Al-Quds University
Deanship of Graduate Studies

Genetic Characterization *of Anaplasma* and *Ehrlichia* in *Ixodid* Ticks and Animals from Palestine: Spatial Distribution

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M.Sc. Thesis

Jerusalem-Palestine

1438/ 2017

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A thesis submitted in partial fulfillment of requirement for the degree of Master of Biochemistry and Molecular Biology/ Department of biochemistry/Deanship of Graduate Studies /Al-Quds University

1438/2017

Al-Quds University
Deanship of Graduate Studies Biochemistry and Molecular Biology

Thesis Approval

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Master thesis submission and acceptance date: 16.05.2017

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Jerusalem-Palestine
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Dedication

This work is dedicated to my beloved family.

Taher Mohammad Taher Zaid

Declaration

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the it) has not been submitted for a higher degree to any other university or Institution.

Signed: _____

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Date:

Acknowledgment

I would like to express my deep gratitude to Dr. Suheir Ereqat and Dr. Abedelmajeed Nasereddin for their great help and support to achieve this study.

I would like also, to express my honest regards to Prof. Ziad Abdeen, director of Al-Quds Nutrition and Health institute and my colleague Mr. Ahmad Abdul-Qader. Finally, I would like to thank the Netherlands Ministry of Foreign Affairs, The Hauge, Netherlands, for funding this research project.

Genetic Characterization of *Anaplasma* and *Ehrlichia* in *Ixodid* Ticks and Animals from Palestine: Spatial Distribution

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Abstract

Background: Tick-borne anaplasmosis and ehrlichiosis are rickettsial diseases caused by various species in the genera *Ehrlichia* and *Anaplasma* causing different emerging diseases worldwide with significant negative impacts on both human and animals. This study aimed at detecting and genetically characterizing of *Ehrlichia* and *Anaplasma* species in *ixodid* ticks and their animal hosts from Palestine.

Methodology/ Principal Findings: A total of 723 *ixodid* ticks belonging to seven species (*Rhipicephalus sanguineus, Rhipicephalus turanicus, Rhipicephalus bursa, Hyalomma dromedarii, Hyalomma impeltatum, Haemaphysalis parva, Haemaphysalis adleri*) were collected from dogs, sheep, goats and camels. In addition, 189 blood samples were collected from different animal hosts including; dogs, sheep, camels, horses and a goat residing in nine districts in Palestine. DNA was extracted from all samples (ticks and blood) and screened for the presence of *Anaplasma* and *Ehrlichia* targeting a 345-partial sequence of the *16s rRNA* gene followed by sequence analysis. *Anaplasma* DNA was detected in 6.5% (47/723) of the tested ticks. Among them,13 were *Anaplasma platys* and 34 were *Anaplasma spp.* Using short *msp4*-PCR, 24 samples were further identified as *Anaplasma ovis*. Four ticks were infected with *Ehrlichia* (4/723; 0.6%), three of them were *Ehrlichia canis* and one was *Ehrlichia spp.*

Among canine blood samples (n=135), 11.1% (15/135) were positive. Of which, 13 (9.6%) samples were infected with *A. platys* and two samples (1.5%) were infected with *Ehrlichia canis*. Out of 47 sheep blood samples, 19 (40.4%) samples were positive; 6 of them were identified as *Anaplasma spp*. and 13 samples were found to be infected with *A. ovis* using our newly designed primers targeting a partial sequence of *msp4* gene.

Implementation of purely-spatial analysis by saTScan for the pooled cases of *Anaplasma* revealed two statistically significant clusters in two districts in Palestine; Tubas and Majdal-

Bani-Fadil village on the western hills of the Jordan Valley. Most cases (83%) were in rural areas where life cycle components abundantly interact.

Conclusions: This study revealed the presence of *Anaplasma* and *Ehrlichia* in Palestine. Public health workers should be aware of emerging tick-borne diseases in Palestine, particularly infections due to *A. ovis*.

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Table of Abbreviations

Abbreviation	Full Word
Spp.	Species
Rh.	*Rhipicephalus*
H.	*Haemaphysalis*
Hy.	*Hyalomma*
A.	*Anaplasma*
E.	*Ehrlichia*
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
msp	Major surface protien
sodB	Superoxide dismutase gene
rRNA	Ribosomal Ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
IFA	Immuno- florescence assay
ICD-10	The International Classification of Diseases Version 10
µL	Microliter
M	Molar
PBS	Phosphate buffer saline
EDTA	Ethylenediamintetraacetic acid
TAE	Tris-acetate-EDTA
V	Volt
bp	Base pair
F	Forward
R	Revearse
°C	Celsius or degrees centigrade
min	Minute
sec	Second
n	Number
BLAST	Basic local alignment search tool
GIS	Geographic information system
P-value	Probability value
RR	Relative risk value

Chapter 1:

Introduction

Ticks are small arachnids, part of the super-order *Parasitiformes*, with mites they constitute the subclass *Acari*. Ticks are obligate hematophages ectoparasites, requiring a host animal for food and development.

Tick-borne bacterial diseases like ehrlichiosis and anaplasmosis are emerging in human and animal worldwide; they are caused by various species in the genera *Ehrlichia* and *Anaplasma* which are obligatory intracellular Gram negative bacteria that infect blood cells of mammals, the reservoir-hosts include numerous wild and domesticated animals (Dumler et al., 2001; Nazari et al., 2013; Razmi et al., 2006).

Ticks from the *Ixodidae* family usually transmit these pathogens to mammals; in addition, they can be transmitted via blood transfusions (Dawson et al., 1991; Jafarbekloo et al., 2014).

1.1 Tick Biology, Behavior and Life Cycle

Ticks life cycle consists of four stages: egg, 6-legged larva, 8-legged nymph and adult which may be male or female. Mlting or shedding the cuticle by digesting a blood meal is necessary for both larvae and nymphs to change to the next stage.

During their life cycle, *Ixodidae* may have one, two or three animal hosts (Figure 1.1). Larvae and nymphs must feed once to engorge before molting. Single host-ticks molt twice on the same host, from larva to nymph then from nymph to adult. Two host-ticks molt once from larvae to nymph on the host, the engorged nymph drops off, molts off the host and the resulting adult must find a second host. Three-host ticks do not molt on the host, the

engorged larva drops off, molts to a nymph, which must find a second host animal to engorge and drop off again, then molts to the adult stage and attach to a third host. Usually Ixodidae adults mate on the host, then the female feeds to engorgement, drops off, lays a large batch of eggs (which could range roughly from 1,000 to 18,000 eggs.) before it dies, but males might remain on the host for several months (Figure 1.2) (Jongejan & Uilenberg, 2004; Stafford III, 2004).

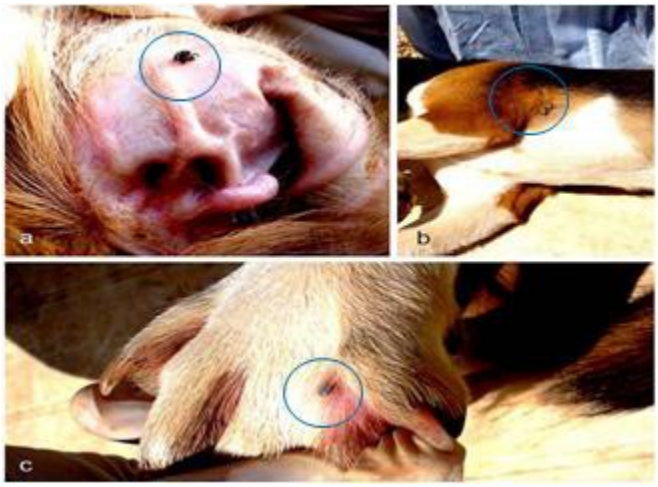

Figure 1.1: Attachment of an *Ixodid* tick (*Rh. sanguineus*) to their animal host's skin (Dantas-Torres et al., 2010).

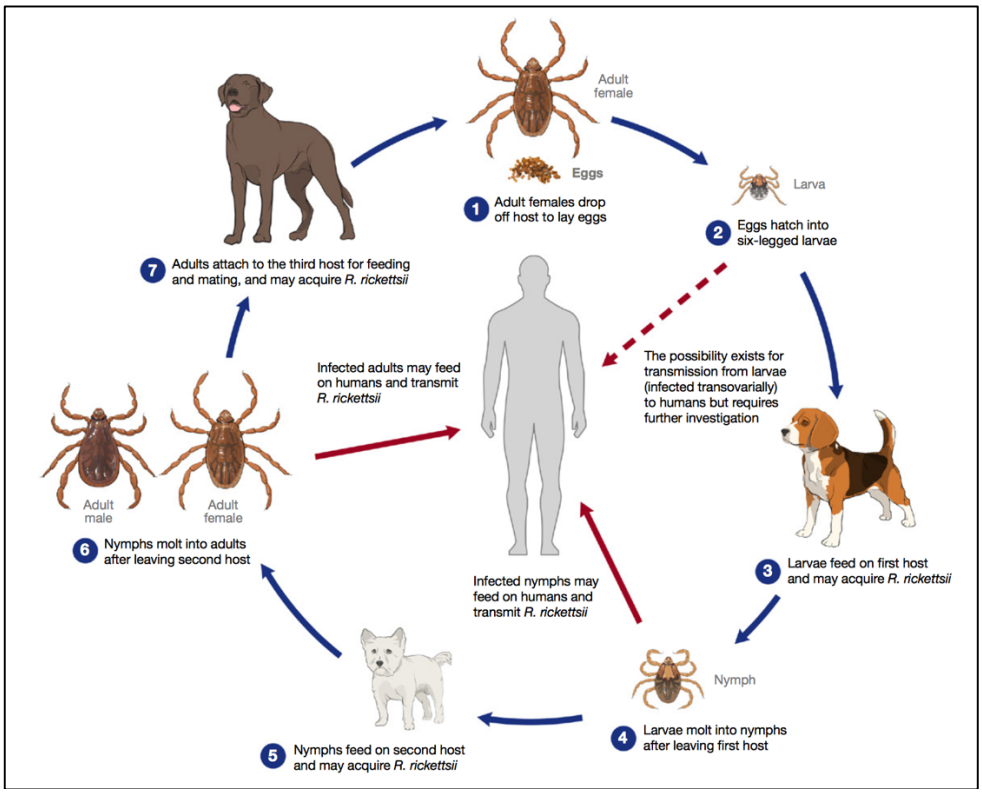

Figure 1.2: Life cycle of *Rh. sanguineus* (species of hard ticks) and the transmission of *Rickettsia rickettsia* (tick-brone disease). The Centers for Disease Control and Prevention (CDC, 2016), Retrieved from https://www.cdc.gov/ticks/life_cycle_and_hosts.html

1.1.1 Tick Species in Palestine

Family *Ixodidae* consists of five subfamilies *(Ixodinae, Amblyomminae, Haemaphysalinae, Hyalomminae, and Ripicephalinae*) (Wilamowski et al., 1999). Several *Ixodidae* species had been previously reported from different ticks and animal hosts residing in different districts throughout Palestine. *Rh. sanguineus*, *Rh. turanicus* and *H. parva* were among the most common species (Ereqat et al., 2016a; Ereqat et al., 2016b). These ticks were also among the most common ticks circulating ticks in neighboring countries (Guberman et al., 1996; Jafarbekloo et al., 2014; Jongejan & Uilenberg, 2004; Mumcuoglu et al., 1993).

1.2 Tick-brone Pathogens

Tick-borne diseases comprise a group of globally distributed and rapidly spreading illnesses caused by a range of pathogens of veterinary and public health importance. A comprehensive current listing of bacterial pathogens, their respective tick hosts and geographical distributions is presented in (Table 1.1).

Table 1.1: Description of tick-borne protozoal and bacterial pathogens, their vectors, geographical distribution and their host species.

Pathogen	Disease	Tick vectors	Geographical distribution	Host species affected	References
Protozoa; genus *Babesia*					
B. beliceri		*Hyalomma* spp.	Russia	Cattle	(G. Uilenberg, 2006)
B. bigemina	Cattle babesiosis	*Boophilus* spp., *Rhipicephalus* spp.	Africa, America, Asia, Australia	Cattle, buffalo	Uilenberg, 2006)
B. bovis	Cattle babesiosis	*Boophilus* spp.	Africa, America, Asia, Australia	Cattle, buffalo	Uilenberg, 2006)
B. major	Cattle babesiosis	*Haemaphysalis* spp.	Europe	Cattle	Uilenberg, 2006)
B. ovata		*Haemaphysalis* spp.	Asia	Cattle	Uilenberg, 2006)
B. occultans		*Hyalomma* spp.	Africa	Cattle	Uilenberg, 2006)
B. divergens	Cattle babesiosis	*Ixodes* spp.	Europe	Cattle, Human	Uilenberg, 2006)
B. microti		*Ixodes scapularis*	USA, Canada	Human, rodents	Uilenberg, 2006)
B. canis	Dog babesiosis	*Rhipicephalus sanguineus*, *Dermacentor reticulatus*, *D. marginatus*	Tropical and semitropical worldwide	Dogs	Uilenberg, 2006), (Shaw et al., 2001), (Jongejan & Uilenberg, 2004)
B. vogeli	Dog babesiosis	*Rhipicephalus sanguineus*	Tropical and semitropical worldwide	Dogs	Uilenberg, 2006)
B. rossi	Dog babesiosis	*Haemaphysalis leachi*	Southern Africa	Dogs	Uilenberg, 2006), (Shaw et al., 2001)
B. gibsoni	Dog babesiosis	*Haemaphysalis bispinosa, H. longicornis*, *Rhipicephalus sanguineus*	Africa, Asia, USA, southern Europe	Dogs	Uilenberg, 2006), (Shaw et al., 2001), (Jongejan & Uilenberg, 2004)
B. ovis	Sheep babesiosis	*Rhipicephalus bursa, R. turanicus*	Africa, Asia, Europe	Sheep	Uilenberg, 2006)
B. motasi	Sheep babesiosis	*Haemaphysalis* spp.	Africa, Asia, Europe	Sheep	Uilenberg, 2006)
B. caballi	Horse babesiosis	*Dermacentor* spp., *Rhipicephalus evertsi evertsi*	Africa, America, Asia, Europe	Horses, mules, donkeys	Uilenberg, 2006), (Jongejan

					& Uilenberg, 2004)
B. felis		Unknown	Africa	Cats	Uilenberg, 2006)
B. bicornis		Unknown	Southern Africa	Black rhinoceros (*Diceros bicornis*)	(Nijhof et al., 2003)
B. odocoilei		*Ixodes scapularis*	America	Cervidae and wild Bovidae	(Schoelkopf et al., 2005)
Protozoa; genus *Theileria*					
T. annulata	Tropical theileriosis	*Hyalomma* spp.	Eurasia, Africa, Central Asia	Cattle, Camels	(G Uilenberg, 1981)
T. orientalis		*Haemaphysalis* spp.	Asia	Cattle, Asian buffalo	(Gubbels et al., 2000)
T. parva	East Coast Fever	*Rhipicephalus appendiculatus*	Africa	Cattle	(Perry et al., 1991)
T. lawrencei	Corridor disease	*Rhipicephalus zambeziensis*	Africa	Cattle	(A. R. Walker, 2003)
T. velifera	Apathogenic	*Amblyomma* spp.	Africa	Cattle, African buffalo	(Bockarie, 2002)
T. buffeli	Apathogenic	*Haemaphysalis* spp.	Asia	Cattle, Asian buffalo	(Bockarie, 2002)
T. mutans	Benign Theileriosis	*Amblyomma hebraeum, A. lepidum, A. variegaum*, *A. cohaerens*, *A. gemma*	Africa	Cattle	(Jongejan & Uilenberg, 2004), (A. R. Walker, 2003)
T. taurotragi	Benign Theileriosis	*Rhipicephalus appendiculatus, R. pulchellus, R. zambeziensis*	Africa	Cattle	(Gubbels et al., 2000)
T. ovis	Sheep theileriosis	*Hyalomma* spp., *Rhipicephalus bursa*	Africa, Asia	Sheep	(Aktas et al., 2006)
T. lestoquardi	Sheep theileriosis	*Hyalomma* spp.	Mediterranean region, Asia, Middle East, India	Sheep, Goats	(Bockarie, 2002)
T. separata	Non-pathogenic	*Rhipicephalus evertsi*	Sheep, goats	Africa	(Bockarie, 2002)
T. bicornis	Not named	Unknown	Black rhinoceros (*Diceros bicornis*)	Southern Africa	(Nijhof et al., 2003)
T. equi	Equine biliary fever	*Dermacentor* spp., *Rhipicephalus* spp., *Hyalomma* spp., *Boophilus* spp.	Horses, mules, donkeys	Southern Euro pe, Africa, Asia	(Jongejan & Uilenberg, 2004), (Bockarie, 2002)
T. cervi	Non-pathogenic	*Amblyomma americanum*	White-tailed deer (Odocoileus virginianus)	Nearctic	(Laird et al., 1988)
Protozoa; genus *Hepatozoon*					
H. canis	Hepatozoonosis	*Rhipicephalus sanguineus, Haemaphysalis longicornis*	Southern Europe, Middle East, Far East, Africa	Dogs	(Ashford, 2001)
H. americanum	Hepatozoonosis	*Amblyomma maculatum*	Southern USA	Dogs	(Ashford, 2001)
Protozoa; genus *Cytauxzoon*					
Cytauxzoon felis	Cytauxzoonosis	*Dermacentor variabilis*	USA, Brazil	Domestic cats and wilds felids	(Birkenheuer et al., 2006), (Peixoto et al., 2007)
Bacteria; genus *Aegyptianella*					
A. pullorum	Aegyptianellosis	*Argas walkerae, A. persicus*, *A. reflexus*	Africa, southern Europe, Middle Asia, Indian subcontinent	Domestic poultry	(Jongejan & Uilenberg, 2004), (Hoogstraal, 1985)
Bacteria; genus *Rickettsia*					
R. rickettsii	Rocky Mountain spotted fever	*Dermacentor andersoni, D. variabilis, Amblyomma cajennense, A. aureolatum, Rhipicephalus sanguineus*	Americas	Human, dog	(Parola & Raoult, 2001), (Parola et al., 2005b), (Parola et al., 2005a)
R. amblyommii	Spotted fever rickettsiae group; no disease name	*Amblyomma americanum, A. neumanni, A. cajennense, A. coelebs*	Americas	Human	(Billeter et al., 2007)
R. conorii conorii	Mediterranean spotted fever	*Rhipicephalus sanguineus*	Europe, Africa, Asia	Human, dog	Philippe Parola & Raoult, 2001), (Parola et al., 2005b), (Parola et al., 2005a)
R. conorii israelensis	Israeli spotted fever	*Rhipicephalus sanguineus*	Israel	Human	(Parola et al., 2005b), (Parola et al., 2005a)
R. conorii caspia	Astrakhan fever	*Rhipicephalus sanguineus, R. pumilio*	Africa, Asia	Human	(Parola et al., 2005b), (Parola et al., 2005a)
R. conorii indica	Indian tick typhus	*Rhipicephalus sanguineus*	India	Human	(Parola & Raoult, 2006)
R. sibirica sibirica	Siberian or North Asian tick typhus	*Dermacentor nuttalli, D. marginatus, D. silvarum, D. sinicus,*	Asia	Human	Philippe Parola & Raoult,

		Haemaphysalis concinna			2001), (Parola et al., 2005b), (Parola et al., 2005a)
R. sibirica mongolotimonae	Unnamed	*Hyalomma asiaticum, H. truncatum, H. anatolicum excavatum*	Africa, China, France	Human	Philippe Parola & Raoult, 2001), (Parola et al., 2005b), (Parola et al., 2005a)
R. australis	Queensland tick typhus	*Ixodes holocyclus, I. tasmani*	Australia	Human	Philippe Parola & Raoult, 2001), (Parola et al., 2005b), (Parola et al., 2005a)
R. japonica	Oriental or Japanese spotted fever	*Ixodes ovatus, Dermacentor taiwanensis, Haemaphysalis longicornis, H. flava*	Japan	Human	Philippe Parola & Raoult, 2001), (Parola et al., 2005b), (Parola et al., 2005a)
R. africae	African tick-bite fever	*Amblyomma hebraeum, A. variegatum*	Africa, Reunion Island, West Indies	Human	Philippe Parola & Raoult, 2001), (Parola et al., 2005b), (Parola et al., 2005a)
R. honei	Flinders island spotted fever	*Bothriocroton hydrosauri, Amblyomma cajennense, Ixodes granulatus*	Australia, USA, Thailand	Human	Philippe Parola & Raoult, 2001), (Parola et al., 2005b), (Parola et al., 2005a)
R. slovaca	Tibola, debonel	*Dermacentor marginatus, D. s reticulatus*	Europe, Asia	Human	Philippe Parola & Raoult, 2001), (Parola et al., 2005b), (Parola et al., 2005a)
R. helvetica	Pathogenicity suspected in humans	*Ixodes ricinus*	Europe	Human	(Fournier et al., 2004)
R. heilongjiangensis	Unnamed	*Dermacentor silvarum*	China	Human	(Parola & Raoult, 2006)
R. aeschlimannii	Unnamed	*Hyalomma marginatum marginatum, H. m. rufipes, Rhipicephalus appendiculatus*	Europe, Africa	Human	Philippe Parola & Raoult, 2001), (Parola et al., 2005b), (Parola et al., 2005a)
R. parkeri	Unnamed	*Amblyomma. maculatum, A. triste, A. dubitatum*	USA, Uruguay, Brazil	Human	(Parola et al., 2005b), (Parola & Raoult, 2006)
R. massiliae	Unnamed	*Rhipicephalus sanguineus, R. turanicus, R. muhsamae, R. lunulatus, R. sulcatus*	Europe, Asia, Argentina, USA	Human	(Parola et al., 2005b), (Eremeeva et al., 2006), (Vitale et al., 2006)
R. marmionii	Australian spotted fever	*Haemaphysalis novaeguineae, Ixodes holocyclus*	Australia	Human	(Parola et al., 2005b), (Parola & Raoult, 2006)
R. monacensis	Unnamed	*Ixodes ricinus*	Europe	Human	(Parola et al., 2005b)
Bacteria; genus *Ehrlichia*					
E. chaffeensis	Human monocytic ehrlichiosis	*Amblyomma americanum, Dermacentor variabilis*	USA	Human and various mammals	(Shaw et al., 2001), (Parola et al., 2005a)
E. ewingii	Canine granulocytic ehrlichiosis, Human ehrlichiosis	*Amblyomma americanum*	USA	Human, dogs	(Shaw et al., 2001), Philippe Parola & Raoult, 2001)
E. ruminantium	Heartwater	*Amblyomma hebraeum, A. astrion*, *A. cohaerens*, *A. gemma*, *A. marmoreum*, *A. lepidum*, *A. pomposum*, *A. variegatum* *A. americanum*	Africa, Caribbean	Mainly cattle	(Jongejan & Uilenberg, 2004), (J. B. Walker & Olwage, 1987)
E. canis	Canine ehrlichiosis	*Rhipicephalus sanguineus*	Southern USA, southern Europe, Africa, Middle East, eastern Asia	Dogs	(Shaw et al., 2001)
Bacteria; genera *Anaplasma, Francisella* and *Coxiella*					
Anaplasma phagocytophilum	Human granulocytic anaplasmosis	*Ixodes scapularis, I. pacificus, I. ricinus, I. hexagonus*	USA, Europe	Human and various mammals	(Parola et al., 2005a),

					(Estrada-Peña & Jongejan, 1999), (Grzeszczuk et al., 2006)
A. marginale	Bovine anaplasmosis	Various	Worldwide	Cattle	(Ashford, 2001)
A. centrale	Bovine anaplasmosis	Various	Worldwide	Cattle	(Ashford, 2001)
A. ovis	Ovine anaplasmosis	Various	Worldwide	Sheep	(Ashford, 2001)
A. platys	Canine ehrlichiosis	*Rhipicephalus sanguineus*		Dog	(Aguirre et al., 2006)
F. tularensis	Tularemia	Various	Eurasia, Nearctic	Human and various mammals	Philippe Parola & Raoult, 2001)
C. burnetii	Q fever	Various	Worldwide	Human and various mammals	Philippe Parola & Raoult, 2001)
Bacteria; genus *Borrelia*					
B. burgdorferi	Lyme disease	*Ixodes pacificus, I. persulcatus, I. ricinus, I. scapularis*	USA, Canada, Europe, Asia, northern Africa	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999)
B. garinii	Lyme disease	*Ixodes persulcatus, I. ricinus*	Europe, Asia, northern Africa	Human	(Estrada-Peña & Jongejan, 1999)
B. afzelii	Lyme disease	*Ixodes persulcatus, I. ricinus*	Europe, Asia, northern Africa	Human	(Estrada-Peña & Jongejan, 1999)
B. valaisiana	Lyme disease	*Ixodes ricinus*	Europe, Asia	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999)
B. lusitaniae		*Ixodes ricinus*	Europe		(Estrada-Peña & Jongejan, 1999)
B. spielmani	Lyme disease	*Ixodes ricinus*	Europe	Human	(Richter et al., 2004)
B. japonica	Lyme disease	*Ixodes ovatus*	Japan	Human	(Estrada-Peña & Jongejan, 1999)
B. lonestari		*Amblyomma americanum*	USA	Human	(Varela et al., 2004)
B. theileri	Bovine borreliosis	*Boophilus* spp., *Rhipicephulus evertsi*	Africa, Central and South America, Australia	Cattle	(Barbour & Hayes, 1986)
B. turcica		*Hyalomma aegyptium*	Parts of Turkey		(Güner et al., 2004)
B. miyamotoi		*Ixodes persulcatus*	Asia		(Fukunaga et al., 1995)
B. hermsii	New World tick-borne relapsing fever	*Ornithodoros hermsi*	USA, Canada	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999), (Barbour & Hayes, 1986)
B. turicatae	New World tick-borne relapsing fever	*Ornithodoros turicata*	USA, Mexico	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999), (Barbour & Hayes, 1986)
B. parkeri	New World tick-borne relapsing fever	*Ornithodoros parkeri*	USA	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999), (Barbour & Hayes, 1986)
B. mazzottii	New World tick-borne relapsing fever	*Ornithodoros talaje*	USA, Mexico		Philippe Parola & Raoult, 2001), (Barbour & Hayes, 1986)
B. venezuelensis	New World tick-borne relapsing fever	*Ornithodoros rudis*	Central and South America	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999), (Barbour & Hayes, 1986)
B. duttonii	Old World tick-borne relapsing fever	*Ornithodoros moubata*	Africa	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999), (Barbour & Hayes, 1986)
B. crocidurae	Old World tick-borne relapsing fever	*Ornithodoros erraticus*	Europe, Africa	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999),

					(Barbour & Hayes, 1986)
B. persica	Persian relapsing fever	*Ornithodoros tholozani*	Asia	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999), (Barbour & Hayes, 1986)
B. hispanica	Old World tick-borne relapsing fever	*Ornithodoros erraticus*	Spain, Portugal	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999), (Barbour & Hayes, 1986)
B. latyschevii	Old World tick-borne relapsing fever	*Ornithodoros tartakovskyi*	Iran, Central Asia	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999), (Barbour & Hayes, 1986)
B. caucasica	Old World tick-borne relapsing fever	*Ornithodoros aspersus*	Asia (Caucasus and Iraq)	Human	Philippe Parola & Raoult, 2001), (Estrada-Peña & Jongejan, 1999), (Barbour & Hayes, 1986)
B. graingeri		*Ornithodoros graingeri*	Africa	Human	Philippe Parola & Raoult, 2001)
B. anserina	Avian borreliosis	*Argas* spp.	Worldwide	Birds	(Barbour & Hayes, 1986)
B. tillae		*Ornihodoros zumpti*	Africa	Human	(Rebaudet & Parola, 2006)
B. coraciae	Bovine epizootic abortion	*Ornithodoros coriaceus*	USA	Cattle	(Estrada-Peña & Jongejan, 1999), (Barbour & Hayes, 1986)
B. parkeri		*Ornithodoros parkeri*	USA	Human	(Rebaudet & Parola, 2006)
Bacteria; genus *Dermatophilus*					
D. congolensis	Dermatophilosis	*Amblyomma variegatum*	Africa	Ruminants	(Jongejan & Uilenberg, 2004)

1.2.1 Tick Pathogens Detected in Palestine

In Palestine, tick fever was firstly reported by F. D. Nicholson, at 1919. In 1918, eight male soldiers from Suffolk, UK, were admitted to hospital suffering from relapsing fever several days after getting tick bites during their occupation of Palestine. Later investigations proved these soldiers were infected with *Spirillum recurrentis* bacteria transmitted by the soft tick *Argas persicus*. Since then few studies were published, several of which were performed and reported recently from our institute at Al-Quds University. Confirming the presence of different pathogens; *Bartonella, Rickettsiae, Theileria, Babesia* and *Hepatozoon* in both ticks and their animal hosts from Palestine (Azmi et al., 2017; Azmi et al., 2016; Ereqat et al., 2016a; Ereqat et al., 2016b)

1.3 Ticks as vectors of *Anaplasma* and *Ehrlichia* genera and their related diseases

The genus *Ehrlichia* contains five recognized species: *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris* and *E. ruminantium*. In the United States of America (USA), the primary agents

of human ehrlichiosis are *E. chaffeensis* which is transmitted by the Lone Star tick, *Amblyomma americanum, E. ewingii* the known cause of canine granulocytic ehrlichiosis (Christenson et al., 2016; Dawson et al., 1991; Jafarbekloo et al., 2014; Wright et al., 2014), The currently recognized species in the genus *Anaplasma* are, *A. platys, A. marginale, A. bovis, A. ovis* and *A. phagocytophilum* which cause human granulocytic anaplasmosis (Dumler et al., 2001; Goethert & Telford, 2003).

The common tick vector for *Anaplasma* and *Ehrlichia* is *Rh. sanguineus*, which has been reported from India, the USA, all regions of Africa, and around the Mediterranean Basin (Dantas-Torres, 2008; Harrus et al., 2011; Razmi et al., 2006). However, several tick species including *Rh. turanicus* and *Rh.* bursa have been incriminated as vectors worldwide including neighboring countries (Friedhoff, 1997; Harrus et al., 2011; Loftis et al., 2006a, 2006b).

1.3.1 Anaplasmosis and Ehrlichiosis Infections

The clinical manifestations of ehrlichiosis and anaplasmosis are similar in both human and animals. The consequences of infection vary from asymptomatic infections or mild symptoms to a severe, potentially fatal illness. In human, these diseases are characterized by the acute onset of a nonspecific febrile illness, often accompanied by thrombocytopenia, leukopenia and elevated levels of hepatic enzymes in the blood (Doudier et al., 2010; Dumler et al., 2001). Cattle can be infected by several *Anaplasma* species, like *A. marginale*, *A. phagocytophilum*, *A. centrale*, and *A. bovis* (Geurden et al., 2008; Liu et al., 2012). *A. marginale* is known to be highly pathogenic in cattle, whereas *A. centrale* is less pathogenic and is being used for immunization against *A. marginale* since 1900s in many countries (Rymaszewska & Grenda, 2008; Theiler, 1911). *A. ovis* is moderately pathogenic in sheep, goats, and wild ruminants (Kuttler, 1984; Lew et al., 2003) and causes acute disease in animals exposed to stress or other predisposing factors such as hot weather, deworming, tick infestation, and animal movement (Friedhoff, 1997). In dogs, different pathogenic *Anaplasma* and *Ehrlichia* species have been reported. *Ehrlichia canis* is responsible for the most common and clinically severe form of canine ehrlichiosis, and may also be a cause of human granulocytic ehrlichiosis (HGE) (Perez et al., 2006; Perez et al., 1996). *A. platys* infection in dogs is reported to be either with few or no clinical symptoms (Gaunt et al., 2010; Harvey, 2006) or more virulent (Aguirre et al., 2006; Brouqui, 2002; M'Ghirbi et al., 2009; Ulutaş et al., 2007). Moreover, *A. phagocytophilum* infection has been also reported in horses, cattle, small ruminants and human causing human granulocytic

anaplasmosis (HGA) (Dumler et al., 2001; D. H. Walker & Dumler, 1996). In camels, there are new studies from Saudi Arabia, Tunis and Nigeria reporting the presence of *Anaplasma* and *Ehrlichia* (Bastos et al., 2015; Belkahia et al., 2015; Lorusso et al., 2016).

1.4 Laboratory Diagnosis

Microscopic examination of Giemsa-stained blood smears is used as a rapid method of diagnosis for anaplasmosis and ehrlichiosis. However, this method is useful for detecting clinically suspected animals during the acute phase of the disease (M'Ghirbi et al., 2016). *A. phagocytophilum* as an example, has elementary bodies or morulae which are usually found within the cytoplasm of granulocytes in the periphery and in the "feather-edge" of the blood film. (Figure 1.3) (Kirtz & Leidinger, 2015). Currently, serological methods such as competitive enzyme-linked immunosorbent assay (cELISA) are used as a reliable screening test to identify cattle infected with *A. marginale* and to reveal their carrier state (Knowles et al., 1996). However, cross-reactivity with antigenically similar *Anaplasma* species has been reported (Dreher et al., 2005). Therefore, molecular methods have been developed to detect and identify *Anaplasma* and *Ehrlichia* species targeting different genes. According to CDC, HGE (ICD-10 code: A77.40) has could be diagnosed by either of the following lab criteria:

- Demonstration of a four-fold change in antibody titer to *E. phagocytophila* antigen by IFA in paired serum samples.
- Positive PCR assay and confirmation of *E. phagocytophila* DNA.
- Identification of morulae in leukocytes, and a positive IFA titer to *E. phagocytophila* antigen.
- Immunostaining of *E. phagocytophila* antigen in a biopsy or autopsy sample.
- Culture of E. phagocytophila from a clinical specimen. (Retrieved from: https://wwwn.cdc.gov/nndss/conditions/human-granulocytic-ehrlichiosis/case-definition/2000/).

Also, HGA (ICD-10 code: A79.8) has could be diagnosed by either of the following lab criteria:

1. Supportive:
    - Serological evidence of elevated IgG or IgM antibody reactive with *A. phagocytophilum* antigen by IFA, enzyme-linked immunosorbent assay (ELISA), dot-ELISA, or assays in other formats (CDC uses an IFA IgG cutoff of

≥1:64 and does not use IgM test results independently as diagnostic support criteria).

- Identification of morulae in the cytoplasm of neutrophils or eosinophils by microscopic examination

2. Confirmed:
   - Serological evidence of a four-fold change in IgG-specific antibody titer to *A. phagocytophilum* antigen by indirect immunofluorescence assay (IFA) in paired serum samples (one taken in first week of illness and a second 2-4 weeks later).
   - Detection of *A. phagocytophilum* DNA in a clinical specimen via amplification of a specific target by polymerase chain reaction (PCR) assay.
   - Demonstration of anaplasmal antigen in a biopsy/autopsy sample by immunohistochemical methods.
   - Isolation of *A. phagocytophilum* from a clinical specimen in cell culture. (Retrieved from: https://wwwn.cdc.gov/nndss/conditions/ehrlichiosis-and-anaplasmosis/case-definition/2008/)

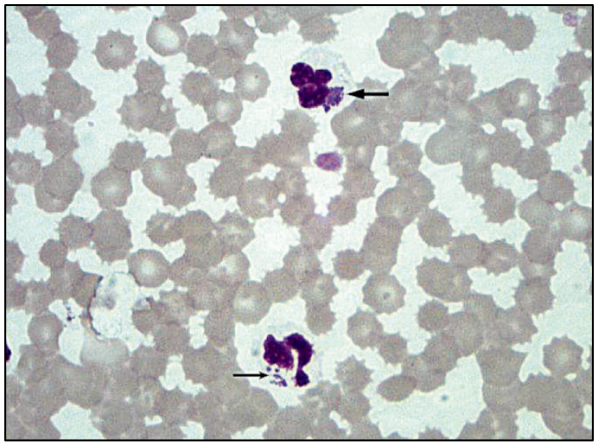

Figure 1.3: Blood smear: elementary bodies (thin arrow) and Morulae (thick arrow) of *A. phagocytophilum* in two neutrophils in a dog's blood (Kirtz & Leidinger, 2015).

1.5 Prevention and Treatment

Control methods for anaplasmosis and ehrlichiosis include ticks control, chemoprophylaxsis and vaccination.

The best way to prevent these infections is to avoid tick bites through integrated vector management rather than depending on a single method of vector control such as using acaricide, this is a labor intensive, expensive, pollutant, it may lead to development of resistant ticks as well. Therefore, if an infection occurs, carrier animals should be isolated

from the rest and treated with antibiotic, oxytetracycline and imidocarb have shown to be effective. Vaccines against anaplasmosis and ehrlichiosis which are derived from the blood of infected animals are available (Coetzee et al., 2005; Fourie et al., 2015; Kocan et al., 2000). In human, doxycycline is the first line treatment for both anaplasmosis and ehrlichiosis in adults and children of all ages and should be initiated immediately after suspection. (CDC (2016), https://www.cdc.gov/anaplasmosis/symptoms/ and https://www.cdc.gov/ehrlichiosis/symptoms/)

1.6 Economic Burden of Anaplasmosis among Animals

Diseases caused by *Anaplasma* species revealed worldwide serious constraints on the health and production of domestic animals. *A. marginale* is considered to be one of the most important pathogen in ruminants especially in cattle , causing significant economic losses (Kocan et al., 2000). Also, ovine anaplasmosis caused by *A. ovis*, which mainly infects sheep and goats, is widely distributed. Although, it is assumed that this bacterium causes only mild clinical symptoms (Friedhoff, 1997), however, its adverse effect is aggravated in infected animals especially if they were stressed by other factors such as co-infection, poor health conditions, vaccination, hot weather, heavy tick infestation or deworming (Khayyat & Gilder, 1947; Manickam, 1987). Ovine anaplasmosis is considered to contribute in economic losses to the livestock industry in many countries including Portugal (Santos et al., 2004) and Iraq (Renneker et al., 2013). In Turkey 42 million sheep were reported to have relatively low productivity of local breeds due to infection with various tick-borne pathogens, as *A. ovis* and *A. marginale*, having an impact on health of sheep and thus on their milk and meat production (Ndung'u et al., 1995). A study performed in California USA, the economic loss due to anaplasmosis of beef cattle was included in a total estimated loss of $300 million due to all livestock diseases of economic importance (Goodger et al., 1979).

The socioeconomic impact of this disease and the restrictions on trading infected animals internationally led the Office International des Epizooties (OIE) Animal Health Code to categorize anaplasmosis as a disease that required a notification of its presence (Commission & Committee, 2008).

1.7 Molecular Epidemiology of *Anaplasma* and *Ehrlichia*

Obligatory intracellular bacteria from both genera of *Anaplasma* and *Ehrlichia* are presented in different animal hosts and transmitting ticks. Many molecular epidemiological

surveys were conducted worldwide to understand the prevalence and geographical distribution of these pathogens.

The prevalence of *A. platys* in canine blood was reported to be high in different studies from Northern Parana, Brazil (19.4%), Lara, Venezuela (16%), Kenya, Ivory Coast (12.5%) (da Silva et al., 2012), (Huang et al., 2005), (Matei et al., 2016)). On the other hand, lower prevalences were reported from Okinawa Prefecture, Japan (7.5%) (Inokuma et al., 2002) and Sicily, Italy (4%) (de la Fuente et al., 2006). While in ticks from dogs, *A. platys* prevalence in Panama (21.4%) (Santamaria et al., 2014) was higher than in Israel (1.2%), Kenya and Ivory Coast (4.38%) ((Harrus et al., 2011), (Matei et al., 2016)).

Prevalence of *A. ovis,* the agent of ovine anaplasmosis was investigated by different molecular epidemiological surveys in several countries, such as; northwest China reports it was (40.5%) (Yang et al., 2015), Iran (80%) (Razmi et al., 2006), Iraq (66.6%), Sudan (41.6%), Portugal (84.2%) and Turkey (31.4%) (Renneker et al., 2013).

The prevalence of *E. canis* in blood of dogs varies too, it is relatively low as reported from Japan (1.5%) (Kubo et al., 2015), higher in northern Parana, Brazil (16.4%) (da Silva et al., 2012) and significantly high in Panama (64.2%) (Santamaria et al., 2014). In ticks collected from different localities in the vicinity of human habitations, *E. canis* prevalence is reported to be 10% in Israel (Harrus et al., 2011), and 16.66% in Iran (Khazeni et al., 2013).

1.8 Literature Review

1.8.1 Molecular Diagnosis of *Anaplasma* and *Ehrlichia*

Polymerase chain reaction (PCR)-based assays are excellent choices for detection and identification of tick-brone pathogens. Conventional PCR, nested PCR, multiplex PCR, real-time PCR have been developed and successfully used for the identification of various species of *Anaplasma* and *Ehrlichia*.

Most epidemiological studies start the screening process for the presence of *Anaplasma* and *Ehrlichia* using conventional PCR, targeting a 345bp fragment of *16s rRNA* gene using EHR 16SD and EHR 16SR genus specific primers (Harrus et al., 2011; Martin et al., 2005; Matei et al., 2016). These primes are reported to be able to amplify DNA of various species including *E. canis*, *E. chaffeensis*, *E. muris*, *E. ruminantium*, *A. phagocytophilum*, *A. platys*, *A. marginale* and *A. centrale* (Inokuma et al., 2000), followed by sequencing and comparison with sequences deposited in the GenBank (BLAST analyses) (Martin et al., 2005). Another set of primers targeting 600bp fragment of *groEL* gene (GroEL belongs to

the chaperonin family of molecular chaperones, and it is found in many bacteria (Zeilstra-Ryalls et al., 1991)) are also used to screening for *Anaplasma/ Ehrlichia spp.* (Barber et al., 2010).

Co-infections of *Anaplasma* and *Ehrlichia* is commonly investigated by applying either *Anaplasma* or *Ehrlichia* specific primers. To identify the canine *A. platys* a 679bp fragment of the *16s rRNA* gene is targeted using "Platys" forward primer combined with previously mentioned reverse primer EHR 16SR (Motoi et al., 2001). Moreover, to identify bovine *A. marginale,* ovine *A. ovis* and *A. centrale* a 851bp fragment of *msp4* gene (major surface protein 4) is targeted for amplification (de la Fuente et al., 2003). Another 849bp fragment of *msp4* gene is amplified to identify the presence of *A. phagocytophilum* (de la Fuente et al., 2005a). Other sequences from different genes are also targeted to identify *Anaplasma* species like *msp5* (major surface protein 5) and *msp2* (major surface protein 2) genes ((Torioni de Echaide et al., 1998), (Prakash et al., 2009) respectively). On the other hand, a 300bp fragment of the *sodB* gene (Superoxide dismutase gene) which is specific for *Ehrlichia* could be targeted too to investigate the presence of co-infections (Qurollo et al., 2013).

Nested PCR assay was also applied in some studies to investigate the presence of *Ehrlichia* and *Anaplasma* (Kubo et al., 2015; Matei et al., 2016), one of these studies targeted the *16s rRNA* gene using EHR 16SD and EHR 16SR primes in the first PCR reaction, then amplified a shorter sequence in the second PCR reaction using *A. platys* specific primers EPLAT5/EPLAT3 (Matei et al., 2016).

Other previous studies applied the multiplex PCR assay, targeting multiple sequences of different genes using specific primers for each species of *Anaplasma* and *Ehrlichia* (Corales et al., 2014; Rodriguez et al., 2015).

Real-time PCR based on the same target genes in conventional PCR, were also applied in different studies (de la Fuente et al., 2003; Machado et al., 2016). Real-time PCR has several advantages, including higher sensitivity and specificity, usefulness as a quantitative assay, and is able to deliver faster results than end point PCR.

Finally, to estimate the evolutionary relationships among amplified gene sequences and to compare them with other sequences reported worldwide, phylogenetic analysis is commonly performed for all used genes (de la Fuente et al., 2003; Ereqat et al., 2016a; Machado et al., 2016).

1.9 Objectives

Anaplasmosis and ehrlichiosis, despite being emerging diseases worldwide with significantly negative impacts on human and animal health and economy; they have not been investigated and no previous data are available on both diseases in Palestine. Therefore, the main objectives of this study were:

1. To investigate the prevalence of *Anaplasma* and *Ehrlichia* in hard ticks and different animal hosts.
2. To establish an epidemiological database on anaplasmosis and ehrlichiosis in Palestine including the geographic distribution of tick species and animal hosts.
3. To perform a phylogenetic analysis of Palestinian strains and published sequences deposited in GeneBank.

1.10 The Significance of the Study

The results of this study demonstrate the geographic distribution of *Anaplasma* and *Ehrlichia* and clearly indicate the presence of *A. platys, A. ovis* and *E. canis* in Palestinian domestic animals and their ticks which put us under high risk for outbreaks specially form *A. ovis* the causative agent of ovine anaplasmosis. So, clinicians, veterinarian and the Palestinian ministry of health should be aware of emerging tick-borne diseases in the West Bank, particularly infections due to *Anaplasma* and *Ehrlichia*.

1.11 Hypothesis

Different *Anaplasma* and *Ehrlichia* species were detected worldwide and in neighboring countries. The emergence of several major tick-borne diseases have been attributed to several factors including specific human activities that disrupt ecosystems, persistence of ticks in the environment and movement of animal hosts. Therefore, we hypothesized that *Anaplasma* and *Ehrlichia* are present in animals and ticks in Palestine. This molecular survey provided additional information for the pathogenesis and molecular epidemiology of *Anaplasma* and *Ehrlichia spp.* and identifying the main suspected tick vectors implicated in the transmission of these pathogens.

Chapter 2:

Materials and Methods

2.1 Ticks and Blood Samples Collection

Tick samples (n=723) were collected from different host animals in nine districts in Palestine; Jenin, Tulkarm, Tubas, Nablus, Salfit, Ramallah, Jericho, Bethlehem, and Al-Khalil. All ticks were microscopically identified to the genus and species level using standard taxonomic keys (Guglielmone et al., 2009), split into different Eppendorf tubes containing 70% ethyl alcohol and kept frozen at −20°C until DNA extraction. In addition, blood samples (n=189) were collected from different domestic animals including, dogs, sheep, camels, horses and a goat. All these animals were from the same areas mentioned above.

2.2 DNA Extraction

Prior to DNA extraction, individual ticks were washed with phosphate-buffered saline (PBS), air dried for 10 min on tissue paper and separately sliced into small pieces by a sterile scalpel blade then manually homogenized with a sterile micro pestle. The sliced sample was re-suspended in 200 µl of lysis buffer and 20 µl of proteinase K followed by DNA extraction using QIAamp animal blood and tissue Kit procedure (QIAGEN GmbH, Hilden, Germany). DNA was extracted from whole blood samples (300 µl) using the same kit mentioned above. DNA concentration was measured by Nanodrop (Thermo Scientific NanoDrop 1000) and kept frozenat -20 until further use.

2.3 Gel electrophoresis:

All PCR products were loaded onto 2 % agarose gel (Agarose LE, Analytical gradient, Promega, Spain). The gel was prepared by dissolving 2g of agarose in 100 ml solution of 1X Tris-acetate EDTA buffer (TAE) (40 mM of Tris acetate and 1mM EDTA). The agarose was dissolved in Erlenmeyer flask using microwave for about 1min till completely dissolved, and then 3.5µl of 10 mg/ml (0.35µg/ml) of Ethidium Bromide was added for DNA staining. The gel was poured in the gel tray in the casting chamber (Bio-Rad Laboratories Inc., USA). Five µl of PCR products were loaded onto the gel. DNA marker ladder of 100bp (Thermo scientific Lithuania) was used in each run. The gel was run at 100V for 45min. The gel images were captured using MiniLumi 1.4 gel

documentation system from (DNR Bio-Imaging Systems Ltd, Israel).

2.4 PCR Amplification and Sequence Analysis

Detection of *Anaplasma* and *Ehrlichia* was run by *16S rRNA*-PCR (Parola et al., 2000). All DNA samples were screened by PCR using a primer pair, EHR16SD and EHR16SR, targeting a 345bp fragment of the *16S rRNA* gene (Table 2.1). These primers can detect both *Ehrlichia* and *Anaplasma* DNA. PCR was performed as described previously (Parola et al., 2000) with the modification: the PCR reactions were performed in a total volume of 25 µl using ready mix (Thermo scientific Lithuania) containing 1 µM of each set of primers and 5 µl of the extracted DNA. All PCR Positive samples were subjected to a second PCR using the primers *sodB*-F and *sodB*-R, designed to amplify a 300bp fragment of the *sodB* gene specific for *Ehrlichia* (Qurollo et al., 2013) to rule out the possibility of double infection with *Anaplasma spp.* (Table 2.1), The PCR amplification were set up within a 25 µl reaction mixture containing 5 µl of DNA template and 20 µl of master mix (Thermo scientific Lithuania). The thermal cycling procedure was applied as described previously (Qurollo et al., 2013).

Table 2.1: Targeted genes and sequences of the oligonucleotide primers used:

Pathogen	Target gene	Primer names	Oligonucleotide sequences (5′-3′)	Amplicon size (bp)	Reference
Anaplasma spp./ Ehrlichia spp.	*16S rRNA*	EHR16SD	GGTACCYACAGAAGAAGTCC	345	(Parola P, 2000)
		EHR16SR	TAGCACTCATCGTTTACAGC		
Ehrlichia spp.	*sodB*	sodB-F	TTTAATAATGCTGGTCAAGTATGGA ATCAT	300	(Qurollo BA, 2013)
		sodB-R	AAGCGTGTTCCCA TACATCCATAG		
Anaplasma marginale/ A. centrale/ A. ovis	*msp4*	longmsp4F	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	851	(de la Fuente, 2003)
		longmsp4R	CCGGATCCTTAGCTGAACAGGAATCTTGC		
Anaplasma marginale/ A. centrale/ A. ovis	*msp4*	shortmsp4F	TTACAGCCCAGCGTTTCC	480	This study
		shortmsp4R	GYGGTTACTTGCTTAGADATGTC		

2.5 Identification of *Anaplasma spp.* Targeting *msp4* Gene

A fragment of 851bp of the major surface protein gene (*msp4*) was amplified to differentiate between *A. marginale, A. centrale* and *A. ovis*, designated as long *msp4*-PCR in this study. PCR reactions were performed in 25µl PCR-ready Supreme mix (Syntezza Bioscience-Jerusalem), containing 1 µM of each set of primers and 5 µl of the extracted DNA. The thermal cycling procedure was run as described previously (de la Fuente et al., 2003).

A new set of specific primers for detection of *Anaplasma spp.* targeted a shorter fragment of *msp4* (480bp) was designed. Partial sequences of the *msp4* gene were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov/) in FASTA format, organized and aligned using Clustal software (http://www.ebi.ac.uk/clustalw/) (KJ782403.1, AY829459.1, AF428090.1: from different species of *A. ovis, A. marginale, A. centrale,* respectively). Two degenerate primers with sequences conserved for all the species; forward (TTACAGCCCAGCGTTTCC) and reverse (GYGGTTACTTGCTTAGADATGTC) were selected that contain heterogenic sequences to generate amplicons with appropriate size (480bp) to differentiate *Anaplasma spp.* Optimization of PCR components and amplification conditions for the short-*msp4* PCR allowed a successful amplification of the specific amplicons for DNA controls (*A. centrale* and *A. marginale* were thankfully provided by Dr. Monica L. Mazuz, Division of Parasitology, Kimron Veterinary Institute, Bet Dagan, Israel). PCR reactions were performed in a total volume of 25 µl using PCR ready mix (Thermo scientific Lithuania) at 1 µM final concentration of each primer and 5µl of DNA template. The amplification conditions started with initial denaturation at 95°C for 5 min, followed by 38 cycles of denaturation at 94°C for 20s, annealing at 53°C for 30s and elongation at 72°C for 45s and final elongation at 72°C for 6 min.

In all amplification reactions, negative controls (without DNA) were included. PCR amplifications were carried out in a (Biometra TProfessional basic 96 gradient thermocycler) and the obtained PCR products visualized under UV illumination after electrophoresis on 2% agarose gels stained with ethidium bromide using different DNA ladders as a molecular-weight size markers (Thermo Scientific Lithuania GeneRuler 100bp and 1kb DNA Ladders).

2.6 DNA Sequence Analysis

All amplified products were sequenced from both directions (forward and reverse) (hylabs, Israel). The obtained sequences were analyzed and evaluated with (The Sequence Manipulation Suite (Stothard, 2000) and Multiple sequence alignment with hierarchical clustering (Corpet, 1988)) (Figure 2.1).

Figure 2.1: Sequence analysis and arrangement. **(a)** The Sequence Manipulation Suite was used to generate reverse complement (RC) sequences, then multiple sequence alignment with hierarchical clustering **(b)** was used to obtain full sequences, then for analysis and arrangement before BLAST analysis step.

Species identity for each obtained sequence was assessed based on the closest BLASTn match (identity ≥ 99%) using the MegaBLAST and a query cover no smaller than 99%) with homologous sequences deposited in NCBI database (National Center for Biotechnology Information, U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda MD, 20894 USA) (Figure 2.2).

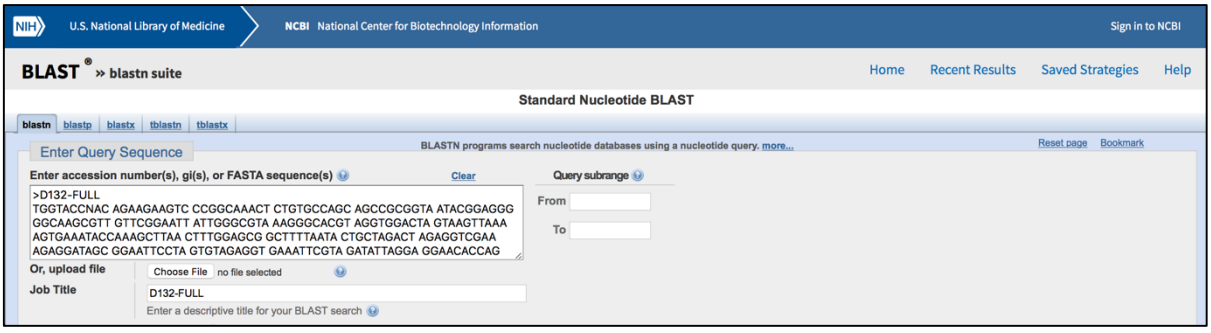

Figure 2.2: Detection of *Anaplasma* and *Ehrlichia* based on sequence homology using BLAST.

CLUSTALW program (http://www.genome.jp/tools/ clustalw/) (Figure 2.3) and Gblocks Version 0.91b (Castresana, 2000; Talavera & Castresana, 2007) were used for the multiple sequence alignment. Phylogenetic tree construction was carried out using a Maximum Likelihood (ML) approach, using the Kimura's 2-P (K2P) evolutionary model with 1000 bootstrap, by MEGA software version 7 (Kumar et al., 2016). Final trees were manipulated

for display using FigTree 1.4.3 “http://tree.bio.ed.ac.uk/software/figtree/”).

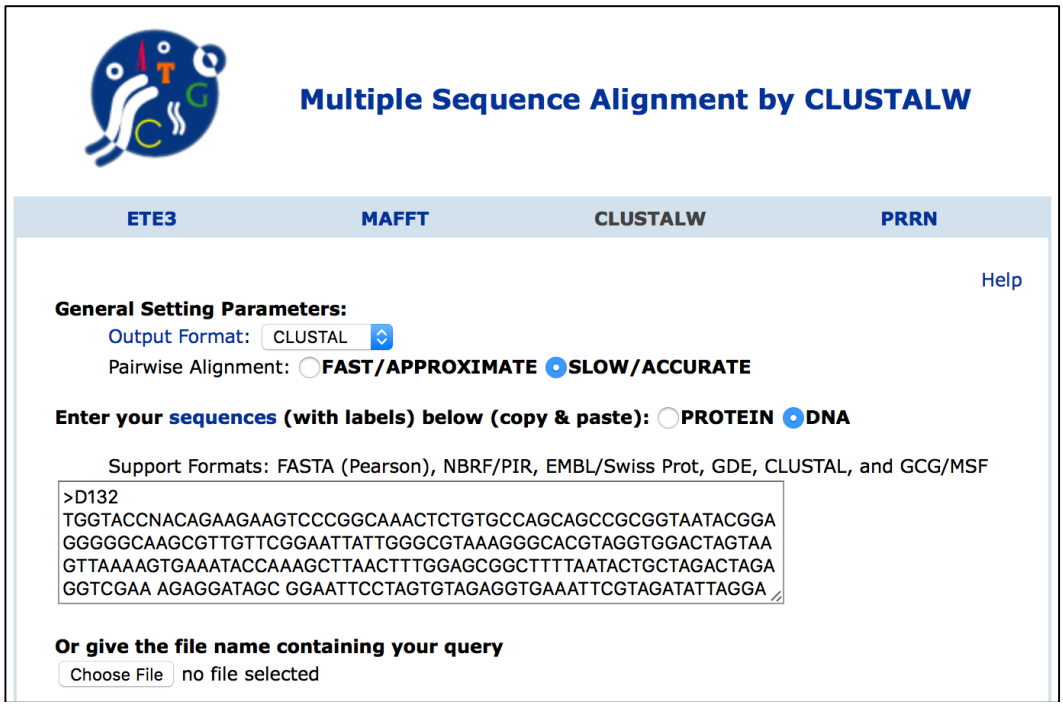

Figure 2.3: Alignment of obtained sequences using CLUSTAL-W. Sequence obtained from present study were aligned with those deposited in GeneBank by CLUSTAL-W method for the purpose of constructing specific phylogenetic trees.

2.7 GIS Analysis

Epi Info™ statistical package (CDC free-software) was used for spot mapping of cases of *Anaplasma* and *Ehrlichia*. SaTScan™ v8.0 Freeware was used to detect statistical evidence for purely-spatial clustering of cases caused by *Anaplasma*. Analysis was done on two levels; the first included segregation of cases based on host, while the second was based on pooling of cases regardless of host. SaTScan™ v8.0 input files included number of cases per locality, year of infection, host population size of location in the year of infection, and the exact latitude-longitude coordinates of each location. Data were analysed based on discrete Poisson model with level of statistical significance considered at P-value ≤ 0.05 (Kulldorff, 1997).

2.8 Statistical Analysis

Chi-square test was used to determine the statistical significance. For all analyses, significance was indicated by a P-value< 0.05. Analysis was performed by IBM SPSS statistics software version 23.

2.9 Ethics statement

The animal population was residing in different farms throughout the West Bank. Prior to ticks sampling, the animal owners were verbally informed about the goals of the project and the sampling protocol. All owners gave their verbal informed consent to collect ticks from their animals. The study was approved by the ethics committee at the Faculty of Medicine in Al-Quds University-Palestine (EC number: ZA/196/013).

Chapter 3:

Results

3.1 Sampling and Tick Identification

A total of 723 hard ticks were collected from different districts and host animals as shown in (Table 3.1). The ticks comprised three genera *Rhipicephalus*, *Hyalomma* and *Haemaphysalis*. Among them: 508 were *Rh. sanguineus*, 108 *Rh. turanicus*, 11 *Rh. bursa*, 32 *Rh.* spp., 32 *Hy. dromedarii*, five *Hy. impeltatum*, six *Hyalomma* spp, 16 *H. parva* and five *H. adleri*.

Blood samples were collected from different outdoor domestic dogs (n=135) and camels (n=4) simultaneously at the time of tick sampling. Moreover, 47 blood samples were taken from sheep, horses (n= 2) and one blood sample from a goat (Table 3.2). All animals were apparently healthy and did not show any clinical signs at the time of sampling.

3.2 Molecular Detection of *Ehrlichia* and *Anaplasma* in Ticks

All tick samples (n=723) were screened for the presence of *Anaplasma* or *Ehrlichia* DNA using *16s rRNA* -PCR. The sample was considered positive if a fragment of 345bp was observed on 2% agarose gel (Figure 3.1).

Among 723 tested ticks, 51 (7.1%) were positives. To confirm the identity of the amplified products, all positive samples were sequenced. Based on BLAST analysis, 6.5% (47/723) of samples belonged to *Anaplasma* and only four samples (0.6%) were identified as *Ehrlichia*. All *Ehrlichia*-infected ticks (n=4) were taken from dogs (Table 3.1). Three sequences obtained from *Rh. sanguineus* were identified as *E. canis* (Appendix A, sequence A10) whereas one sequence from *H. parva* (Appendix A, sequence A11) belonged to *Ehrlichia spp.* (Table 3.1). Among *Anaplasma*-infected ticks (n=47), 13 samples obtained from dogs were identified to harbour *A. platys* (Appendix A, sequence A1) and 34 samples harboured *Anaplasma spp*. (Appendix A, sequence A13) The tick species and their animal hosts are shown in (Table 3.1).

Table 3.1: The overall prevalence of *Anaplasma* and *Ehrlichia* DNA in ticks using *16s* rRNA PCR.

***Ixodid* Tick Species**	**Number of Tested Ticks**	**EHR *16s* PCR Positives (%)**	**Pathogen Detected (n)**	**Animal Hosts**	**District**
Rh. sanguineus	508	26 (5.1)	*Anaplasma platys* (13), *Anaplasma spp* (10) *E. canis* (3)	Dogs, Sheep, Goats	Jenin, Ramallah, Al-Khalil, Tubas, Nablus, Jericho, Tulkarm
Rh. turanicus	108	21 (19.4)	*Anaplasma spp* (21)	Sheep, Dogs	Nablus, Tubas, Jenin, Ramallah, Jericho.
Rh. bursa	11	0	0	Sheep, Goats, Dogs	Tubas, Jenin, Ramallah
Rh. spp.	32	3 (9.4)	*Anaplasma spp* (3)	Sheep, Dogs	Nablus, Jenin, Ramallah, Al-Khalil, Tubas, Jericho
Hy. dromedarii	32	0	0	Camels	Jericho
Hy. impeltatum	5	0	0		
Hy. spp.	6	0	0		
H. parva	16	1 (6.3)	*Ehrlichia spp* (1).	Dogs	Ramallah, Jenin, Tulkarm
H. adleri	5	0	0	Dogs	Jenin, Ramallah
Total	723	51 (7.1)			

3.3 *Ehrlichia* and *Anaplasma* in Blood

Out of 189 animal blood samples screened by *16s rRNA* –PCR (Figure 3.1), 34 samples (18%) were positives (Table 3.2).

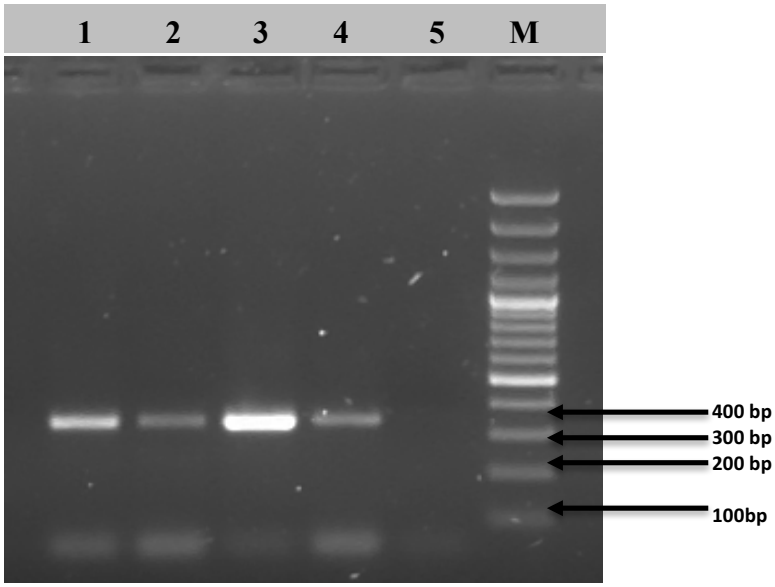

Figure 3.1: Molecular detection of *Ehrlichia* and *Anaplasma* using *16s rRNA* primers. For both *Anaplasma* and *Ehrlichia* a fragment of 345bp was observed on 2% agarose gel; lanes 1-3: A. platys, lane 4: *E. canis*, lane 5: negative control, M: DNA marker (100bp).

Among canine blood samples (n=135), 11.1% (15/135) were positives (Table 3.2). Of which, 13 (9.6%) samples were infected with *A. platys* (Appendix A, sequence A2) and two (1.5%) infected with *E. canis* (Appendix A, sequence A10). Of the 47 sheep blood samples, 40.4% (19 /47) were positive; all of which were identified as *Anaplasma spp*. (Appendix A, sequence A4), (Table 3.2). None of the blood samples from camels (n =4), horses (n=2) and a goat were positive for *Anaplasma* or *Ehrlichia* (Table 3.2).

Table 3.2: The overall prevalence of *Anaplasma* and *Ehrlichia* DNA in animal blood samples using 16s rRNA PCR.

Animal Species	Number of Animals	EHR *16s* PCR Positives (%)	Pathogens Detected (n)	Districts
Dogs	135	15 (11.11)	*E. canis* (2), *A. platys* (13)	Jenin, Al-Khalil, Ramallah, Jericho, Salfit, Tulkarm, Nablus
Sheep	47	19 (40.43)	*Anaplasma spp.* (19)	Jericho, Bethlehem
Camels	4	0	0	Jericho
Goats	1	0	0	Bethlehem
Horses	2	0	0	Jericho
Total	189	34 (18)		

3.4 Identification of *Anaplasma spp.* by *msp4* Gene

To confirm the *16s rRNA* -PCR results and to determine the species of *Anaplasma*, long *msp4* PCR (Figure 3.2) was applied to all samples that were identified as *Anaplasma spp*. by *16s rRNA* -PCR (n=53; 34 ticks and 19 sheep blood samples).

Among ticks (n=34), the detection rate of *Anaplasma* by long *msp4* PCR was 38.2%, (13/34), all of them were identified as *A. ovis* (Appendix A, sequence A5), (Table 3.3). Out of sheep blood samples (n=19), *A. ovis* (Appendix A, sequence A6) was identified in 26.3% (5/19) of samples (Table 3.3).

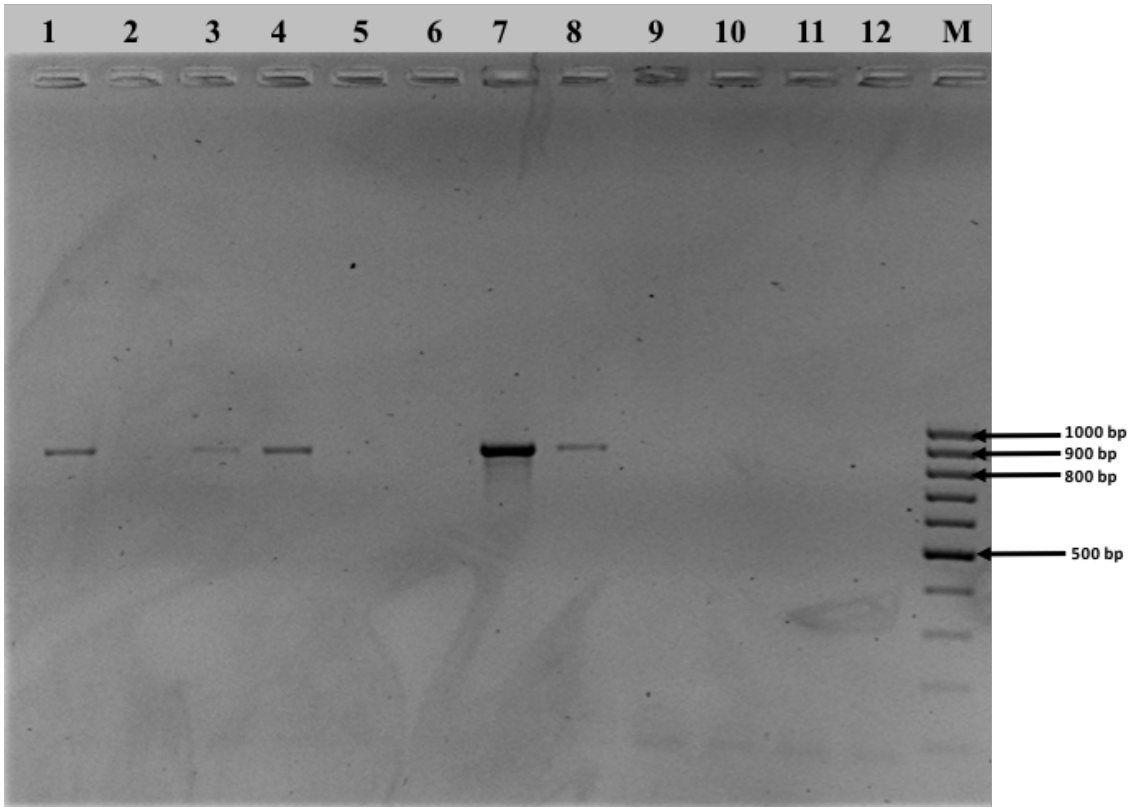

Figure 3.2: Identification of *Anaplasma spp.* using long *msp4* primers. For *A. ovis* a fragment of 851bp was observed on 2% agarose gel; lanes 1,3,4,7,8: *A. ovis*, lane 12: negative control, lane M: DNA marker (100bp).

The detection rate of *Anaplasma* by short *msp4* PCR increased to 70.6% (24/ 34) in ticks and to 68.4% (13/19) in sheep. The positive samples were found to be infected with *A. ovis* (Table 3.3). The overall prevalence of *A. ovis* in sheep and their corresponding ticks were 27.7% (13/47) and 18.6% (24 positive ticks out of 129 ticks collected from sheep), respectively.

The specificity of short *msp4*-PCR was investigated using pure DNA of *A. marginale, A.*

centrale and *A. ovis* as positive controls, the sequencing results proved that short *msp4*-PCR can differentiate between all species. Moreover, none of *A. platys* samples were detected by short *msp4*-PCR indicating the specificity of these primers for *A. marginale, A. centrale* and *A. ovis* (Figure 3.3).

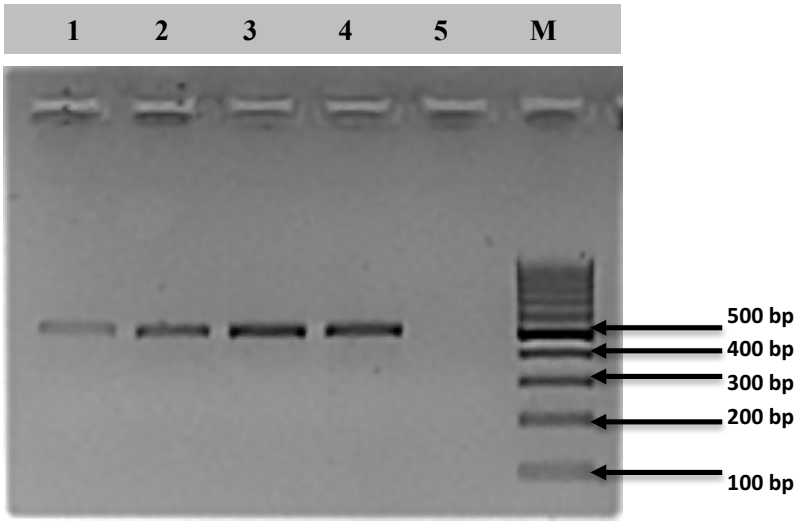

Figure 3.3: Identification of *Anaplasma spp.* using short *msp4* primers and specificity of short *msp4*-PCR; For short *msp4* a fragment about 500bp was observed on 2% agarose gel; Lanes 1 & 2 represent two *A. ovis* samples, lane 3 represent *A. centrale* positive control, lane 4: *A. marginale*, lane 5: represent a previously positive sample for *A. platys* using *16s* rRNA. M lane: 100bp DNA marker.

Table 3.3: Molecular identification of *Anaplasma* species in ticks and blood samples targeting *msp4* gene.

Tested Samples (n)	Long *msp4* positives (%)	Short *msp4* positives (%)	Animal/tick Species	Pathogens Detected (n)
Ticks (34)	13 (38.2)	24 (70.6)	*Rh. turanicus* (16), *Rh. sanguineus* (8) (6), *Rhipicephalus spp.* (2)	*A. ovis* (24)
Blood (19)	5 (26.3)	13 (68.4)	Sheep (19)	*A. ovis* (13)
Total (53)	18 (34)	37 (69.8)		

3.5 Detection of Co-infections of *Anaplasma* and *Ehrlichia*

Co-infections of *Anaplasma* and *Ehrlichia* were tested in 67 *16s rRNA* positives (n=37 from ticks and n=30 from blood) samples were screened for the presence of *Ehrlichia* using genus specific primers (*sodB*-F and *sodB*-R). *Ehrlichia* was not detected in any *Anaplasma* positive samples (Figure 3.4). Three *Ehrlichia* positive samples were used as positive controls and confirmed by sequencing.

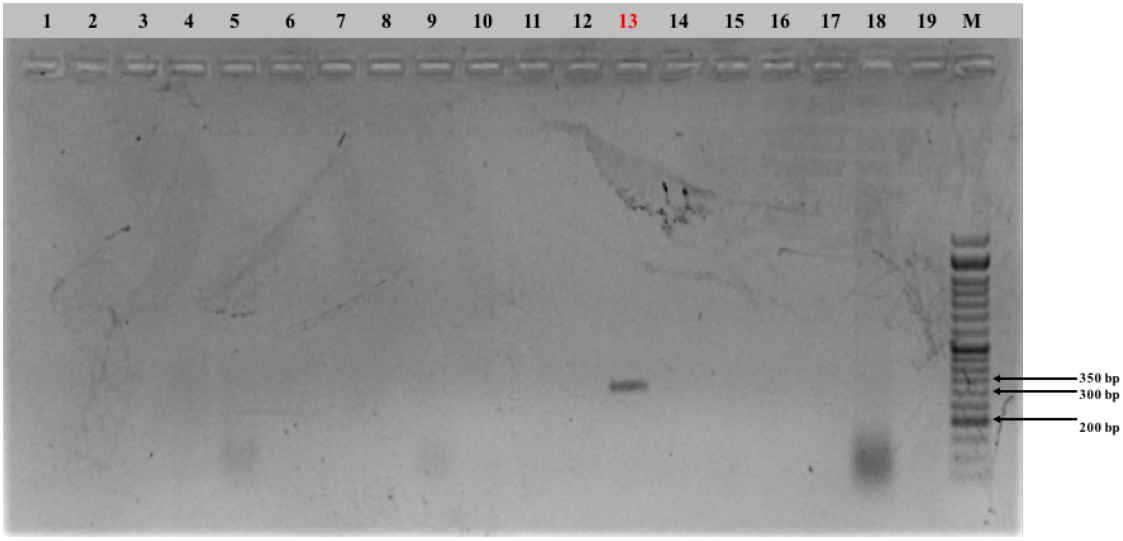

Figure 3.4: Screening for double infections using *Ehrlichia* specific *sodB*-PCR targeting a fragment of 300bp; All Lanes (except of 13) represent *Anaplasma* positive showed negative by *sodB*-PCR excluding double infection; Lane 13: represents positive *E. canis* sample used as positive control. Lane M: 50bp DNA marker.

3.6 Phylogenetic Analysis

Phylogenetic analysis based on partial sequences of *16s rRNA* gene revealed two main clusters. Cluster I represents the strains of *Ehrlichia* (n=6); the obtained sequences from ticks (n=3) and those from dogs (n=2) were identical to each other and to the *E. canis* reference strain deposited in the Gene bank (KP182942.1) (Appendix B, B1). One sequence obtained from *Haemaphysalis parva* tick formed a separate branch and showed 99% sequence identity to the reference strain of *Ehrlichia* spp. (KJ410253.1), (Appendix B, B3) (Figure 3.4). Cluster II which represents the strains of *Anaplasma* was separated into two sub-clusters: Cluster IIA, represents the strains of *A. platys* (n=26) were obtained from dogs (13) and ticks (13) and showed 99-100% sequence identity to each other and to the reference sequence of *A. platys* deposited in the Gene bank (KU500914.1), (Appendix B, B2). Cluster IIB representing the strains of *Anaplasma spp.* (n=53), shared at least 99% sequence identity to each other and to the reference sequences of *A. centrale*, *A. marginale* and *A. ovis* (KC189842.1, KU686794.1 and KJ410246.1, respectively) (Appendix B, B3). The DNA sequence of *A. phagocytophilum* (accession no. KR021165.1) was used as an outgroup in this analysis (Figure 3.5).

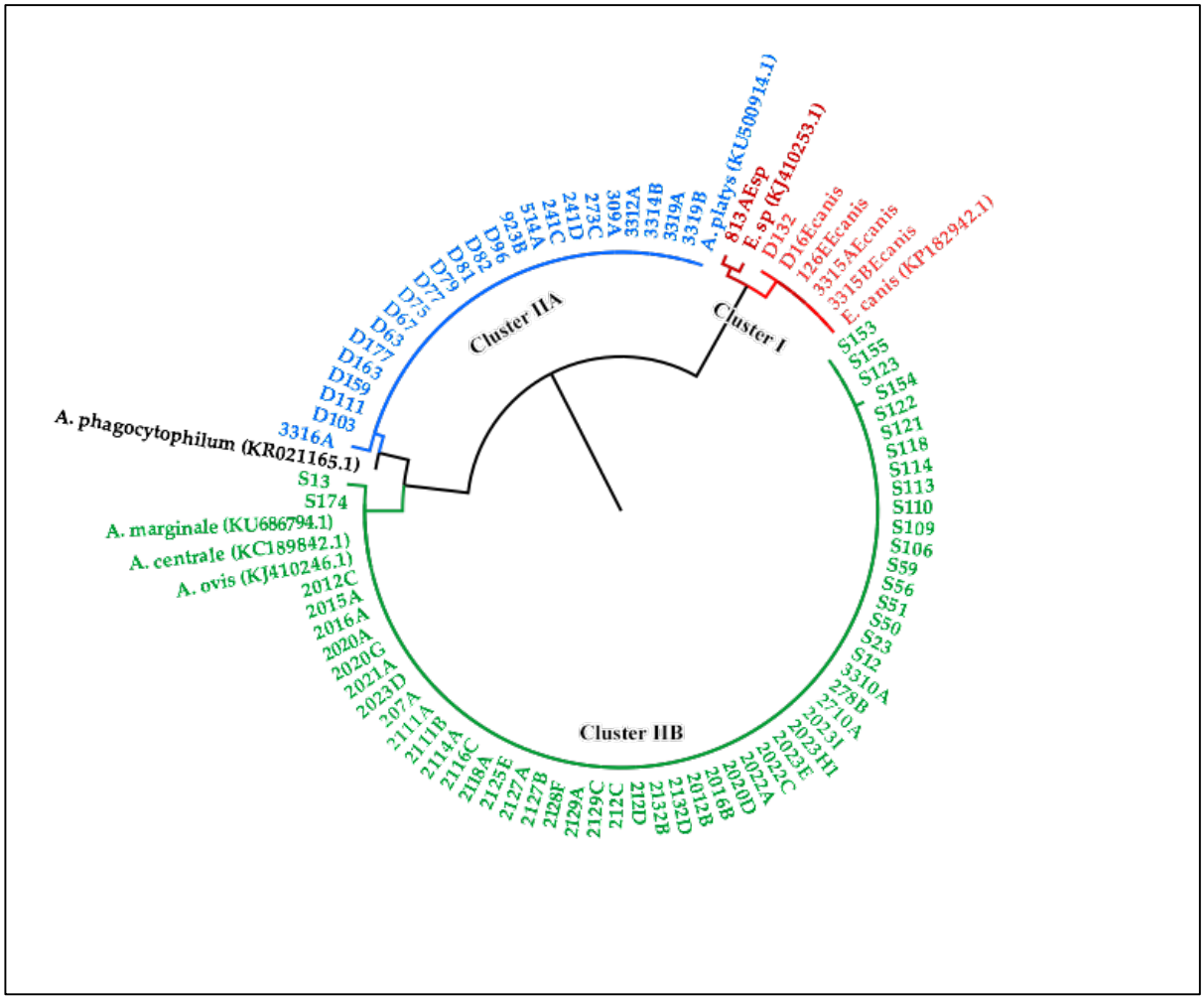

Figure 3.5: Phylogenetic analysis for *Anaplasma* and *Ehrlichia* based on EHR *16s* sequences. Cluster I (in red) represents two *Ehrlichia* strains (*E. canis* and *Ehrlichia spp.*). Cluster II is separated into two sub-clusters: Cluster IIA (in blue) represents *A. platys* and Cluster IIB (in green) represents *Anaplasma spp. A. phagocytophilum* was used as an outgroup.

The samples of cluster IIB (n=37) were further characterized using short *msp4* primers, the partial sequences of these samples shared 100% coverage and 99% similarity to *A. ovis* reference strains (KM285220.1 and LC126849.1), (Appendix B, B5) (Figure 3.6). DNA sequences of two samples had few nucleotide differences and showed 97% coverage and 98-99% identity to the *msp4* sequences of *A. ovis* identified in this study and to the reference strain of *A. ovis* (KM285220.1).

The DNA sequences of the strain references of *A. marginale* and *A. centrale* (accession no. LC126846.1, AY054383.2, respectively) showed different branches in this phylogenetic analysis (Figure 3.6).

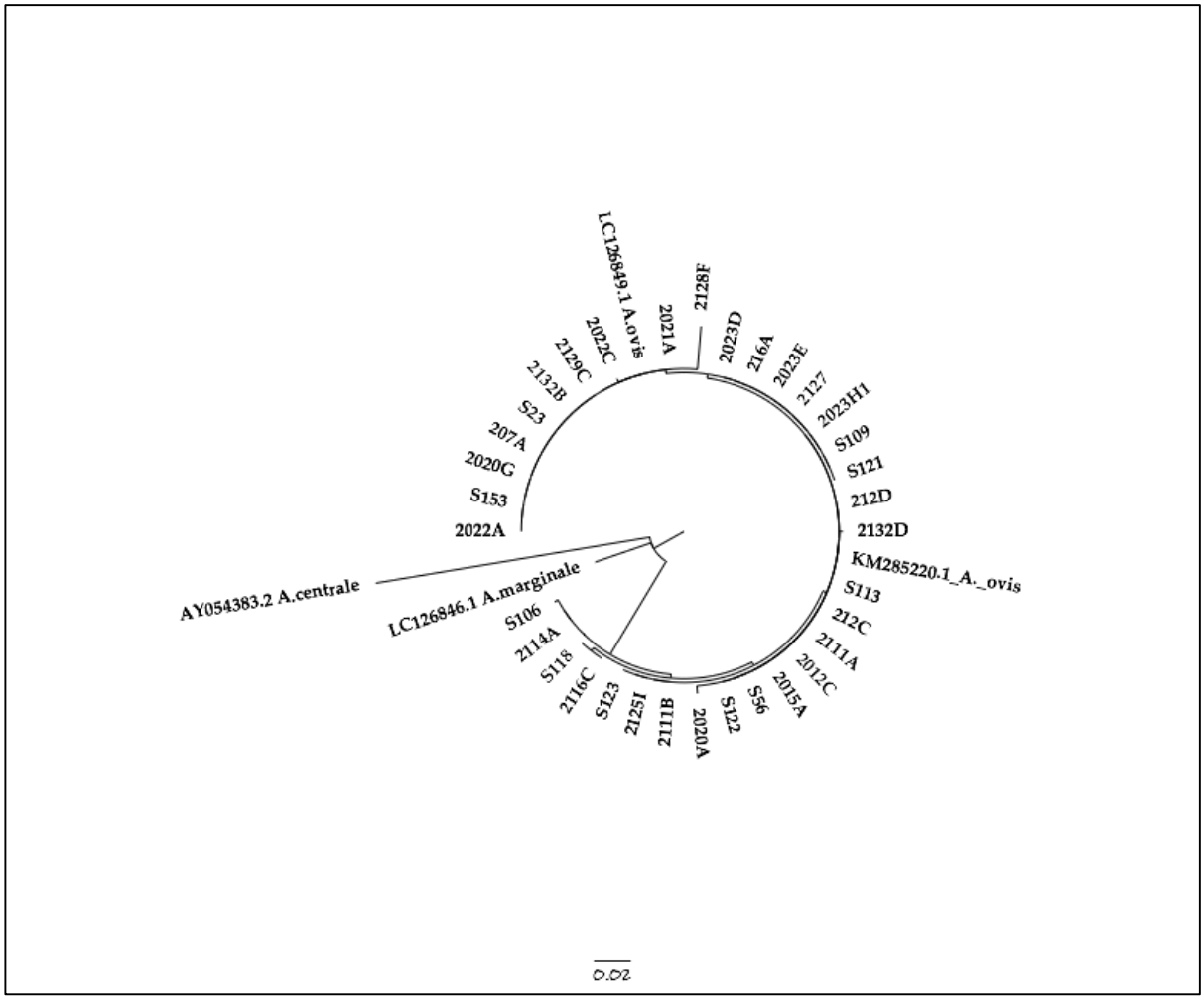

Figure 3.6: Phylogenetic analysis for *A. ovis* short *msp4* sequences. All *A. ovis* sequences grouped into one cluster, two *A. ovis* reference strains are included (KM285220.1, LC126849.1) in addition to two reference strains; *A. marginale* (LC126846.1) and *A. centrale* (AY054383.2) which showed two separate clusters.

3.7 Spot Mapping and Statistically Significant Clusters

Fifty Palestinian localities in the West Bank were targeted for the collection of *Anaplasma* and *Ehrlichia* cases. Cases of both infections in the study originated from 18 (36%) localities, which included villages (11/18=66%), refugee camps (2/18=11%) and cities (6/18=33%). Most of them (12/18=67%) were from rural areas. Cities like Jericho (Ariha), Tubas and Salfit are by standards considered towns rather than well-established cities with rural activities on the margins. *Anaplasma* was isolated from dogs in 10 (29%) out of 35 localities, while from ticks in 8 (23%) localities out of 39. At the same time, it was isolated from sheep in all 7 targeted localities. Six cases of *Ehrlichia* were isolated, four of which were from ticks and 2 from dogs. Cases of *Ehrlichia* were from the districts of Al-Khalil, Jericho, Tulkarem, and Jenin (Figure 3.7).

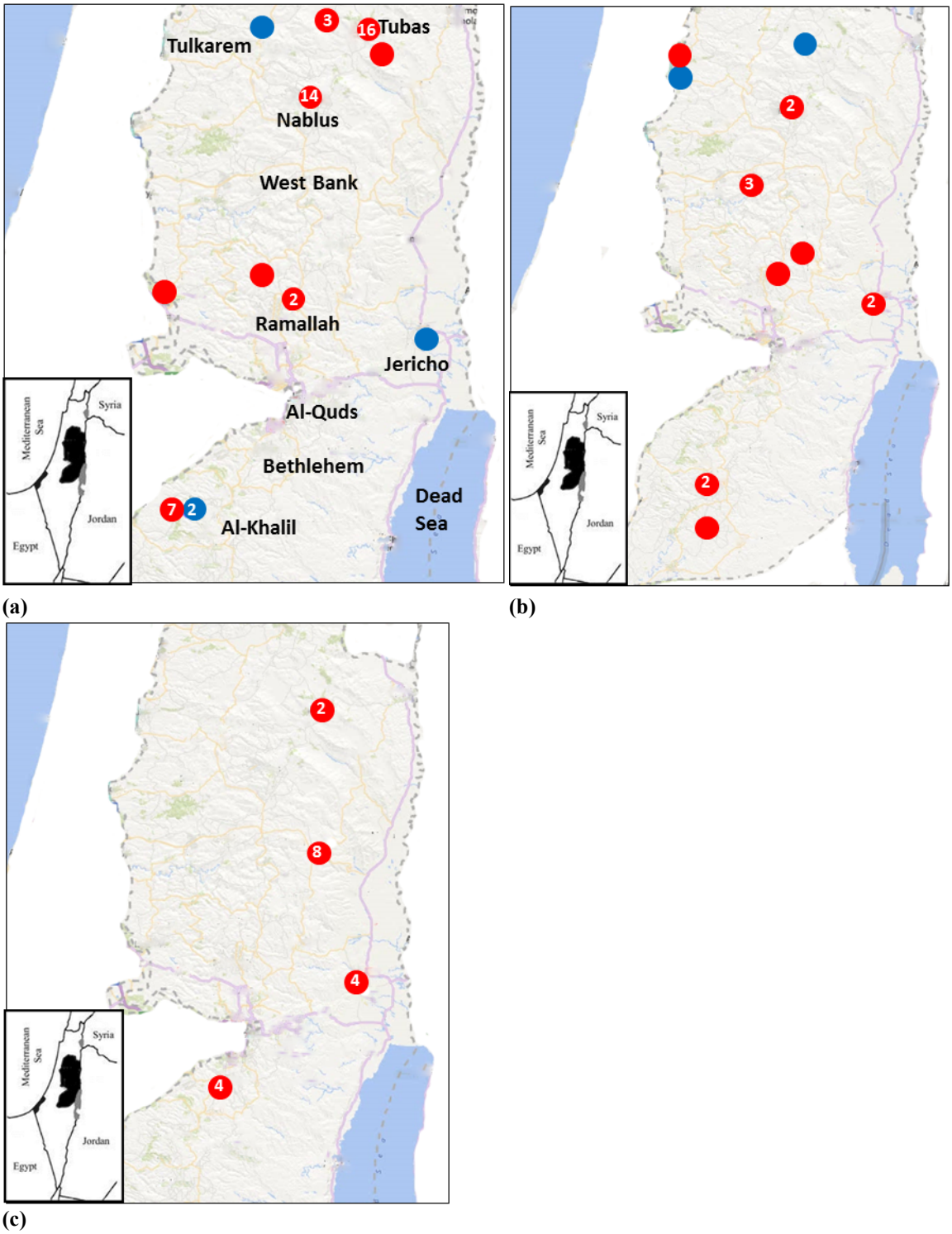

Figure 3.7: Spot maps of cases of *Anaplasma* (red circles) and *Ehrlichia* (blue circles) isolated from ticks **(a)**, dogs **(b)** and sheep **(c)**. The number within the circles indicate cases spotted in the area, empty circles indicate one case.

Implementation of purely-spatial analysis by saTScan for the pooled cases of *Anaplasma* cases revealed two statistically significant clusters (P<0.05) (Figure 3.8). However, implementing the same analysis on segregated bulks of cases did not reveal any significant clusters. Significant clusters were in Tubas (P=0.00005, RR=4.3) (RR: Relative risk value)

and Majdal-Bani-Fadil village in Ramallah (P=0.0012, RR=6.5) (Figure 3.8). In Al-Khalil district south of the West Bank, Hitta village close to the green line border had 10 cases of *Anaplasma* which was close to forming a cluster (P=0.08; RR=3).

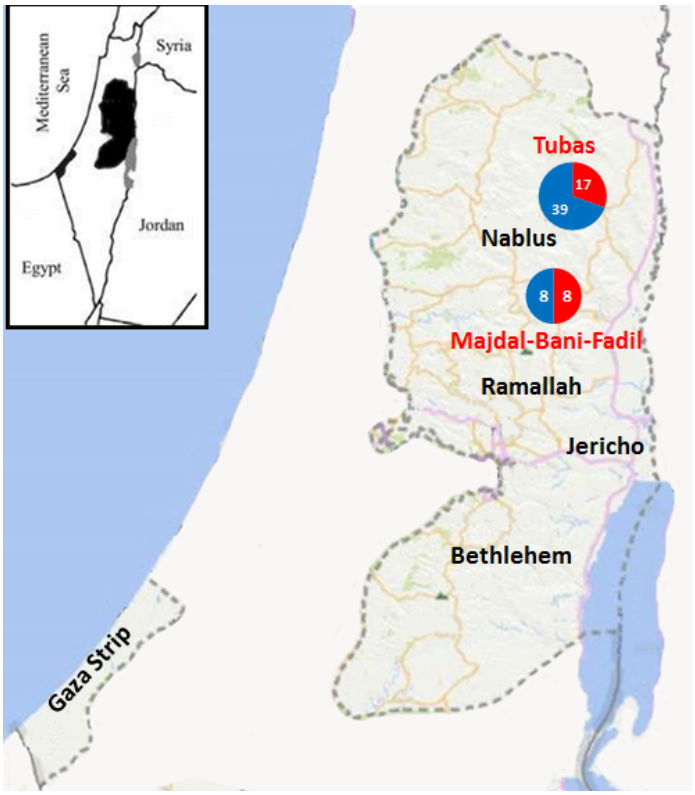

Figure 3.8: Geographical distribution of statistically significant foci of *Anaplasma* cases in Palestine (excluding Gaza strip) on implementing purely spatial analysis by saTScan; Red sectors indicate positive cases while blue sectors indicate negative cases. The numbers within the sectors indicate the number of cases.

Chapter 4:

Discussion

Tick-borne bacteria are important pathogens which affect the health of both humans and animals globally. In this study and for the first time in Palestine, we have confirmed the presence of *Anaplasma* and *Ehrlichia* in *ixodid* ticks and blood samples from different domestic animals. These pathogens have been reported from different neighboring countries including, Egypt, Iran, and Israeli areas (Harrus et al., 2011; Jafarbekloo et al., 2014; Loftis et al., 2006a, 2006b). Two pathogens were detected in canine blood samples: *A. platys* and *E. canis.* The prevalence of *A. platys* (the causative pathogen of canine cyclic thrombocytopenia) determined in this study (9.6%) was higher than in other studies reported from Italy (de la Fuente et al., 2006) and Japan (Inokuma et al., 2002), but lower than in Kenya (Abdel-Shafy et al., 2012), Brazil (da Silva et al., 2012) and Venezuela (Huang et al., 2005).

The results of this study were consistent with these from Japan; *E. canis* was detected in 1.5% of the tested canine blood samples compared to 1.5% in this study (Kubo et al., 2015). In contrast, two studies conducted in Brazil (da Silva et al., 2012) and Panama (Santamaria et al., 2014) reported much higher prevalence; 16.4% and 64.2%, respectively. Co-infection of *A. platys* and *E. canis* was not detected in the sampled animals. Despite that *E. canis* is well-known as a dog pathogen; it has been reported in domestic ruminants (Zhang et al., 2015). However, in this study, *E. canis* was not detected in sheep, camels, goat and horses. *A. platys* and *E. canis* were identified in *Ixodid* ticks obtained from the same infected dogs. Although the blood and tick samples were collected from dogs simultaneously, the results showed that the prevalence of *E. canis* in ticks (0.55%) was also lower than in dogs (1.5%). This can be partially explained by the tick infection behavior in which one infected tick can spread the infection among more than one dog, However, higher infection rates of *E. canis* in ticks were reported from Israeli areas (10%) (Harrus et al., 2011) and Iran (16.66%) (Khazeni et al., 2013). In contrast, the prevalence of *A. platys* in ticks (1.8%) was lower than in dogs (9.6%). The discrepancy between the prevalence of *A. platys* and *E. canis* infections in dogs and the prevalence in ticks could be explained by the unequal number of the tested tick populations per animal host (Ereqat et al., 2016b). Our findings showed that *A. platys* and *E. canis* infected ticks were mainly from the species of *Rh. sanguineus* which is considered the main vector in Palestine and the neighboring countries (Dantas-Torres, 2008; Ereqat et al., 2016b; Harrus et al., 2011). However, the presence of *A. platys* in other

species of *Rhipicephalus*, such as un-engorged *R. turanicus* and *R. bursa* were reported in Turkey and Israeli areas (Aktas et al., 2009; Harrus et al., 2011). On the basis of *16s rRNA* phylogenetic analysis, high genetic homology was observed among the sequences of *A. platys* identified in this study. heterogeneity was observed among *Ehrlicjia* group. However, distinct *Ehrlichia* sequence found to be 99% similar to the corresponding sequence of improperly identified *Ehrlichia spp*. reported from China (Dong et al., 2014; Kang et al., 2014). Further characterization with additional genes is needed to reveal this species.

In this study, *A. ovis*, the agent of ovine anaplasmosis, was identified for the first time in Palestine. The overall prevalence of *A. ovis* in sheep (27.7%) and their corresponding ticks (18.6%) was lower than reported by other studies conducted in Iran (Razmi et al., 2006), northwest China (Yang et al., 2015), Iraq, Sudan, Portugal and Turkey (Renneker et al., 2013). However, blood samples and ticks were not simultaneously collected.

Our findings provide molecular evidence for the presence of *A. ovis* in *Rh. turanicus* and *Rh. sanguineus* ticks which was in line with previous studies showing that *Rhipicephalus spp.* is one of the most important vectors of diseases in sheep (de la Fuente et al., 2005a; De La Fuente et al., 2005b; de la Fuente et al., 2004; Hosseini-Vasoukolaei et al., 2014; Jafarbekloo et al., 2014; Renneker et al., 2013).

Amplification of *16s rRNA* gene is commonly used for detection of *Anaplasma*/ *Ehrlichia* DNA and thus further testing is required to investigate co-infections by the two pathogens and for species identification of the same genus such as *A. marginale* and *A. ovis*. In the present study, and since *16s rRNA* PCR was unable to definitively identify most of the *Anaplasma species; A. ovis* infections have been identified using the newly designed short *msp4* primers, the detection rate increased by 50% compared to the previously published long *msp4* primers (de la Fuente et al., 2003).

This study confirmed the specificity of the short *msp4*- PCR in the identification of different *Anaplasma* species (*A. marginale*, *A. centrale* and *A. ovis*). However, the short *msp4*- PCR was unable to detect all of the *Anaplasma spp.* that were positive by *16s rRNA* with 4.7% of the *16s rRNA* positive samples not successfully amplified by short *msp4*- PCR indicating different sensitivities of the PCRs targeting different genes in *Anaplasma spp.*, farther optimization steps may be required (eg. Number of cycles, annealing temperature, adding of additives …etc) to improve the sensitivity of this reaction. The phylogenetic tree based on short *msp4* -PCR showed high genetic homology among the sequences of *A. ovis* identified in this study.

The presence of *A. ovis* is confirmed in Palestine. Although this bacterium is supposed to cause only mild clinical signs (Friedhoff, 1997), its effect may be worsen if the animals were under stress by different factors such as poor health conditions, hot weather, co-infection, heavy tick infestation, vaccination or deworming is aggravated in infected ruminants (Khayyat & Gilder, 1947; Manickam, 1987). Since the small ruminants are major source of meat, milk, and wool in Palestine, ovine anaplasmosis, caused by *A. ovis*, may present an economic loss, if stress occurs during infection. Therefore it is important to better understand this disease which justifies the necessity of further investigations.

Spot mapping of cases confirmed that most of the cases of anaplasmosis and ehrlichiosis (83%) were in rural areas where the vast majority of livestock and accompanying dogs are located and the optimal habitat of *Ioxdid* ticks exists. Cases of *Ehrlicha* were in north, south and east of the West Bank (Figure 3.8). This is consistent with the distribution of livestock and open wilderness with caves and wild vegetation. Kulldorf's saTScan revealed two main statistically significant foci for *Ananplasma* infection regardless of the host or vector (Figure 3.9). These were in Tubas district north of the West Bank and in Majdal-Bani-Fadil; a village on the western border of the Jordan Valley. In both areas people lead a rural life with extensive farming and livestock activities. The two statistically significant foci lie on the migration route of birds on the great Syria-African rift valley extending from East Africa until Syria. The Jordan Valley lying in the middle of the rift is a major point of attraction for these birds to rest in on their way during the spring and autumn annual migration. Although not proved in Palestine, but migrating birds have been found to carry infected ticks and transfer tick-borne diseases from one area to another as found in Sweden (Bjoersdorff et al., 2001). Another plausible explanation for the two foci is the climate change. The vector-borne diseases are climate-sensitive and the vectors have been found to move north as far as Norway, Sweden and Russia as well as to higher altitudes (Andreassen et al., 2012; Jore et al., 2011; Lindgren et al., 2000; Ostfeld & Brunner, 2015). The increasing temperature in Palestine and the Jordan Valley as an arid area in particular could have participated in the increase of the disease rates in the two foci. However, the climate change affects not only the ticks, but also the hosts responsible for supporting the ticks and microorganisms causing the disease. Furthermore, the two areas are considered under-privileged rural areas with low socioeconomic status and leading extensively active life style of farming and livestock-raising which again could have contributed to the high disease rate (Campbell-Lendrum et al., 2015). In anyhow, this is far from being a single factor event, but largely multifactorial with complex interactions of several variables such

as climate change, environmental change and human behavior.

4.1 Conclusions

1. This study highlights for the first time the presence of *A. platys, A. ovis* and *E. canis* in the Palestine (West Bank).
2. The detection of *A. platys, A. ovis* and *Ehrlichia* DNA in *Rhipicephalus* ticks indicates its probable vectorial role.
3. *A. ovis* the causitive agent of ovine anaplasmosis was detected in sheep indicating a potential risk for adverse more aggravate disease.
4. It is important to better understand this disease and further investigations are necessary.

4.2 Recommendations

1. Before getting in rular areas, wear covering clothes and apply ticks repellents on skin and clothes.
2. Local farmers can use pesticides. Isolate suspected animals and ask for help from professionals.
3. Veterinarians in Palestine should put into consideration the presence of *Anaplasma* and *Ehrlichia* during clinical examination of sick animals particularly when clinical signs are suggestive of with anaplasmosis or ehrlichiosis.
4. Increasing the level of education and awareness of ticks and tick -borne diseases.

4.3 Study Limitations

1. Number of blood samples collected from goats, horses and camels were not representative for investigation the prevalence of anaplasmosis and echrlichiosis in these animals.
2. The possibility of double infections by the three-close species of *Anaplasma* (*ovis, centrale* and *marginale*) which may present in sheep blood and their ticks was not investigated.

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Appendix A: Representative sequences of *Anaplasma* and *Ehrlichia* genera

Sequence A1: *A. platys* partial *16s rRNA* DNA sequence originated from *Ixodid* ticks collected from a dog and identified according to BLAST:

>3319B
ACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG
GGCAAGCTTGTTCGGAATTATTGGGCGTAAAGGGCATGTAGGCGGTTCGGTA
AGTTAAAGGTGAAATGCCAGGGCTTAACCCTGGAGCTGCTTTTAATACTGCCA
GACTCGAGTCCGGGAGAGGATAGCGGAATTCCTAGTGTAGAGGTGAAATTCG
TAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTATCTGGTCCGGTACTG
ACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCTGTAAACGAT

Sequence A2: *A. platys* partial *16s rRNA* DNA sequence originated from dog blood and identified according to BLAST:

>D77
ACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG
GGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCATGTAGGCGGTTCGGT
AAGTTAAAGGTGAAATGCCAGGGCTTAACCCTGGAGCTGCTTTTAATACTGCC
AGACTCGAGTCCGGGAGAGGATAGCGGAATTCCTAGTGTAGAGGTGAAATTC
GTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTATCTGGTCCGGTACT
GACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCTGTAAACGAT

Sequence A3: *Anaplasma spp.* partial *16s rRNA* DNA sequence originated from *Ixodid* ticks collected from sheep and identified according to BLAST:

>3310A
ACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG
GGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCATGTAGGCGGTTTGGT
AAGTTAAAGGTGAAATACCAGGGCTTAACCCTGGGGCTGCTTTTAATACTGCA
GGACTAGAGTCCGGAAGAGGATAGCGGAATTCCTAGTGTAGAGGTGAAATTC

GTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTGTCTGGTCCGGTACT
GACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCTGTAAACGAT

Sequence A4: *Anaplasma spp.* partial *16s rRNA* DNA sequence originated from sheep blood and identified according to BLAST:

>S50
ACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG
GGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCATGTAGGCGGTTTGGT
AAGTTAAAGGTGAAATACCAGGGCTTAACCCTGGGGCTGCTTTTAATACTGCA
GGACTAGAGTCCGGAAGAGGATAGCGGAATTCCTAGTGTAGAGGTGAAATTC
GTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTGTCTGGTCCGGTACT
GACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCTGTAAACGAT

Sequence A5: *A. ovis* partial *msp4* DNA sequence (using *msp4* long primers) originated from *Ixodid* ticks collected from sheep and identified according to BLAST:

>207A
TTACAGAGAATTGTTTACAGGGGGCCTGTCAGCAGCCACAGTTCTGCGCCTGC
ACCCCGTGACTTGTTAGTGGGGCCGTAGTGGCGTCTCCCATGAGTCATGAAGT
GGCTTCTGAAGGGAGCGGGGTCATGGGAGGTAGCTTTTATGTGAGTGCGGCT
TACAGCCCAGCGTTTCCCTCTGTTACCTCATTCGACATGCGTGAGTCAAGCAG
AGAGACCTCGTATGTTAGAGGCTATGACAAGAGTGTTGCAACAATTGATGTG
AGTGCGCCAGCAAACTTTTCCAAATCCGGCTACACTTTTGCTTTCTCTAAGAA
TTTACTCACATCTTTCGACGGCGCTGTGGGATATTCTCTGGGAGGAGCTAGAG
TGGAACTAGAAGCAAGCTACAGAAGGTTTGCTACTTTAGCGGACGGGCAGTA
CGCAAAAAGTGGTGCAGAGTCCCTGGCTGCAATTACTCGCGACGCTGCCATT
ACTGAGAACAATTACTTTGTGGTCAAAATCGATGAAATCACAAACACTTCAGT
CATGCTAAATGGCTGCTATGACGTGTTGCACACAGATTTGCCTGTGTCCCCAT
ATGTGTGTGCCGGAATAGGTGCTAGCTTTGTCGACATTTCTAAGCAAGTAACC
ACAAAGCTAGCCTACAGGGGCAAGGTTGGAATCAGCTACCAGTTTACTCCAG
AAATATCTTTGGTGGTAGGTGGGTTCTACCACGGACTCTTTGACGAGTCTTAC

AAGGACATACCCGCACATAACAGTGTAAAGTTCCCCGGAGAAGCAAAATCGC
TCGGTCCAGGCAACATAATGCTGATTACGGTTTTAGACCTTGGAGCAAGATTC
CTGTTCA

Sequence A6: *A. ovis* partial *msp4* DNA sequence (using *msp4* long primers) originated from sheep blood and identified according to BLAST:

>S153
TTACAGAGAATTGTTTACAGGGGGCCTGTCAGCAGCCACAGTCTGCGCCTGCT
CCCTACTTGTTAGTGGGGCCGTAGTGGCGTCTCCCATGAGTCATGAAGTGGCT
TCTGAAGGGAGCGGGGTCATGGGAGGTAGCTTTTATGTGAGTGCGGCTTACA
GCCCAGCGTTTCCCTCTGTTACCTCATTCGACATGCGTGAGTCAAGCAGAGAG
ACCTCGTATGTTAGAGGCTATGACAAGAGTGTTGCAACAATTGATGTGAGTGC
GCCAGCAAACTTTTCCAAATCCGGCTACACTTTTGCTTTCTCTAAGAATTTACT
CACATCTTTCGACGGCGCTGTGGGATATTCTCTGGGAGGAGCTAGAGTGGAA
CTAGAAGCAAGCTACAGAAGGTTTGCTACTTTAGCGGACGGGCAGTACGCAA
AAAGTGGTGCAGAGTCCCTGGCTGCAATTACTCGCGACGCTGCCATTACTGAG
AACAATTACTTTGTGGTCAAAATCGATGAAATCACAAACACTTCAGTCATGCT
AAATGGCTGCTATGACGTGTTGCACACAGATTTGCCTGTGTCCCCATATGTGT
GTGCCGGAATAGGTGCTAGCTTTGTCGACATTTCTAAGCAAGTAACCACAAA
GCTAGCCTACAGGGGCAAGGTTGGAATCAGCTACCAGTTTACTCCAGAAATA
TCTTTGGTGGTAGGTGGGTTCTACCACGGACTCTTTGACGAGTCTTACAAGGA
CATACCCGCACATAACAGTGTAAAGTTCCCCGGAGAAGCAAAAGCCTCAGTC
AAGGCACATATTGCTGATTACGGTTTTAACCTTGGAGCAAGATTCCTGTTCA

Sequence A7: *A. ovis* partial *msp4* DNA sequence (using *msp4* short primers) originated from *Ixodid* ticks collected from sheep and identified according to BLAST:

>207A
CTTACAGCCCAGCGTTTCCCTCTGTTACCTCATTCGACATGCGTGAGTCAAGC
AGAGAGACCTCGTATGTTAGAGGCTATGACAAGAGTGTTGCAACAATTGATG
TGAGTGCGCCAGCAAACTTTTCCAAATCCGGCTACACTTTTGCTTTCTCTAAG
AATTTACTCACATCTTTCGACGGCGCTGTGGGATATTCTCTGGGAGGAGCTAG
AGTGGAACTAGAAGCAAGCTACAGAAGGTTTGCTACTTTAGCGGACGGGCAG

TACGCAAAAAGTGGTGCAGAGTCCCTGGCTGCAATTACTCGCGACGCTGCCA
TTACTGAGAACAATTACTTTGTGGTCAAAATCGATGAAATCACAAACACTTCA
GTCATGCTAAATGGCTGCTATGACGTGTTGCACACAGATTTGCCTGTGTCCCC
ATATGTGTGTGCCGGAATAGGTGCTAGCTTTGTCGACATTTCTAAGCAAGTAA
CCAC

Sequence A8: *A. ovis* partial *msp4* DNA sequence (using *msp4* short primers) originated from sheep blood and identified according to BLAST:

>S153
CTTACAGCCCAGCGTTTCCCTCTGTTACCTCATTCGACATGCGTGAGTCAAGC
AGAGAGACCTCGTATGTTAGAGGCTATGACAAGAGTGTTGCAACAATTGATG
TGAGTGCGCCAGCAAACTTTTCCAAATCCGGCTACACTTTTGCTTTCTCTAAG
AATTTACTCACATCTTTCGACGGCGCTGTGGGATATTCTCTGGGAGGAGCTAG
AGTGGAACTAGAAGCAAGCTACAGAAGGTTTGCTACTTTAGCGGACGGGCAG
TACGCAAAAAGTGGTGCAGAGTCCCTGGCTGCAATTACTCGCGACGCTGCCA
TTACTGAGAACAATTACTTTGTGGTCAAAATCGATGAAATCACAAACACTTCA
GTCATGCTAAATGGCTGCTATGACGTGTTGCACACAGATTTGCCTGTGTCCCC
ATATGTGTGTGCCGGAATAGGTGCTAGCTTTGTCGACATTTCTAAGCAAGTAA
CCAC

Sequence A9: *E. canis* partial *16s rRNA* DNA sequence originated from *Ixodid* ticks collected from a dog and identified according to BLAST:

>126E
ACAGAAGAAGTCCCGGCAAACTCTGTGCCAGCAGCCGCGGTAATACGGAGGG
GGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCACGTAGGTGGACTAGT
AAGTTAAAAGTGAAATACCAAAGCTTAACTTTGGAGCGGCTTTTAATACTGCT
AGACTAGAGGTCGAAAGAGGATAGCGGAATTCCTAGTGTAGAGGTGAAATTC
GTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTATCTGGTTCGATACT
GACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCTGTAAACGAT

Sequence A10: *E. canis* partial *16s rRNA* DNA sequence originated from dog blood and identified according to BLAST:

>D132

ACAGAAGAAGTCCCGGCAAACTCTGTGCCAGCAGCCGCGGTAATACGGAGGG
GGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCACGTAGGTGGACTAGT
AAGTTAAAAGTGAAATACCAAAGCTTAACTTTGGAGCGGCTTTTAATACTGCT
AGACTAGAGGTCGAAAGAGGATAGCGGAATTCCTAGTGTAGAGGTGAAATTC
GTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTATCTGGTTCGATACT
GACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCTGTAAACGAT

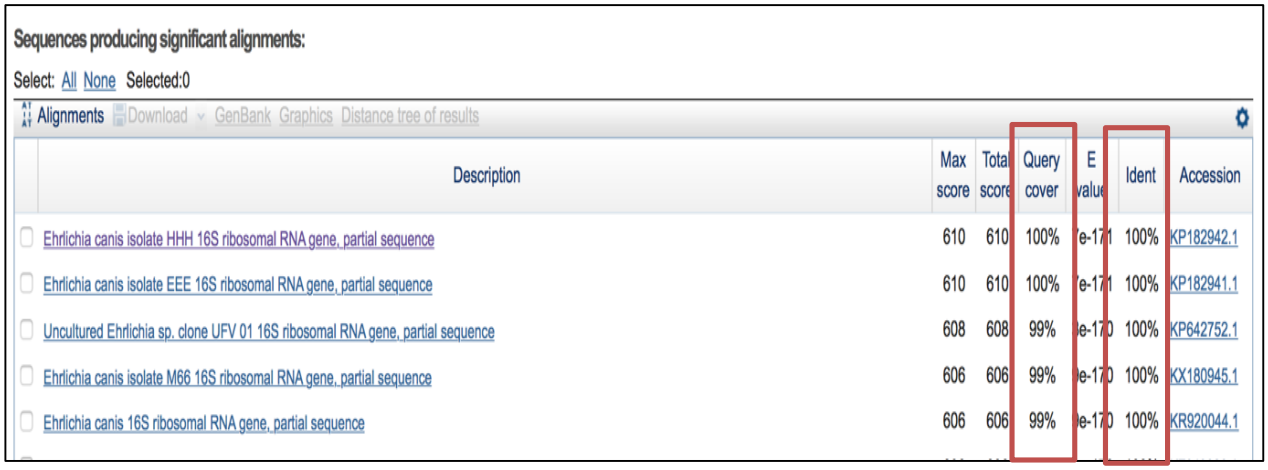
Sequence A11: *Ehrlichia spp.* partial *16s rRNA* DNA sequence originated from *Ixodid* ticks collected from a dog and identified according to BLAST:

>2128F

CCCAGCGTTTCCCTCTGTTACCTCATTCGACATGCGTGAGTCAAGCAGAGAGAC
CTCGTATGTTAGAGGCTATGACAAGAGTGTTGCAACAATTGATGTGAGTGCGC
CAGCAAACTTTTCCAAATCCGGCTACACTTTTGCTTTCTCTAAGAATTTACTCA
CATCTTTCGACGGCGCTGTGGGATATTCTCTGGGAGGAGCTAGAGTGGAACTA
GAAGCAAGCTACAGAAGGTTTGCTACTTTAGCGGACGGGCAGTACGCAAAAA
GTGGTGCACAGTCCCTGGCTGCAATTACTCGCGACGCTGCCATTACTGACAAC
AATTACTTTGTGGTCAAAATCGATGAAATCCCAAACACTTCAGTCATGCTAAAT
GGCTGCTATGACGTGTTGCACACAGATTTGCCTGTCTCCCCATATGTGTGTGCC
GGATAGGTGCTAGCTTTGTCGACCATTAACAACCAGTAA

Appendix B: BLAST analysis of *16s rRNA* sequences obtained in this study compared with reference DNA sequences deposited in the GeneBank

B1: BLAST of *E. canis 16s rRNA* sequence against reference strains sequences. (Coverage and identity are marked with red rectangle).



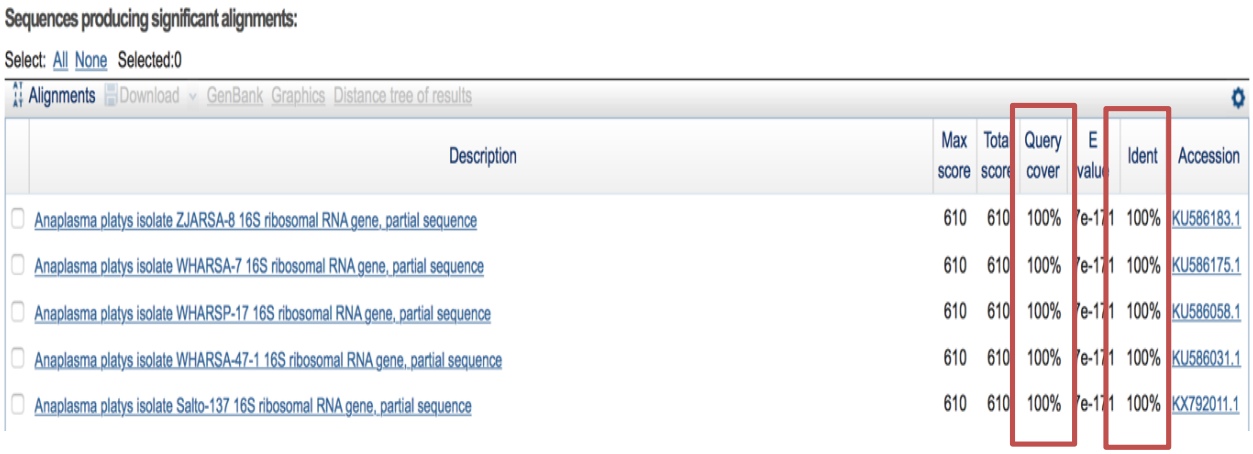
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Ehrlichia canis isolate HHH 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KP182942.1
Ehrlichia canis isolate EEE 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KP182941.1
Uncultured Ehrlichia sp. clone UFV 01 16S ribosomal RNA gene, partial sequence	608	608	99%	3e-170	100%	KP642752.1
Ehrlichia canis isolate M66 16S ribosomal RNA gene, partial sequence	606	606	99%	3e-170	100%	KX180945.1
Ehrlichia canis 16S ribosomal RNA gene, partial sequence	606	606	99%	3e-170	100%	KR920044.1

B2: BLAST of *A. platys 16s rRNA* sequence against reference strains sequences (Coverage and identity are marked with red rectangle).



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Anaplasma platys isolate ZJARSA-8 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KU586183.1
Anaplasma platys isolate WHARSA-7 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KU586175.1
Anaplasma platys isolate WHARSP-17 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KU586058.1
Anaplasma platys isolate WHARSA-47-1 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KU586031.1
Anaplasma platys isolate Salto-137 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KX792011.1

B3: BLAST of *Anaplasma spp. 16s rRNA* sequence against reference strains sequences (Coverage and identity are marked with red rectangle).

Anaplasma ovis strain Tuva-Dn4420 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KC484562.1
Anaplasma marginale strain KNP/M8/a 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KC189852.1
Anaplasma marginale strain KNP/M7/a 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KC189847.1
Anaplasma marginale strain KNP/M12/a 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KC189846.1
Anaplasma centrale strain HIP/A8/e 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KC189842.1
Anaplasma centrale strain HIP/A8/c 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KC189840.1
Anaplasma centrale strain KNP/G26/a 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	KC189838.1
Uncultured Anaplasma sp. clone ZXG-18 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	JX898992.1
Uncultured Anaplasma sp. clone Ceb6-9 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	JX860288.1
Anaplasma marginale isolate C6A 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	JQ839012.1
Anaplasma marginale isolate C7D 16S ribosomal RNA gene, partial sequence	610	610	100%	7e-171	100%	JQ839011.1

B4: BLAST of *A. ovis msp4* long primers sequence against reference strains sequences (Coverage and identity are marked with red rectangle).

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Anaplasma ovis isolate Yuzhong major surface protein 4 (msp4) gene, complete cds	1555	1555	100%	0.0	100%	JQ456348.1
Anaplasma ovis strain Italy 147 major surface protein 4 (msp4) gene, complete cds	1555	1555	100%	0.0	100%	AY702924.1
Anaplasma ovis isolate 395 surface protein Msp4 (msp4) gene, complete cds	1550	1550	100%	0.0	99%	EU497698.1
Anaplasma ovis msp4 gene for major surface protein 4, complete cds, clone: msp4_clone_12	1550	1550	100%	0.0	99%	LC141088.1
Anaplasma ovis msp4 gene for major surface protein 4, complete cds, clone: msp4_clone_8	1550	1550	100%	0.0	99%	LC141084.1

B5: BLAST of *A. ovis msp4* short primers sequence against reference strains sequences (Coverage and identity are marked with red rectangle).

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Anaplasma ovis msp4 gene for major surface protein 4, partial cds, isolation_source: blood of game animals V76	883	883	100%	0.0	100%	LC126849.1
Anaplasma ovis msp4 gene for major surface protein 4, complete cds, clone: msp4_clone_12	883	883	100%	0.0	100%	LC141088.1
Anaplasma ovis isolate YC26 major surface protein 4 (msp4) gene, complete cds	883	883	100%	0.0	100%	KJ782404.1
Anaplasma ovis strain GBK1 major surface protein (msp4) gene, partial cds	883	883	100%	0.0	100%	KC432641.1
Anaplasma ovis isolate A major surface protein 4 (MSP4) gene, partial cds	883	883	100%	0.0	100%	JQ621903.1

التوصيف الوراثي للبكتيريا من الجنسين *Ehrlichia* و*Anaplasm* في القراد *Ixodid* والحيوانات العائلة لها في فلسطين: التوزيع المكاني

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المشرف الثاني : د. عبد المجيد ناصر الدين

الملخص

معلومات أساسية: ينقل القراد الامراض البكتيرية Anaplasmosis وEhrlichiosis تسببها بكتيريا تنتمي الى عائلة Rickettsiales من جنسي *Ehrlichia* و*Anaplasma* وهذه تسبب الكثير من الامراض ذات الاهمية المتزايدة في جميع أنحاء العالم مع آثار سلبية كبيرة على صحة الإنسان والحيوان. هدفت هذه الدراسة إلى الكشف عن التوصيف الوراثي للجنسين *Ehrlichia* و*Anaplasma* في القراد *Ixodid* الحيوانات لعائلة لها في فلسطين.

المنهجية والنتائج الرئيسية: تم جمع 723 قراد *Ixodid* تنتمي إلى سبعة أنواع (*Rhipicephalus sanguineus, Rhipicephalus turanicus, Rhipicephalus bursa, Hyalomma dromedarii, Hyalomma impeltatum, Haemaphysalis parva, Haemaphysalis adleri)* من الكلاب والأغنام والماعز والجمال. وبالإضافة إلى ذلك، تم جمع 189 عينة دم من مضيفاتها من الحيوانات المختلفة بما في ذلك؛ والكلاب، والأغنام، والجمال، والخيول، والماعز المقيمين في تسع مناطق في فلسطين. ثم تم استخراج الحمض النووي من جميع العينات (القراد والدم) وفحصها للكشف عن *Anaplasma* و*Ehrlichia* عن طريق استهداف تسلسل 345 جزئية من الجيناتrRNA *16s* تليها تحليل التسلسل الجيني. كشفت الدراسة عن وجود الحمض النووي المستهدف في 6.5٪ (723/47) من القراد التي تم اختبارها. من بينها، حددت الدراسة 24 عينة *A. ovis* و13 عينه *A. platys* وأخيرا عشر عينات ك *Anaplasma spp.*
من بين عينات الدم التي جمعت من الكلاب (عددها = 135)، 11.1٪ (135/15) كانت إيجابية. من بينها كانت: 13 (9.6٪) عينة *A. platys* وعينتين (1.5٪) ايجابيتين لل *E. canis*. من أصل 47 عينة دم من الأغنام، 19 عينة (40.4٪) كانت إيجابية. تم التعرف على 6 منها على أنها *Anaplasma spp* و13 عينة كانت ايجابيه ل *A. ovis* وذلك باستخدام بادئات تفاعل PCR التي صممت في هذه الدراسة خصيصا لاستهداف تسلسل جزئي للجين *msp4* بفعالية عالية.
كشف التحليل المكاني باستخدام saTScan لحالات *Anaplasma* المجمعة عن وجود موقعين هامين إحصائيا لوجود اعداد كبيره من هذه المسببات المرضية فيهما وذلك في منطقتين في فلسطين هما: طوباس وقرية مجدل بني فاضل في رام الله على التلال الغربية لوادي الأردن. وكانت معظم الحالات (83٪) في المناطق الريفية حيث تتفاعل مكونات دورة حياة هذه البكتيريا بشكل كبير.

الاستنتاجات: كشفت هذه الدراسة عن وجود *Anaplasma* و *Ehrlichia* في فلسطين. على العاملين في مجال الصحة العامة ان يكونوا على دراية بطبيعة وبينة هذه الأمراض المنقولة بوساطة القراد في فلسطين، وخاصة الإصابات الناجمة عن بكتيريا *A. ovis*.