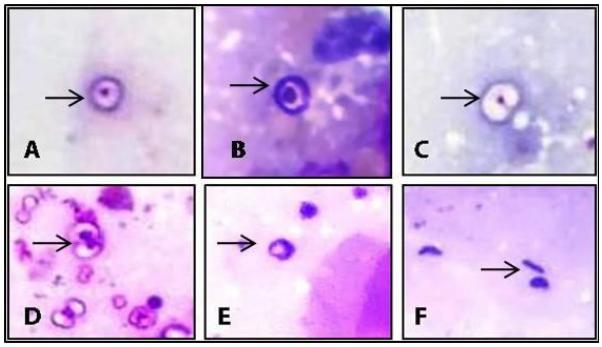


**Fig. 5:** Developmental stage of *Babesia canis* in Giemsa stained smears from gut, salivary gland and egg of *R. sanguineus.* (A and B) zygote in gut, (C) gut smear showing developing kinete inside zygote, (D) salivary gland smears showing binary fission of sporont to form sporozoites, (E and F) *Babesia* stages in egg smears (X 1000).

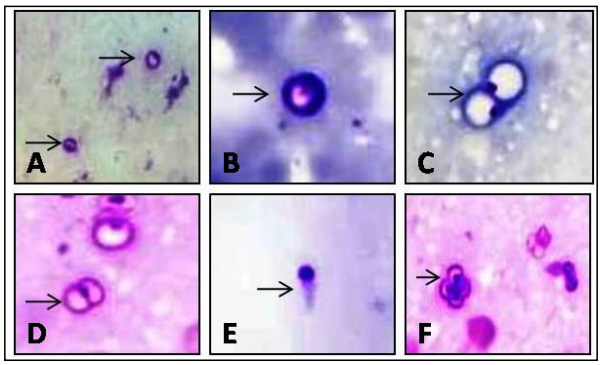


**Fig. 6:** Developmental stage of *Theileria* spp. in Giemsa stained smears from gut, hemolymph and salivary gland of *R. sanguineus.* (A) Macro gamete. (B) Early zygote with chromatin material at cell margins. (C) Older zygote with distinct peripheral nucleus. (D) Developing kinete in zygote. (E) Club shaped mature kinete with dense polar cap in hemolymph smears. (F) Multiple fission of sporont to form sporozoites in salivary gland smears (X 1000).

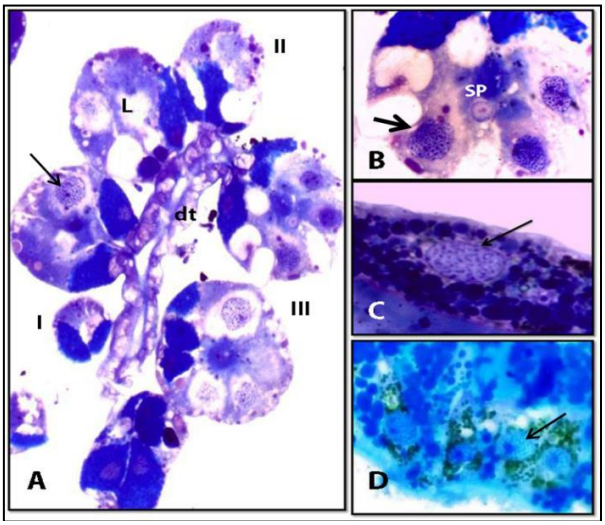


**Fig. 7:** Toludine blue stained semithin section through salivary gland and midgut of *R. sanguineus* showing (A and B) Some salivary acini filled with *Babesia* sporozoites, (C) *A. phagocytophilum* colony in midgut section, (D) Early oocyst of *H. canis* in midgut sections. (I: acini type I, II: acini type II, III: acini type III, L: acinus lumen, dt: duct, SG: salivary granules, Sp: spront (A at X 400 and B, C, D at X 1000).

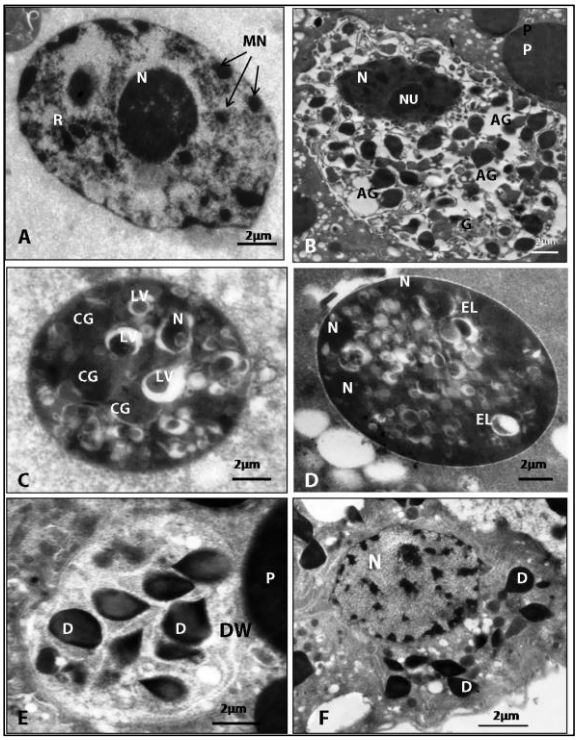


**Fig. 8:** Electron photograph of ultrathin cross sections through gut of *R. sanguineus* showing (A) *H. canis* early oocyst; few rhopteris (R) and micronemes (MN), (B) Young oocyst of *H. canis* has many amylopectin granules (AG), electron-dense bodies, and folded wall, (C) Young sporocyst filled with crystalloid granular bodies (CG) and lipid vacuoles (LV), (D) Older Sporocysts with multiple divided nuclei (N), and exhausted lipid vacuoles (EL), (E) *A. phagocytophilum c*olonies in double membrane bounded vacuole (DW) and contained electron-dense granules (D), (F) Ruptured vacuole and dense form of *A. phagocytophilum* are free in cytoplasm of infected cells.

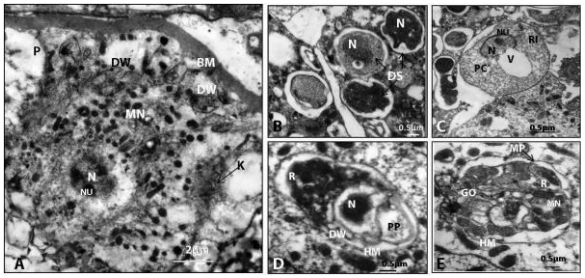


**Fig. 9:** Electron photographs of ultrathin cross sections through salivary gland of *R. sanguinus* showing (A) Penetrating kinete(K), (B) Dividing stages(DS), (C) Higher magnification of dividing stages almost pyriform in shape with cytoplasm filled with ribosomes, (D) Sporozoites longitudinal section, (E) Cross section in sporozoite apical part: ((N) nucleus, (Nu) nucleolus, (P) pellicle, (BM) basal membrane of host cell, (DW) double-membraned structures, (V) vacuoles, (RI) ribosomes, (PC) pale cytoplasm, (R) rhoptries, (MN) micronemes, (MP) micropores, (PP) posterior polar ring, (GO) golgi apparatus and (HM) mitochondria of host cell).

## DISCUSSION

Tick infestation is considered a serious problem in tropical and subtropical areas, where hard ticks are responsible for transmitting various pathogens as viruses, rickettsia, bacteria, and protozoa. Several tick-borne agents have now been associated with diseases to human and dogs (Pereira *et al.*, 2018). In the current study, ticks were identified as *R. sanguineus* which came in accordance with Abdullah *et al.* (2016) who reported that dogs in Egypt are primarily infested by *R. sanguineus.*

The present study elucidated the development of different pathogens in *R. sanguineus* and followed the morphological changes of *B. canis, H. canis, Theileria spp.* and *A. phagocytophilum* that occur during their development inside the vector.

As the previous works on the incidence of *B. canis, A. phagocytophilum* and *Theileria spp*. in salivary glands, hemolymph, midgut and egg smears from *R. sanguineus* were scant; the present study was discussed with any works done on other tick species even from different animals.

Incidence of *B. canis* infection in hemolymph smears was 8.1% which was much lower than that detected by Fahmy *et al.* (1983) in Giza, 75.76% of hemolymph smears from *Boophilus annulatus* collected from cattle infected by *Babesia* spp. Also, Okon *et al.* (2011) found a higher incidence (35%) of *Babesia bigemina* in *Boophilus decoloratus* collected from cattle.

In addition, the midgut smears showed *Babesia* spp. infection 10.99% which was higher than that recorded by El-Kamah*et al.* (2007) who found that *B. canis* infection was 2.4% in midgut and salivary gland smears prepared from *R. sanguineus*. The variations in results may be regarded as some authors performed experimental infection to the vector by rearing ticks on infected splenectomized calves. Also, variation in results could be due to variations in examined tick species and their vertebrate host. Unlike Fahmy *et al.* (1983) who recorded percentage of *Babesia* stages in egg smears prepared from *Boophilus annulatus* were 80 and 90% in the 6$^{th}$ and 7$^{th}$ day of egg laying, the present study showed lower transovarian transmission of *Babesia* spp. (15%). But, Mahoney and Mirre (1971) agreed with the present results as they found the prevalence of bovine *Babesia* within the progeny of infected female *Boophilus microplus* was very low.

Prevalence of *H. canis* stages recovered from hemolymph and midgut smears were recorded to be 35.89 and 49.82% respectively. Wahba and El-Refaii (2003) recorded higher rates as 62.7 and 6.6% in hemolymph and gut of *Hyalomma dromedarii* collected from camels. Baneth *et al.* (2001) found the oocyst of *H. canis* in 85% of hemolymph smears of *R. sanguineus*.

Also, smears from adult tick collected from a naturally infected dog, *H. canis* never detected in salivary gland smears, a fact which supported with what reported by Mathew *et al.* (1998) in *H. americanum* in its vector (*Amblyomma maculatum*). This report provides further evidence that tick biting is not the rout of transmission of *Hepatozoon* spp. to vertebrate host but the oral intake of ticks containing mature oocysts with infective sporozoites is the main route of infection (Smith, 1996; Ewing and Panciera, 2003). In addition, the incidence of *Theileria* spp. was very low (0.73, 2.93 and 1.83%) in heamolymph, midgut and salivary gland smears of *R. sanguineus* respectively. While, Fahmy *et al.* (1983) recorded much higher incidence of *Theileria* spp. (59.5%) in hemolymph smears prepared from female *Hyalomma anatolicum* collected from Egyptian cattle.

Mixed infection in same tick was observed in 4.76% of examined midgut smears. These results are in accordance with Chen *et al.* (2014) who recorded co-

infection of *Babesia* spp. with Rickettsia spp. in ticks collected from domestic animals in China. They likewise showed that *Theileria* spp. might be more similar to co-exist with other agents in ticks.

In the present study, *H. canis* stages in gut fits well the observations of Baneth *et al.* (2007) in hemocoel smears of experimentally infected *R. sanguineus* after injection with blood from infected dogs. But, Wahba and El-Refaii (2003) detected *Hepatozoon* spp. in gut of *Hyalomma dromedarii* collected from slaughtered camel but with smaller sizes of oocysts than in the present results. Such difference in dimensions might be attributed to species of the vector and the infection load within the vector. The only stage of *B. canis* detected in midgut was zygote and neither macro nor microgamete was detected. This was contradictory to Gough *et al.* (1998) where they cultivated *B. bigemina in-vitro* after addition of tick gut extract obtained from fully engorged females of *B. microplus*. The explanation of this result may be due to fusion of *B. canis* gametes occurred very early in the host erythrocytes before lysis in the gut of the vector (*Dermacentor reticulatus*) as reported by Mehlhorn and Schein (1984) and Mehlhorn *et al.* (1980). Morphometric similarities were found between *Theileria* stages in gut and hemolymph of *R. sanguineus* as recorded also in previous reports (Schein *et al.*, 1975; 1977; Warnecke *et al.*, 1979; El-Refaii *et al.*, 1998) to *T. annulata* (in gut of *Hyalomma a.excavatum*) and *T. parva* (in gut of *R. appendiculatus)*, *T. velifera* (in gut of *Amblyomma variegatum)*, and *T. camelensis* (in gut of *Hyalomma dromedarii)* respectively. However, there were distinct differences in morphology of macro and microgamete in the present study and that of *T. mutans* (Warnecke *et al.*, 1980). The morphological features of the infectious sporozoites in salivary smears and salivary gland sections, there was no basic difference between those in *Babesia* and those in *Theileria* however, *Theileria* sporozits were half the size of *Babesia* (Schein *et al.*, 1979).

In the present study, the recorded sporogenic development of *H. canis* in gut of *R. Sanguineus*, agreed with Paperna *et al.* (2002) who reported *Hepatozoon kisrae* in gut of *Hyalomma cf. aegyptium.* But, Desser *et al.* (1995) and Hervas *et al.* (1997) reported parasitophorous vacuoles surrounding *Hepatozoon* stages. The reason for absence of parasitophorus vacuoles could be that the recorded *Hepatozoon* stages were free in gut lumen (Paperna *et al.*, 2002).

In the current study, the ultrastructure of *A. phagocytophilum* (dense form) in gut of *R. sanguineus* was similar to canine ApMuc01c strain in IRE/CTVM20 cells, *Anaplasma phagocytophilum* from white-tailed deer (Dyachenko *et al.*, 2013). *A. phagocytophilum* and *A. marginale* from equine in the *Ixodes scapularis*-derived IDE8 and ISE6 cell lines (Munderloh *et al.*, 1996, 2003; Blouin and Kocan, 1998).

In salivary gland tissue, all developmental forms described by electron microscope in the present study were previously reported for *B. canis* in *Dermacentor reticulatus* salivary gland (Schein *et al.*, 1979). The ultrastructure of sporozoites resembles that recorded by Riek (1966) for *B. argentina* in salivary gland of *Boophilus microplus* in Australia and by Friedhoff *et al.* (1972) for *B. ovis*, furthermore, they were closely resembling to the intra erythrocytic merozoites. On the other hand, Potgieter and Els (1976, 1977) found fissions in *B. bovis* and *B. bigemina* in salivary gland of *Boophilus microplu*s and *Boophilus decoloratus* as schizogony not binary fission as in the present study. This disagreement might be due to these authors considered the granular cytoplasm of the altered host cell as cytoplasm of a "schizont", within which the "merozoites" were already present.

The ultra-structure of penetrating kinetes of *B. canis* recorded in the present study was very similar to *Theileria ovis* kinetes (Mehlhorn *et al.*, 1979), but there was difference in size. They also recorded that nuclear division of *T. ovis* kinetes started before it reached to the salivary gland where multiple-fission took place in the parasites. The SEM could not detect *Theileria* stages in salivary gland. Although, sporogeny in *Theileria* was similar to *Babesia canis*, *Theileria* sporogny appeared as multiple fissions of the large cytomeres and their stretched nuclei simultaneously give rise to several smaller sporozoites than that of *Babesia canis*, as described by Schein and Friedhoff (1978) for *Theileria annulata* in salivary gland of *Hyalomma a. excavatum* and Mehlhorn *et al.* (1979) for *Theileria ovis* in *Rhipicephalus evertsi*.

## Conclusion

The present study concluded that *R. sanguineus* collected from naturally infested dog were infected with *Babesia, Theileria, Hepatozoon, Anaplasma phagocytophilum* pathogens. Furthermore, mixed infections with multiple pathogens were recorded which lead to more complicated signs of diseases and increase failure in accurate diagnosis.

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