



Cairo University
Faculty of Veterinary Medicine
Department of Parasitology



Advanced Studies on Tick Borne Blood Parasites Among Pets (Dogs)

A thesis Presented by

Asmaa Abdelwadod Mohamed Hegab

B.V.Sc. Benha University (2004)

M.V.Sc. Cairo University (2017)

For Ph.D. degree

(Parasitology)

Under Supervision of

Dr. Magdy Mostafa Fahmy

Professor of Parasitology

Faculty of Veterinary Medicine

Cairo University

Dr. Hussein Mohamed Omar

Professor of Parasitology

Faculty of Veterinary Medicine

Cairo University

Dr. Mai Abuowarda Mohammed

Assistant Professor of Parasitology

Faculty of Veterinary Medicine

Cairo University

Dr. Souzan Girgis Ghattas

Chief Researcher

Department of Parasitology

Animal Health Research Institute

2021



Cairo University
Faculty of Veterinary Medicine
Department of Parasitology



Name: Asmaa Abdelwadod Mohamed Hegab.

Nationality: Egyptian.

Date and place of birth: 16-10-1982 (Kaliobia Governorate).

Scientific Degree: Ph.D. in Veterinary Medical Sciences.

Spatiality: Parasitology.

Title of thesis: Advanced studies on tick borne blood parasites among pets (dogs).

Supervisors:

Dr. Magdy Mostafa Fahmy

Professor of Parasitology, Faculty of Veterinary Medicine, Cairo University

Dr. Hussein Mohamed Omar

Professor of Parasitology, Faculty of Veterinary Medicine, Cairo University

Dr. Mai Abuowarda Mohammed

Assistant Professor in Parasitology Department, Faculty of Veterinary Medicine, Cairo University

Dr. Souzan Girges Gattas

Chief Researcher, Parasitology Department, Animal Health Research Institute, Agricultural Research Center

Abstract

The present study screened house hold and kenneled dogs with its attached ticks for tick-borne pathogens (TBPs) by traditional and molecular techniques. Blood samples were collected from 208 dogs from sexes, different ages and breeds in Cairo and Giza governorates during period between March 2018 to February 2019. Additionally, 1386 ticks were collected from 144 infested dogs then, divided to 546 ticks were dissected for preparation of hemolymph, mid gut and salivary gland smears, 120 female ticks were kept in lab till egg laying for preparation of 360 egg smears and 720 engorged ticks were used for preparation of 144 tick pools for PCR. Results showed that, all ticks collected in the present study were identified as *Rhipicephalus sanguineus*. TBPs were detected microscopically in 35.58% (74/208) of examined blood samples including, *Theileria equi* in 25/208 (12.02%) dogs followed by *Anaplasma* and *Ehrlichia* in 23/208 (11.1%) finally, *Babesia canis* in 17/208 (8.2%). While *Hepatozoon canis* was not detected microscopically in blood smears. Co-infections were observed in 9/208 (4.33%). The total prevalence rates of infection with TBPs in ticks were 44.69%, 68.50% and 15.75% in hemolymph, mid gut and salivary gland respectively. Significant difference in total TBPs rate of infection between different seasons and age groups with P value < 0.05 . While breed and sex have no significant effect on rate of infection. Ultrastructure of different TBPs stages were described in details within mid gut and salivary gland of *R. sanguineus* by TEM as, the early oocyst and sporocysts of *H. canis*, *A. phagocytophilum* colony, penetrating kinetes and sporozoites (infective stages) of *Theileria equi* and *Babesia canis*. PCR amplified a monomorphic DNA fragment of 560 bp size in case of *Babesia* and *Theileria* spp, 670 bp in case of *H. canis* and 450 bp in case of *Anaplasma* and *Ehrlichia* spp. Overall molecular prevalence rate of TBPs was 51.61% and 36.8% of examined dog's blood and ticks. *Theileria* spp. recorded the highest prevalence rate in (25.81% and 10.42%) dogs followed by, *Anaplasma* and *Ehrlichia* in (19.35% and 20.83%), then *Babesia canis* in (6.45% and 5.55%). While, *H. canis* recorded the lowest prevalence rate (0% and 2.8%) in examined blood and Tick respectively. Sequence analysis identified seven different species of TBPs, namely *Theileria equi*, *B. canis vogeli*, *H. canis*, *E. canis*, *A. phagocytophilum*, *A. marginale* and *A. Platys*. The identified TBPs were accessed on the GenBank under accession number: MT533853, MT533854, MT533857, MW237710, MW237711 and MT533884 for *Theileria equi* isolates, MW432533 for *B. canis*, MZ203845 for *H. canis*. While, *Anaplasmataceae* family accession numbers were MZ068099 and MZ203829 for *A. platys* and *A. phagocytophilum* respectively, MZ203830, MZ203832, MZ203831 and MZ203834 for *A. marginale*. MZ191504, MZ191505 and MW433608 for *E. canis* from dog's blood and ticks (*R. sanguineus*).

Keywords: *Anaplasma*, *Babesia*, *Hepatozoon*, PCR, *Rhipicephalus sanguineus*, *Theileria*, TEM, TBPs.

Table of Contents

Chapter	Title	Page
1.	Introduction	1
2.	Review of literature	4
	2.1. Identification of ticks collected from dogs	4
	2.2. Prevalence of tick-borne pathogens in dogs	7
	2.3. Prevalence of tick-borne pathogens in <i>R. sanguineus</i>	13
	2.4. Risk factors associated with Tick-borne pathogens in dogs	15
	2.5. Light microscopic examination of TBPs in dog's blood and ticks.....	20
	2.6. Transmission electron microscope examination (TEM) of TBPs in ticks	27
	2.7. Polymerase chain reaction (PCR) and DNA sequencing	34
3.	Published paper	40
4.	Submitted papers	48
	4.1. First submitted paper.....	48
	4.2. Second submitted paper	83
5.	Discussion	130
6.	Conclusions and Recommendations	143
7.	Summary	145
8.	References	148
9.	Appendix	170
10	Arabic summary	١

List of Tables

NO.	Title	Page
<u>Chapter (3). Published paper</u>		
Table (1)	Incidence rates of infection with tick-borne blood parasites in smears prepared from Brown dog ticks.	43
<u>Chapter (4). Submitted papers</u>		
<u>4.1. First submitted paper</u>		
Table (1)	Prevalence of <i>Theileria</i> spp. in blood and ticks collected from dogs using different techniques.	74
Table (2)	Prevalence of <i>Theileria</i> spp. infection among household and sheltered dogs (based on blood smears).	74
Table (3)	The identity percentage of <i>T. equi</i> sequences from dog blood and <i>R. sanguineus</i> detected in the current study (MT533853, MT533854, MT533857, MW237710, MT533884, and MW237711) in <i>T. equi</i> strains retrieved from animals and ticks.	75-76
<u>4.2. Second submitted paper</u>		
Table (1)	Primers sequences used in the current study.	113
Table (2)	Thermal profile used in PCR procedures.	113
Table (3)	Prevalence of Tick-borne pathogens infection related to some risk factors (season, age, sex and breed) by microscopic examination of blood smears.	114
Table (4)	Prevalence of tick -borne pathogens infection in dog's blood and associated ticks by traditional and molecular techniques.	115
Table (5)	The identity percentage of <i>Babesia canis vogeli</i> sequences from dog blood detected in the current study (MW432533) in relation to <i>Babesia canis vogeli</i> strains retrieved from animals and vector	115

List of Tables (continued).

NO.	Title	Page
Table (6)	The identity percentage of <i>Hepatozoon canis</i> sequences from <i>R. sanguineus</i> collected from dogs detected in the current study (MZ203845.1) concerning <i>Hepatozoon canis</i> strains retrieved from animals and ticks.	116
Table (7)	The identity percentage of <i>Anaplasma platys</i> , <i>A. phagocytophilum</i> , and <i>A. marginale</i> sequences from dog blood and <i>R. sanguineus</i> detected in the current study (MZ068099, MZ203829, MZ203831, MZ203834, MZ203832, and MZ203830) in <i>Anaplasma</i> species retrieved from animals and ticks.	117-118
Table (8)	The identity percentage of <i>E. canis</i> sequences from dog blood and <i>R. sanguineus</i> detected in the current study (MW433608, MZ191505, and MZ191504) in <i>E. canis</i> retrieved from animals and ticks.	119
<u>Chapter (9). Appendix</u>		
Table (1)	The current isolates Accession numbers deposited on the GenBank	174

List of Figures

NO.	Title	Page
Chapter (3). Published paper		
Figure (1)	Infection rates of tick borne protozoan parasites in smears prepared from <i>R. sanguineus</i> .	38
Figure (2)	An adult of male and female <i>R. sanguineus</i> 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: accesoryadanal plate SC: scutum, E: Eye).	39
Figure (3)	Predilection attachment sites of <i>R. sanguineus</i> on infested dogs	39
Figure (4)	Giemsa stained gut smears of <i>R. sanguineus</i> showed developmental stages of <i>H. canis</i> (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 (X1000).	39
Figure (5)	Developmental stage of <i>Babesia canis</i> in Giemsa stained smears from gut, salivary gland and egg of <i>R. sanguineus</i> . (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): <i>Babesia</i> stages in egg smears (X 1000).	39
Figure (6)	Developmental stage of <i>Theileria</i> spp. in Giemsa stained smears from gut, hemolymph and salivary gland of <i>R. sanguineus</i> . A: macro gamete. (B): early zygote with chromatin material at the margins of the cell. (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).	39

List of Figures (continued).

NO.	Title	Page
Figure (7)	Toluidine blue stained semi thin section through salivary gland and midgut of <i>R. sanguineus</i> showing (A and B): Some salivary acini filled with <i>Babesia</i> sporozoites, (C): <i>A. phagocytophilum</i> colony in midgut section, (D): Early oocyst of <i>H. canis</i> 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).	39
Figure (8)	Electron photograph of ultrathin cross sections through gut of <i>R. sanguineus</i> showing (A): <i>H. canis</i> early oocyst has few rhopteris (R) and micronemes (MN), (B): young oocyst of <i>H. canis</i> 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): <i>A. phagocytophilum</i> colonies in double membrane bounded vacuole (DW) and contained electron-dense granules (D), (F): ruptured vacuole and dense form of <i>A. phagocytophilum</i> are free in cytoplasm of infected cells.	40
Figure (9)	Electron photographs of ultrathin cross sections through salivary gland of <i>R. sanguineus</i> showing (A): penetrating kinete(K), (B): dividing stages(DS), (C): higher magnification of dividing stages begins to be almost pyriform in shape with cytoplasm filled with ribosomes, (D): sporozoites longitudinal section, (E): cross section through apical part of sporozoite. ((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).	40

List of Figures (continued).

NO.	Title	Page
Chapter (4). 4.1. first submitted paper		
Figure (1)	Male and female <i>R. sanguineus</i> . (A) Male dorsal view, (B) male ventral view, (C) female dorsal view, (D) female ventral view, (E) male spiracular plate, (F) six-legged larva (A, B, C, and D: 20×; F, 200×. (Mp : Mouth part: BC Basis capituli, E: eye, F: festoons, GO: genital opening, AO: anal opening, AP: adanal plate, CA: caudal appendage, C: Coxa.	73
Figure (2)	Sites and signs on dogs: (A and B) predilection sites of ticks. (C, D) Signs of <i>Theileria equi</i> infection in dogs. (C): Pale mucosal membrane. (D): Corneal opacity.	73
Figure (3)	Giemsa-stained thin blood and buffy coat smears from dogs showing <i>Theileria</i> spp. (A and B) Erythrocytic forms (signet ring form). (C) Maltese cross form. (D, E, and F) Schizont in lymphocyte. (D) Microschizont. (E) Macroschizont. (F) <i>Theileria</i> schizont in buffy coat smears (1000×).	74
Figure (4)	Giemsa-stained <i>R. sanguineus</i> smears. (A and B) Midgut smears showing <i>Theileria</i> zygotes and a developing kinetes inside zygotes. (C and D) Salivary gland smears showing sporonts before and during multiple fission. (E, F, and G) Hemolymph smears showing sporokinetes (E), rod-shaped multinucleated vermicule (F), And round and amoeboid stages of <i>Theileria</i> (G) 1000×).	74
Figure (5)	Toluidine-blue-stained semi-thin section through the midgut and salivary gland of <i>R. sanguineus</i> showing (A and B) a <i>Theileria</i> spp. zygote within parasitophorous vacuoles in midgut tissue; (C) rounded penetrating ookinetes inside salivary gland tissue; (D) sporoblast of <i>Theileria</i> spp. filled with salivary acini. PK: penetrating ookinete, SG: secretory granules, HN: host cell nucleus) (1000×).	75

List of Figures (continued).

NO.	Title	Page
Figure (6)	Electron micrographs of ultra-thin cross sections through the gut and salivary gland of <i>R. sanguineus</i> showing <i>Theileria</i> spp. (A) Zygote and (B) the apical part of sporozoites. (N) nucleus, (R) rhoptries, (MN) micronemes, (GC) granular cytoplasm, (PV) parasitophorous vacuole, (DM) double membrane, (GA) Golgi apparatus, (ER) endoplasmic reticulum, and (HM) host-cell mitochondria).	75
Figure (7)	Phylogenetic relationships based on ssu-rRNA sequences of the current strains of <i>T. equi</i> in Egypt. The trees were constructed and analyzed using a neighbor-joining method. Similarity (percent identity) and genetic divergence of the ssu-rRNA sequences of the current strains of <i>T. equi</i> from dogs and <i>R. sanguineus</i> ticks in Egypt were compared with each other.	76
Figure (8)	Similarity (percent identity) of ssu-rRNA sequences of <i>T. equi</i> obtained from dogs and <i>Rhipicephalus sanguineus</i> ticks in Egypt.	77
Figure (9)	Phylogenetic relationships based on the small subunit ribosomal RNA (ssu-rRNA) sequences of <i>T. equi</i> . The trees were constructed and analyzed using a neighbor-joining method	78
Chapter (4). 4.2. Second submitted paper		
Figure (1)	Male and female <i>R. sanguineus</i> showed (A) Male dorsal view, (B) Male ventral view, (C) Female dorsal view, (D) Female ventral view (bare=200 μ m).	115
Figure (2)	Giemsa-stained dog blood smears showing, (A and B) <i>B. canis</i> , (C) <i>Anaplasma platys</i> inclusion inside thrombocytes, (D) <i>Anaplasma</i> inclusion inside RBCs. (E) <i>A. phagocytophilum</i> merulae inside neutrophil, (F) <i>E. canis</i> merulae inside monocyte (X1000 bare= 5 μ m).	115

List of Figures (continued).

NO.	Title	Page
Figure (3)	Giemsa stained hemolymph smears of <i>R. sanguineus</i> showed (A): Spherical and amoeboid form of <i>Babesia canis</i> . (B): Mature club shaped vermicules of <i>B. canis</i> . (C): <i>Hepatozoon canis</i> mature oocysts. (D): Non infected plasmacyte cell. (E): plasmacyte cell after phagocytosis of <i>Anaplasma</i> spp. (F): plasmacyte eliminate <i>Anaplasma</i> spp by nodulation (X 1000, bare= 5 µm).	116
Figure (4)	Signs of TBPs infection in dogs (A): jaundice in sclera of eye (B): icteric mucosal membrane.	116
Figure (5)	Phylogenetic relationships based on small subunit ribosomal RNA (ssu-rRNA) sequences of <i>Babesia canis vogeli</i> . The tree was constructed and analyzed using a neighbor-joining method.	117
Figure (6)	Similarity (percent identity) and genetic divergence of small subunit ribosomal RNA (ssu-rRNA) sequences of <i>Babesia canis vogeli</i> isolated from Blood of dogs in Egypt (representing number, 1) compared with the most similar reference sequences (GenBank).	118
Figure (7)	Phylogenetic relationships based on 18S ribosomal RNA (18S-rRNA) sequences of <i>Hepatozoon canis</i> . The tree was constructed and analyzed using a neighbor-joining method	119
Figure (8)	Similarity (percent identity) and genetic divergence of 18S ribosomal RNA (18S-rRNA) sequences of <i>Hepatozoon canis</i> isolated from <i>Rhipicephalus sanguineus</i> ticks collected from dogs in Egypt (representing number, 1) compared with the most similar reference sequences (GenBank).	119
Figure (9)	Phylogenetic relationships based on 16S ribosomal RNA (16S- rRNA) sequences of <i>Anaplasmataceae</i> . The trees were constructed and analyzed using a neighbor-joining method.	120

List of Figures (continued).

NO.	Title	Page
Figure (10)	Similarity (percent identity) and genetic divergence of 16S ribosomal RNA (16S- rRNA) sequences of <i>A. platys</i> , <i>A. phagocytophilum</i> and <i>A. marginale</i> isolated from dogs and <i>Rhipicephalus sanguineus</i> ticks in Egypt (representing number, 1-4, 14, 25) compared with the most similar reference sequences (GenBank). The 16S rRNA of <i>A. marginale</i> sequenced in this study is marked with oval shape and represented as numbers 1-4, <i>A. phagocytophilum</i> is marked with triangular shape and represented as number 14 and <i>A. platys</i> is marked with square shape and represented as number 25.	121
Figure (11)	Phylogenetic relationships based on 16S ribosomal RNA (16S- rRNA) sequences of <i>Ehrlichia canis</i> . The trees were constructed and analyzed using a neighbor-joining method	122
Figure (12)	Similarity (percent identity) and genetic divergence of 16S ribosomal RNA (16S rRNA) sequences of <i>Ehrlichia canis</i> isolated from dogs and <i>Rhipicephalus sanguineus</i> ticks in Egypt (representing number, 1, 9, 15) compared with the most similar reference sequences (GenBank). The 16S rRNA of <i>Ehrlichia canis</i> sequenced in this study is marked and represented as numbers 1, 9, 15	123
<u>Chapter (9). Appendix</u>		
Figure (1)	Developmental stages of <i>B. canis</i> in dog's blood and attached ticks smears. (A): pear-shaped merozoites in RBCs, (B): Clup-shaped vermicules in hemolymph smears, (C): Zygote in midgut smears and (D): Sporont during binary fission in salivary gland of ticks (X1000).	162

List of Figures (continued).

NO.	Title	Page
Figure (2)	Developmental stages of <i>Theileria</i> spp. in dog's blood and attached ticks smears. (A): ring-shape merozoites in RBCs, (B): micro schizonts in lymphocyte, (C): Zygote in midgut smears, (D): developing kinite inside the zygote in midgut smears, (E): Clup-shaped ookinite in hemolymph smears and (E): Sporont during multiple fission in salivary gland of ticks (X1000).	163
Figure (3)	Developmental stages of <i>Anaplasma</i> spp. in dog's blood and attached ticks smears. (A): dark basophilic inclusions in RBCs, (B): <i>A. phagocytophilum</i> merulae in neutrophil, (C): <i>A. platys</i> inclusions inside blood platelets, (D, E and F) hemolymph smears (D): Non infected plasmacyte cell. (E): plasmacyte cell after phagocytosis of <i>Anaplasma</i> spp. (F): plasmacyte eliminate <i>Anaplasma</i> spp by nodulation (X 1000).	163
Figure (4)	(A, B and C): Developmental stages of <i>H. canis</i> in midgut, and hemolymph smears prepared from tick's smears. (A): <i>H. canis</i> macrogametes, (B): <i>H. canis</i> zygote merulae in neutrophil, (C): <i>H. canis</i> mature oocyst, (D): <i>Ehrlichia canis</i> morulae inside monocyte in blood of dogs (X 1000).	164
Figure (5)	Agarose gel electrophoresis of amplified products obtained from <i>Theileria</i> spp. M, DNA size marker is indicated on the left; lane P is the positive control, lane N is the negative control, and lanes 1–4 are <i>Theileria equineus</i> -positive samples at 560 bp.	164
Figure (6)	Agarose-gel electrophoresis of amplified products obtained from (A): <i>Babesia canis</i> . M, 100 bp DNA ladder is indicated at left; lane P indicated control positive, lane N indicated control negative, lane 1- 6 indicated positive samples at 560 bp. (B): <i>Anaplasma</i> and <i>Ehrlichia</i> spp. M, 100 bp DNA size marker is indicated at left; lane1 indicated control negative, lane 2, 3, 5 and 6 positive samples at 450 bp lane 4 and 7 negative samples. (C): <i>Hepatozoon canis</i> M, 100 bp DNA ladder is indicated at left; lane 1 indicated control negative, lane 2,3 indicated positive samples at 670 bp.	165

List of Abbreviations

Abbreviations	Description
ME	Microscopic Examination
PCR	Polymerase Chain Reaction
TBPs	Tick-Borne Pathogens
TBDs	Tick-Borne Diseases
EDTA	Ethylene Diamine Tetra Acetic Acid
PH	Degree of acidity and alkalinity
TBE buffer	Tris borate ethylene diamine tetra acetic acid buffer
μl	Micro liter
%	Percent
DNA	Deoxy Ribo Nuclie Acid
RPM	Revelution Per Minute
μm	Micrometer
χ^2	Pearson chi-square
TEM	Transmission Electron Microscope
bp	Base Pair
ELISA	Enzyme Linked Immune Sorbent Assay
IFAT	Indirect Flourcent Antibody Tichnique
16S-rRNA	16 Small Ribosomal Ribo Nuclie Acid
ssu-rRNA	Small SubUnite Ribosomal Ribo Nuclie Acid
18S-rRNA	18Small Ribosomal Ribo Nuclie Acid