Al-Quds University

Deanship of Graduate Studies

Molecular Detection and Identification of *Bartonella* Species in Fleas (Insecta: *Siphonaptera*) collected from Animal Hosts in Palestinian Cities

Ahmed 'Mohammad Rasheed' Sadi Rishiq

M.Sc. Thesis

Jerusalem-Palestine

1436/ 2015

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Prepared by:

Ahmed 'Mohammad Rasheed' Sadi Rishiq

B. Sc. Biology and Biotechnology

Arab American University Jenin (AAUJ) - Palestine

Supervisor: Professor Ziad Abdeen

Co-supervisor: Dr. Abedelmajeed Nasereddin

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

1436/2015

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

Thesis Approval

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Prepared by: Ahmed 'Mohammad Rasheed' Sadi Rishiq

Student ID No: 21120190

Supervisors: Professor Ziad Abdeen

Co-supervisor: Dr. Abedelmajeed Nasereddin

Master thesis submission and acceptance date:

The names and signatures of examining committee members:

1. Head of committee: Professor Ziad Abdeen Signature…….
2. Co- Supervisor: Dr. Abedelmajeed Nasereddin Signature…….
3. Internal Examiner: Dr. Ibrahim Abbasi Signature…….
4. External Examiner: Dr. Amer Al-Jawabreh Signature…….

Jerusalem- Palestine
1436/2015

Dedication

This work is dedicated to my family, father, mother, brothers and sisters.

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 same) has not been submitted for a higher degree to any other university or Institution.

Signed: _____

Ahmed 'Mohammad Rasheed' Sadi Rishiq

Date:

Acknowledgment

I wish to express my deep gratification to my supervisor Professor Ziad Abdeen and to co-supervisor Dr. Abedelmajeed Nasereddin as well as Dr. Kifaya Azmi, and Dr. Suhair Ereqat for their kind help. I am grateful to Prof. Kosta Y. Mumcuoglu for training me on fleas' classification as well as some of samples confirmation. In addition, I would like to thank the Dutch Government for funding my research work. Many thanks to members of Al-Quds Nutrition and Health Research Institute (ANAHRI) for their endless support.

Abstract:

For an extended period of time, researchers have considered small ectoparasite insects that belong to *Siphonaptera* order as vectors for many microorganisms as *Bartonella* and *Yersinia* bacteria. Bacteria from genus *Bartonella* are gram-negative, haemotrophic and fastidious were suspected to be transmitted through fleas. However, *Bartonella* can cause a wide range of diseases according to their species type. No data on *Bartonella* were ever recorded in Palestine before.

This study investigated the prevalence of *Bartonella* organisms in fleas from cats, dogs, rats, and hyraxes in Palestine and characterized their genetic composition. Collected fleas from different districts in Palestine were characterized. The identified species were subjected to traditional molecular methods (DNA extraction, PCR and RFLP) followed by DNA sequencing for the aim of *Bartonella spp* identification. Based on the previous steps, dendrogram trees have been constructed using three different genetic loci.

Fleas (n=289) were collected from cats (121), dogs (135), hyraxes (26) and rats (7) from northern (n=165), central (n=113), and southern (n=11) regions of Palestine. The prevalent flea species were: *Ctenocephalides felis* (n=119/289; 41.2%), *Ctenocephalides canis* (n=159/289; 55%), and *Xenopsylla spp.* (n=7/289; 2.4%). Targeting the Intergenic Transcribed Spacer (*ITS*) locus, DNA of *Bartonella* was detected in 22% (64/289) of all fleas. Fifty percent of the *C. felis* and 57% of the *Xenopsylla spp*. contained *Bartonella* DNA. DNA sequencing showed the presence of *Bartonella clarridgeiae* (46.7%), *Bartonella henselae* (25%), and *Bartonella koehlerae* (3.1%) in *C. felis*. *Xenopsylla spp*. collected from *Rattus rattus* were infected with *Bartonella tribocorum*, *Bartonella elizabethae,* and *Bartonella rochalimae*. By using the 16S-23S ribosomal RNA gene (*ITS*) for constructing the phylogenetic tree; *ITS* DNA sequence analysis showed four genetic clusters with unique sublcusters: cluster 1 includes *B. henselae* and *B. koehlerae* as its two subclusters, cluster 2 includes *B. clarridgeiae.* However, *B. tribocorum* and *B. elizabethae* formed two more out-group clusters. On the other hand, citrate synthase (*glt*A) showed two main clusters and RNA polymerase β subunit (*rpo*β) genes displayed two main clusters. All confirms the effectiveness of the *ITS* gene in discriminating between *Bartonella spp*.

These findings showed the important role of cat and rat fleas as vectors of zoonotic *Bartonella* species in our region. It is anticipated that this study will raise awareness among Palestinian physicians, veterinarians, and other health workers of the high prevalence of *Bartonella spp*. in fleas and the potential risk of these pathogens to humans and animals in this region.

This work has been published in a peer reviewed journal: Journal of Vector Ecology; 2014; 39 (2): 261-270.

التحليل الجزيئي والتعريف لفصائل بكتيريا البارتونيلا من براغيث تم جمعها من حيوانات حاضنه من مدن فلسطينية

إعداد: أحمد "محمد رشيد" سعدي رشق

إشراف: أ.د. زياد عابدين و د. عبد المجيد نصر الدين

الملخص:

لفترة طويلة من الزمن خمن العلماء ان الحشرات هي الناقل الرئيسي لبعض الكائنات الدقيقة مثل بكتيريا البارتونيلا *Bartonella* و*اليرسينيا Yersinia*. البكتيريا من نوع البارتونيلا *Bartonella* هي سالبة جرام عند الصبغ،لها احتياجات استزراعية وتغذوية معقدة ويعتقد انها تنتقل بوساطة البراغيث. وتعتبربكتيريا البارتونيلا *Bartonella* ا لمسؤول الرئيسي عن احداث العديد من الامراض بناًء على نوع البكتيريا. لا يوجد ابحاث تركز على جانب الكشف عن بكتيريا *Bartonella* والامراض المتعلقة بها في فلسطين.

في هذه البحث تم دراسة انتشار بكتيريا *Bartonella* في فلسطين وكل من محتواها الجيني في عينات من البراغيث جمعت من قطط، كلاب، جرذان ومن حيوان الوبر الصخري. من اهم اهداف هذه الدراسة هو تصنيف عينات من البراغيث التي تم جمعها من مناطق مختلفة في فلسطين باستخدام طرق البحث الجزيئية التقليدية (DNA extraction, PCR & RFLP) ومن ثم عمل تسلسل للمادة الوراثية للبكتيريا. بناًء على ما تقدم تم بناء شجرة سلالات لهذه الانواع التي تم الكشف عنها والتي تعود لدولة فلسطين، باستخدام ثلاثة جينات مختلفة.

عدد العينات من البراغيث التي جمعت كانت 289 عينة تم جمعها على النحو التالي: 121 من القطط، 135 من الكلاب، 26 من حيوان وبر الصخر، 7 من الجرذان. وقد تم الجمع من عدة مناطق في فلسطين حيث توزعت كالاتي: من شمال فلسطين 165 عينة من الوسط 113 عينة ومن الجنوب 11عينة. ولقد كان توزيع فصائل البراغيث كما يلي: براغيث القطط (*C. felis*) 119/289 (42.2%) براغيث الكلاب(*C. canis*) 159/289(55%) وبراغيث الجرذان(*Xenopsylla spp.*) 7/289 (2.4%).عند استخدام جين *ITS* للكشف عن وجود المادة الوراثية DNA للبارتونيلا اظهرت 64 عينة من 289 (22%) نتائج ايجابية منهم 50%من براغيث القطط و 57% من براغيث الجرذان. من اجل اثبات النوع الدقيق للبارتونيلا *Bartonella* تم فحص تسلسل المادة الوراثية للعينات الايجابية وبناًء عليها اظهرت النتائج ان 46.7 %من البارتونيلا من براغيث القطط هي من نوع *B. clarridgeiae* و 25% من نوع *B. henselae* 3.1% من نوع *B. koehlerae* . اما براغيث

الجرذان *Xenopsylla spp* فقد كشفت عن وجود كل من *B. elizabethae* و*B. tribocorum* و *B. rochalimae*.

عند تحليل جين *ITS* لانشاء شجرة ارتباطات سلالات *لل*Bartonella، اظهر التحليل اربع عنقودات جينية رئيسة: تحت عنقود1 ويشمل كل من *B. henselae* و*B. koehlerae* وتحت عنقود 2 ويشمل *B. clarridgeiae* واعتبرت كل من *B. tribocorum* و*B. elizabethae* كمجموعة خارجية. من ناحية اخرى، عند استخدام جين *glt*A عنقودين رئيسيين وللجين *rpoβ* اظهرت عنقودين اثنين، وهذا يؤكد اهمية استخدام جين*ITS* للتفريق بين هذه الانواع.

اظهرت نتائج هذا البحث انواع *Bartonella* وانتشارها وتنوعها الجيني.ويضاف الى ذلك،تطوير فحص تشخيصي في المختبرات العامة يساهم بالكشف عن بعض الامراض التي تتعلق بال *Bartonella* والتي لم تكتشف مسبقا نظرا للافتقار لهذا النوع من الدراسات. وأظهرت هذه النتائج أهمية دور القطط والفئران والبراغيث الناقلة لأنواع *Bartonella* في فلسطين. ومن المأمول أن يساهم هذا البحث بزيادة الوعي بين الأطباء، والبيطريين، والعاملين بالصحة العامه في معدل ارتفاع انتشار ال *Bartonella* في البراغيث في فلسطين والمخاطر المحتملة لهذه الكائنات الممرضة للإنسان والحيوان في هذه المنطقة.

لقد تم نشر هذا البحث في مجلة Journal of Vector Ecology في سنة 2014 العدد 39 الفصل الثاني بين الصفحات 261-270.

Table of Abbreviations

Abbreviation	Full Word
Spp.	Species
C. felis	*Ctenocephalides felis*
C. canis	*Ctenocephalides canis*
BA	Bacillary angiomatosis
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
gltA	Citrate synthase gene
rRNA	Ribosomal Ribonucleic acid
ITS	Intergenic transcribed spacer
ELISA	Enzyme linked immunosorbent assay
IFA	Immuno- florescence assay
µL	Microliter
M	Molar
PBS	Phosphate buffer saline
EDTA	Ethylenediamintetraacetic acid
TAE	Tris acetate EDTA
V	Volt
bp	Base pair
°C	Celsius or centigrade degree
HIV	Human immundeficency virus
AIDS	Acquired immundeficency syndrom
rpoβ	RNA polymerase beta-subunit gene
VNTR	Variable number tandem repeats
BLAST	Basic local alignment search tool

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Chapter 1

Introduction

1.1 Flea classification, life cycle, nutrition, and general properties.

Fleas are insects belonging to *Siphonaptera* order; they are wingless, highly sclerotized, laterally compressed and 2-10 mm in length [1]. About 2575 species belong to the same order; they are divided into 15-18 families and about 245 genera [2]. Generally, fleas identified as ectoparasites and depend on blood meals to obtain heme from special hosts like mammals to complete their life cycle (Fig. 1.1) [3].

The flea lifecycle begins when females laying their eggs deep in the animal fur. The eggs will proceed in development based on the environmental conditions such as temperature and humidity and other factors like shelter. After around 2-14 days, the eggs will hatch allowing the larvae to emerge. Larvae are eyeless, legless, with small head bearing thin and short antenna and are approximately 1.5-10 mm in length. Moreover, the main sources of food are feces of adult fleas where the organic debris is found, to a lesser extent, in small arthropods or even other larvae. After two successful molts/instars, larvae will be converted into the next stage of development called pupa. This stage is characterized by the structure that covers the pupa called cocoon which is a sheath of silk acting as a chamber of pupation [4 and 5].

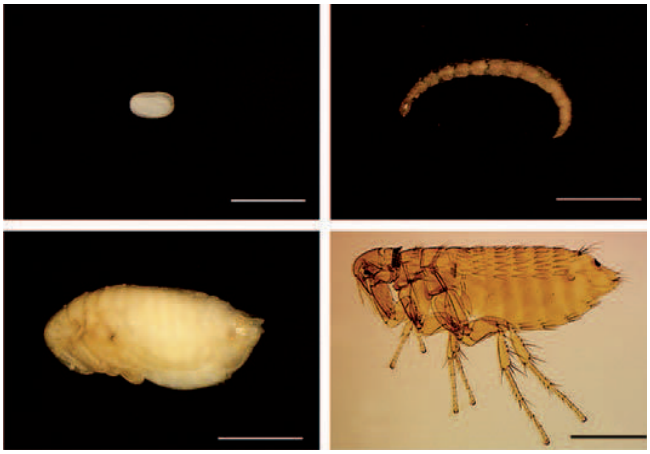


Figure 1.1: Flea life cycle. From left to right, the egg, larvae, pupa and adult stages [2].

The dormant stage lasts for 1-2 weeks until specific stimuli like carbon dioxide (CO_2), temperature or vibrations are available to trigger the molting of pupa and the emergence of the adult. This is considered the last stage and characterized by the segmented body: head, thorax and abdominal region. The head usually contains two black dots not recognized as compound eyes, an antenna, and a mouth with parts (Stylets) modified for blood sucking (Fig. 1.2) [2, 4 and 6]. The thorax part is found in the middle region bearing three pairs of legs. Body segments extend to reach the hind part with a total of ten segments, three of these segments are found in the abdominal parts that are modified for reproduction purpose [7].

The movement of fleas is controlled by two body parts; the first part contains the legs that are intended for jumping and clinging to the host. The second is Metathorax (coxa region) which participates in the movement by the action of special protein called Resilin. This

protein is compressed in the flexion body mode and eventually will relax rapidly to push the body parts toward a jump of about 30 cm in height and 20 cm in distance [4 and 7].

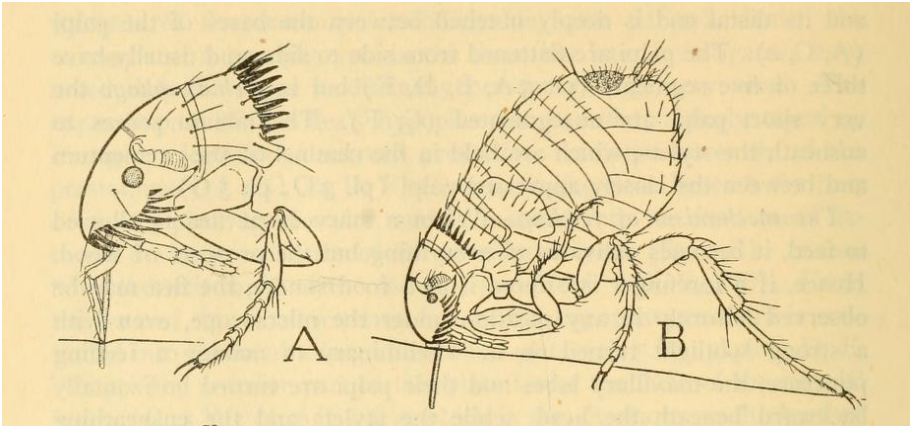


Figure 1.2: Morphology of the Flea's head. The head parts contain the mouth part equipped with needle-like structure (stylets) (A) intended for blood sucking (B) from host animal [6].

Cat flea (*Ctenocephalides felis)* can be distinguished from dog flea (*Ctenocephalides canis*) using a hair-like structures (spines) found in the head region, in the mouth part called genal combs (*Ctenidia*, which give the species their name *"Ctenocephalides"*) and in the first thoracic segment called pronotal combs (7 to 8 spines) as shown in (Fig. 1.3). As a key for distinguishing the cat flea through the genal comb, the first hair is approximately equal in length to the second hair. For dog fleas; the first one is only half the length of the second hair. In addition to combs, there is another marker that used to differentiate between cat flea and other fleas. This marker is called mesopleural rod which is a rod-like structure found in the first thoracic segment of the body [1, 4, 5 and 8].

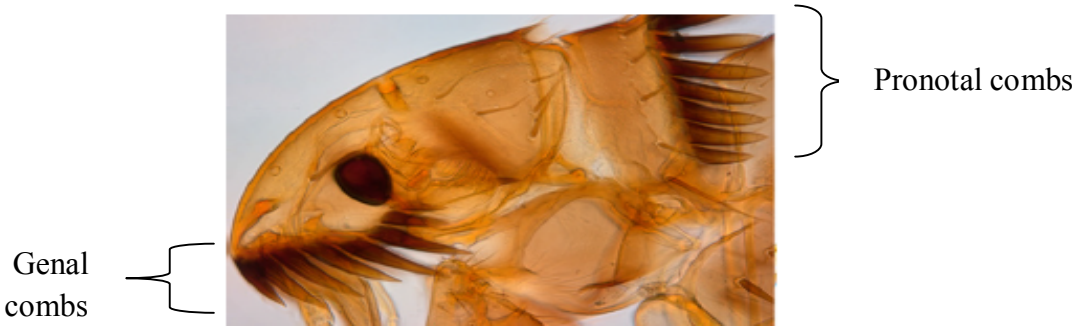


Figure 1.3: Flea genal and pronotal combs (*Ctenidia*). Genal combs localized in the mouth part, and pronotal combs in the first thoracic segment [8].

Genitalia parts located in the hind part can be used to differentiate between males (which have a bean-like structure and thread in the last abdominal part (aedeagus) and females spermatheca (a sperm collecting chamber in the hind part of flea body) [2 and 6].

1.1.1 *Ctenocephalides felis*

C. felis or cat flea (Fig. 1.4a) is ectoparasites of cats and dogs, reddish-brown in color and 10 mm in length. The *C. felis* and *C. canis* (dog flea) have many similar morphological features, except for some features like elongated head in cat fleas which is rounded in dog fleas [8 and 9].

(a) (b)

Figure 1.4: *Ctenocephalides spp*. including: Cat flea *C. felis* (a) on the right panel and *C. canis* on the left panel (b) [9].

1.1.2 *Ctenocephalides canis*

C. canis are the flea species that mainly inhabit dogs (Fig. 1.4b). They are morphologically similar to cat fleas except for some features as aforementioned in C. *felis*. At least 15 different species of fleas affect domestic dogs [5 and 8].

1.1.3 *Xenopsylla spp*

Many species belong to *Xenopsylla* fleas with main type called *X. cheopis* or the oriental rat flea. *Xenopsylla spp*. are characterized by the absence of both genal combs and pronotal combs with slightly rounded head. In addition, the presence of mesopleural rod and ocular bristle (one hair found under flea's eye) separates it from the closely related *Pullex irritants* or so called the human flea. In the case of sex determination, the same rule is followed for determining sex of *C. canis* and *C. felis* [6 and 8].

1.2 Literature review

1.2.1 Fleas as vector of *Bartonella spp*. and their related diseases

In addition to their nuisance of biting humans, fleas are believed to serve as vectors; as they have the ability to transmit special types of pathogenic bacteria. *Bartonella* species are the main types of bacteria transmitted by fleas. They were recognized as zoonotic agents in human [3 and 8]. Eleven species of *Bartonella* can cause disease to human. Among them *B. henselae* and *B. clarridgeiae* which are transmitted by cat fleas and cause cat scratch disease (CSD) which results from infection by *Bartonella* either by cat scratch, bite or touching the cat fur contaminated with the bacteria found in fleas feces. It is also documented that dog bite can transmit the disease from dogs to human. The diseases are attributed to the infection from the *Bartonella spp.* called Bartonellosis [5, 10, 11, and 12].

The oriental rat flea *X. cheopis* has the ability to transmit *Yersinia pestis* bacteria that causing the plague disease (Black Death). In addition, *Rickettsia typhi*; considered as the causative agent of murine typhus. This pathogen also can be transmitted through rats via ectoparasites [1, 8, 10, and 13].

Bartonella is an aerobic, fastidious, hemotropic, gram negative bacilli (0.3-1µm) bacteria which belongs to the alpha-proteobacteria subgroup 2 [14 and 15]. More than 20 species are found, five of them are zoonotic [16]. *Bartonella spp*. parasitizes red blood cells and endothelial cells of their mammalian hosts [17]. In addition to cat Scratch Disease (CSD), many diseases could be manifested as an infection with *Bartonella* species. These include encephalopathy, neuroretinitis, arthritis, lytic bone lesions and infectious endocarditis [18, 19, and 20]. *Bartonella spp*. are distributed worldwide (Fig. 1.5) and transmit Bartonellosis from animals to humans [15]. The current species of *Bartonella* that have been studied are summarized in Table 1.1.

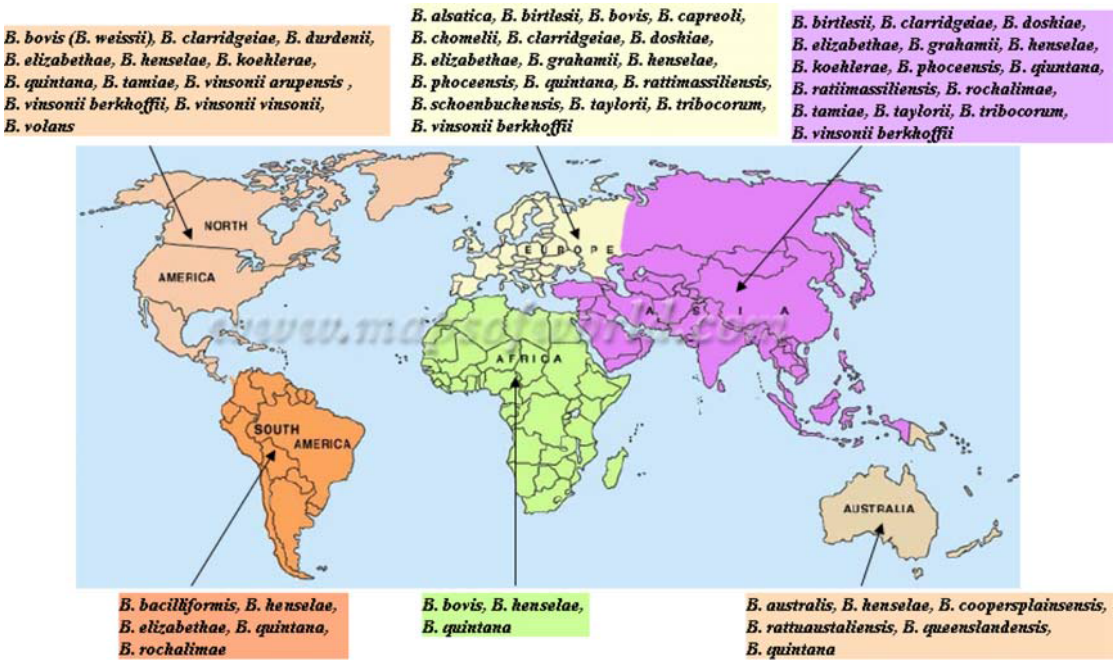


Figure 1.5: The distribution of *Bartonella* species throughout the world [15].

Table 1.1: Summary of some *Bartonella spp.* main reservoir, vector, hosts and their related diseases.

Bartonella spp.	**Reservoir**	**vector**	**Accidental host**	**Diseases**	**References**
B. bacilliformis	Human	Sand fly *(Lutzomyia Verrucarum)*	None	Carrión's disease: Oroya fever and verruga peruana	21, 22, 23
B. quintana	Human	Body louse (*pediculus humanis*)	Cats and dogs	Trench fever, endocarditis, bacillary angiomatosis	21, 22
B. elizabethae	Rat	Oriental rat flea(*Xenopsylla chepois*)	Human, dog	Endocarditis, neuroretinitis	22
B. grahamii	Wild mice	Rodent flea	Human	Neuroretinitis	21, 22
B. henselae	Cat	Cat flea (*C. felis*)	Human, dogs, birds	Cat-scratch disease,endocarditis, bacillary angiomatosis, bacillary peliosis , neuroretinitis, bacteremia with fever	21, 22, 24
B. clarridgeia	Cat	Cat flea	Human, dog	Cat-scratch disease	21, 22
B. koehlerae	Cat	Cat flea	Human, Dogs, birds	Endocarditis	22, 24, 25, 26
B.vinsonii berkhoffii	Coyote and dog	Tick	Human	Endocarditis	27, 28
B. tribocorum	Rat	Rodent flea *Xenopsylla spp.*	Human	Endocarditis	13, 29
B. alsatica	Rabbit	Flea (*Spilopsyllus cunicul*)	Human	Endocarditis, lymphadenitis	30
B. rochalimae	Rat,Fox, raccoon, coyote	Flea (*Pulex spp.*)	Human, Dogs	Fever, rash, splenomegaly, mild anemia,	31, 32, 33, 34,35

1.3 Diagnostic methods for *Bartonella* infection

Many symptoms appear on patients who have Bartonellosis. Symptoms can be easily observed, fever, enlarged lymph nodes (lymphadenopathy), or the possibility of arising of small vesicles or granuloma at inoculation site which develop after exposure to cat scratch or bite that has been contaminated with fleas' feces. Samples can be obtained from blood, lymph nodes by fine needle aspirate as well as from spleen, hepatic and skin biopsies. Recent reports indicate that serological tests and culture are more accurate in confirming diagnosis [21, 36, 37, and 38].

There are many diagnostic methods that are used to detect *Bartonella spp*. infection. Two serological tests were used to detect *Bartonella spp*. antibodies; are the Enzyme-linked immunoassay (EIA) and immunofluorescent assay (IFA). Immunoperoxidase assay, an immunohistochemistry method in case of cat scratch disease (CSD) lymphadentitis biopsy and western blot are also considered as good methods for diagnosis [15 and 39]. Molecular methods, mainly PCR based methods, followed by sequencing or RFLP of PCR amplified genes [38]: Target genes include citrate synthase (*gltA*) [41], 16S-23S rRNA intergenic transcribed spacer sequence (*ITS*) [40] and RNA polymerase beta-subunit gene (*rpoβ*) [42 and 43]. Recently, use of multiplex SYBR green qPCR detection method showed high specificity compared to traditional PCR when tested on blood specimens from cats that were previously suspected to be infected with *B. henselae* and *B. clarridgeiae*. The method detected co-infection with both species [44].

Bartonella spp. have been detected in animal blood samples including rodents (black rats), human blood such as the first case with *B. koehlerae* in Israel, and from fleas collected from rodents [25, 45 and 46]. For isolation of *Bartonella* from blood, samples were collected in EDTA- plastic tubes and kept at -20 °C. They were then plated onto blood agar or chocolate agar (*Bartonella* alpha Proteobacteria Growth Medium, BAPGM) followed by incubation for 4 weeks at 5% CO_2 and temperature of 35°C. The bacteria can also grow in broth with fetal calf serum [21, 37, and 38]. Bacteriological culture failed to isolate *Bartonella* from patients with endocarditis, and instead DNA amplification showed a positive result toward *B. henselae* [47].

1.4 Treatment

Bartonella infection showed a variety of clinical diseases. The treatment options are numerous and tend to be complex. The medications provided to patients, containing antibiotics (Table 1.2) are based on the statements made by these patients such as if the patients are immune-competent or immune-compromised. Moreover, patients affected with cat scratch disease (CSD), bacteremia, endocarditis, encephalopathy, bacillary angiomatosis (BA), Peliosis hepatis and other Bartonellosis related symptoms; caused by species specific *Bartonella* will facilitate determination of the appropriate therapy for those patients [48].

Table 1.2: Summary of recommended antibiotics against *Bartonella spp.*

Disease/symptoms	***Bartonella spp***	**Recommended antibiotics**	**References**
Trench fever Bacteremia	*B. quintana*	Gentamicin combined with doxycycline, ceftriaxone and amoxicillin	50 , 51
Cat scratch disease and lymphadenopathy	*B. henselae* and *B. clarridgeia*	Azithromycin	52, 53
Bacillary angiomatosis and peliosis hepatis	*B. henselae* and *B. quintana*	Erythromycin or doxycyclin	54
Endocarditis	*B. henselae*, *B. koehlerae*, *B. elizabethae* and others	Gentamicin, ceftriaxone and doxycycline	55
Carrion disease, Oroya fever and verruga peruana	*B. bacilliformis*	Chloramphenicol, Ciprofloxacin, Rifampin and Streptomycin	56

Patients who are immune-compromised (like HIV/AIDS infected patients) had special treatment follow up; where the dose, rout of administration and treatment duration are very important factors that should be taken into consideration. Rolain and colleagues *et al.* [48]; discussed these factors based on previous clinical studies for different *Bartonella* diseases in addition to trials for examining the drugs efficiencies, both *in vivo* and *in-vitro* levels [48]. In addition, antibiotic resistant and efficiency tested

recently by Biswas and Rolain prescribed the drugs that are suitable to animals and humans and emphasized some potent drugs that were prescribed previously [49].

1.5 Local studies and prevalence

No previous study was conducted to evaluate prevalence of Bartonellosis, and to identify *Bartonella* species, and its genetic diversity in Palestine. However; few studies included both investigations of bacteria from fleas, or animal samples or through serology tests from patients suffering generally from lymphadenopathy as a sign of cat scratch disease (CSD) infection as indicated later were done in neighboring countries. These studies describe the importance of revealing this bacterium in our country from the therapeutic point. From these local studies, the first human case was reported to cause endocarditis. However, due to a technical error, the patient was diagnosed with *B. henselae* infection but eventually when new primers designed based on riboflavin synthase fragment (*ribC*) were utilized, the patient indeed showed infection with *B. koehlerae* using a tissue sample from aortic valve. This type of data ensures the need for precise molecular methods that discriminate between species [25 and 57]. Another patient at the Carmel Hospital in Haifa had shown symptoms of bone marrow and skin granulomatous as well as fever. Based on these signs and the negative results from other tests; serology tests detected anti-*B. henselae* in patient serum. But, both bone marrow and skin were negative for presence of *Bartonella spp*. Although the positivity for cat scratch disease (CSD) was observed, this case showed no lymphadenopathy despite it was immune-competent patient [58].

At Schneider Children's Medical Center in Petah Tikva, Israel, asymptomatic 7.5 year- old- girl showed lymph node enlargement behind her left ear lobe (Fig. 1.6). She was exposed to scratch from a kitten and a serological test revealed infection with *B. henselae* [52].

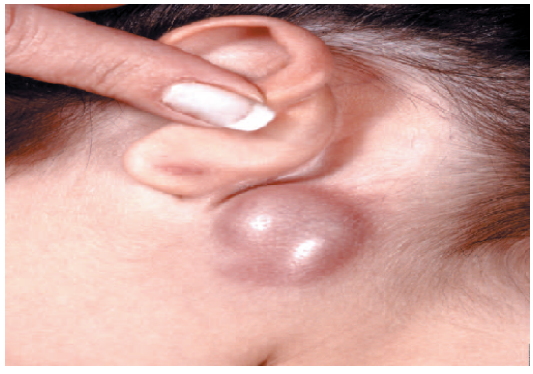


Figure 1.6: Enlarged lymph node in girl infected with *B. henselae* [52].

A similar case was seen for a 17 years old male patient admitted to Tel Aviv Medical Center for a cervical mass. Serological examination showed infection with *B. henselae* but failed to be cultured. Aspiration of pus from this mass and using the *Bartonella spp*. discrimination assay (PCR followed by RFLP) using *Taq1* restriction enzyme confirmed *Bartonella* infection [59]. In addition, a 9- year- old boy was scratched by a dog which developed later to osteomyelitis in left hand metacarpal. In

fact, the boy was diagnosed with *B. henselae* infection based on enzyme immunoassay (EIA) tool [60].

Table 1.3: Summary of local Bartonellosis cases including symptoms and diagnostic procedures.

Case	Symptoms	Diagnosis procedure	Diagnosed *Bartonella spp*	References
60-year-old man	Endocarditis	PCR and RFLP using *rib*C primer	*B. koehlera*	25
54- year- old woman	Bone marrow inflammation and skin granulomatous and fever	Serology tests immunoassay (EIA)	*B. henselae*	58
7.5 year- old- girl	Lymph node enlargement	Serology tests	*B. henselae*	52
17 years old male	Cervical mass	ELISA, PCR and RFLP	*B. henselae*	59
9- year- old male	Osteomyelitis	Immunoassay (EIA)	*B. henselae*	60

1.6 Study significance and importance

Due to the development in diagnostic methods for Bartonellosis, sharp increase in cat scratch disease (CSD) infection rate or arthropathy was reported [20, 61, 62, and 63]. Based on this, there is a need for developing new methods for the diagnosis and *Bartonella* species identification to help in conducting epidemiological studies and reduce misdiagnosis of Bartonellosis. Furthermore, this will encourage the medical service providers in Palestine to implement such diagnostic techniques.

The use and implementation of molecular detection tools as part of this study shows the significance of these methods. Additionally, obtaining *Bartonella spp*. and making comparison with previously detected species will allow comparison between these species or how they are related based on DNA sequence homology. In addition, molecular methods will allow the study of genetic heterogeneity and polymorphism between and within the same species from isolates in Palestine compared to other countries. Genetic diversity and geographical distribution will also be explored.

Bartonellosis is a neglected disease in Palestine. This study will increase awareness among medical staff and Public health workers towards the disease and the potential risk of these pathogens pose to humans and animals in Palestine.

Objectives:

The main objectives of this study are:

- To classify fleas collected from three regions in Palestine based on their species and gender using classical methods.

- To detect and discriminate *Bartonella spp*. in fleas using molecular-based methods such as Polymerase chain reaction (PCR), Restriction fragment length polymorphism (RFLP) and DNA sequence analysis.

- To study the genetic heterogeneity of *Bartonella* species that have been detected and identified in Palestine.

Chapter 2

Materials and methods:

2.1 Samples collection

Fleas were collected between December 2011 to April 2012 from infested animals by a fine-toothed metal comb (11 teeth\per cm) (Lochdan, Regev, Israel) as well as hair forceps. Samples were then transferred into sterile microfuge tubes (1.5 ml) (SARSTEDT, Nümbrecht, Germany) containing 70% ethanol to Al-Quds Nutrition and Health Research Institute (ANAHRI) laboratory at Al-Quds University. Samples were stored at −20˚ C for future use.

2.2 Fleas classification

Using stereomicroscope, fleas were classified according to published identification keys by sex and species under the supervision of Dr. Kosta Y. Mumcuoglu laboratory, Hebrew University, Hadassah Medical School. [1, 4, 5, 6, 8 and 64]. Flea species were determined based on the presence of combs (*Ctenidia*). Combs, spine-like structures, are found in the mouth (genal) and on the dorsal (pronotal). However, other markers that were described and documented for fleas classification are sophisticated. Of these, the hairs found on hind tibia of fleas and the size of genal combs was unclear during examination. Absence of combs ensured that fleas were related either to *Xenopsylla spp* or *Pullex spp*. In addition, the Mesopleural rod and the ocular bristle location used as definitive markers for discrimination of *Xenopsylla spp* from *Pullex spp* [1, 4, 5, 6, 8 and 64].The flea reproductive system organs were the best markers in sex determination. In male, the presence of the thread-like structure (aedeagus internal part) or the bean-like structure (aedeagus external part). In female, the absence of male's genitlia and the presence of spermatheca (sperm collecting chamber) were used as accurate markers [1, 4, 5, 6, 8 and 64]. Each individual flea was then placed in a separate tube for DNA extraction and then identification of *Bartonella spp.*

2.3 DNA extraction

Each flea was removed from the alcohol tube and dried on tissue paper, placed into a new microfuge tube, digested by mechanical grinding using plastic pestles, and was then subjected to DNA extraction using GeneJet Genomic DNA purification Kit (Thermoscientific, Lithuania) following manufacturer's instructions with some modifications: 200µl of 1X (PBS) followed by mechanical grinding using pipette's tip to homogenize the sample. Then, flea was lysed with 400µl of the kit lysis solution that contained 20 µl (20 mg/ml, 400 µg/reaction) Proteinase K, ground, vortexed, incubated at 56˚C overnight, purified with GeneJet columns, and finally eluted in 100µl of kit elution buffer then stored at -20^{o}C for PCR analysis.

2.4 Amplification of DNA for:

2.4.1 *Bartonella* diagnosis

The *Bartonella gltA* gene [16 and 41] and *ITS* locus [40] were targeted for the bacterium DNA detection. To identify positive controls; thirty samples were amplified using the intergenic *ITS* PCR and *gltA* PCR systems. PCR amplified DNA sequencing using forward and reverse primers.

The *Bartonella gltA* gene (379bp) was amplified using the following primers: BhCS.1137n (5′AATGCAAAAAGAACAGTAAACA-3′) and BhCS.781p (5′-GGGGACCAGCTCATGGTGG-3′) [41]. The *ITS* locus was amplified using the primers: 321s (5′-AGATGATGATCCCAAGCCTTCTGG3') and H493as (5′TGA ACCTCCGACCTC ACGCTTATC-3') as previously described [40] (Table 2.2). PCR reactions were performed in 25-µl PCR ready mix (Syntezza, Jerusalem), containing 0.8 µM of each set of primers and 10 µl of the extracted DNA. The PCR conditions were as described previously [40, 41 and 43]. All PCR products of the positive samples were sent for DNA sequencing using forward and reverse primers. Positive and negative control were included in each run Nuclease-free water were used as negative control.

To evaluate the best system for diagnosis based on high sensitivity and specificity, the first sequence-confirmed *B. henselae* isolate was used as positive control. Ten (10µl) of extracted DNA followed by serial dilution 1:5 were applied. A *Wolbachia endosymbiont of Nasonia longicornis* positive sample previously identified by DNA sequencing of the *gltA* using forward and reverse primers was used for specificity evaluation.

2.4.2 Gel Electrophoresis

All PCR products were loaded into 1.5% agarose gel (Agarose LE, Analytical gradient, Promega, Spain). The gel was prepared by dissolving 1.5 g 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 1 minute till completely dissolved, and then 3µl of 10 mg/ml (0.3µg/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). 10µl of PCR products were loaded into the gel. The size of DNA ladder of 100bp (Thermo scientific Lithuania) was used in each run. The gel was run at 100V for 45 minutes. The gel images were captured using MiniLumi 1.4 gel documentation system from (DNR Bio-Imaging Systems Ltd, Israel) .

2.4.3 PCR for genetic cluster analysis

All samples positive for *ITS*-PCR or *gltA* were sequenced at HyLab sequencing service (Rehovot, Israel) using forward and reverse primers. A third locus called *rpoB* (825bp) was amplified using primers 1400F (5′-CGCATTGGCTTACTTCGTATG-3′) and 2300R (5′-GTAGACTGATTAGAACGCTG-3′) [43] (Table2.4). The PCR reactions and conditions were performed as described previously [40, 41 and 43]. A non-

experimental *Rickettsia rickettsii* 16S rRNA gene, GenBank: U11021.1 ribosomal RNA intergenic spacer was used as an out-group.

2.5 Genetic data analysis: Alignment, BLAST and dendrograms.

2.5.1 Alignment

The CLUSTALW program (http://www.genome.jp/tools/clustalw/) was used for the multiple sequence alignment of *Bartonella* sequences obtained in this study with those of known *Bartonella* species deposited in the GenBank/EMBL/DDBJ databases.

Table 2.4: The main properties of the primers used in this study.

Gene name	Primer	Primer sequence	Primer size bp	Amplicon size bp	Annealing temperature °C
gltA	BHCS.1137n (F)	5'-AAT GCA AAA AGA ACA GTA AACA-3	22	379	42
	BHCS.781p(R)	5'-GGG GAC CAG CTC ATG GTG G-3'	19		
ITS	H439as (F)	5'-TGA ACC TCC GAC CTC ACG CTT ATC-3'	24	190	57
	S321(R)	5'-AGA TGA TGA TCC CAA GCC TTC TGG-3'	24		
*Rpo*β	1400F (F)	5′-CGCATTGGCTTACTTCGTATG-3′	21	825	53
	2300 (R)	5′GTAGACTGATTAGAACGCTG-3′	20		

2.5.2 BLAST and Cluster analysis

Sequencing results were modified and arranged by placing the appropriate nucleotides using BioEdit sequence alignment editor software [65] (Fig. 2.1).

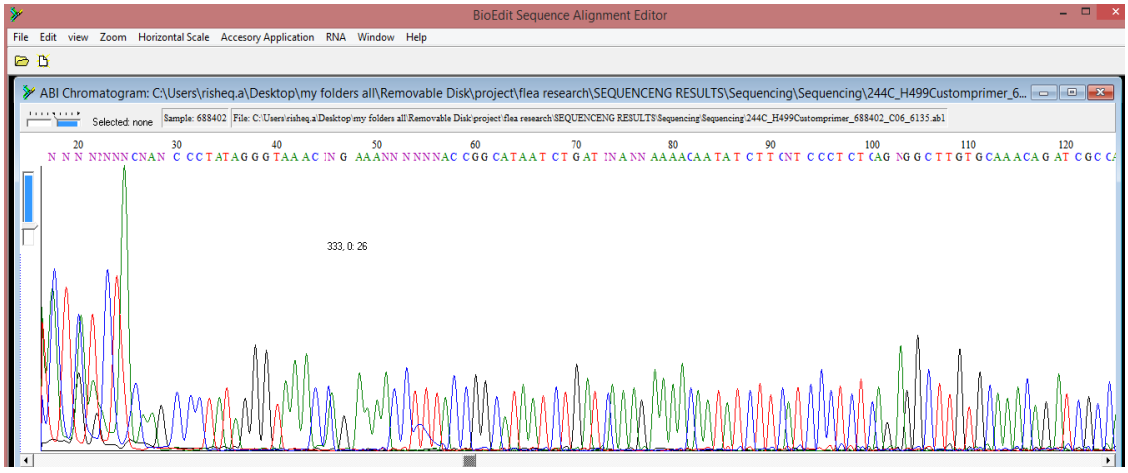


Figure 2.1: BioEdit software for analysis of extracted DNA sequences. The DNA sequences were analyzed and arranged to prepare them for BLAST analysis step [65].

BLAST software (http://blast.ncbi. nlm. nih.gov/Blast.cgi) (Fig. 2.2) was used for a comparison among present study *Bartonella spp* DNA sequences and those already found previously in the database.

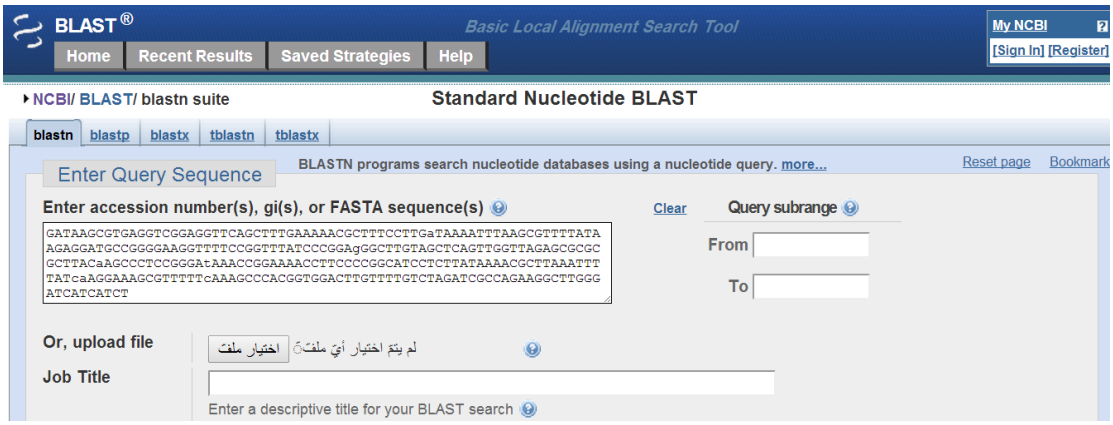


Figure 2.2: Detection of *Bartonella spp* based on sequence homology using BLAST website. (http://blast.ncbi.nlm. nih.gov/ Blast.cgi).

Sequences were compared to other local strains using BLAST alignment (Fig. 2.3). However, the first sequences were related to *B. henselae* and the strain was acquired from GeneBank accession number (http://www.ncbi.nlm.nih.gov/ nuccore/FJ832091) (FJ832091) from aortic valves of Boxer dogs with infective endocarditis from Israel [26].

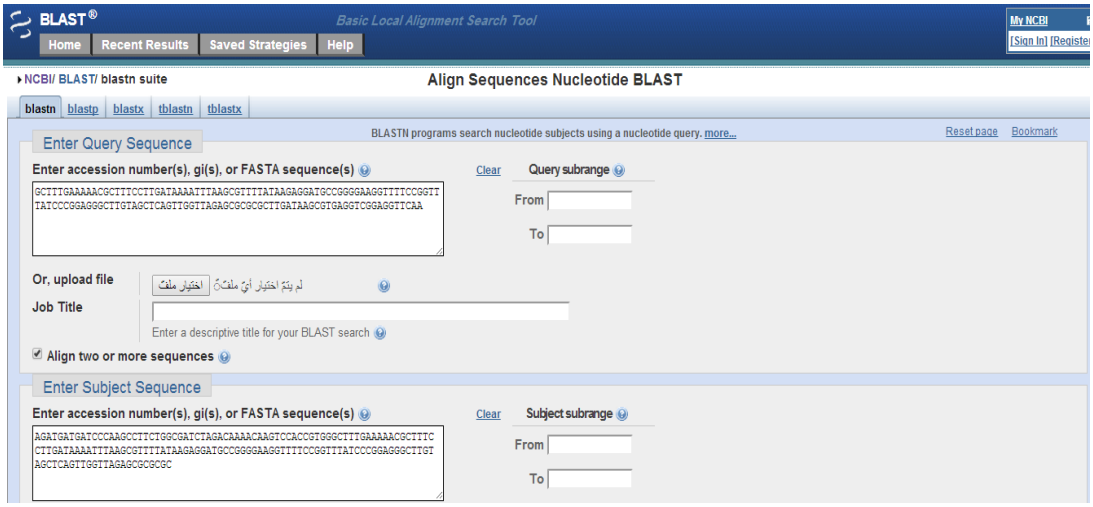


Figure 2.3: Comparison of *B. henselae* isolates from two studies: present and another local one. The upper sequence refers to *B. henselae* obtained from present study and the lower sequence is from previous local study (FJ832091) [26].

For the purpose of amplifying *Bartonella spp* DNA, *gltA* primers were used. *Wolbachia endosymbiont of Nasonia longicornis* was detected in few samples, which rendered the PCR system to be less specific.

Phylogenetic trees were constructed based on the three gene sequences, namely: *gltA* (379 bp), *rpoB* (825 bp), and *ITS* (192 bp) and IUB (DNA weight matrix) scoring matrix BESTFIT was used for comparison of nucleic acid sequences. The consensus trees were constructed by bootstrap and neighbor-joining method with default repeat

number =1000 (http://www.genome.jp/tools/ clustalw/) [66]. Alignment of *Bartonella spp* sequences with those found in the GenBank/EMBL /DDBJ databases using CLUSTAL_W program (http://www.genome.jp/tools/ clustalw/) (Fig. 2.4) was then carried out.

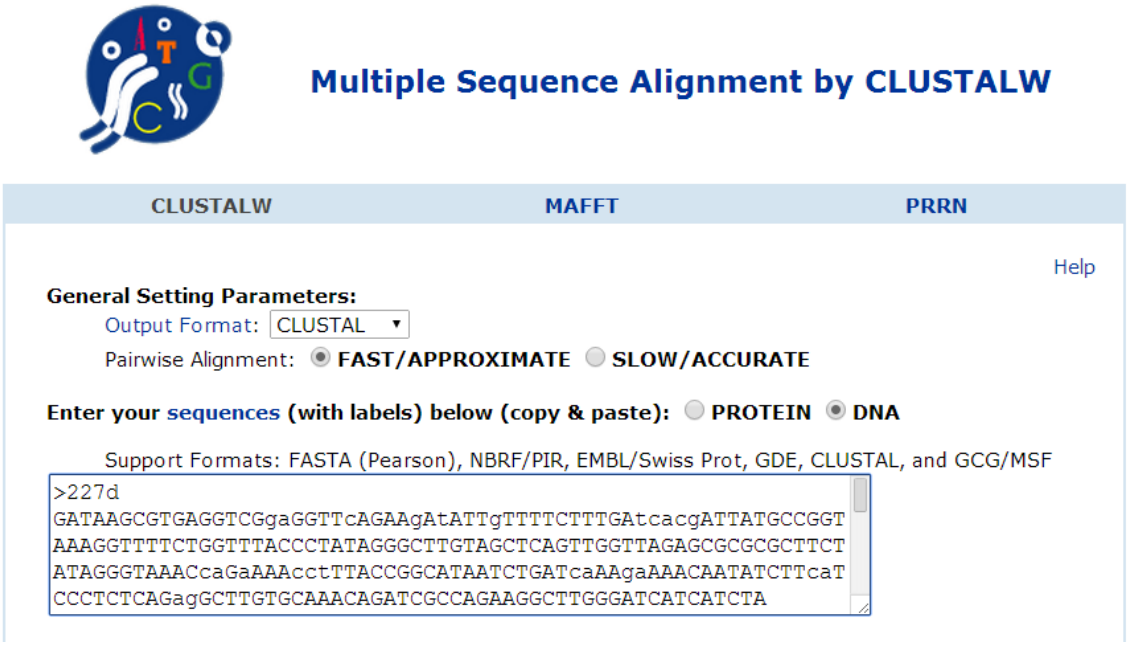


Figure 2.4: Alignment of *Bartonella spp* sequences obtained from present study with those deposited in GeneBank by CLUSTAL-W method for the purpose of constructing specific phylogenetic trees related to *Bartonella spp* distributed in Palestine (http://www.genome.jp/tools/ clustalw/).

2.6 PCR for Restriction fragment length polymorphism (RFLP) analysis

A special gel-based PCR-RFLP system was prepared for the differentiation of *B. henselae* and *B. koehlerae*. *ITS* loci were scanned for differential restriction sites using the nebcutter website (http://tools.neb.com/NEBcutter2/). Restriction enzyme (*PsiI*) (New England biolabs, Ipswich MA,USA), was incubated for 2 h at 37 ^{o}C which was then run on 2% agarose gel. MiniLumi 1.4 (DNR Bio-Imaging Systems Ltd, Israel) gel documentation system was used gel image capture.
Sequenced samples from both *Bartonella* species were used to confirm the virtual digestion.

2.7 Statistical tests

Statistical analysis was carried out using the SPSS program v13. Two- tailed t-test was performed to find association between *Bartonella* positivity and animal host, gender, location and presence/characteristics of ectoparasites. The level of statistical significance (P-value) was considered ≤ 0.05.

Chapter 3

Results:

3.1 Animal sampling and classification

A total of 289 fleas were collected from 46 animals from Nablus, Tubas, Bethlehem and Jerusalem. Overall, 119 *C. felis* (Fig. 3.1) were collected from 27 cats, 133 *C. canis* (Fig. 3.2) and two *Xenopsylla spp*. (Fig. 3.3) from nine dogs, 23 *C. canis* were sampled from five hyraxes, and seven *Xenopsylla spp*, three *C. canis* and two *C. felis* were isolated from five rats (Table 3.5). These included 212 female and 73 male fleas (Fig 3.4). The sex of four fleas was not determined due to their damage during handling. The most prevalent species were *C. canis* (n=159/289; 55%), *C. felis* (n=119/289; 41.2%), and *Xenopsylla spp.* (n=7/289; 2.4%) (Table 3.5).

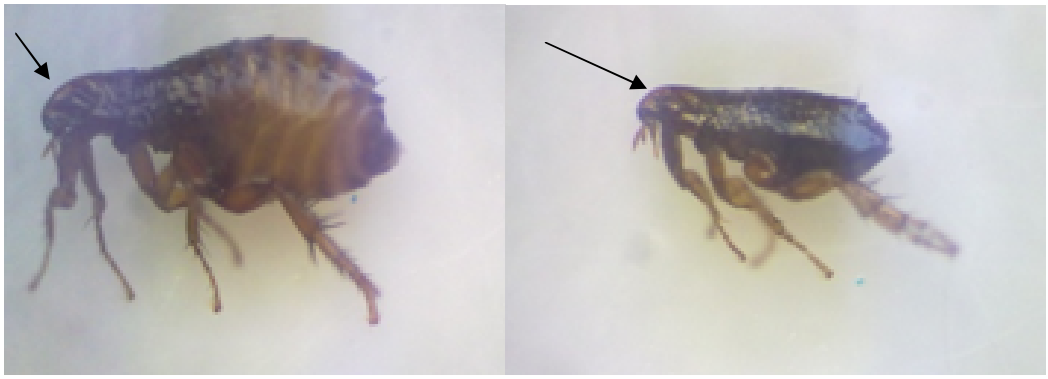
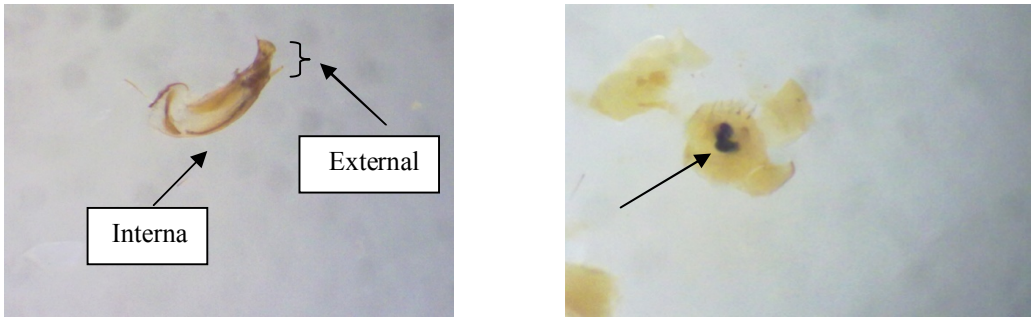


Figure 3.1: Cat flea, *C. felis.* It can be classified based on combs found in the mouth part for flea's type and presence of bean-like shape structure in the hind part of male for gender determination (Left: female, right: male). Note: the arrow shows the elongated head in contrast to dog fleas (10X magnification).

Figure 3.2: The dog flea, *C. canis* (left is female and right is male). Note the shape of head which is slightly rounded not elongated as in cat flea. The arrow (in hind region) in right figure indicates the male genital part (external aedeagus) which is used as key discriminator between male and female, (10X magnification).

Figure 3.3: Fleas from genus *Xenopsylla spp*. Female on the left panel and male on the right panel. The male and female genital parts are obvious as indicated by the arrows showing the spermatheca in female and thread-like structure (internal aedeagus) in male. The genal and pronotal combs are absent in *Xenopsylla spp*. as a differentiated from other *Ctenocephalides spp*. The presence of mesopleural rod in the first thoracic segment used as a signature for these species as well (10X magnification).



(a) **(b)**

Figure 3.4: Flea's reproductive system. (a) Thread-like structure (aedeagus) found in males which consist of two parts: internal and external genitalia. (b) The female spermatheca (the black arrow shows the sperm collecting chamber part) these parts are obtained from *Xenopsylla spp*. (10X magnification).

Table 3.5: Distribution of collected fleas by animal host and type of fleas.

Animal host (#)	**Number of collected fleas**	**Species**
Cats (27)	119	*C. felis*
Dogs (9)	135	*C. canis*, *Xenopsylla spp.*
Rats (5)	12	*Xenopsylla spp.*, *C. canis* and *C. felis*
Hyraxes (5)	23	*C. canis*
Total (46)	289	

We investigated the relationship between *Bartonella* PCR positivity in fleas and different epidemiological factors. A significant correlation was observed between *Bartonella ITS* PCR positivity and the animal host (cats, $p<0.01$), flea species (*C. felis*; $p<0.01$), flea sex (female; $p=0.012$), and the percentage of cats that hosted *Bartonella*-positive fleas which was strongly associated with the number of fleas from each cat ($p=0.008$). More than half of the tested cats (16/27, 59.3%) carried at least one infected flea. The cat sex, host's geographic location, and fleas collected from east and west Jerusalem (neighborhood ethnicity) showed no significant correlation ($p>0.05$) with *Bartonella* presence (Table 3.6).

Table 3.6: Relationship between *Bartonella* infection and animal host, gender, location and presence/characteristics of ectoparasites

Variables	***ITS* PCR**	**P- value**
Animal host (cat)	(16/27) 59.3%	0.001*
Flea species/cat	*C. felis*	<0.01*
Flea gender (females)	(M:F) 8:54	0.0012*
Fleas #/cat	0.5	0.008*
Cat Gender	No correlation	0.067
Host location	No correlation	>0.05
Neighborhood ethnicity	No correlation	>0.05

* Significant correlation.

**Out/indoor correlation was not suitable for analysis, since most of the samples were outdoor and few were both in/outdoor.

3.2 Selection of appropriate diagnostic system

Of the first 30 randomly-screened samples, four were positive for *ITS* PCR by showing a band of ~192bp. DNA sequencing of these four positive samples and subsequent BLAST analysis showed 100% sequence identity with *B. henselae* as the bacterial organism present in these fleas. On the other hand, eight samples were shown to be positive for *gltA* by amplifying a band at the level of 379 bp. Four of these positive samples (all originating from *C. felis* collected from cats) were *B. henselae* with 100% sequence identity by BLAST. The other four *gltA* sequences matched with *Wolbachia* endosymbiont of *Nasonia longicornis* with 81% sequence identity, and originated from *C. canis* fleas collected from dogs.

Sensitivity of the *ITS* PCR assay was higher than that of the *gltA* with capability of detection at the dilution of 1:125, while the *gltA* PCR system was only positive with the undiluted DNA sample. The *gltA* was not specific because it detected *Wolbachia* DNA in addition to *Bartonella* (Fig. 3.5).

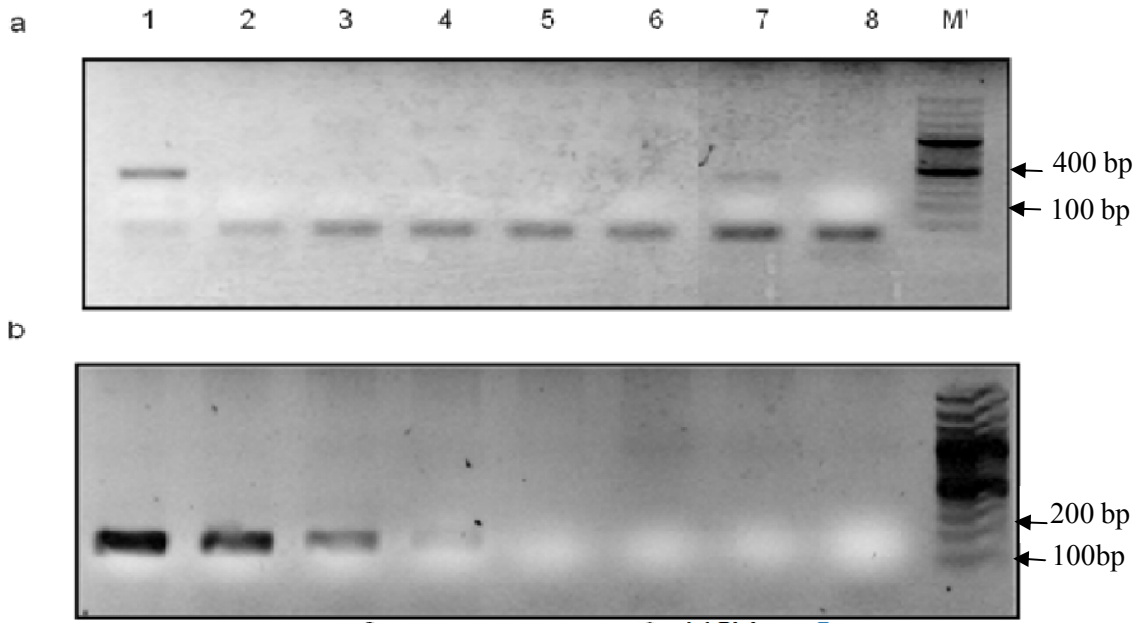


Figure 3.5: PCR sensitivity of *gltA* (a) and *ITS* (b). Lanes 1-6 represent serial dilutions of DNA template with distilled water. 1: 10µl of undiluted DNA template ; 2, 1:5 ; 3, 1:25; 4, 1:125; 5, 1:625; 6, 1:3125; 7, *Wolbachia spp.* as positive control for *gltA* and 8, negative control, M: DNA marker (bp).

3.3 Detection of *Bartonella spp.* by *ITS* PCR system

Since the *ITS* PCR showed better sensitivity and specificity than the *gltA* PCR, it was used to detect *Bartonella spp* in all 289 flea samples. *Bartonella* DNA was detected in 22% of fleas as shown in Table 3.7. Four band patterns were observed on agarose gel using the *ITS* locus PCR. The PCR product of all samples were purified and sent for DNA sequencing using forward and reverse primers. The sequencing and BLAST analysis showed that a band at size level of 192 bp belonged to *B. henselae* and *B. koehlerae* which cannot be distinguished by gel electrophoresis. A band at the 182 bp level belonged to *B. clarridgeiae*, while a 250 bp band belonged either to *B. elizabethae* or *B. tribocorum,* and a dual infection shown with the presence of a 250 bp and a 182 bp bands (Fig. 3.6)

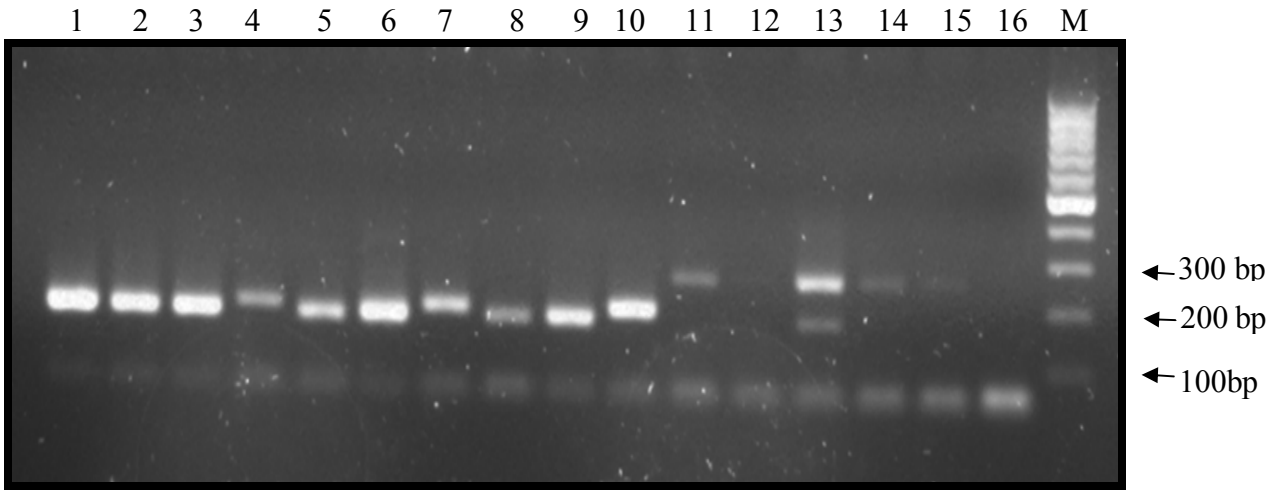


Figure 3.6: Detection of *Bartonella* DNA based on *ITS*-PCR; the bands pattern was different among tested samples. Lanes 1, 2, 3, 5, 6, 8, 9: *B. clarridgeaie,* Lane4: *B. Koehlerae, Lane* 7, 10: *B. henselae*, Lane 11: *B. tribocorum*, Lane 13 co-infection (*B. elizebetha*e and *B. rochalimae*), Lane 14, 15:*B. elizebetha*e and Lane 16: Negative control, M: DNA marker (bp)

One sample (lane 13, in Fig. 3.6) showed a two-band pattern indicating co-infection with two different bacterial species; therefore each band was cut from the gel, purified and re-amplified separately using *ITS* PCR. The PCR product of each sample was purified and sent for DNA sequencing using forward and reverse primers. Sequence and BLAST analysis revealed the presence of *B. elizabethae* and *B. rochalimae* in the same sample (Fig. 3.7).

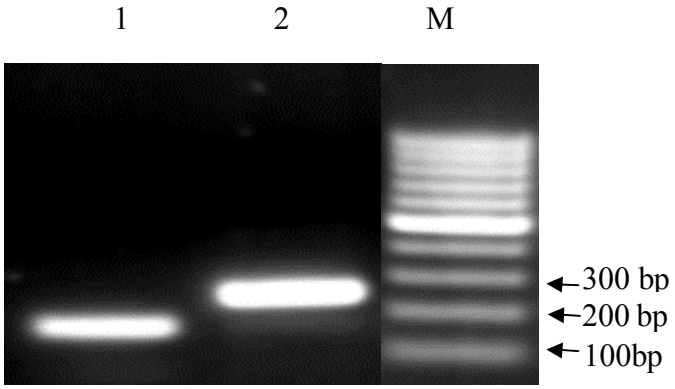


Figure 3.7: Detection of co-infection with two *Bartonella* species. Lane 1: a band of 182 bp represents *B. rochalimae* and lane 2: a band of 250 bp represents *B. elizabethae,* M: DNA marker (100bp).

To identify the *Bartonella* species of the tested samples, the PCR products of the positive samples (n=64) were purified and sent for DNA sequencing using forward and reverse primers, BLAST analysis of the positive *Bartonella* DNA sequences showed the following species distribution; *B. clarridgeiae* 30/64 (46.7%), *B. henselae* 16/64 (25%), *B. koehlerae* 2/64 (3.1%), and *B. elizabethae* 3/64 (4.6%). In addition, both *B. tribocorum* and *B. rochalimae* were detected in one out of 64 samples (1.6%). Since both *B. henselae* and *B. koehlerae* showed a fragment of 192 bp that cannot be distinguished by gel electrophoresis, we developed restriction fragment length polymorphism (RFLP) to differentiate between the two species without the need for DNA sequencing as described in section 2.6 in the materials and methods. *Bartonella*

spp. were only observed in *C. felis* and *Xenopsylla spp*. fleas, of which 60/119 (50.4%) and 4/7 (57%) were infected, respectively. *Wolbachia* endosymbionts DNA was only detected by *gltA* PCR in *C. canis* fleas of which 25/135 (18.5%) were infected (Table 3.7). *Wolbachia* endosymbionts-infected *C. canis* fleas originated only from dogs. No *Bartonella* DNA was obtained from fleas that originated from dogs and hyraxes. Out of seven fleas which originated from rats, one was positive for *B. tribocorum*, two for *B. elizabethae*, and one showed co-infection with *B. elizabethae* and *B. rochalimae* as showed by extracting the band from gel, re-amplified and then sequenced using forward and reverse primers.

Table 3.7: Prevalence of *Bartonella spp.* in fleas from different animals in Palestine.

	***ITS* PCR results (number; %)**		
Flea source (No.)	**Negative (%)**	**Positive (%)**	**Species (No., %)**
Cats (119)	59 (49.6)	60 (50.4)	*B. clarridgeiae* (30; 46.7%); *B. henselae* (16; 25%); *B. koehlerae* (2; 3.1%)
Rats (7)	3 (43)	4 (57)	*B. elizabethae* (3; 5%); *B. tribocorum* (1; 1.6%); *B. rochalimae* (1; 1.6%)
Dogs (135)	135 (100)	0 (0)*	
Hyraxes (23)	23 (100)	0 (0)	
Unknown (5)	5 (1.7)	0 (0)	
Total (289)	225 (78)	64 (22)	

* By sequencing of the *gltA* DNA products, 18.5% (25/135) of the dog fleas were found to harbor *Wolbachia* DNA

3.4 Restriction fragment length polymorphism (RFLP) analysis

The digestion of the *ITS* locus was clear as the RFLP was able to differentiate between the closely related species, *B. henselae* and *B. koehlerae*. *Bartonella henselae* produced two bands at the DNA sizes of 97 bp and 94 bp, which appeared as one band since it is difficult to show a 3 bp difference on 2% agarose gel. *B. koehlerae* showed two bands at the DNA sizes of 114 bp and 74 bp (Fig. 3.8). These two patterns were easily distinguishable on the agarose gel (Fig. 3.8).

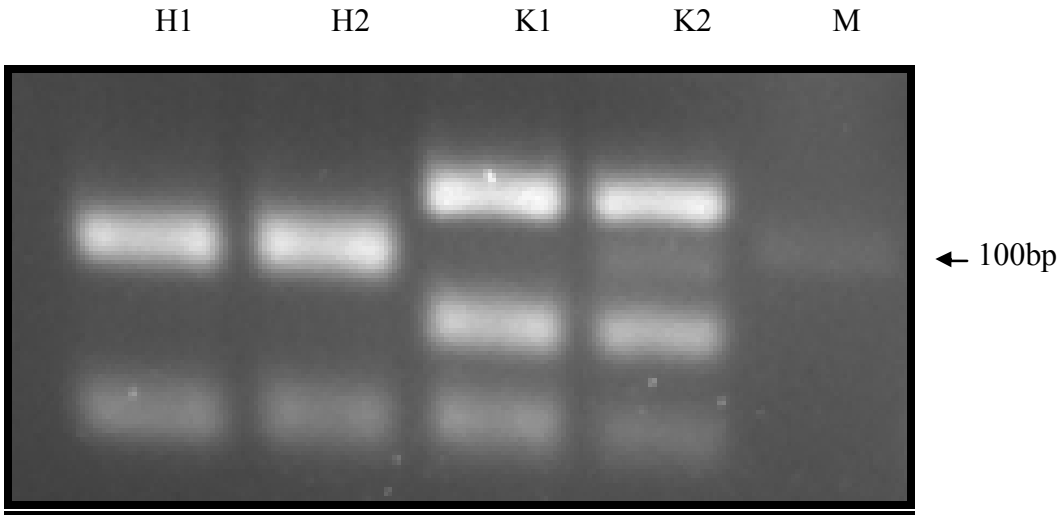
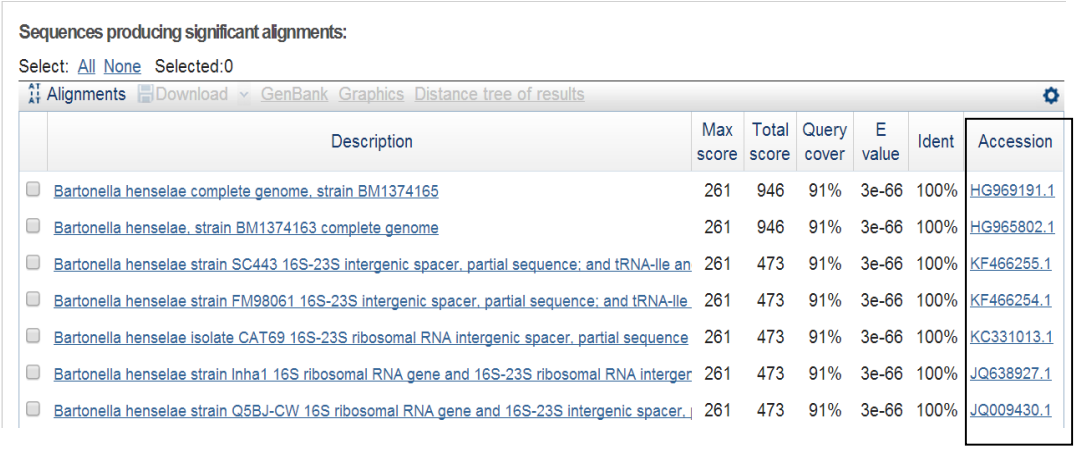


Figure 3.8: Restriction fragment length polymorphism (RFLP) analysis after digestion of the *ITS* entire product using *PsiI* enzyme. Product was loaded on 2% agarose gel. H1 and H2 represent samples sequence-proven *B. henselae*, while K1 and K2 are sequence-proven *B. koehlerae*, both were from fleas originating from cats, M: DNA marker (bp).

3.5 BLAST analysis of DNA sequences and genetic clusters

To study the genetic relationship and microheterogenitiy between *Bartonella* samples, a partial sequence of three genes: *glta*, *ITS* and *rpoβ* were studied. Successful DNA sequencing was obtained from 64 positive fleas as follows: *ITS* (52/64) 81% (Appendix A, sequences A1-A6), *gltA* (48/64) 75% (Appendix A, sequences A7-A10) and *rpoB* (33/64) 52% (Appendix A, sequences A11and A12). Not every *ITS* positive *Bartonella* gave positive PCR results for the other two genes.
BLAST analysis revealed that 25% of the tested samples had 100% homology with reference sequences of *B. henselae* deposited in the GeneBank (Fig. 3.9). Other samples (46.7%) showed 100% homology with *B. clarridgeiae* (Fig. 3.10). The remaining sequences showed similarity to *B. koehlerae*, *B. tribocorum*, *B. elizabethae* and *B. rochalimae* as shown in Appendix B1-4. All DNA sequences were multi-aligned with each other using website software: http://multalin.toulouse-.inra.fr/multalin/ (See Appendix C2).



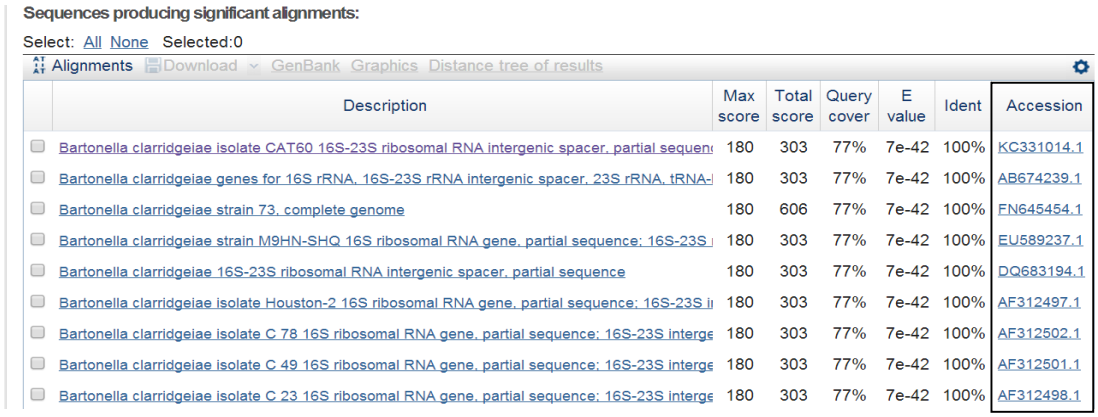
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bartonella henselae complete genome, strain BM1374165	261	946	91%	3e-66	100%	HG969191.1
Bartonella henselae, strain BM1374163 complete genome	261	946	91%	3e-66	100%	HG965802.1
Bartonella henselae strain SC443 16S-23S intergenic spacer, partial sequence; and tRNA-Ile an	261	473	91%	3e-66	100%	KF466255.1
Bartonella henselae strain FM98061 16S-23S intergenic spacer, partial sequence; and tRNA-Ile	261	473	91%	3e-66	100%	KF466254.1
Bartonella henselae isolate CAT69 16S-23S ribosomal RNA intergenic spacer, partial sequence	261	473	91%	3e-66	100%	KC331013.1
Bartonella henselae strain Inha1 16S ribosomal RNA gene and 16S-23S ribosomal RNA interger	261	473	91%	3e-66	100%	JQ638927.1
Bartonella henselae strain Q5BJ-CW 16S ribosomal RNA gene and 16S-23S intergenic spacer,	261	473	91%	3e-66	100%	JQ009430.1

Figure 3.9: BLAST of *B. henselae ITS* sequence against reference strains sequences (the accession numbers are marked with black square).



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
Bartonella clarridgeiae isolate CAT60 16S-23S ribosomal RNA intergenic spacer, partial sequen	180	303	77%	7e-42	100%	KC331014.1
Bartonella clarridgeiae genes for 16S rRNA, 16S-23S rRNA intergenic spacer, 23S rRNA, tRNA-I	180	303	77%	7e-42	100%	AB674239.1
Bartonella clarridgeiae strain 73, complete genome	180	606	77%	7e-42	100%	FN645454.1
Bartonella clarridgeiae strain M9HN-SHQ 16S ribosomal RNA gene, partial sequence; 16S-23S	180	303	77%	7e-42	100%	EU589237.1
Bartonella clarridgeiae 16S-23S ribosomal RNA intergenic spacer, partial sequence	180	303	77%	7e-42	100%	DQ683194.1
Bartonella clarridgeiae isolate Houston-2 16S ribosomal RNA gene, partial sequence; 16S-23S i	180	303	77%	7e-42	100%	AF312497.1
Bartonella clarridgeiae isolate C 78 16S ribosomal RNA gene, partial sequence; 16S-23S interge	180	303	77%	7e-42	100%	AF312502.1
Bartonella clarridgeiae isolate C 49 16S ribosomal RNA gene, partial sequence; 16S-23S interge	180	303	77%	7e-42	100%	AF312501.1
Bartonella clarridgeiae isolate C 23 16S ribosomal RNA gene, partial sequence; 16S-23S interge	180	303	77%	7e-42	100%	AF312498.1

Figure 3.10: BLAST of *B. clarridgeiae ITS* sequence against reference strains sequences (the accession numbers are marked with black square).

Furthermore, one *ITS* sequence of *B. henselae* was compared with other local strain of *B. henselae* (Accession number FJ832091.1) reported by Ohad *et al* [26]. Homology of 100% with 81% query coverage was observed between the two sequences (Fig. 3.11). The same procedure was applied to detect sequence homology between *B. koehlera*e with the local strain of *B. koehlerae* (Accession number FJ832087.1) described by the same study (Appendix C1).

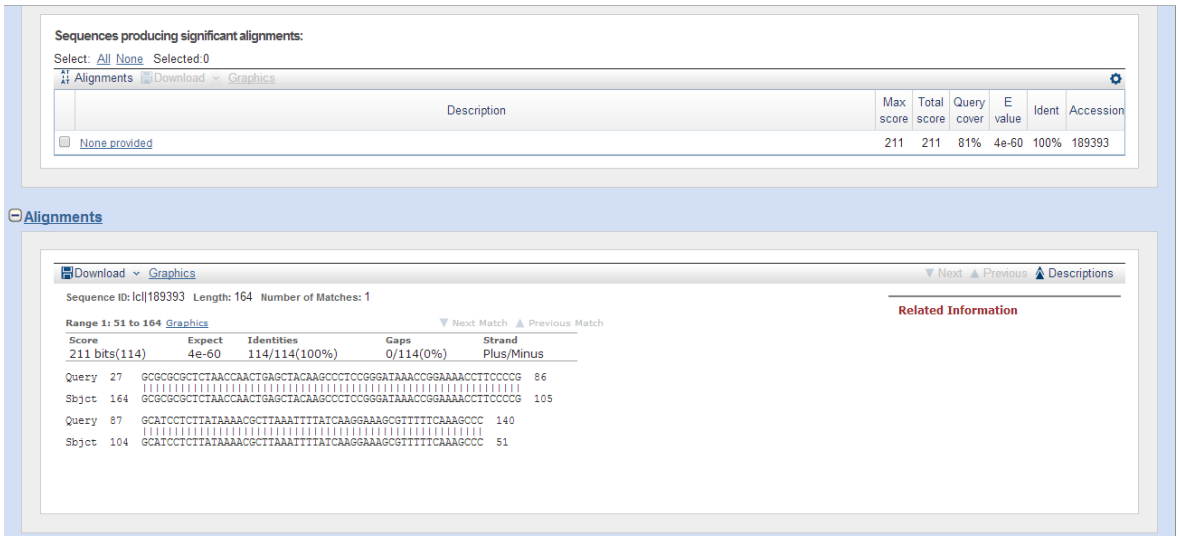


Figure 3.11: Sequence homology of *B. henselae* isolated in Palestine compared with local strain of *B. henselae* from Israel [53].

The *ITS* sequence of *B. tribocorum* from *Xenopsylla spp* flea obtained from rat was only 89% homologous (203/229 bp) to a previously reported DNA sequence accession number (FJ577653) from blood sample obtained from rat (*R. rattus*) captured in north-central Tel Aviv (Fig. 3.12) [45], whereas *B. elizabethae ITS* sequence showed 94% homology to the same novel strain (Fig. 3.13) [45].

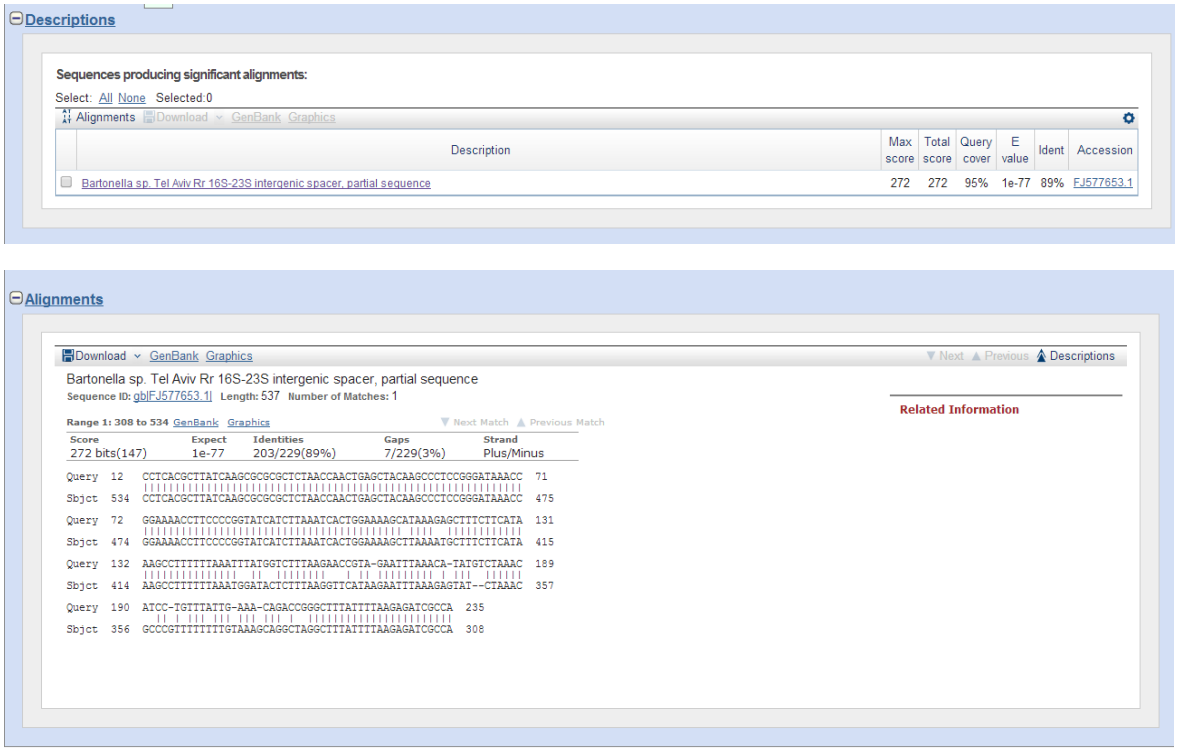


Figure 3.12: Alignment of the amplified *ITS* sequence of the Palestinian *B. tribocorum* against *Bartonella spp* described by Harrus *et al* [45].

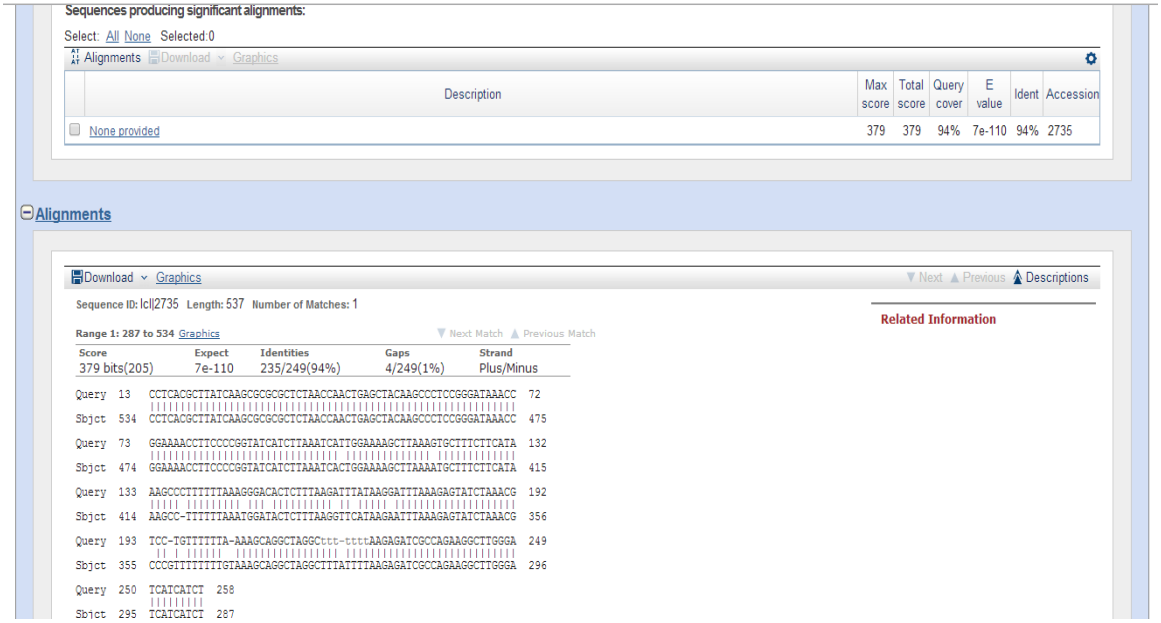


Figure 3.13*:* Alignment of the amplified *ITS* sequence of the Palestinian *B. elizabethae* against *Bartonella spp* described by Harrus *et al* [44].

In this study, *B. rochalimae* was identified for the first time in our region. Since the 16S-23S *ITS* region DNA sequences of other local *B. rochalimae* strains are not yet available in the database; we compared our strain with non local strain of *B. rochalimae* (accession number DQ676491) from blood sample obtained from dog from California, USA [67]. Multiple DNA sequence alignment of the *ITS* region demonstrated that DNA from our strain was 98% homologous (120/123 bp) to the *B. rochalimae* isolate obtained from the Californian dog (Fig. 3.14).

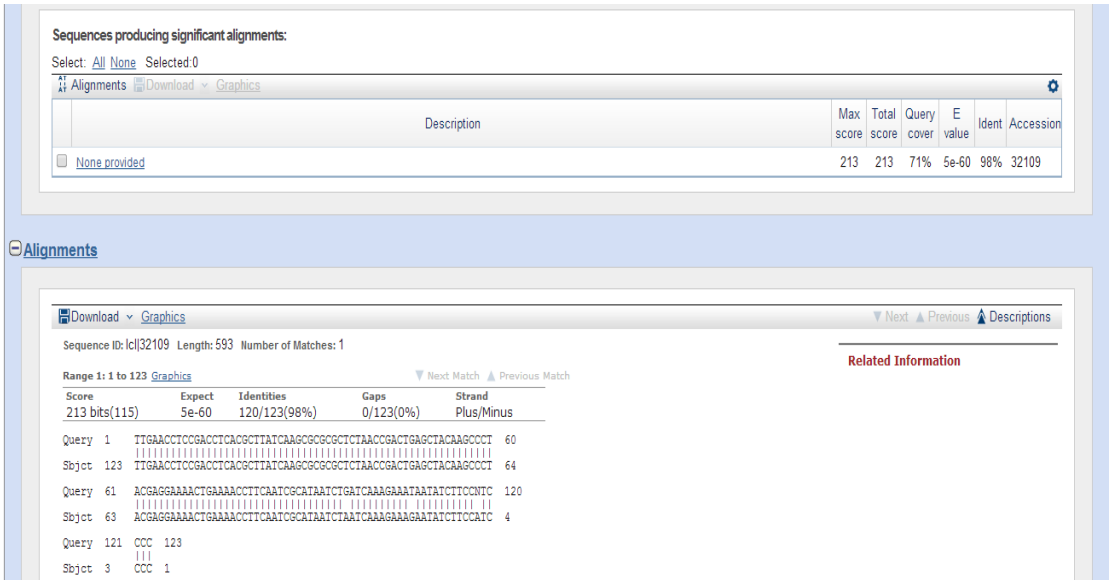
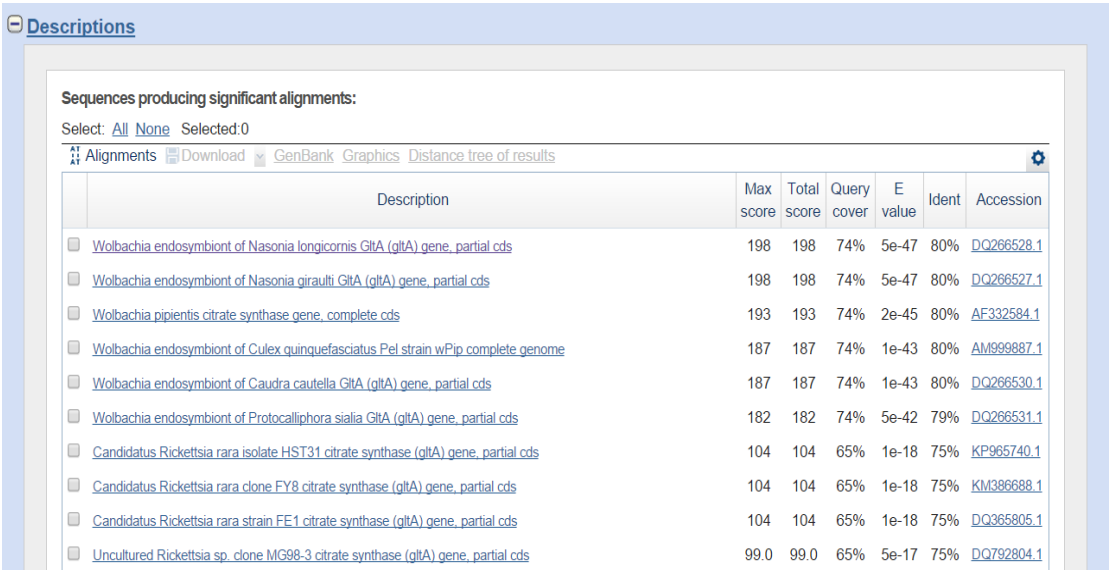


Figure 3.14: Multiple DNA sequence alignment of the *ITS* region of *B. rochalimae* (this study) against *B. rochalimae* isolate obtained from dog from California, USA [67]. Homology of 98% was observed between the two sequences.

The *gltA* gene used in our study has frequently been used to test flea samples from different hosts. A 379 bp sequence of *gltA* gene was obtained from dog flea (*C. canis)*. Sequencing analysis revealed detection of *Wolbachia endosymbiont of Nasonia longicornis* illustrating the low specificity of these primers for detection of *Bartonella* infection (Fig. 3.15).



Descriptions

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
Wolbachia endosymbiont of Nasonia longicornis GltA (gltA) gene, partial cds	198	198	74%	5e-47	80%	DQ266528.1
Wolbachia endosymbiont of Nasonia girault GltA (gltA) gene, partial cds	198	198	74%	5e-47	80%	DQ266527.1
Wolbachia pipientis citrate synthase gene, complete cds	193	193	74%	2e-45	80%	AF332584.1
Wolbachia endosymbiont of Culex quinquefasciatus Pel strain wPip complete genome	187	187	74%	1e-43	80%	AM999887.1
Wolbachia endosymbiont of Caudra cautella GltA (gltA) gene, partial cds	187	187	74%	1e-43	80%	DQ266530.1
Wolbachia endosymbiont of Protocalliphora sialia GltA (gltA) gene, partial cds	182	182	74%	5e-42	79%	DQ266531.1
Candidatus Rickettsia rara isolate HST31 citrate synthase (gltA) gene, partial cds	104	104	65%	1e-18	75%	KP965740.1
Candidatus Rickettsia rara clone FY8 citrate synthase (gltA) gene, partial cds	104	104	65%	1e-18	75%	KM386688.1
Candidatus Rickettsia rara strain FE1 citrate synthase (gltA) gene, partial cds	104	104	65%	1e-18	75%	DQ365805.1
Uncultured Rickettsia sp. clone MG98-3 citrate synthase (gltA) gene, partial cds	99.0	99.0	65%	5e-17	75%	DQ792804.1

Figure 3.15: A BLAST of the amplified sequences obtained by *gltA* primers shows query coverage of 74% and 80% maximal identity specific to *Wolbachia endosymbiont of Nasonia longicornis* sequence. The result indicates the low specificity of these primers.

ITS genetic diversity analysis showed three main clusters, a *B. henselae* and *B. koehlerae*-like cluster, a *B. clarridgeiae*-like cluster, and a rat *Bartonella*-associated cluster (including *B. elizabethae* and *B. tribocorum*) (Fig. 3.16). The *B. henselae* clade apparently belonged to a separate sub-cluster (*B. koehlerae*), while the *B. clarridgeiae*-like cluster separated into three sub-clusters (Fig. 3.16A). A non-experimental *Rickettsia rickettsii* 16S- rRNA gene (GenBank: U11021.1) was used as an out-group. Previous studies [68 and 69] indicated that the *ITS* phylogenetic analysis was a useful tool to identify *Bartonella* species.

Two main clusters for *gltA* and two clusters for *rpoB* genes were observed (Fig. 3.16 B and C). Each cluster of *rpoB* correlated with one of the clusters of the *gltA* and *ITS* loci. All *Bartonella* genotypes seemed to follow a pattern in which the genotype in cluster I of the *rpoB* also belonged to cluster I of *gltA* and *ITS* (*B. henselae* group) (Fig. 3.16A, B, C).

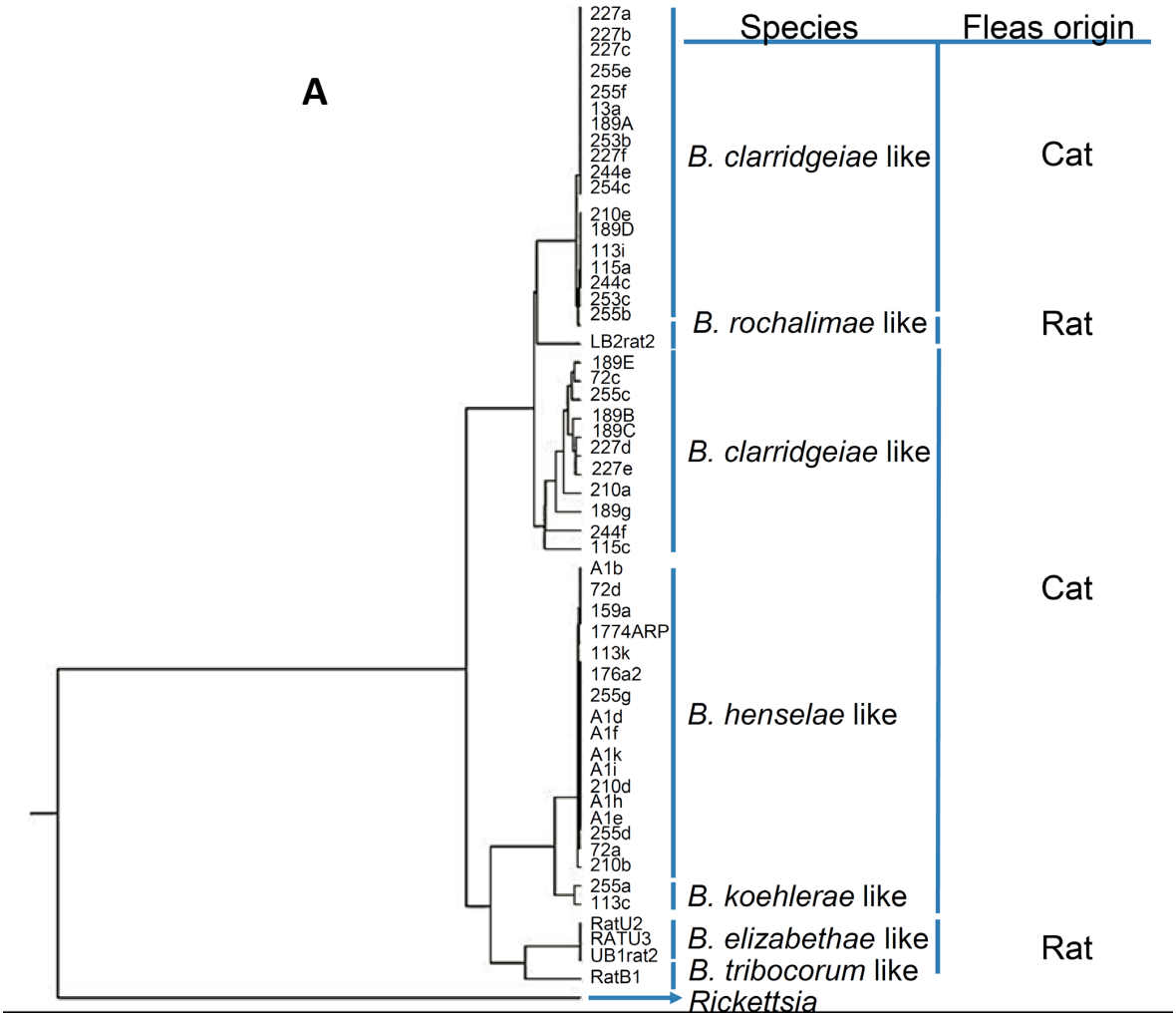
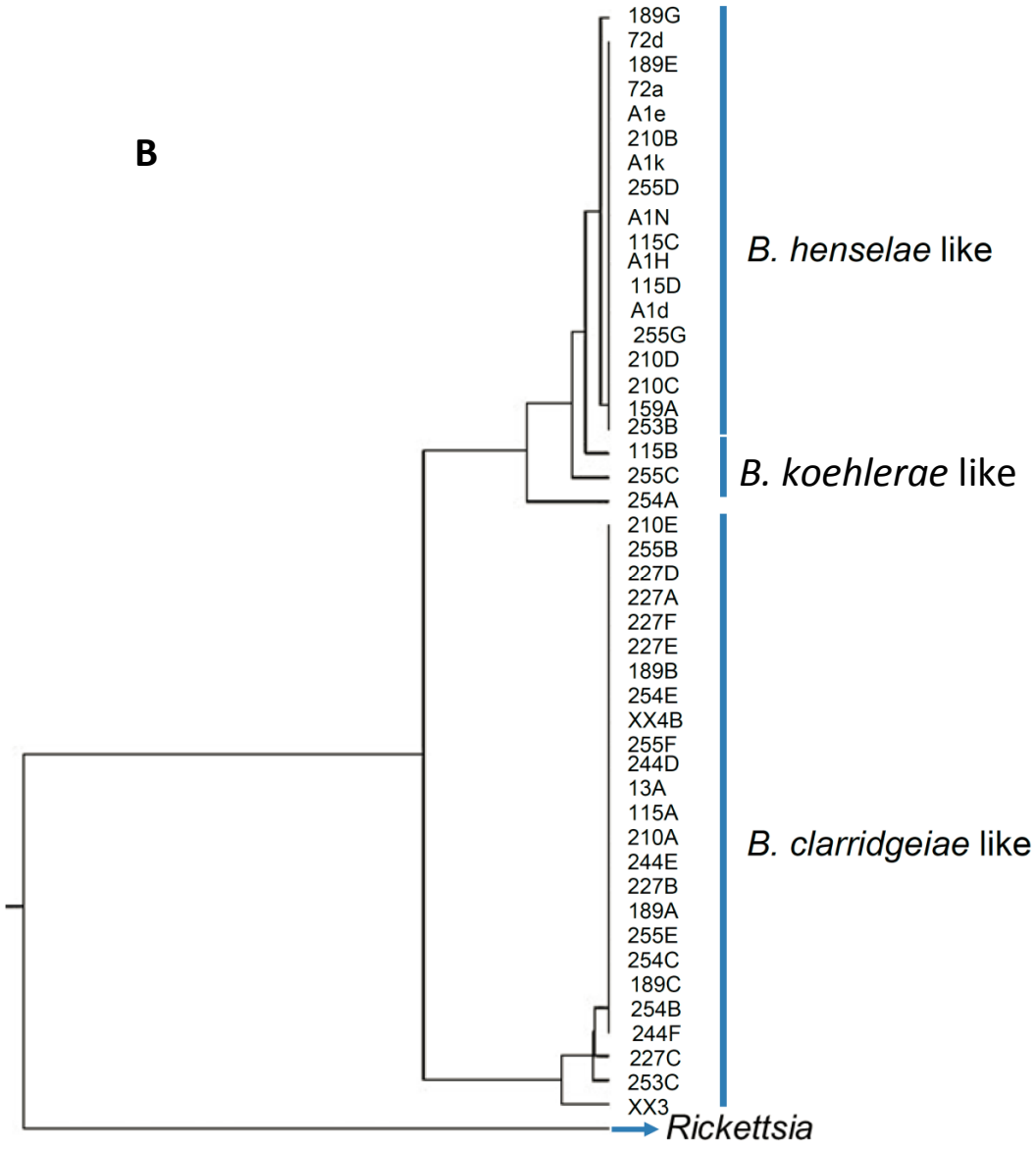
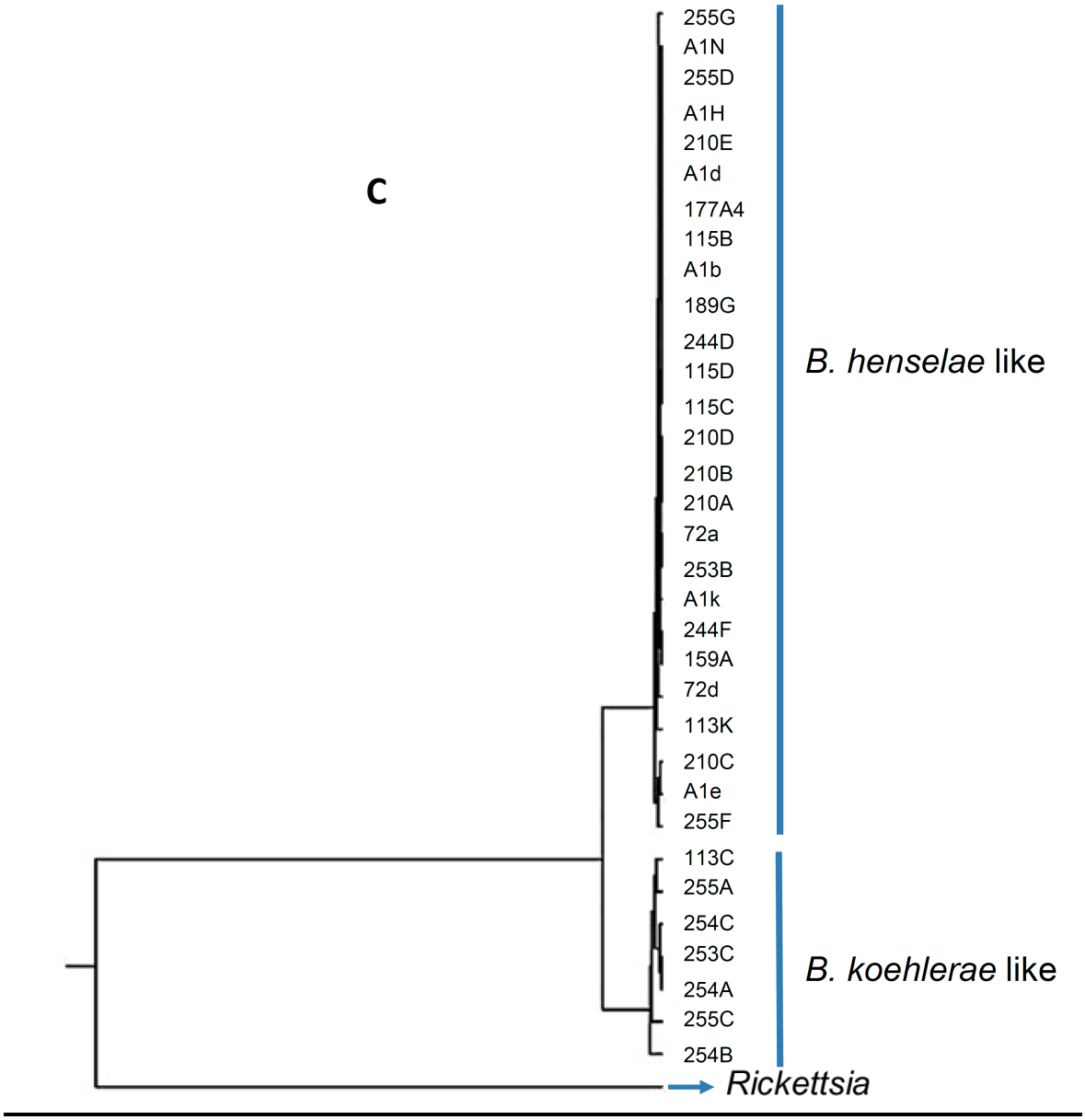


Figure 3.16: Phylogenetic classification of Palestinian *Bartonella* based on sequences of A: *ITS*, B: *gltA* and C: *rpoβ* loci. The trees were constructed by the neighbor-joining method using the CLUSTAL-X program (http://www.genome.jp/tools/ clustalw/) for the alignment of *Bartonella* sequences obtained in this study with those of known *Bartonella* species deposited in the GenBank/EMBL/DDBJ databases. They were drawn using the IUB scoring matrix used by BESTFIT for the comparison of nucleic acid sequences.



(Continued Fig. 3.16 B)



(Continued Fig. 3.16 C)

Chapter 4

Discussion :

This study investigated the prevalence of *Bartonella* organisms in fleas from cats, dogs, rats, and hyraxes in Palestine and characterized their genetic composition. The collected fleas mainly contained *C. canis*, *C. felis* and *Xenopsylla spp.* Moreover, fleas' sex was determined based on special keys. More female's fleas were observed than males (212:73). This bias may be due to the requirement of blood for offspring feeding. The sex of four fleas was undetermined because of damage caused during handling.

Previous study showed that dogs are an incidental host for *C. felis* and cats are a carrier for *C. canis* [14]. However, such results were not observed in our study. Despite rats are the main reservoir of *Xenopsylla spp*., *C. canis* was found in rat samples in this study. In addition, all fleas collected from hyraxes were *C. canis*.

The overall prevalence of *Bartonella* infection in fleas from studied animals (cats, dogs, rats and hyraxes) was 22% (64/289). These results were similar to those found in Israeli cats, 25.1% (84/334) were infected with *Bartonella* species [70].

In the present study, no *Bartonella* DNA was detected in fleas collected from dogs (n=135) or hyraxes (n=23). However, fleas collected from dogs in other Mediterranean countries, including Greece and Italy, were reported to be infected with *Bartonella spp.* with a prevalence of 4% and 11.7%, respectively [71]. On the other hand, dog fleas (*C. canis)* in other regions previously showed positive results for *Bartonella spp*., for example, *B. henselae* detected in *C. felis* and *C. canis* from infested Korean dogs with percentages of 54.8% and 45.2%, respectively [9]. In Jordan, positive sero-prevalence of cats with *B. henselae* was 32% [72], while in Lebanon; the PCR positivity for *B. henselae* was 2.9%, all were collected from *C. felis* [73]. In northern and western Iraq as well as Baghdad; blood samples from cats showed a sero-prevalence of 15% for *B. henselae* and 12.6% for *B. clarridgeiae* [74]. In addition, the sero-prevalence of *Bartonella spp*. in canids was investigated using dogs, Jackales and red foxes blood samples. The results showed 47% sero-positivity for the followed *spp*.: *B. henselae*, *B. clarridgeiae*, *B. vinsonii subsp. berkhoffii* and *B. bovis* [75]. In Egypt, *B. clarridgeiae* was the main species of *Bartonella* detected from rat fleas (*X. cheopis*) which is similar to our finding *C. felis* [76].

In a study conducted in Tunisia on 40 sera of blood from patients suffering from infective endocarditis, 12 of them were positive for *B. quintana* and one case showed infection with *B. henselae*. It showed about 10% infection rate while the rate in UK is 1%, 1% in Germany and 3% in France [77]. In Algeria; blood samples of 211 stray cats were collected and tested for *Bartonella* infection; the study revealed that *Bartonella spp* was isolated from 36/211(17%); all were from genus *B. henselae* [78]. Another study in Algeria showed a sero-prevalence in dogs of 31% for *Bartonella spp*. including *B. vinsonii* subsp. *berkhoffii*, *B. clarridgeiae* and/or *B. henselae* antigens [79]. In Morocco; of 147 dogs, 56 (38%) showed seropositive for *Bartonella* from genus *visonii* subspecies *berkhoffii* [80]. In Bahrin, blood sera were obtained through donation from HIV infected patients revealed the sero-positivity of 16% (9/56) for *B. henselae* antibodies [81]. Of the 110 samples of saliva collected from cats in Iran, 12 (10.9%) harbored *B. henselae* DNA [82]. In Turkey; a sample population of blood sera was collected from 298 cats, the seropositivity ratio using

immune-fluorescent assay against anti-*B. henselae* IgG was 27.9% (83/298) distributed on five regions [83].

The prevalence of different *Bartonella spp*. found in cat fleas in this study, i.e., *B. clarridgeiae* (46.7%), *B. henselae* (25%), and *B. koehlerae* (3.1%). It is worthy to note that the same three *Bartonella* species were detected in cats from Israel [70].
B. clarridgeiae distribution (46.7%) was higher than those obtained from other previous studies in Papeete, Tahiti (7.4%) and New Zealand (7%) [84 and 85]. This may be due to the sample size or due to the wide distribution of this *spp*. in the country. *B. henselae* percentage was (25%) which was found to be higher than that shown in Lebanon (2.9%) obtained from fleas and lower than detected in Jordan 32% [72 and 73]. In case of *B. koehlerae* (3.1%), this percentage was found to be close to that found in France (3.7%) [86]. On the other hand, *B. tribocorum* (1.6%) of present study was lower than other study percentages obtained from rat blood samples 18.5% (37/200) and rat fleas (*X. cheopis*) 27.5% (25/91) from California [87 and 88]. Likewise, *B. elizabethae* (5%) and *B. rochalimae* (1.6%) also showed lower percentage which may due to low rat-flea sample size (n=7) examined in this study, further studies on these animals should be conducted. *Rattus rattus* rats and cats captured in the Palestine were highly infested with fleas infected with *Bartonella spp*. (57% and 50.4%, respectively), suggesting that those mammals might be major reservoirs of *Bartonella* species in Palestine. *Rattus rattus* captured in this study were infected with *B. tribocorum*, *B. elizabethae*, and *B. rochalimae*. As *B. rochalimae* was reported to infect humans and animals, its potential zoonotic role is emphasized [33 and 89]. In addition, the rat flea (*Xenopsylla spp*.) showed to be infected with *B. rochalimae* was accompanied with *B. elizabethae* infection. Other species of *Bartonella* (i.e. *B. bacilliformis, B. quintana, B. grahamii, B. doshiae*) was not detected in our samples due to host specificity to these specific microbes or absence of these microbes in our region. As an example for host specificity is the presence of *B. quintana* solely in body louse [21 and 22] and *B. vinsonii berkhoffii* solely in dogs and coyotes [21 and 22].
Co-infection with more than one *Bartonella spp*. was documented in this study in 6% (4/64) of the infected fleas and in 16 (59.3%) of the cats harboring at least one *ITS* PCR-positive flea. This was shown through sequencing of different individual fleas that originated from the same cat. The percentage of cats that hosted *Bartonella*-positive fleas was strongly associated with the number of fleas from each cat (p=0.008) where more fleas on cats correlated positively with the infection. In the present study, different fleas collected from the same animal showed mixed *Bartonella* species (*B. henseale*, *B. clarridgeaie* and *B. koehlerae*) and genotypes.

Co-infection of fleas with different *Bartonella* species has already been described. A previous study conducted in Israel showed co-infection with two or more different *Bartonella spp*. in 2.1% of stray and domestic cats [70]. Domestic cats from Thailand were also demonstrated to be co-infected with more than one species of *Bartonella* as described earlier [90]. In our study, the co-infection of *B. henselae* and *B. clarridgeaie* or the other *Bartonella spp*. was not observed within the same flea.

The two *spp*. *B. rochalimae* and *B. clarridgeaie* previously shown very closely related to each other [33]; were also proved to be so in our study after *ITS* amplification showed the same band pattern (182bp). Then, DNA sequencing revealed it belongs to *B. rochalimae* (Fig 3.16). This shows the important role of using DNA sequencing in identification of *Bartonella spp*. Additionally, *B. tribocorum* is more genetically related to *B. elizabethae* as shown in other studies [32 and 91].

There was significant correlation between flea *Bartonella* infection rate and the flea species examined. The high *Bartonella* infection rate of *C. felis*, was not surprising because it is well known that cats are the main reservoir of some *Bartonella spp*. and the main host of this flea species [78, 92 and 93].

This study emphasizes that fleas are the vectors of different *Bartonella spp*. and responsible for transmission of variable diseases collectively known as Bartonellosis. Cats and rats are the two animals' harboring fleas at a higher infection rate of *Bartonella* than others.

The DNA amplification and DNA sequencing of the *ITS* loci has been shown to offer a sensitive means of species and intra-species differentiation of the *Bartonella* organism as shown by others [68] and confirmed by the present study.

Several advantages for the *ITS* PCR system over the *gltA* PCR system were found in our study: higher sensitivity for *ITS* PCR (192 bp) possibly due to a shorter PCR product than the *gltA* (379 bp) as shown in a previous report [94]. The *ITS* locus PCR was shown to be more specific for *Bartonella spp*., than *gltA* PCR. The *gltA* PCR amplified *Wolbachia spp*. in addition to *Bartonella*. The *ITS* locus RFLP was able to differentiate the closely related species *B. henselae* and *B. koehlerae* in our samples. However, it needs to be applied on a sufficiently large sample size for system approval for molecular diagnostics and epidemiological purposes. Our study showed genetic clustering in the dendrograms, with interspecies heterogeneity, evident more in *B. clarridgeiae* than *B. henselae* (Figure 3.16A) based on *ITS* DNA sequence. Six bacterial clades were evident as *B. clarridgeaie, B. rochalimae, B. henselae-like, B. koehlerae-like, B. elizabethae-like,* and *B. tribocorum*-like cluster with *Rickettsia* as an out group. *ITS*-based clustering was shown to split each species in a specific group and, as expected, *B. henselae* and *B.koehlerae* were shown to closely cluster together than others as demonstrated previously [42, 69 and 95].
Rodent *Bartonella spp*. was shown to be in a new clade branch as expected [42 and 95]. *Bartonella clarridgeiae* presented a higher heterogeneous group than the other species. The genetic dendrogram based on DNA sequences of *gltA* (Fig. 3.16B) divided the *Bartonella* species into two main clades: *B. clarridgeiae* and *B. henselae* with less inter-species genetic variability in both species than the *ITS* locus. The DNA sequences of *rpoB* (Fig. 3.16C), clustered the *Bartonella* species into two main ones: *B. henselae,* and *B. koehlerae* species. Higher genetic variability of *B. henselae* was observed using this gene than the *gltA* and *ITS* loci.

BLAST tool analysis revealed high similarity between the DNA sequences of *B. henselae* and *B. koehlerae* isolated in this study and those isolated in previous study [26]. In addition, a previous study by Harrus *et al*. on rodent bacteria showed close relationship between *B. elizabethae* and *B. tribocorum* [45]. In this study, *B. elizabethae* showed 94% similarity to Harrus *et al* [45] isolate. Also, in this study *B. tribocorum* sequence showed 89% similarity to that isolated by Harrus *et al* [45]. It is crucial to note that the presence of *B. clarridgeaie* and *B. rochalimae* was first documented in this study. The sequencing of *B. rochalimae* shown in this study has not been previously reported in any local or regional studies. Detection of *Wolbachia endosymbionts* DNA only in *C. canis* fleas and *Bartonella* only in *C. felis* showed the specificity of these vectors to their bacteria and the role of these vectors in transmission of the diseases.

A study by Flexman *et al*, examined a case from Australia infected with cat scratch disease (CSD) and had no history of cat bite or scratch, but his cat was infested with

fleas. Cat owner's blood and cat fleas showed the infection with *B. henselae* [96]. Although there is no strict evidence that flea-bite can transmit *Bartonella* to human directly, but, in a rare reported case, woman had been bitten many times was infected with *B. rochalimae* [33]. In addition, a study revealed veterinarian woman co-infection with *B. koehlerae* and *B. berkhoffii subsp vinsonii* [97]. In previous study, from eight infected patients with *B. koehlerae*, four of them were actually veterinarians and one had a history of fleas and other insect bites because the frequent animal contact as well as insects, however, this could indicate disease transmission by animals or at least by fleas [98]. This supports the idea that *B. henselae*, *B. rochalimae* and *B. koehlerae* can cause direct infection to the Palestinian population.

Infection with *B. henselae*, *B. clarridgeiae* and *B. koehlerae* causes cat scratch disease (CSD), endocarditis and other diseases. Such infections have been caused by the inoculation of flea's feces through cat scratch by the contaminated claws. In *B. quintana,* the mode of transmission is the same as in the aforementioned species, but by inoculation with the human lice (*Pediculus humanis)* feces through scratching the patient his head [21, 22 and 99].

B. tribocorum, B. elizabethae and *B. rochalimae* cause endocarditis, neuroretinitis and other diseases. Such bacteria are transmitted mainly by rat fleas *Xenopsylla spp*. and *pulex spp*., or their host animals such as rats, foxes, dogs and coyotes. Mode of transmission to man is not fully elucidated, but the presence of infected animals in proximity to human is one of the risk factors associated with infection [13, 22, 32 , 33 , 29 and 96]. However, homeless people as well as drug users are recently found to be more prone to infection [100 and 101].However, cat's behavior like mating, playing with each other and sharing the place support such infestation. Moreover, the infested mother transmits fleas to kittens during milk feeding. Indoor furniture i.e. carpet also causes flea transmission between animals and to human as well. Keeping indoor animal/s clean of fleas and away from other infested animals contribute for sanitary and reducing the risk of disease transmission. The infection rate that was observed in fleas raises a question about whether the infested animal is infected. But, previous studies revealed that animals such as cats can harbor the bacteria without showing any symptoms (asymptomatic). Also, the transmission of bacteria in fleas showed evidence of a vertical transmission(from mother to child) pattern after examining the digestive and reproductive systems of fleas [102]. The transmission of bacteria should include other source and it is the instars or larvae of fleas as they actually fed on fleas' feces that were contaminated with the bacteria. Therefore, the proposed model here is that bacteria may be transmitted to larvae through the flea's feces.

The information that was obtained from the present study would pave the way towards more investigations of *Bartonella spp*. in the future. Furthermore, the insufficient data in Palestine about the *Bartonella spp*. may encourage other researchers to continue investigating the disease and its causative agent. Currently, data on disease serology in Palestine is not available. The number of samples collected in this study was low. Increasing the number of samples would give more sufficient statistical data for a more robust detection of other species. In this study, genetic variability using multi-locus variable number tandem repeats (VNTR) typing technique was conducted [103]. This step was aimed to discriminate between *B. henselae* isolates harvested in our study. Unfortunately, the data using the VNTR technique was limited and we failed to amplify all the VNTR markers**.**

In our study, no significant correlation was observed between *Bartonella spp* infection and cat gender, host location and neighborhood ethnicity. This may due to the low number of indoor animals compared to outdoor ones. For that out/indoor correlation was not suitable for analysis.

In conclusion, a high infection rate with different *Bartonella spp*. was found in fleas from animal hosts in Palestine. Three flea species were identified in our study, *C. felis*, *C. canis* and *Xenopsylla spp*. In addition, six *Bartonella spp*. were detected, *B. henselae*, *B. clarridgeaie*, *B. koehlerae, B. elizabethae, B. tribocorum and B. rochalimae.* Our findings are of potential public health importance and should alert local physicians and public health authorities to the possibility of human infections with these *Bartonella* species. *ITS-PCR* was recommended for diagnostic purposes and identification of *Bartonella spp*.

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Appendix A

Representative sequences of *Bartonella spp*.

Sequence A1: *B. henselae ITS* DNA sequence originated from flea collected from cat and identified according to BLAST

```
TGAACCTCCG   ACCTCACGCT  TATCAAGCGC GCGCTCTAAC CAACTGAGCT  ACAAGCCCTC        60
CGGGATAAAC   CGGAAAACCT  TCCCCGGCAT CCTCTTATAA AACGCTTAAa  TTTTAtCAAG       120
GAAAGCGTTT   TTCAAAGCCC                                                   140
```

Sequence A2: *B. clarridgeiae ITS* DNA sequence originated from flea collected from cat and identified according to BLAST

```
TGAACCTCCG   ACCTCACGCT  TATCAAGCGC GCGCTCTAAC  CAACTGAGCT ACAAGCCCTC        60
CGGGATAAAC   CGGAAAACCT  TCCCCGGCAT CCTCTTATAA  AACGCTTAAa TTTTAtCAAG        120
GAAAGCGTTT   TTCAAAGCCC                                                   140
```

Sequence A3: *B. koehlerae ITS* DNA sequence originated from flea collected from cat and identified according to BLAST

```
TTGAACCTCC GACCTCACGC TTATCAAGCG CGCGCTCTAA CCAACTGAGC TACAAGCCcT            60
CCGGGATAAA CCGGAAAACC TTCCCCGGCA TCCTCTTACA AAACGCTCAA TTTTATAAAG           120
GAAAGCGCTT TTCAAAGTCC ACGGTGGACT TGTTTGTGTA GAT                             163
```

Sequence A4: *B. tribocorum ITS* DNA sequence originated from flea collected from rat and identified according to BLAST

```
TGAACCTCCG  ACCTCACGCT TATCAAGCGC GCGCTCTAA CCAACTGAGC TACAAG               60
CCCTCCGGGA  TAAACCGGAA AACCTTCCCC GGTATCATC TTAAATCACT GGAAAA              120
GCATAAAGAG  CTTTCTTCAT AAAGCCTTTT TTAAATTTA tGGTCTTTAA GAACCG              180
TAGAATTTAA  AcatAtGTCT AAACatCCtg TTtAtTGAA ACAGACCGGG CT                   240
TTATTTTAAG  AGATCGCCA                                                      262
```

Sequence A5: *B. elizabethae ITS* DNA sequence originated from flea collected from rat and identified according to BLAST

```
TGAACCTCCG ACCTCACGC TTATCAAGC GCGCGCTCT AACCAACTGA GCTACAAG            60
CCCtCCGGGA TAAACCGGA AAACCTTCC CCGGTATCA TCTTAAATCA TTGGAAAA            120
GCTTAAAGTG CTTTCTTCA TAAAGCCCT TTTTTAAAG GGACACTCTT TAAGATTT            180
ATAAGGATTT AAaGaGTAT CTAAACGTC CtGTTTtTT AAAAGCAGGC TAGGC               240
TTTTTTTAAG AGATCGCC                                                    260
```

Sequence A6: *B. rochalimae ITS* DNA sequence originated from flea collected from rat and identified according to BLAST

```
TTGAACCTCC GACCTCACG CTTATCAAG CGCGCGCTC  TAACCGACT GAGCTACAA           60
GCCCTACGAG GAAAACtGA AAACCTTCA ATCGCATAA  TCTGATcaA AGAAATAAT           120
ATCTTCcNTC CCCCTTAGA GGCTTGTAT AAACAGATC  GCCAGAAGG CTTGGGATC           180
ATNATCT
```

Sequuence A7: *B. henselae gltA* DNA sequence originated from flea collected from cat and identified according to BLAST

```
TGTTTACTGT TCTTTTTGCA TTAGGTTCTG TTGAAAGAAT TCCtGactTC aTTGCACGTG        60
CAAAAGATAA AAATGATTCT TTCCGCCTTA TGGGTTTTGG TCATCGAGTC TATAAAAATT       120
ATGATCCACG CGCAAAAATC ATGCAACAAA CCTGCCATGA GGTTTTAAAA GAATTGAACA       180
TTCAAAATGA TCCACTTCTT GATATTGCTA TCACGCTTGA AAATATTGCT CTAAATGATG       240
AATATTTTAT TGAAAAAAAA CTTTACCCTA ATGTCGATTT CTATTCTGGC ATTACATTAA       300
AAGCTCTAGG ATTTCCAACA GAAAAAGTTT TTTTTCaATA AAATATTCaT CATTTAGAGC       360
AaTATTTTCA AGCGTGATAG CAATATCAAG AAGTGGATCA TTTTGAATGT TCAATTCTTT       420
TAAAACCTCA TGGCAGGTTT GTTGCATGAT TTTTGCGCGT GGATCATAAT TTTTATAGAC       480
TCGATGACCA AAACCCATAA GGCGGAAAGA ATCATTTTTA TCTTTTGCAC GTGCAATGAA       540
TTCAGGAATT CTTTCAACAG AACCTATTTC TTGTAACATT TTTAGGCATG CTTCATTANC       600
TCCACCATGA GCTGGTCCCC A                                                 621
```

Sequuence A8: *B. koehlera gltA* DNA sequence originated from flea collected from cat and identified according to BLAST

```
GGGGACCAGC TCATGGTGGA GCCAATGAAG CATGCCTAAA AATGTTNCAA GAAATAGGTT        60
CTGTTGAAAG AAT-CCCT-C AATTCATTGC ACGTGCAAAA GATAAAAATG ATTCTTTCCG       120
CCTTATGGGT TTTGGTCACC GAGTCTATAA AAATTATGAT CCACGTGCAA AAATTATGCA       180
ACAAACTTGT CATGAGGTTT TAAAAGAACT AAACATTCAA GATGATCCAC TTCTTGATAT       240
TGCTATCGCA CTTGAAAATA TTGCCCTGAA TGATGAATAT TTTGTTGGAA AAAAACTTTA       300
CCCTAATGTC GATTTCTATT CTGGTATTAC ATTAAAAGCT TTAGGTTTTC CAACTGAAAT       360
GTTTACTGTT CTTTTTGCAT TA                                                382
```

Sequuence A9: *B. clarridgeiae gltA* DNA sequence originated from fleas collected from rat and identified according to BLAST

```
TGGGGACCAG CTCATGGTGG TGCTAATGAA ACATGTCTAA AAATGCTGCA AGAAATAGGC        60
ACTGTTCAAA AAATTCCTGA GTTTATTGCA CGCGCAAAAG ACAAAAATGA TCGTTTCCGT       120
CTTATGGGTT TTGGTCATCG TGTCTATAAA AATTATGATC CACGTGCGAA AATTATGCAA       180
CAAACTTGCC ATGAAGTCTT AAAAGAACTC AATATCCAAG ATGATCCACT TCTTGATATC       240
GCTATGGAAC TTGAAAAAAT TGCTTTGAAT GATGAATACT TTATTGAAAA AAAGCTTTAT       300
CCTAATGTTG ATTTCTATTC TGGTATTACA TTAAAAGCCT TAGGCTTCCC GACTGAAATG       360
TTTACTGTTC TTTTTGCATT A                                                 381
```

Sequence A10: *Wolbachia endosymbiont of Nasonia longicornis gltA* DNA sequence originated from fleas collected from dog and identified according to BLAST

```
TGTTTACTGT TCTTTTTGCA TTGTtACAAA GGCCaAAGAT GACAAGGATC CTTTCaAGTT        60
AATGGGATTT GGTCATCGCG TTTACAAAAA TTATGATCCT CGTGCACTCA TATTGAAAGA       120
CGCGTGCGAT GAAATTCTAA ATAAATCCAC ACAAAACAAT AAACTGCTTG AGATTGCAAT       180
AGAGCTTGAG AAAATTGCTT TAAGAGATGA GTATTTTGTC GAGCGCAAGT TATACCCAAA       240
TGTTGATTTT TATTCAGGTA TAATAATGAA TGTTATTGGC ATTCCTGCAA GCAAACTTGC       300
GCTCGACAAA ATACTCATCT CTTAAAGCAa TTTTCTCAAG CTCTATTGCA ATCTC            355
```

Sequence A11: *B. henselae rpoβ* DNA sequence originated from flea collected from cat and identified according to BLAST

```
GNTTACTTNG TATGGAGCNT GCGATAAAAG AGNGTATGTC CTCGGTTGAA ATTGATACCG        60
TCATGCCACA GGATTNGATT AACGCAAAGC CAGCTGCTGC AGCTGTTCGC GAATTTTTTG       120
GGTCTTCGCA ATTATCACAG TTTATGGATC AAACCAACCC ATTATCGGAA ATTACCCATA       180
AGCGGCGTCT TTCTGCTCTT GGTCCAGGTG GTTTAACCCG TGAACGGGCA GGTTTTGAAG       240
TTCGTGACGT ACATCCTACA CATTATGGTC GTATTTGCCC GATTGAAACG CCGGAAGGTC       300
CTAATATTGG TCTGATTAAT TCCTTAGCAA CCTTTGCGCG TGTTAATAAA TATGGTTTTA       360
TTGAAAGTCC ATACCGCAAA ATTATTGATG GGAAAGTGAC AACGGAAGTT ATTTATCTTT       420
CTGCTATGGA AGAGTCAAAA CACTATGTGG CTCAGGCTAA TTCTTCCTTA GATGCTGAAG       480
GACGTTTGTC AGAAGAGTTT GTTGTTTGCC GTCATGCAGG TGAAGTTTTG ATGGCTCCGC       540
GCGATCATGT TGATTTGATG GATGTTTCAC CAAAACAGTT GGTTTCAGTA GCTGCTGCTC       600
TTATTCCGTT TTTGGAAAAT GATGATGCGA ATCGTGCGTT GATGGGATCT AATATGCAGC       660
GTCAGGCAGT TCCACTTGTA CGTGCTGAAG CACCATTTGT TGGTACGGGA ATGGAGTCAA       720
TAGTAGCTCG CGATTCAGGG GCTGCTGTTG CTGCAAGGCG TAGTGGTATT GTTGATCAAG       780
TTGATGCAAC ACGTATTGTT ATTCGTGCGA CAGAAGATTT AGATCCTTCA AAATCTGGTG       840
TTGATATTTA TCGNTNGNAG AAATTTCAGC GTTCTAATNN GTNCTACN                    888
```

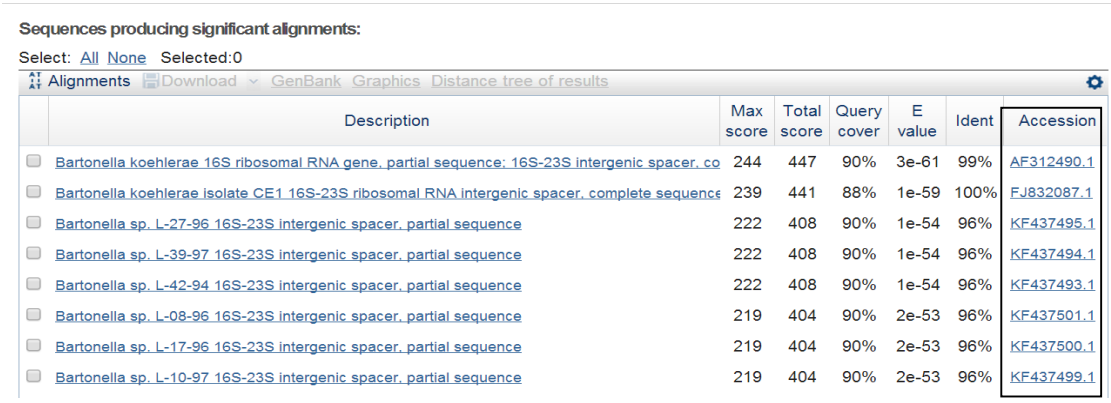
Sequence A12*:B. koehlerae rpoβ* DNA sequence originated from flea collected from cat and identified according to BLAST

```
CTTNGTATGG AGCGTGCGAT AAAAGAACGT ATGTCCTCGG TTGAAATTGA TACCGTCATG        60
CCACAGG-AT TTGATTAACG CAAAGCCAGC CGCTGCAGCT GTTCGCGAAT TTTTTGGATC       120
TTCGCAATTA TCGCAGTTTA TGGACCAAAC CAACCCCTTG TCAGAAATTA CCCATAAGCG       180
CCGTCTTTCT GCTCTTGGTC CAGGTGGTTT AACCCGCGAA CGAGCAGGTT TTGAAGTTCG       240
TGATGTACAT CCTACGCATT ATGGTCGTAT TTGCCCAATT GAAACTCCGG AGGGTCCTAA       300
TATTGGTCTG ATTAATTCCT TAGCAACCTT TGCGCGTGTT AATAAATATG GTTTTATTGA       360
AAGTCCATAC CGCAAAATTA TTGATGGAAA AGTGACAACA GAAGTTATTT ATCTTTCTGC       420
TATGGAAGAG TCAAAACATT ATGTGGCTCA AGCTAATTCT TCCTTAGATG TTGAAGGACG       480
TTTTTCAGAA GAGTTTGTTG TTTGCCGTCA TGCAGGTGAA GTTTTGATGG CACCACGCGA       540
TCATGTCGAT TTGATGGATG TTTCACCAAA ACAGTTGGTT TCAGTAGCTG CTGCTCTTAT       600
TCCGTTTCTG GAAAATGACG ATGCGAATCG TGCGTTAATG GGATCGAATA TGCAGCGTCA       660
GGCAGTACCA CTTGTACGCG CTGAGGCACC ATTTGTTGGT ACGGGAATGG AATCAATAGT       720
TGCACGCGAT TCAGGAGCTG CTGTTGCTGC AAAACGTAGT GGTATTGTTG ATCAAGTTGA       780
TGCAACGCGT ATTGTTATTC GTGCGACAGA AGATTTAGAT CCTTCAAAAT CTGGTGTTGA       840
TATTTATCGN TTGCAGAANT TTCANCGTTC TAATCAGTCT ACA                         883
```

Appendix B

BLAST analysis of *ITS* sequences of different *Bartonella* species obtained in this study compared with reference DNA sequences of *Bartonella spp* deposited in the GeneBank

B1: BLAST of *B. koehlera ITS* sequence against reference strains sequences. (The accession numbers are marked with black square).



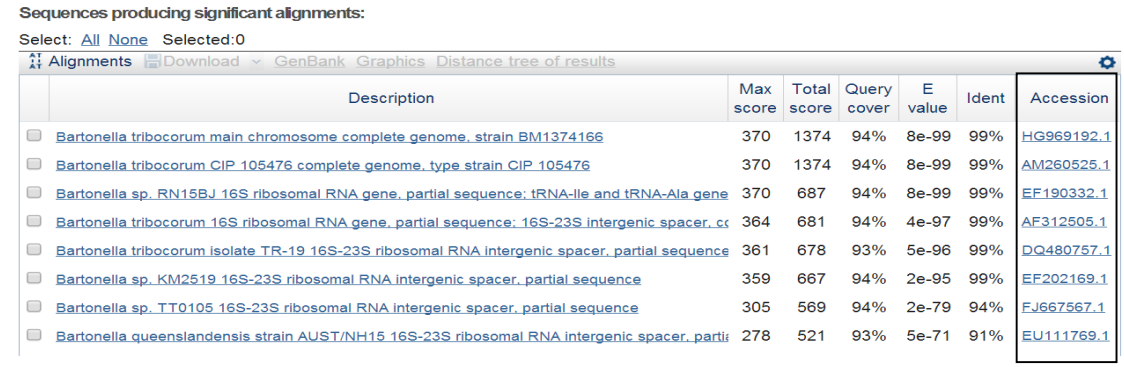
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bartonella koehlerae 16S ribosomal RNA gene, partial sequence; 16S-23S intergenic spacer, co	244	447	90%	3e-61	99%	AF312490.1
Bartonella koehlerae isolate CE1 16S-23S ribosomal RNA intergenic spacer, complete sequence	239	441	88%	1e-59	100%	FJ832087.1
Bartonella sp. L-27-96 16S-23S intergenic spacer, partial sequence	222	408	90%	1e-54	96%	KF437495.1
Bartonella sp. L-39-97 16S-23S intergenic spacer, partial sequence	222	408	90%	1e-54	96%	KF437494.1
Bartonella sp. L-42-94 16S-23S intergenic spacer, partial sequence	222	408	90%	1e-54	96%	KF437493.1
Bartonella sp. L-08-96 16S-23S intergenic spacer, partial sequence	219	404	90%	2e-53	96%	KF437501.1
Bartonella sp. L-17-96 16S-23S intergenic spacer, partial sequence	219	404	90%	2e-53	96%	KF437500.1
Bartonella sp. L-10-97 16S-23S intergenic spacer, partial sequence	219	404	90%	2e-53	96%	KF437499.1

B2: BLAST of *B. tribocorum ITS* sequence against reference strains sequences. (The accession numbers are marked with black square).



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
Bartonella tribocorum main chromosome complete genome, strain BM1374166	370	1374	94%	8e-99	99%	HG969192.1
Bartonella tribocorum CIP 105476 complete genome, type strain CIP 105476	370	1374	94%	8e-99	99%	AM260525.1
Bartonella sp. RN15BJ 16S ribosomal RNA gene, partial sequence; tRNA-Ile and tRNA-Ala gene	370	687	94%	8e-99	99%	EF190332.1
Bartonella tribocorum 16S ribosomal RNA gene, partial sequence; 16S-23S intergenic spacer, c	364	681	94%	4e-97	99%	AF312505.1
Bartonella tribocorum isolate TR-19 16S-23S ribosomal RNA intergenic spacer, partial sequence	361	678	93%	5e-96	99%	DQ480757.1
Bartonella sp. KM2519 16S-23S ribosomal RNA intergenic spacer, partial sequence	359	667	94%	2e-95	99%	EF202169.1
Bartonella sp. TT0105 16S-23S ribosomal RNA intergenic spacer, partial sequence	305	569	94%	2e-79	94%	FJ667567.1
Bartonella queenslandensis strain AUST/NH15 16S-23S ribosomal RNA intergenic spacer, parti	278	521	93%	5e-71	91%	EU111769.1

B3: BLAST of *B. elizabethae ITS* sequence against reference strains sequences. (The accession numbers are marked with black square).

Bartonella sp. RN28BJ 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA inter	346	641	94%	1e-91	98%	EF213776.1
Bartonella sp. RN26-SBJ 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA in	344	637	94%	5e-91	98%	EF407570.1
Bartonella sp. RN25BJ 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA inter	303	563	94%	8e-79	94%	EF407566.1
Bartonella sp. RN17BJ 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA inter	303	563	94%	8e-79	94%	EF213772.1
Bartonella sp. Rr238ug 16S-23S ribosomal RNA intergenic spacer, partial sequence	300	552	78%	1e-77	98%	JX428752.1
Uncultured Bartonella sp. clone Ac 16S-23S ribosomal RNA intergenic spacer, partial sequence	300	554	80%	1e-77	97%	FJ686049.1
Bartonella sp. Tel Aviv Rr 16S-23S intergenic spacer, partial sequence	298	552	94%	4e-77	93%	FJ577653.1
Bartonella sp. RN29BJ 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA inter	298	552	94%	4e-77	93%	EF407569.1
Bartonella sp. RN27BJ 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA inter	298	552	94%	4e-77	93%	EF213775.1
Bartonella sp. RN14BJ 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA inter	298	552	94%	4e-77	93%	EF213770.1
Bartonella sp. RN3BJ 16S ribosomal RNA gene, partial sequence; tRNA-Ile and tRNA-Ala genes.	298	552	94%	4e-77	93%	EF190331.1
Bartonella elizabethae 16S-23S intergenic spacer region, transfer RNA-Ile and transfer RNA-Ala g	298	552	94%	4e-77	93%	L35103.1
Bartonella sp. RN34BJ 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA inter	292	547	94%	2e-75	93%	EF407568.1

B4: BLAST of *B. rochalimae ITS* sequence against reference strains sequences. (The accession numbers are marked with black square).

Sequences producing significant alignments:

Select: All None Selected:0

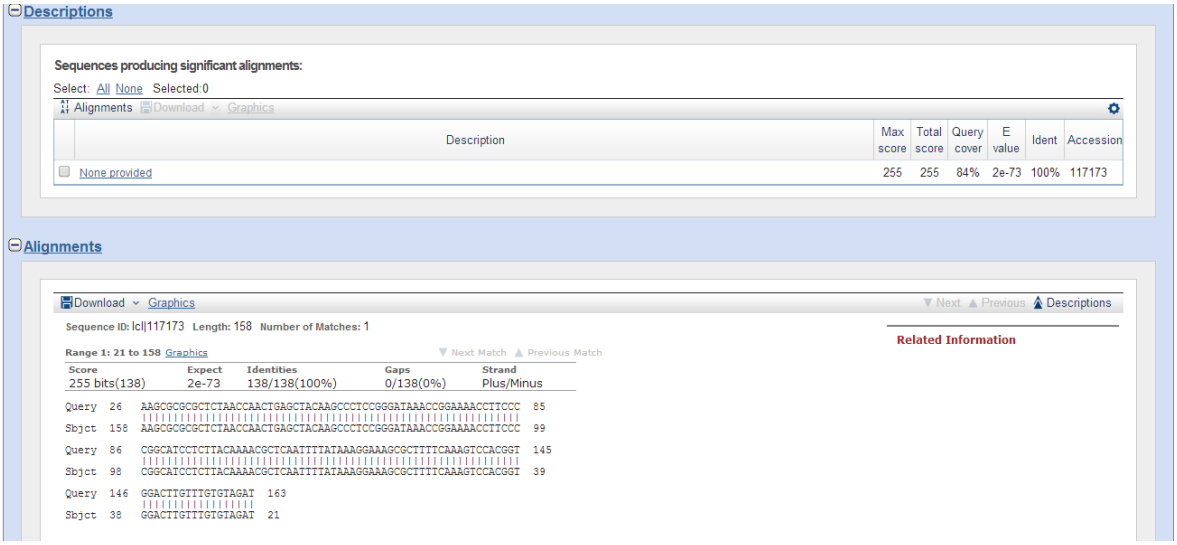
Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bartonella sp. 1-1C contig19, whole genome shotgun sequence	305	305	100%	8e-80	98%	FN645504.1
Bartonella sp. 1-1C contig18, whole genome shotgun sequence	305	305	100%	8e-80	98%	FN645503.1
Bartonella rochalimae ATCC BAA-1498 Contig12, whole genome shotgun sequence	300	900	100%	4e-78	98%	FN645466.1
Uncultured Bartonella sp. 16S-23S ribosomal RNA intergenic spacer, partial sequence; and tRNA	300	300	100%	4e-78	98%	EU191619.1
Uncultured Bartonella sp. clone BFI7688 16S-23S ribosomal RNA intergenic spacer, partial seque	298	298	96%	1e-77	99%	AF415211.1
Bartonella rochalimae 16S-23S ribosomal RNA intergenic spacer, partial sequence; tRNA-Ile gene	261	261	84%	2e-66	99%	HQ185696.1
Bartonella sp. LEV 16S-23S ribosomal RNA intergenic spacer, partial sequence	261	261	84%	2e-66	99%	EF614392.1
Bartonella sp. AR 15-3 contig18, whole genome shotgun sequence	255	511	100%	9e-65	93%	FN645485.1

Appendix C

BLAST analysis and multiple DNA sequence alignment of *ITS* sequences of *Bartonella* species obtained from this study.

C1: Sequence homology of *B. koehlerae* (this study) compared with local strain of *B. henselae* from Israel (Ohad *et al* 2010[53]).



C2: Multiple alignments of all *ITS* sequences obtained in this study. Red dots represent similarities between nucleotides. Blue and black colors show nucleotides differences between *Bartonella spp*.