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 *Anaplasmaphagocytophilum* 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). *Anaplasmaphagocytophilum* 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).

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 4th day till the 6th 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, axila 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 hemolymph, 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 Hepatozoon 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).
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, Anaplasmaphagocytophilum 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 amilopeptin 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 worthy, no parasitophorous vacuole surrounding Haptozoon 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 pyriform 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 micro pores and had some organelles as golgi apparatus, rhoptries and some microneme-like structures (Fig. 9E).

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

<table>
<thead>
<tr>
<th>Tick borne pathogens</th>
<th>Hemolymph Infected No. (%)</th>
<th>Midgut Infected No. (%)</th>
<th>Salivary gland Infected No. (%)</th>
<th>Three egg smears from each of 120 females</th>
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<tbody>
<tr>
<td><strong>Hepatozoon canis</strong></td>
<td>35.89 (196)</td>
<td>49.82 (272)</td>
<td>(0) (0)</td>
<td>(48.50 (272))</td>
</tr>
<tr>
<td><strong>Babesia canis</strong></td>
<td>10.99 (44)</td>
<td>13.55 (74)</td>
<td>15.63 (54)</td>
<td>13.55 (54)</td>
</tr>
<tr>
<td><strong>Theileria spp.</strong></td>
<td>2.93 (4)</td>
<td>1.83 (10)</td>
<td>0.73 (0)</td>
<td>0.73 (0)</td>
</tr>
<tr>
<td><strong>Anaplasmaphagocytophilum</strong></td>
<td>0.00 (0)</td>
<td>0.00 (0)</td>
<td>0.00 (0)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td><strong>Mixed infection</strong></td>
<td>2.86 (0)</td>
<td>2.86 (0)</td>
<td>2.86 (0)</td>
<td>2.86 (0)</td>
</tr>
<tr>
<td><strong>Non infected tick</strong></td>
<td>0.00 (0)</td>
<td>0.00 (0)</td>
<td>0.00 (0)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>35.89 (196)</td>
<td>49.82 (272)</td>
<td>15.63 (54)</td>
<td>48.50 (272)</td>
</tr>
</tbody>
</table>

Fig. 1: Infection rates of tick borne protozoan parasites in smears prepared from R. sanguineus.
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: Toluidine 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: sporont (A at X 400 and B, C, D at X 1000).
nullatus. The variations in results may be
(C) Young sporocyst filled with
molymph
thories, (MN) micronemes, (MP) micropores,
onlaries in double
agents have now been associated with
responsible for transmitting various pathogens as viruses,
mitochondria of host cell). (PP) posterior polar ring, (GO) golgi apparatus and (HM)
cytoplasm, (R) rhopt
temned structures, (V) vacuoles, (RI) ribosomes, (PC) pale
(P) pellicle, (BM) basal membrane of host cell, (DW) double
section in sporozoite apical part: ((N) nucleus, (Nu) nucleolus,
kinete
salivary gland
phagocytophilum
granules (D), (F) Ruptured vacuole and dense form of
membrane bounded vacuole (DW) a
lipid vacuoles (EL), (E)
Older Sporocysts with multiple divided nuclei (N), and exhausted
bodies, and folded wall
canis
rhopteris (R) and micronemes (MN), (B) Young oocyst
of
Fig. 8: Electron photograph of ultrathin cross sections through
gut of R. sanguineus showing (A) H. canis early oocyst; few
rhopetris (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 colonies 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.
fication 10.99% which was higher than that recorded by
El-Kamahet 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 6th and 7th 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
midgut smears, a fact which supported with what reported
by Mathew et al. (1983) who recorded percentage of
Babesia spp. to vertebrate host but the oral intake of
within the progeny of infected female
Boophilus microplus was very low.

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 route 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 hemolymph,
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 coexist 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-Refai (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-Refai 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 sporozoits 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, Dessier et al. (1995) and Hervas et al. (1997) reported parasitophorous vacuoles surrounding Hepatozoon stages. The reason for absence of parasitophorous 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 phagocytophium from white-tailed deer (Dyachenko et al., 2013). A. phagocytophium 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 microplus and Boophilus decoloratus as schizonygot 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, sporogony in Theileria was similar to Babesia canis, Theileria sporogy 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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