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Metagenomic Profile of the Bacterial Communities Associated With Ornithodoros Muesebecki (Acari: Argasidae) Ticks On Socotra Cormorant Colony in the United Arab Emirates and Presence of Three Important Pathogenic Groups in Them

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United Arab Emirates University

College of Science

Department of Biology

METAGENOMIC PROFILE OF THE BACTERIAL COMMUNITIES ASSOCIATED WITH *ORNITHODOROS MUESEBECKI* (ACARI: ARGASIDAE) TICKS ON SOCOTRA CORMORANT COLONY IN THE UNITED ARAB EMIRATES AND PRESENCE OF THREE IMPORTANT PATHOGENIC GROUPS IN THEM

Raheel Nasser Mohammed Hmoud Alkayyoomi

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Environmental Sciences

Under the Supervision of Dr. Mohammad Ali Al-Deeb

April 2018

Declaration of Original Work

I, Raheel Nasser Mohammed Hmoud Alkayyoomi , the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Metagenomic Profile s s Ornithodoros Muesebecki (Acari: Argasidae) Ticks on Socotra Cormorant Colony in the United Arab Emirates and Presence of Three Important Pathogenic Groups in Them*", hereby, solemnly declare that this thesis is the original research work that has been done and prepared by me under the supervision of Dr. Mohammad Ali Al-Deeb, in the College of Science at UAEU. This work has not previously been presented or published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Ticks are well known to transmit various pathogens including bacteria, viruses and protozoa to humans and animals. The soft tick (*Ornithodoros muesebecki*) was common on a breeding colony of the Socotra cormorant (*Phalacrocorax nigrogularis*) in Siniya Island, the United Arab Emirates (UAE). The aims of the study were: i) investigating the prevalence and conduct genetic characterization of the important bacterial pathogens Borrelia spp. (causal agents of relapsing fever), *Rickettsia* spp. (causal agents of spotted fever), and *Coxiella burnetii* (causal agent of Q fever); ii) understanding the overall bacterial community associated with *O. muesebecki* by using Illumina-based metagenomic approach; and iii) establishing a molecular record of *O. muesebecki* based on molecular markers. Ticks were collected from the largest breeding colony of Socotra Cormorant in 2013 and 2016. Subsequently, genomic DNA was extracted from each tick, and conventional PCR assays were used to detect certain pathogens. *Borrelia* spp. and *Rickettsia* spp., were not detected. However, PCR assay and metagenomic analysis indicated the presence of the *Coxiella* genus. Sequencing results revealed 809 bacterial operational taxonomic units (OTUs) within the five samples from 2013 and 2686 OTUs within the 5 samples from 2016. Metagenomic analysis showed that *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* are the most dominant phyla. *C. burnetii* was the most prevalent species in all samples in 2013 and 2016. This data provides the complete picture to date of the bacterial communities present within *O. muesebecki* under natural conditions in the UAE using high-throughput sequencing technologies. In addition, this study provided the first DNA molecular record of *O. muesebecki* in GenBank. Further investigations regarding the functional role of *Coxiella* in seabird colonies is needed.

Keywords: Metagenomics; Socotra cormorant; *Ornithodoros muesebecki; Coxiella burnetii* Bacterial community diversity; Next-generation sequencing; Illumina.

Title and Abstract (in Arabic)

توصيف التحليل الميتاجينوميكي للمجتمعات البكتيرية المرتبطة بالقراد اللين اللاذع (*Ornithodoros muesebecki*) على مستعمرة للطائر السقطري في دولة الإمارات العربية المتحدة

الملخص

يُعْرف القراد بأنه ناقل لمسببات الأمراض مثل البكتيريا والفيروسات والطفيليات للإنسان والحيوان. ينتشر القراد اللين اللاذع (*O. muesebecki*) على نطاق واسع في بيئة الطائر السقطري في جزيرة سينية في امارة أم القوين في دولة الإمارات العربية المتحدة. تهدف هذه الاطروحة الى (1) البحث في انتشار ثلاث مجموعات من البكتيريا المسببة للأمراض وتوصيفها جينيا؛ منها بكتيريا (*Borrelia* spp.) المسببة لمرض الحمى الراجعة و (*Rickettsia* spp.) المسببة لمرض الحمى المبقعة وبكتيريا (*Coxiella burnetii*) المسببة لمرض حمى كيو Q؛ (2) التعرف على كافة مجتمعات البكتيريا المرتبطة في القراد وذلك باستخدام تقنية التحليل الميتاجينوميكي؛ و (3) دراسة التوصيف الجيني للقراد اللين اللاذع. من خلال هذه الدراسة، تم جمع عينات القراد من أكبر مستعمرة للطائر السقطري في عامي 2013 و2016. ومن ثم، تم استخلاص الحمض النووي من كل عينة باستخدام التفاعل البولميرزي المتسلسل PCR للكشف عن البكتيريا الممرضة. لم يتم الكشف عن أي بكتيريا من صنف البوريليا والريكتسيا، لكن نتائج التحليل التسلسلي والميتاجينوميكي أشارت إلى وجود بكتيريا من نوع الكوكسيلا. وكشفت نتائج التحليل التسلسلي عن وجود 809 وحدة تصنيفية بكتيرية ضمن الخمس عينات من عام 2013 و2686 وحدة تصنيفية بكتيرية ضمن الخمس عينات من عام 2016. وأظهر التحليل الميتاجينوميكي أن المجموعات البكتيرية Firmicutes، *Proteobacteria* وBacteroidetes كانت أكثر الشعب انتشارا. وأن بكتيريا الكوكسيلا بورنيتي كانت أكثر نوع انتشارا في جميع العينات في عامي 2013 و2016. هذه البيانات وفرت أدق صورة حتى الآن للمجتمعات البكتيرية الموجودة في قراد *O. muesebecki* تحت الظروف الطبيعية لدولة الإمارات باستخدام تقنيات للتحليل التسلسلي عالية الانتاج. بالإضافة إلى ذلك، قدمت هذه الدراسة أول سجل جزيني للحمض النووي لقراد *O. muesebeck* في بنك الجينات Genebank. بناء على هذه الدراسة، هناك ضرورة لإجراء دراسات أخرى بشأن الدور الوظيفي لبكتيريا الكوكسيلا في مستعمرات الطيور البحرية.

مفاهيم البحث الرئيسية: التحليل الميتاجينوميكي؛ القراد اللين اللاذع المسبب للأمراض؛ الطائر السقطري؛ تنوع المجتمع البكتيري؛ الجيل القادم من التحليل التسلسلي؛ إلومينا.

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I would first like to thank both Dr. Mohammad Al-Deeb and Dr. Sabir bin Muzaffur for their help and guidance. I would also like to thank Dr. Mohammad Taher, Dr. Gaber Ramadan, Dr. Mohammad Anan and Ms Raja Al Muskari who assisted me with my laboratory work. I am grateful for your help and support. My sincere thanks extended to Ahmed Taha (Library Research Desk) for his thorough work of editing and proofreading.

I appreciate my wonderful family for their constant encouragement and support, in particular, my mother, Azza Saif AlKayoumi. She is the greatest mom, an extremely good listener and a good thinker too. Whenever I feel I would like to talk to someone, she is the one who immediately comes to my mind. She always cultivates positive thoughts in my mind, and I never start my day without her prayers. She taught me that I should keep on learning and make some mistakes in this life because as she says, 'you won't learn if you haven't made mistakes in your life'. I would like to associate myself with Jubran Khalil Gibran, the Lebanese scholar, who said that '*The three most beautiful women in the world: my mother, her shadow, and her reflection*'. I totally agree with him that you are a truly beautiful woman on the inside and out. May Allah bless you and protect you.

I would like to thank my friends and colleagues. Their contagious energy motivated me every single day. Their precious words were the fuel which enabled me to accomplish my project. I will never forget their empathy toward me when I felt down. They strengthened me and took out the pain inside my soul. *Thank you*!

Acknowledgements

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Dedication

This thesis is dedicated to my beloved family, with love and respect

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List of Abbreviations

AL	Animal Buffer
ATL	Animal Tissue Lysis
BCL	Base Calls
BLAST	Basic Local Alignment Search Tool
CDC	Centre for Disease Control and Prevention, The USA
COI	Cytochrome Oxidase Subunit I
EDTA	Ethylene-Diamine-Tetra-Acetic Acid
ICD	Isocitrate Dehydrogenase Gene
IUNCN	International Union for Conservation of Nature
LB	Lyme Borreliosis
MCS	Miseq Control Software
MSF	Mediterranean Spotted Fever
OTUs	Operational Taxonomic Units
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
QC	Quality Control
RDP	Ribosomal Database Project
RMAF	Rocky Mountain Spotted Fever, The USA
RTA	Real-Time Analysis
SEM	Scanning Electron Microscope
SFG	Spotted Fever Group
STG	Scrub Typhus Group
TBE	Tick-Borne Encephalitis
TrBE	*Tris*-Borate-EDTA
TBP	Tick-Borne Pathogen
NCBI	National Centre for Biotechnology Information
NGS	Next Generation Sequencing

Chapter 1: Introduction

1.1 Overview

Ticks are vectors of many tick-borne pathogens that can affect human and animal health worldwide. Recently, tick-borne diseases have become significant concerns in epidemiological studies especially the bacterial diseases. Tick-borne bacterial diseases are more diverse than any other tick-borne group such as *Borrelia*, *Rickettsia*, *Francisella, Ehrlichia, Anaplasma* and *Coxiella* (Noda *et al.*, 1997). Migratory birds are hosts and act as vectors of tick-borne pathogens. They play a significant role in maintenance and transmission of many infectious diseases including *Borrelia*, *Rickettsia* and *Coxiella burnetii*. The recent development of molecular techniques such as high-throughput sequencing allows us to understand the microbial communities in vectors and reservoirs (Hiergeist *et al.*, 2016).

1.2 Statement of the Problem

Ticks are known disease vectors worldwide. In the UAE Socotra Cormorant birds have been reported to be infested by soft ticks. The hypothesis is that ticks on Socotra Cormorant birds in the UAE have disease-causing agents, which may pose a threat to people living or working in nearby areas. This concern initiated this study to fulfil the following objectives:

1. To investigate the prevalence and conduct genetic characterization of the important bacterial pathogens Borrelia spp. (causal agents of relapsing fever), *Rickettsia* spp. (causal agents of spotted fever), and *Coxiella burnetii* (causal agent of Q fever).

2. Studying metagenomic profile of the bacterial communities associated with argasid ticks (*O. muesebecki*) from a Socotra Cormorant colony in the Emirate of Umm Al Quwain, the United Arab Emirates.
3. Establishing a molecular of the tick record based on molecular markers.

1.3 Tick Taxonomy

Ticks are small obligate blood-feeding ectoparasites that infect vertebrates and distributed in almost every region in the world. More than 850 different species have been discovered grouped in two main families, soft ticks (Argasidae) and hard ticks (Ixodidae) (Black and Piesmant, 1994). The third family is (Nuttalliellidae) with only one single species *Nuttalliella namaqua* found in South Africa and Tanzania share similar structures of both Argasidae and Ixodidae in addition of some unique features (Black and Piesmant, 1994; Estrada-Peña *et al.*, 2010). Interestingly, South Africa alone has been demonstrated 80 ixodid , 25 argasid species and *N. namaqua* since 1908 where 25 of ixodid and two of argasid are restricted to this area (Horak, 2009).

Ticks were classified by using conventional techniques such as morphological features, life histories, and host associations (Black and Piesmant, 1994). Although the molecular analysis and phylogenic approach of *Ixodida* were used, and record but ticks are still classified according to their morphological characteristics (Nava *et al.*, 2009; Guglielmone *et al.*, 2010). Several systematic types of research in term of the genus–level classification of the family Ixodidae have been intensely studied and published in large scale because of its role in the transmission of pathogens (Estrada-Peña *et al.*, 2010); however, the genus–level taxonomy of the family Argasdae, which consist of 193 species still remains unclear due to inadequate studies on stable morphological features and the disagreements at the genus level between taxonomy

schools makes the determination hard to be defined (Estrada-Peña *et al.*, 2010; Estrada-Peña, 2015). Additionally, lots of Argasidae species have been ignored.

This finding clearly explained by (Barros-Battesti *et al.*, 2013) who had proved the difficulty to distinguish between adult and nymphal stages in some *Aragasid* species in particular *Ornithodoros* due to morphological similarity among them and little data at genetic level turn studies to use larval morphological features as standard. Consequently, the phylogeny of the *Argasidae* group is less specific than that of the Ixodidae and most species of *Argasidae* can be categorized into more than one genus. (Guglielmone *et al.*, 2010) remarks that 133 out of 193 *Argasid* species lack correctly generic classification. Ticks based on morphological characteristics are belonging to:

Phylum: Arthropoda

Class: Arachnida (spiders and scorpions)

Subclass: Acari (mites)

Order: Parasitiformes

Suborder: Ixodida

Family-1: Argasidae (soft ticks)

Family-2: Ixodidae (hard ticks)

Family-3: Nuttalliellidae (*N. namaqua*)

1.4 Morphology

The tick body is divided into two sections (Sonenshine, 2009). The capitulum is anterior; contain the mouthparts, and the posterior is idiosoma, which contain the legs, digestive tract and reproductive organs. The capitulum consists of specialized feeding structures called hypostome, used to penetrate host's skin and to suck blood

and the chelicerae, a pair of appendages located in front of the mouth. On the upper surface of each foreleg, a sensorial organ present called Haller's organ used for host identification. In argasid ticks, the capitulum is beneath the anterior end of the body and is not visible from above. Ticks start with three pairs of legs at the larval stage, and it becomes four pairs at adult stage (Brites-Neto *et al.*, 2015).

The Ixodidae can be easily identified by the presence of the sclerotised scutum, a thick plate located on the dorsal body surface that almost covers the entire back of the male but only partly covers the female (Estrada-Peña, 2015). Soft ticks; however, lack of a scutum, so the sexes look alike.

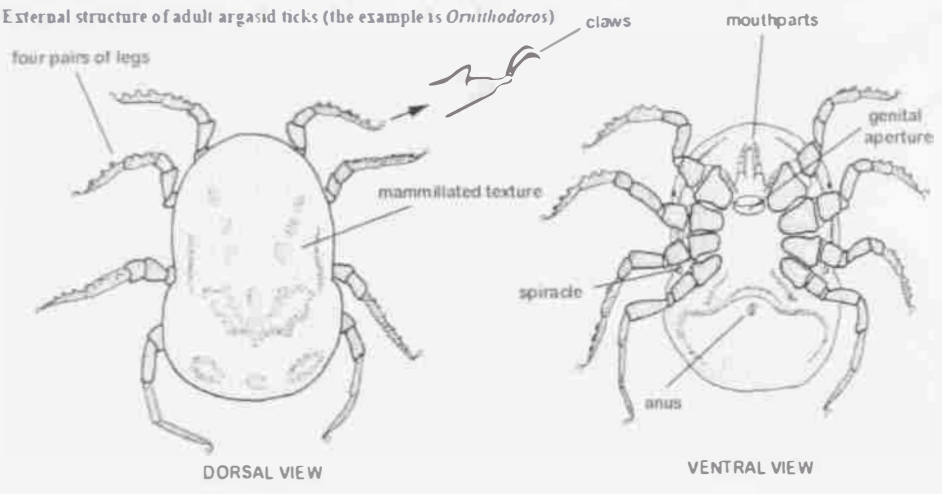

Figure 1: External structure of adult argasid ticks (e.g., *Ornithodoros*)

(Walker, 2003)

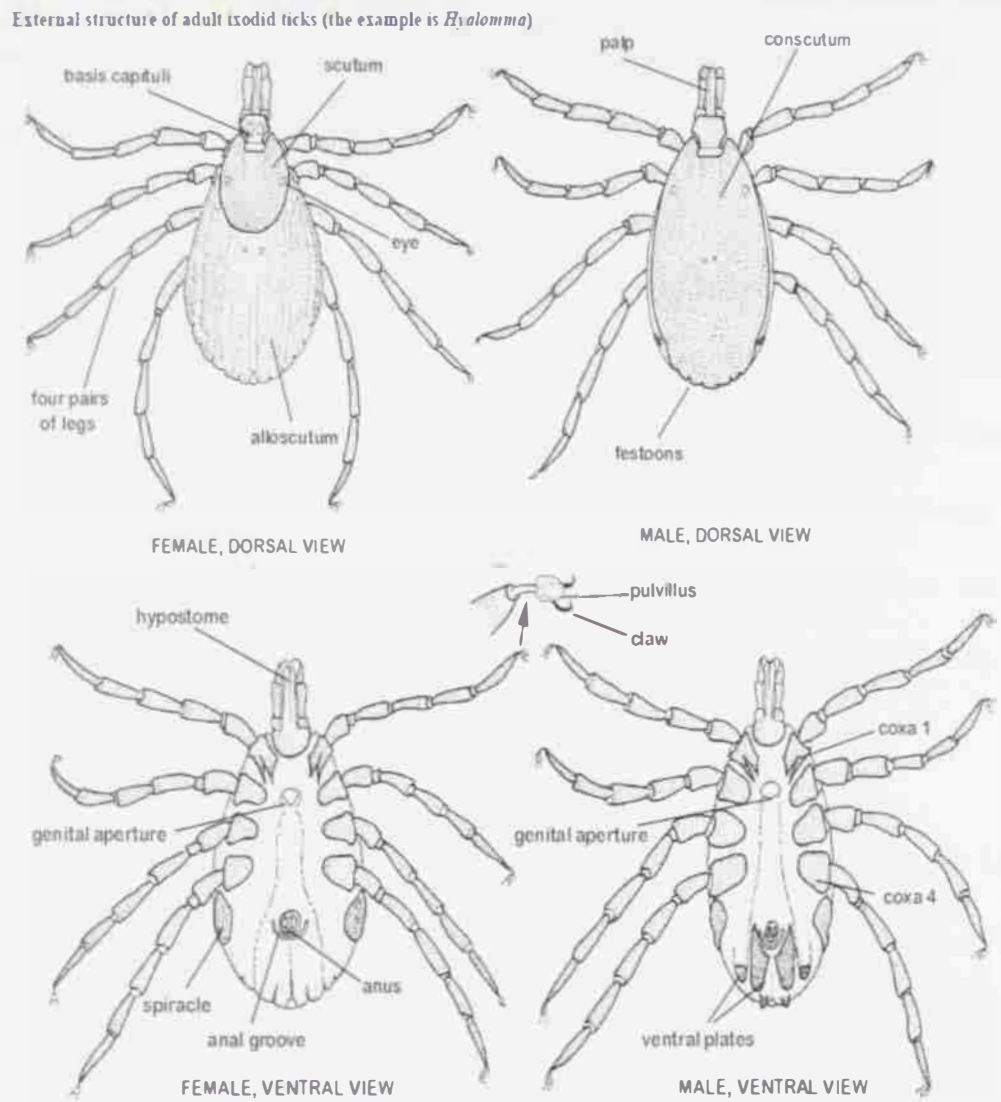

Figure 2: External structure of adult Ixodid ticks (e.g., *Hyalomma*)

(Source: Walker, 2003)

1.5 Tick Biology

Ticks have a complex life cycle, and all life stages of ticks are obligate blood feeders (Estrada-peña *et al.*, 2013). The developmental stages of ticks consist of eggs and three necessary stages namely, larva, nymphal and adult (male and female) (Sonenshine, 2009). Ixodid and argasid ticks differ in life stages. The former has only one single nymphal instar. In contrast, argasid tick life cycle has multiple numbers of nymphal stages (Manzano-román *et al.*, 2012). All ticks obtain the blood meal from the host during some or all stages to moult to the next life stage and for a female tick to lay eggs (Estrada-Peña, 2015). Additionally, at each blood meal, the tick has the opportunity to transmit the pathogens among hosts from infected to the new one. Mostly, males stay on the host and mate with several females, but some species mate in vegetation while questing for a host (Estrada-peña *et al.*, 2013).

1.5.1 Life Cycles of Ixodid Ticks

Generally, Ixodid tick goes through three primary life stages; larva which hatches from eggs, one single nymph and the adult. Most of the Ixodid species exhibit a three-host life cycle which seeks three separate hosts in each active stage (Walker *et al.*, 2003; Estrada-Peña, 2015). A few hard tick species exhibit either a two host-life cycle where the life cycle is completed in two different hosts or only one host-life cycle. After each blood meal, the tick drops to the ground, moults and finds a new host. The feeding process in hard ticks goes slowly, and duration varies from several days too long periods. Usually, the mating occurs during feeding, but some species may mate in vegetation or in the nest. Following mating, the adult females drop from their hosts into the leaf litter to lay thousands of eggs under the suitable environmental condition to ensure their survival and finally the adult female die.

Once oviposition has been completed, the larvae disperse into the vegetation or nest to seek hosts. After they attached to a host, the larvae start feeding slowly, feeding usually takes few days. The engorged larvae drop from their hosts and find a sheltered microhabitat such as soil or leaf litter, or in host nests to moult to nymph. Then, nymph attaches to another host for feeding and return to the ground for a further moult. The final stage is the adult in which it attaches to a third host for feeding and mating. More than 90% of the Ixodid life cycle is spent off the host.

1.5.2 Life Cycles of Argasid Ticks

The *Argasidae* life cycle is entirely different from that of the Ixodidae. Argasid life cycle involves numbers of nymphal instar vary from two to eight depends on the species and the quality and the amount of blood ingested (Vial, 2009). This characteristic and their ability to resist starvation allow the argasid ticks to live for many years (Manzano-román *et al.*, 2012). Thus, the soft tick life cycle may take from 10 to 20 years (Sonenshine, 2009). The feeding behaviour is very rapid among soft tick, especially on nymphs and adults which feed within 15-60 minutes while it takes a longer time on larvae which feed for 12 hours to several days (Vial, 2009).

Argasid ticks are able to survive for long periods between blood meals from months to several years depending on host availability. Each immature stage obtains at least one blood meal on a vertebrate host before moulting, except larvae of some *Ornithodoros* species like *O. moubata*, which directly moult to the nymphal stage without feeding. The soft tick adults have long lifespan reach up to 25 years for some species. The majority of argasid tick larvae seek hosts, feed rapidly, then drop off from their host and moult to the first nymphal instar (Sonenshine, 2009). The first nymph seeks hosts again, feeds rapidly then moults to further nymphal stage. This

continued process of host-seeking, feeding and moulting may create a number of nymphal instars prior to the formation of adults. After the last nymphal moult, an adult is formed, and then it feeds rapidly on an individual host, then produces a small batch of eggs from each blood meal. Usually, mating occurs off the hosts. Figure 3 illustrates the typical life-cycle of the soft tick.

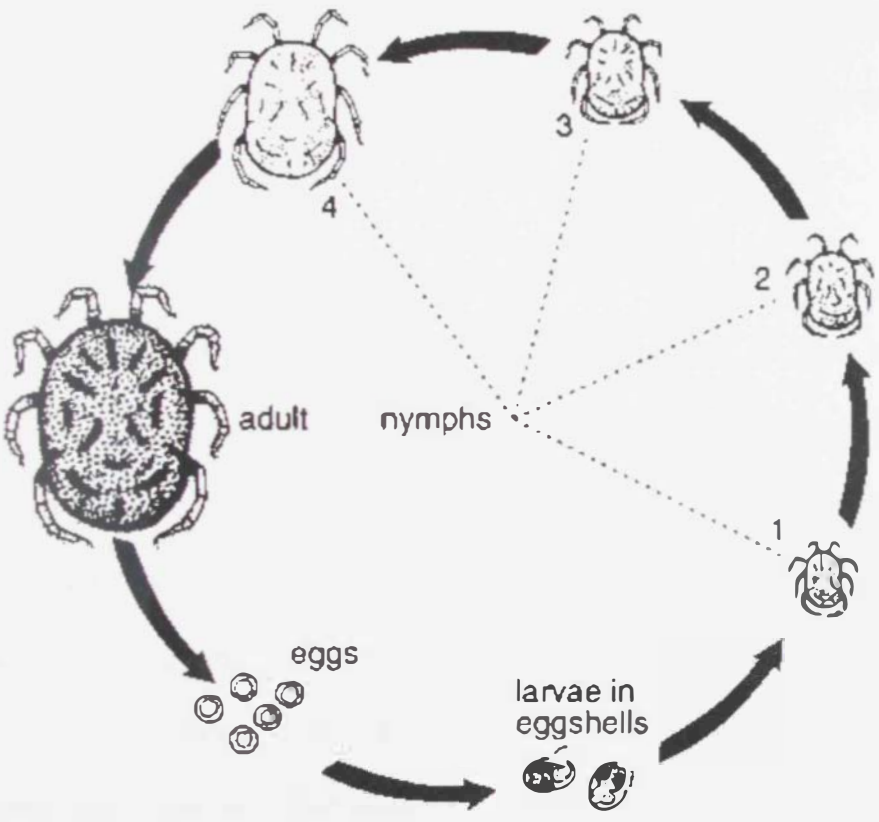

Figure 3: A typical life-cycle of a soft tick

(Source: Vial, 2009)

1.6 Tick Ecology

All Argasids ticks and some Ixodid ticks of the genus *Ixodes* possess endophilic behaviour, where they restricted to the shelters of their hosts (Manzano-román *et al.*, 2012). These species mainly survive away from the weather variables; living in caves, burrows, houses, cracks, and crevices occupied by hosts and feeding when the host arrives. These features give them the ability to complete their life cycle (Vial, 2009). Since endophilic species live in close proximity to their hosts, they do not exhibit seasonal activity, and they show indiscriminate host feeding which they are able to feed in too many hosts. Ixodids, on the other hand, have exophilic behaviour. They live in the open environment, and they don't seek shelter (Parola and Raoult, 2001).

Parola *et al.*, (2001) have pointed that more than 90% of the Ixodid life is spent off the host. Thus, they are likely to be seasonally active, waiting for their hosts when environmental conditions are suitable. Exophilic species typically find their potential hosts by detecting stimuli from them including chemical stimuli such as CO2 and NH3, body heat, humidity, and vibrations. Most Ixodid ticks have two host-seeking s: an ambush strategy in which the ticks climb on the vegetation and wait for any passing host, then cling on the host and hunting strategy in which ticks emerge from their shelters and run toward their hosts when they receive animal stimuli.

More than 85% of ticks are parasitizing specific hosts that considered to be similar to each other or infect different host species which share the same ecological habitat requirements tick habitat (Sonenshine, 2009; Kiewra and Lonc, 2012). Host specificity is a phenomenon in which tick feeds on only a limited species of hosts. For example, *Ixodes uriae* occur in almost all the continent targeting different species

of seabirds in their breeding sites (e.g., Muñoz-Leal and González-Acuña, 2015). This tick adapts in varies habitat as long as the birds are dispersed or congregate in their breeding colonies.

According to of Hoogstraal and Aeschlimann (1982) study on the host preferences of different ticks on their hosts, they examined that at least 700 of 800 species of superfamily *Ixodoidea* are strict to host specificity and half of *Arags* tick species attack on specific birds nesting compared to few of *Ornithodoras* species. Such vital hosts availability in a region may strongly influence the presence of a specific tick that feeds on them (Estrada-Peña and De La Fuente, 2014). Thus, the geographic distribution of such ticks can be readily determined by that of their hosts (Hoogstraal *et al.*, 1982). Host preference varies among tick life stages ranging from very host specialist to broad host generalist. For example, larvae and nymphs are considered as a generalist while adults are restricted in their choice of hosts (Esser *et al.*, 2016).

1.7 Tick Distribution

Various ecological and environmental factors present in ticks' habitat influence their distribution. The diversity of ticks depend on variation within its habitat; this includes vegetation type, host availability, weather, climate change and human activities (Dantas-Torres, 2015).

1.7.1 Europe

Two most popular tick-borne diseases were highly observed during the past two decades in Europe (Randolph, 2004). These are zoonotic tick-borne encephalitis (TBE) and Lyme borreliosis (LB), caused by agents and transmitted by *Ixodes ricinus* and *I. persulcatus,* respectively. Tick-borne encephalitis (TBE) incidence, for

example, showed a 3-fold step increase from 1983 to 1986 in Sweden, doubled in 1993 in the Czech Republic, increased even more dramatically in the same year in Lithuania and Poland, but declined markedly in 1997 in Hungary, Croatia and Slovenia.

In Europe, the tick distribution and abundance are the impact of climate change, and relatively it affects disease prevalence (Gray *et al.*, 2009). For example, *I. ricinus* and *I. persulcatus* prefer certain environmental conditions to adapt where humidity in the area should be at least 80%, high precipitation and dense vegetation to avoid tick mortality. In contrast, low rainfall and high temperature in summer adversely effect on survival, activity and distribution of these ticks. Moreover, the abundance of the host such as reptiles, birds, small and large mammals maintain immature and adult tick population on these habitats as well as contribute to circulating pathogens. In addition to climate change, human activities impact on the incidence of tick disease like crucial European tick species *Rhipicephalus sanguineus* and *Dermacentor reticulatus*.

A long-term study about the dispersal of *I. ricinus* in Sweden and Russia conclude that mild winters affect the expansion of tick from a certain region to another (Dantas-Torres, 2015). The main reason was said is the climate change aid to extend growing season of ticks and the possibility of climate change to affect host population and human activity. Thus, it was suspected to increase the niche of *I. ricinus*. Several tick species in Sweden have been listed by (Jaenson *et al.*, 1994) and their interaction with hosts. Out of these, *I. ricinus* is widespread in most regions, from south to north of Sweden and the primary vector of Lyme borreliosis, tick-borne encephalitis (TBE) and *Babesia divergens* for human and domestic animals.

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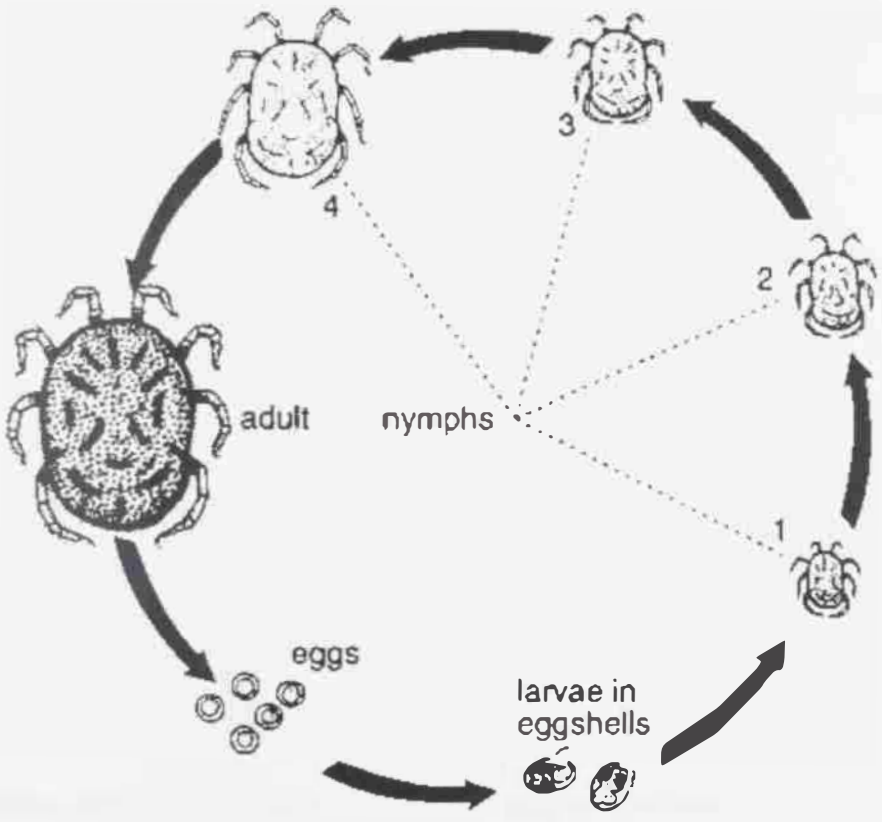
1.5.2 Life Cycles of Argasid Ticks

The *Argasidae* life cycle is entirely different from that of the Ixodidae. Argasid life cycle involves numbers of nymphal instar vary from two to eight depends on the species and the quality and the amount of blood ingested (Vial, 2009). This characteristic and their ability to resist starvation allow the argasid ticks to live for many years (Manzano-román *et al.*, 2012). Thus, the soft tick life cycle may take from 10 to 20 years (Sonenshine, 2009). The feeding behaviour is very rapid among soft tick, especially on nymphs and adults which feed within 15-60 minutes while it takes a longer time on larvae which feed for 12 hours to several days (Vial, 2009).

Argasid ticks are able to survive for long periods between blood meals from months to several years depending on host availability. Each immature stage obtains at least one blood meal on a vertebrate host before moulting, except larvae of some *Ornithodoros* species like *O. moubata*, which directly moult to the nymphal stage without feeding. The soft tick adults have long lifespan reach up to 25 years for some species. The majority of argasid tick larvae seek hosts, feed rapidly, then drop off from their host and moult to the first nymphal instar (Sonenshine, 2009). The first nymph seeks hosts again, feeds rapidly then moults to further nymphal stage. This

continued process of host-seeking, feeding and moulting may create a number of nymphal instars prior to the formation of adults. After the last nymphal moult, an adult is formed, and then it feeds rapidly on an individual host, then produces a small batch of eggs from each blood meal. Usually, mating occurs off the hosts. Figure 3 illustrates the typical life-cycle of the soft tick.



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Parola *et al.*, (2001) have pointed that more than 90% of the Ixodid life is spent off the host. Thus, they are likely to be seasonally active, waiting for their hosts when environmental conditions are suitable. Exophilic species typically find their potential hosts by detecting stimuli from them including chemical stimuli such as CO2 and NH3, body heat, humidity, and vibrations. Most Ixodid ticks have two host-seeking s: an ambush strategy in which the ticks climb on the vegetation and wait for any passing host, then cling on the host and hunting strategy in which ticks emerge from their shelters and run toward their hosts when they receive animal stimuli.

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1.7 Tick Distribution

Various ecological and environmental factors present in ticks' habitat influence their distribution. The diversity of ticks depend on variation within its habitat; this includes vegetation type, host availability, weather, climate change and human activities (Dantas-Torres, 2015).

1.7.1 Europe

Two most popular tick-borne diseases were highly observed during the past two decades in Europe (Randolph, 2004). These are zoonotic tick-borne encephalitis (TBE) and Lyme borreliosis (LB), caused by agents and transmitted by *Ixodes ricinus* and *I. persulcatus,* respectively. Tick-borne encephalitis (TBE) incidence, for

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High host abundance such as that of vertebrates, plays a role in distributing this tick. It is found in 29 mammal species, 56 bird species and two species of lizards.

The similar tick is also a common species along with other 20 endemic species in the UK (Medlock and Leach, 2015). This tick has significantly more densities due to increasing number of wild animals and influence of human activities. Spread of deer and land use and expansion of urban area results in increasing of Lyme disease cases in the UK, more than 1000 confirmed human cases each year. Host individual features and tick-borne pathogen (TBP) epidemiology is an undoubtedly subject in tick abundance. In the northern Iberian Peninsula, *I. ricinus* prevalence is related to climate and environmental factors while the ungulate abundance in particular cattle was considered as a reservoir of *B. burgdorferi* sensu late and *A. phagocytophilum*, the agent of Lyme disease, with *I. ricinus* nymph being its main vector (Ruiz-Fons *et al.*, 2012).

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In Africa, some climatic variation influence on tick distribution and abundance within the continent. Humidity and vegetation type are the significant parameters affecting the distribution of the genus *Rhipicephalus* (Perry *et al.*, 1990). This genus is the most widely distributed ixodid in temperate and subtropical regions where the precipitation and vegetation are common climatic features. *Rhipicephalus evertsi evertsi*, *Rhipicephalus appendiculatus* and *Rhipicephalus (Boophilus) microplus* were frequently reported in livestock and wildlife animals particularly in southern Africa and Mozambique (Horak *et al.*, 2009). Also, many common species of *Rhipicephalus* were found in cattle affecting animal production and their abundances throughout Zambia (Simuunza *et al.*, 2011). Other tick species of *Theileria, Babesia,*

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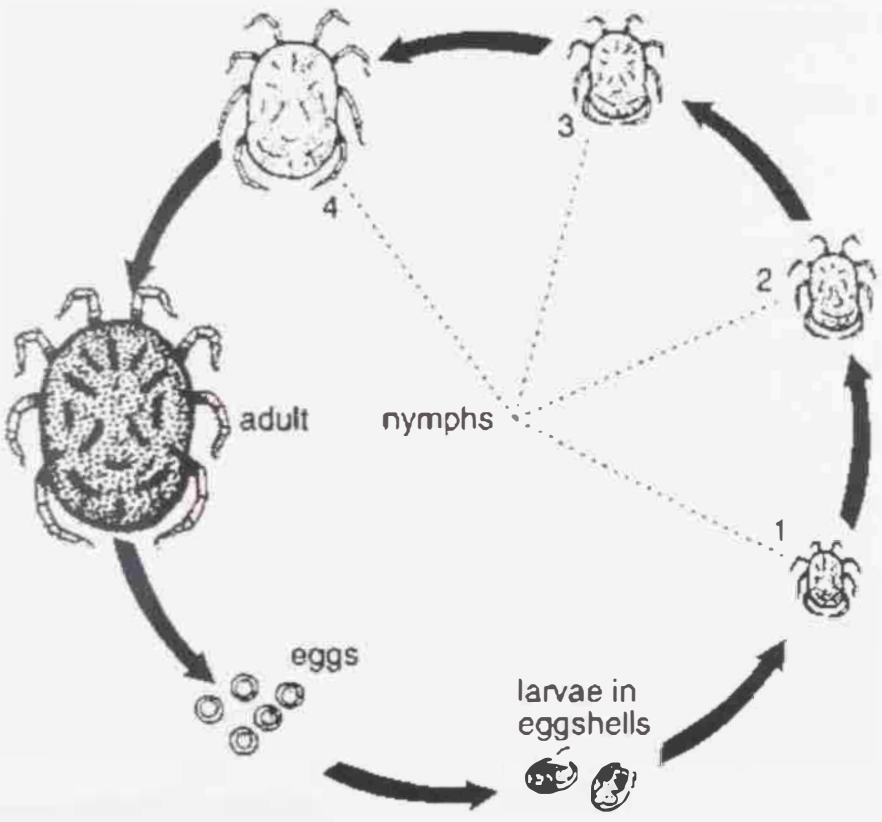

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Anaplasma and *Ehrlichia* are distributed in Sub-Saharan Africa and often identified from domestic animals causing health and economic problems.

According to Fantahun and Mohamed (2012), 60 different species of tick are well recognized in eastern Africa of which *Amblyomma variegatum* and *Boophilus decoloratus* species are widely spread in Ethiopia and adversely affect animal production by transmitting several diseases. Another most wide geographic distribution in south-east Africa is *Amblyomma Hebraeum* the vector and reservoir of Rickettsia Africa, the agent of African bite fever (Snape and Pollard, 2006; Parola *et al.*, 2013; Halajian *et al.*, 2016). In North Africa, notably Algeria and Egypt, *Hyalomma dromedarii* ticks collected from camels (*Camelus dromedarius*) were recorded caused African bite fever. Elsewhere in sub-Saharan Africa, *Amblyomma variegatum*, the tropical Bont tick, is a documented vector of *R. africae*.

1.7.3 The Middle East Region

Tick prevalence across middle east countries has been mainly reported from camels, goats, sheep and cattle. In the Kingdom of Saudi Arabia, tick species were investigated from domestic animals by (Hoogstral and Kaiser, 1959; Banaja and Roshdy, 1978; Banaja and Ghandour, 1994). The *Hyalomma* genus was the highest tick recorded in camels (Banaja *et al.*, 1994) and it has been distributed in many parts of the country causing *Theileria* disease in small ruminants mainly, in sheep (El-Azazy *et al.*, 2001). In addition, *Coxiella burnetii* the causative agent of Q fever has been found in camel, goats and cattle transmitted through faeces, urine and milk and its primary effects reproductive system (Mohammed *et al.*, 2014). In Lebanon, a survey has been conducted of tick species infesting ruminants in six Lebanese provinces and it has reported four different tick genera, among which *Rhipicephalus*

genus represented the highest frequent (72.4%) with respect to the other identified genera: *Heamaphysalis*, *Dermacentor*, and *Hyalomma* (Dabaja *et al.*, 2014).

In Iraq, surveys of tick fauna of domestic and wild animals are well documented from different ecological zones (Shamsuddin and Mohammad, 1988; Mohammad and Jassim, 2011). For instance, different regions of the middle and south of Iraq have examined many ixodid tick species belonging to two genera *Hyalomma* and *Rhipicephalus* in sheep and goat (Mohammad, 2016). In the same region, *Hyalomma* spp. has also constituted the majority of infestation cases in water buffalo (Shubber *et al.*, 2013). Figure 4 shows the geographical locations where the ticks collected in Iraq.

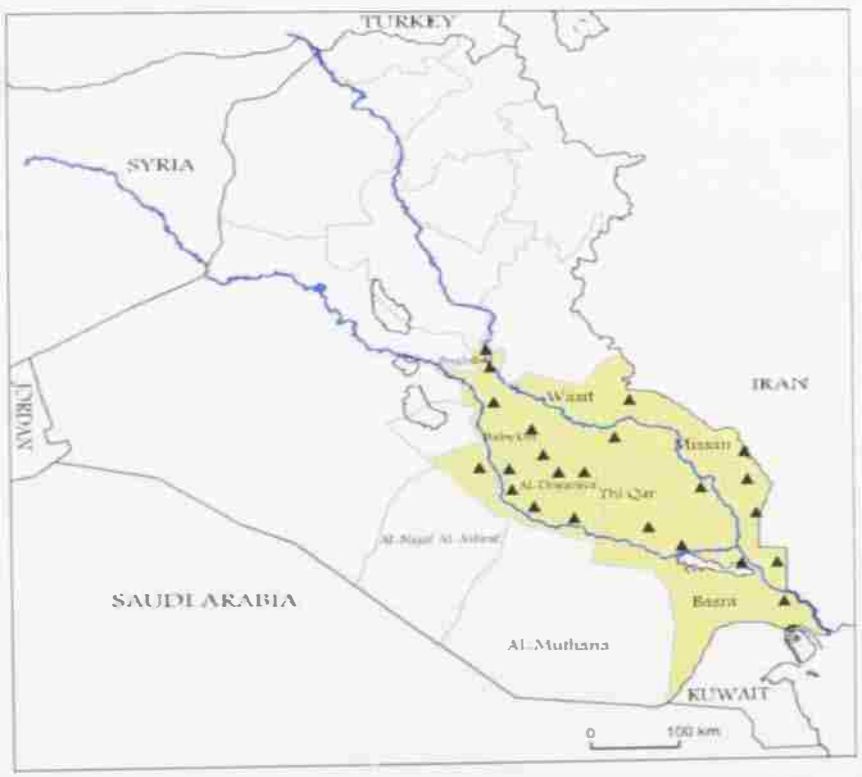

Figure 4: Collection sites of ticks in the middle and south regions of Iraq

(Source: Shubber *et al.*, 2013)

1.8 Tick-Borne Diseases

Ticks are commonly known as a vector for a wide variety of disease causing-pathogens to human and animals including viruses, bacteria and parasites (Aktas, 2014; Maia *et al.*, 2014; Michelet *et al.*, 2016; Liu *et al.*, 2017; Papa *et al.*, 2017). Ticks are a second common arthropod group behind mosquitoes of human diseases and most common vectors of infectious diseases in domestic and wild animals. It has been estimated that most of the vector-borne diseases transmitted to humans in the world were by ticks (de la Fuente *et al.*, 2008). A statistic (Dantas-torres *et al.*, 2012) showed that the most popular disease in the United States from 2000 to 2012 was Lyme borreliosis affecting more than 250,000 humans in addition to more than 50,000 cases in human are reported annually in Europe. Tick has a sure way to transmit the pathogen to the host during a blood meal. In most cases, a tick becomes infected with virus, bacteria or protozoa while feeding on the host that carries the infectious pathogen on its blood (Wilson, 2002). Subsequently, this pathogen concentrates in the gut of ticks where the salivary glands secretion, in turn, transmits the disease to the other hosts.

In the recent decades, tick-borne disease gains more attention in the epidemiological studies because of its importance of deadly transmission diseases to humans and animals. Identification of potential diseases associated with ticks by using molecular biology assay has become widely described. Molecular methods with newly developed tools are assisting in determining tick species and tick-borne pathogens at the genomic and population levels (de la Fuente *et al.*, 2008; de la Fuente and Estrada-Pena, 2012; Dantas-torres *et al.*, 2012; Berggoetz *et al.*, 2014). Among infectious diseases, tick-borne bacterial diseases are most often diagnosed group

compared to other tick-vectored diseases (Mediannikov and Fenollar, 2014). The genera *Borrelia, Rickettsia, Francisella, Ehrlichia, Anaplasma, Cowdria,* and *Coxiella* are most bacterial communities transmitted by a tick (Noda *et al.*, 1997). The significant role of studying the tick pathogens is to prevent tick-borne diseases and to improve control measures (Mediannikov *et al.*, 2014).

1.9 Tick-Borne- Bacterial Diseases

1.9.1 Borreliosis

Borrelia is a genus of Spirochaetes bacteria which causes *Borreliosis,* a group of zoonotic diseases transmitted by ticks or lice (Ehlers *et al.*, 2016; Ehounoud, 2017). *Borreliosis* is primarily classified into two groups of human disease: Lyme disease and relapsing fever (Ras *et al.*, 2017). Lyme borreliosis is transmitted by the hard Ixodes ticks and as for relapsing fevers; they are usually transmitted by soft ticks (Argasidae) of the genus *Ornithodoros.* Lyme disease is among the most important borreliosis caused by members of the *Borrelia burgdorferi sensu lato* complex and other related species mainly concerning North America and Eurasia (Kernif and Leulmi, 2016).

Tick-borne relapsing fever is caused by several *Borrelia* species. For instance, *Borrelia coriaceae* was isolated from the soft tick of the genus *Ornithodoros* and much more of *Borrelia anserine*, the avian borreliosis agent was recognized in *Argas persicus* soft tick (Masuzawa and Asia, 2004). In associated with hard ticks three pathogenic *Borrelia* species were found, namely, *B. theileri* in *Rhipicephalus (Boophilus), B. miyamotoi* in *Ixodes persulcatus* and rodents in Japan and *B.*

lonestari in *Amblyomma* species in the United States (Ras *et al.*, 2017; Ehlers *et al.*, 2016).

1.9.1.1 Lyme Borreliosis

Lyme borreliosis is a zoonotic disease transmitted by hard ticks of the genus Ixodes (Margos *et al.*, 2009; Arco *et al.*, 2017). This disease is caused by many borrelia species belonging to the *Borrelia burgdorferi sensu late* (sl) complex including 22 genospecies (Waindok *et al.*, 2017). In Europe, eleven of them were reported for cases of Lyme borreliosis; the most three common agents are *B. Burgdorferi sensu stricto* (ss), *B. garinii* and *B. afzelii.* Lyme borreliosis is endemic in the Northern Hemisphere, occurs in North America, Europe and Asia (Izac *et al.*, 2017).

Based on many cases, Lyme borreliosis vary from mild to severe symptoms in humans such as fever, *erythema migrans*, cardiac disease, nervous system disorders and other manifestations (Raja *et al.*, 2016; Ms *et al.*, 2017). In 1991 around 10,000 cases of human Lyme disease per year reported in North America, showing higher increased in 2014 to more than 25,000 cases according to the Centers for Disease Control and Prevention (CDC) (Ms *et al.*, 2017). In veterinary medicine, Lyme disease has been documented in canines and equines (Izac *et al.*, 2017). According to Companion Animal Parasite Council, over 250,000 cases of positive canine Lyme disease test were diagnosed based on only 30% of collected test data; however, the actual number of this disease is suspected to reach 800,000 cases.

1.9.1.2 Tick-Borne Relapsing Fever

Many of *Borrelia* spirochetes are considered causing tick-borne relapsing fever a disease transmitted via soft ticks mainly by *Ornithodoros* species (Parola *et al.*,

2011). A soft tick *Ornithodoros* transmits multiple of relapsing fever *borreliae* except for *Borrelia recurrentis* (Dworkin *et al.*, 2008) which is usually vectored by louse (Cutler, 2010). Tick-borne relapsing fever is a worldwide endemic disease. For instance, *B. crocidurae* pathogen transmitted by the endemic ticks *Ornithodoros sonrai* casing relapsing fever was reported in Senegal, Mali, Mauritania, and the Gambia where 2%–70% of animal burrows are inhabited by this tick vector, and an average of 31% of ticks are infected by *B. crocidurae* (Cutler *et al.*, 2009; Parola *et al.*, 2011).

In addition to that, the same pathogen transmitted by the same tick has been recorded in West Africa as high pathogen affecting human population (Vial *et al.*, 2006a; Cutler *et al.*, 2009). This infected tick inhabited on rodents and insectivores affecting people during their sleep causing illness and fever (Vial *et al.*, 2006a). In North America, the spirochete *Borrelia hermsii* also causes the relapsing fever to human which is transmitted by *Ornithodoros hermsi* (Schwan *et al.*, 2007). Dworkin *et al.* (2008) state that this disease spreads in many regions; the western United States, southern British Columbia, the plateau regions of Mexico, Central and South America, the Mediterranean, Central Asia, and throughout much of Africa.

1.9.2 Rickettsiosis and Rickettsiae

Tick-borne rickettsiosis is infectious diseases caused by obligate intracellular, gram-negative bacteria *Rickettisa* (family *Rickettsiaceae*, order *Rickettsiales*) (Eremeeva and Dasch, 2001; Parola *et al.*, 2005). The *Rickettsia* genus is classified into three groups including the spotted fever group (SFG) which has the most common agent *Rickettsia rickettsii* that causing Rocky Mountain spotted fever, the typhus group

consists of two human pathogens, *Rickettsia prowazekii* and *Rickettsia Typhi* and the scrub typhus group (STG) (Wood and Artsob, 2012; Orkun *et al.*, 2014).

1.9.2.1 Mediterranean Spotted Fever

Ricketsia conorii, the causative agent of Mediterranean spotted fever (MSF) is transmitted to humans by a tick bite, mainly by brown dog tick *Rhipicephalus sanguineus* (Mouffok *et al.*, 2009; Kuloglu *et al.*, 2012). The main symptoms of MSF are fever, rash, and skin eschar at the tick bite site. MSF has been known to be endemic in the Mediterranean area, including northern Africa and southern Europe (Papa *et al.*, 2009). In sub-Saharan Africa, all cases of spotted fevers were reported to be MSF with *Rickettsia conorii* as an agent, and many cases of the disease and isolations of the agent were identified in Kenya, Somalia, South Africa, and Chad (Mediannikov *et al.*, 2010). MSF, however, has been observed in Portugal with the highest annual incidence of 9.8 cases per 100,000 persons (Papa *et al.*, 2009; Seixas, 2012). Most cases (87%) were observed during the summer (87%), from July to September which indicates that this pathogen is correlated with high temperatures and attack humans more in warmer temperatures.

1.9.2.2 Rocky Mountain Spotted Fever

Rocky Mountain spotted fever (RMSF) is a zoonotic disease caused by the infection with *Rickettsia rickettsii,* which is a member of the spotted fever group (Nelson, 2015). Hard ticks are the natural reservoirs of *Rickettsia rickettsii* which are transmitted to larger mammals such as humans and dogs (Warner and Marsh, 2002). Today, most cases of RMSF are known in most of the USA. The American dog tick *Dermacentor variabilis* is the primary vector and reservoir of RMSF in the Eastern

US and the Rocky Mountain wood tick, *Dermacentor andersoni*, is the vector in the Western states (Lin and Decker, 2012).

Other tick species contribute in transmitting RMSF is *Rhipicephalus sanguineus*, the brown dog tick that has been recently found in Arizona and Mexico and suggested to display in dog owners homes (Warner *et al.*, 2002; Lin *et al.*, 2012). In addition, *Amblyomma cajennense* and *Amblyomma aureolatum* (Lone Star tick) ticks are primary vectors concerning Latin American countries, such as Argentina, Brazil, Colombia, Panama, and Costa Rica, as shown in Figure 5.

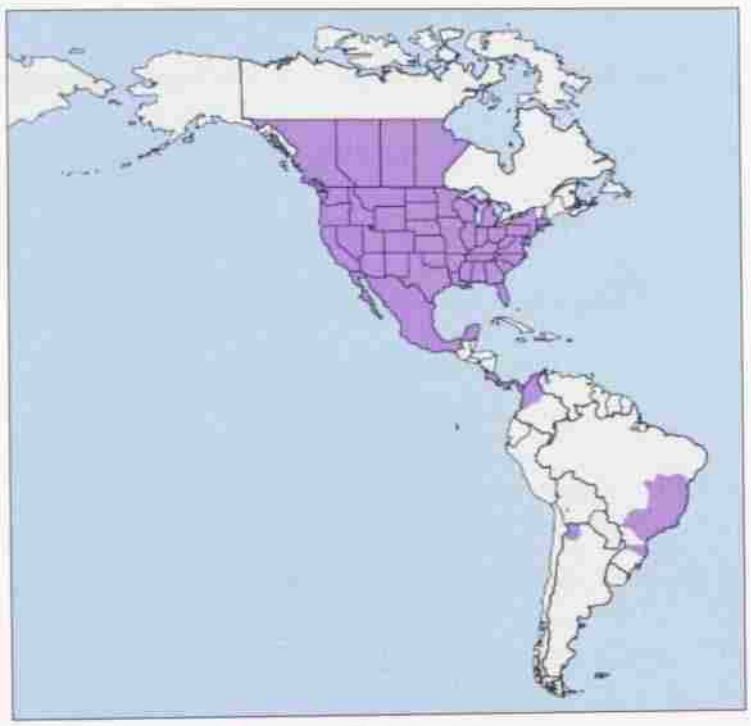

Figure 5: Geographical distribution of RMSF in the American continent (Source: Dantas-Torres, 2007)

The fever, chills, myalgia, and headache are the primary symptoms of the Rocky Mountain spotted fever disease begin after the bite by an infected tick (Socolovschi *et al.*, 2009). The following symptoms are severe health disorders including anorexia, nausea, vomiting, abdominal pain, diarrhoea and cough. Many Rickettsial diseases

are distributed throughout the world causing illness to humans (Dzelalija *et al.*, 2016). In South Africa, three *Rickettsia* species have been detected in humans; *Rickettsia conorii,* the agent of Mediterranean spotted fever, *R. aeschlimannii* and *R. mongolotimonae* (Pretorius and Birtles, 2004).

The *H. truncatum* ticks have been suspected the transmission of these *Rickettsia* species via parasitized migratory birds where they distributed in African countries, including South Africa. In addition, diverse *Rickettsia* species have been reported with potential pathogens in Ethiopia from ticks, fleas, lice, and mites (Pader *et al.*, 2012). *R. africae* species have been documented from hard ticks in Ethiopia as well as high prevalence of *Candidatus R. hoogstraalii* among *Ar. persicus* ticks selected from poultry areas but without recording any infection occurring in livestock and humans. With much of *Rickettsia* diversity in Ethiopia, the same study showed some cases from an indigenous population with numbers of tourists who had infected by *Rickettsia*.

1.9.3 Q fever

Coxiella burnetii is an obligate intracellular gram-negative bacterium, a common agent of Q fever (Wegdam-blans *et al.*, 2012; Tejedor-junco *et al.*, 2016). *C. burnetii* found in several tick species. However the early discovery was in *Dermacentor andersoni* tick (Riemann *et al.*, 1979). Q fever is spread throughout the world, and it reported in many countries especially in the African continent in particular sub-Sahara and West Africa (Kanouté *et al.*, 2017). *C. burnetii* infect wild and domestic mammals, birds, and arthropods and it also affects humans (García *et al.*, 2017). It was highly documented among domestic ungulates such as sheep, goats, cattle and in

wild ungulates including Oryx and gazelle. This agent poses a threat to both humans and domestic animals.

In humans, it considered as an occupational disease in several Mediterranean countries while they are close contact with the numerous domestic animals (Ejercito, 1993; Rizzo *et al.*, 2016). Initially, Fever, headache, myalgias, and anorexia are primary symptoms of acute infection that may affect humans and continues to chronic infection manifested in liver inflammation which appears later. While in ruminants, Q fever is associated with reproductive disorders including abortions, stillbirths and delivery. Tick-borne encephalitis (TBE) is the most distributed viral disease in Europe which is transmitted by the main vector Ixodes spp. (*I. ricinus* and *Ixodes persulcatus*) (Rodríguez *et al.*, 2018). Mammals such as rodents and small ruminants are reservoirs of this agent, in addition to migratory birds which contribute in the circulation of the disease. Another, deadly viral disease transmitted to human is Crimean-Congo hemorrhagic fever. This virus is widely distributed in Asia, Africa, and Europe. The hard tick mainly the genus *Hyalomma,* is the main vector of the disease and some other tick species from the genera *Dermacentor, Amblyomma, Rhipicephalus,* and *Haemaphysalis* have been found to harbour this agent (Estrada-Peña *et al.*, 2014).

1.10 *Ornithodoros muesebecki*

Arabian tick *Ornithodoros muesebecki* has been initially found in Arabian coast from Blue-footed Boobies colony (*Sula dactylatra*) (Hoogstraal and Oliver, 1970). The first recorded of *O. muesebecki* in the UAE was in Zirqa Island in the area of Abu Dhabi, collected from infected birds. It was suggested to serve as a vector of pathogens transmitted to workers in the island, whom typically show different signs

and symptoms like fever, skin irritation, rashes and headaches (Hoogstraal and Oliver, 1970; Estrada-Peña and Jongejan, 1999; Al-Deeb *et al.*, 2016).

O. muesebecki is vectored of *Coxiella*-like endosymbiont bacteria in the largest seabirds colonies in the UAE (Al-Deeb *et al.*, 2016), but no study has uncovered the total bacterial communities harboured by this species or any pathogenic agents yet, which could transmit these pathogens to the animal or human hosts during a bite. Figure 6 shows the morphological features of *O. muesebecki* from both sides.

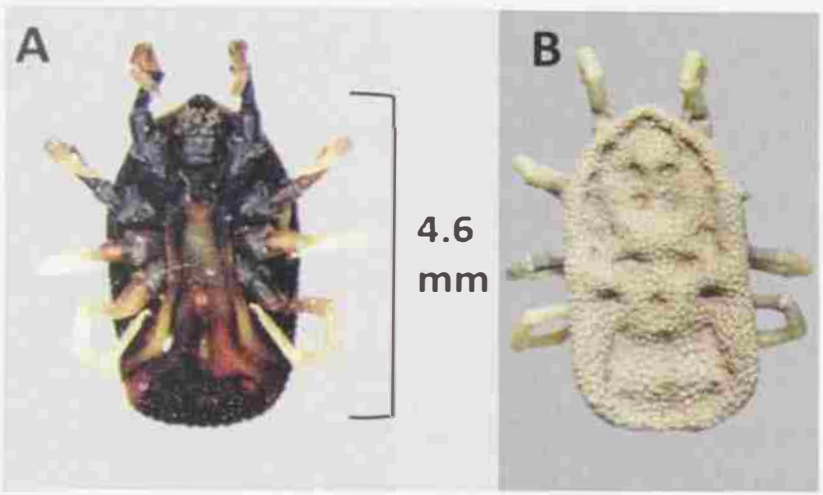

Figure 6: *O. muesebecki* adult female-(A) ventral and (B) dorsal sides (Source: Al-Deeb *et al.*, 2016)

1.11 Migratory Birds and Transmitted Diseases

Generally, birds are defined as reservoirs and disseminators of tick-borne pathogens which they represent a threat to humans and animals health (Estrada-peña *et al.*, 2015). Birds have the capability to transport tick-borne pathogens in different ways including transportation of infected ticks, through being infected with TBP and carried to feeding ticks. In Fact, factors such as years, season, locality and different bird species determine the prevalence of ticks on birds as (Hasle, 2013) state,

whereas the distribution of ticks on different species is associated on the degree of feeding on the ground. This evidence is strongly supported by the high prevalence of tick infestation in *Turdus* spp. especially the blackbird in Europe.

Another study evaluated the prevalence of ticks on wild avian hosts, explaining that birds may harbour both diverse and straightforward infestations (Sparagano *et al.*, 2015). For instance, 37 species of a bird caught from two different sites in Portugal between 2010 and 2011 showed the tick species diversity in infested birds mainly on Eurasian blackbirds, spotless starlings, and European robins. On the other hand, only one single species *I. ricinus* (larvae and nymphs) was detected in 20 bird species captured between 2008 and 2009 in France. Various tick-borne pathogens were observed in birds in Europe such as *Borrelia burgdorferi* sensu lato (s.l.), *Anaplasma phagocytophilum*, *Babesia divergens*, *Babesia venatorum*, *Coxiella burnetii*, various *Rickettsia* species including the most popular *Rickettsia Helvetica* (Capligina *et al.*, 2014). Among these diverse species, *Borrelia* spp. is frequently established in studies to be transported via birds in Europe, North America and Asia (Hildebrandt *et al.*, 2010; Lommano *et al.*, 2014).

Migratory birds contribute to dispersing pathogenic microorganisms as a result of their migratory behaviour. Indeed, through their long seasonal passage and travel across different habitats, birds stop at different sites for rest and feeding, thus various ticks and other organisms have the potential to attach on them, travel with them and detach along the migration route or in breeding areas (Johnson, 1989; Jaenson and Bergstro, 1995; Bjöersdorff *et al.*, 2001). Significantly, seabirds play a role in epidemiological disease and global circulation of tick-borne pathogens since they

travel a long distance, distributed widely and breed in aggregations in specific colony locations (Wilkinson *et al.*, 2014).

The prevalence of Lyme borreliosis-infected ticks was detected highly on migratory birds throughout the world. Birds movement across Europe and the Middle East confirmed their role in the distribution of arboviruses and the Lyme borreliosis agent, *Borrelia burgdorferi* sensu lato via infected ticks (Jaenson *et al.*, 1995). For instance, *B. Burgdorferi sensu strict* pathogen was detected from passerine birds in *Ixodes scapularis* tick in the United States. Likewise, a total of 40 migratory birds species have been determined in North Africa as carriers of ticks, including *Ixodes ricinius* which cause Lyme disease pathogen, *Borrelia brgdorferi* (Johnson, 1989). Similarly, another study demonstrates the occurrence of *Anaplasma* species and *Lyme borreliosis* (LB) spirochetes as vector-borne pathogens in infected birds in North America and Eurasia (Comstedt *et al.*, 2006).

1.11.1 Socotra Cormorant

Cormorants (Phalacrocoracidae) are one of the widely distributed family of water birds worldwide (Threlfall, 1982). They inhabit both freshwater and seacoast environments. In the United Arab Emirates, Arabian endemic Socotra Cormorant (*Phalacrocorax nigrogularis*) is observed on many islands. Years ago, 20 breeding sites in Abu Dhabi and Sharjah existed before they became almost extinct (Wilson, 2012). In 2010, other new colonies were reported from 10 different locations across UAE included few pairs of birds in each location. Currently, the most significant breeding colony of Socotra Cormorants (*P. nigrogularis*) represents on Siniya Island; Umm Al Quwain recorded with 28,000-35,000 pairs (Muzaffar, 2015).

Socotra Cormorant is endemic seabird restricted to the Arabian Gulf and Sea of Oman. The global population is rapidly declining and today is estimated at 110,000 breeding pairs (Muzaffar *et al.*, 2012) thereby, they are listed as Vulnerable on the International Union for Conservation of Nature (IUCN) Red List. A great effort is established to study this species in term of breeding biology, habitat ecology and behavior. Anthropogenic activities and natural threats are behind the decline of their colonies. Despite the limitation, studies on natural threats cause on their colonies, but one assumption was examined to study predation over the thousands mortality of cormorant population on Siniya Island, the UAE. The study showed that two predators fox and feral cats introduced on colony are implicated on praying Socotra Cormorants based on observation of killed birds.

The abundance of (*O. muesebecki*) tick was observed on the same colony suggesting their potential role towards bird mortality. In fact, tick abundance in seabird nests adversely affects chick growth and survival and in most cases is catastrophic to chick health which resulted from diseases transmission (Ramos *et al.*, 2001). Other harmful organisms inhabiting Socotra cormorant such as parasites has not been studied yet. Parasites while they present in the food web, they influence the ecosystem in various ways by affecting the hosts causing mortality and behavioural changes (Moles and Heintz, 2007).

Most studies recorded mortality in marine and shorebirds is primarily caused by parasites. Delayed development and reduce long-term survival of host's offspring were determined in breeding sites (Brown *et al.*, 1995). There are 234 species of tapeworms known from seabirds (Hoberg, 1996), and more than 700 species of helminth parasites were reported in at least 165 seabirds hosts (Muzaffar, 2009). A

full study of parasites in adults and chicks Socotra cormorants with the association of their diet is not done, although of above-discussed evidence determine their impact on seabirds.

1.12 Next Generation Sequencing

The first generation, also known by Sanger sequencing was first described in 1977 by Frederick Sanger (Liu *et al.*, 2012). This sequencing based on chain termination, allowed few base pairs of DNA to be sequenced. Sanger sequencing produces a read length reach to 700bp with low error rate (Adamiak *et al.*, 2016). Following the first generation, the rapid advance of DNA sequencing and data analysis were developed, and this was named next-generation sequencing. NGS platforms adopt parallel DNA sequencing (Behjati and Tarpey, 2013).

The platforms include 454 pyrosequencing Illumina platform and recently established Ion Torrent. DNA sequencing by Roche. The 454 pyrosequencing concept is based on pyrophosphate released during nucleotide incorporation (Liu *et al.*, 2012). This technique generates long read length and relatively high speed. The Illumina platform, however, sequenced DNA by synthesis and uses bridge amplification for polony generation (Buermans and den Dunnen, 2014). It works by detection of the light emitted during synthesis of a complementary DNA strand for each added nucleotide. This method is low cost and produces large numbers of reads compared to 454, but they are short length, only 35 bp long (Van Dijk *et al.*, 2014). Another NGS approach sequenced by synthesis is Ion Torrent. In this method, DNA is sequenced by detection of hydrogen ions concentration (Adamiak *et al.*, 2016). It used in broad range of applications because of its faster and low cost.

Despite many conventional methods which have been used to identify the microbial communities associated with ticks, limitation on detection and analysis of target bacteria still exists (Carpi *et al.*, 2011). Next-generation sequencing technologies are an alternative approach and among the most remarkable and powerful tools for examining microbial communities (Vayssier-Taussat *et al.*, 2013). This technology was used widely for metagenomic profiles of the bacterial communities associated with *Ixodes ricinus* where 16srRNA gene had been amplified and sequenced (Vayssier-Taussat *et al.*, 2013; Bonnet *et al.*, 2014). The 16s ribosomal RNA is a highly conserved bacterial gene used for bacterial identification and usually sequenced using NGS (Fouhy *et al.*, 2016; Hiergeist and Reischl, 2016; Sperling *et al.*, 2016). There are nine significant variable regions of 16srRNA with no studies showed which specific region and which best primer has to be examined and used for bacterial assessment.

Metagenomic analysis is the study of the entire genetic material or the variation of the species isolated from the environmental samples (Thomas *et al.*, 2012). It is a method used in both the molecular biology and genetics in order to identify and characterize the genetic material of the sample. It provides a broad description of the functional genes in the microbial communities associated with the hosts.

1.13 Metagenomic and Tick Gut Microbiota

Recently, so many complex ecosystems have been tested by metagenomics to characterize the microbial communities in the soil, ocean water, and for medical and veterinary purposes (Carpi *et al.*, 2011). An involved ecological community within the organism its gut microbiota, which involves interactions of diverse bacterial species within the host. Many researchers have studied the gut microbial community

structure and its function in different mammalian host species like mouse, human and domestic animals (Mandal *et al.*, 2015). In ticks, diverse pathogens and symbiotic bacteria like *Coxiella*-like bacteria inhibit its gut and these bacteria act as infectious disease or live peacefully with unrecognized role (Qiu *et al.*, 2014; Narasimhan *et al.*, 2015).

In Japan, metagenomic approach facilitated the finding of 163 different genera of bacteria in tick salivary glands, the presence of the prevalent infectious pathogens such as *Ehrlichia*, *Rickettsia,* and *Coxiella* (Qiu *et al.*, 2014).These results were based on three different tick species (*I. ovatus, I.persulcatus, and H. flava*) and evidently, the differences in the bacterial population were clear between tick species as determined by Principal Component Analysis (PCA). Similarly, 454 pyrosequencing had reported a diversity of bacterial phyla and genera from neotropical tick and birds blood DNAs in the US. *Candidatus Rickettsia amblyommii* in infected neotropical ticks carried by migratory birds during seasonal migration (Budachetri *et al.*, 2017a).

Chapter 2: Materials and Methods

2.1 Ticks and Collection Sites

Ticks used in this study were collected from Siniya Island. Siniya Island is a nearshore island located off the Umm Al Quwain Emirate, UAE. The island is about 12 km in total length with a tear-drop shape with numerous lagoons and inlets, as shown in Figure 7. Part of the island contains planted *Prosopis juliflora* and *Acacia tortilis* trees. Scattered throughout are poor communities composed mostly of *Haloxylon / Arthrocnemum* species. Some areas are bordered with mangroves, *Avicennia* marina. The island hosts the largest population of breeding Socotra Cormorants in the UAE, totaling to about 35,000 breeding pairs (Muzaffar, 2014).

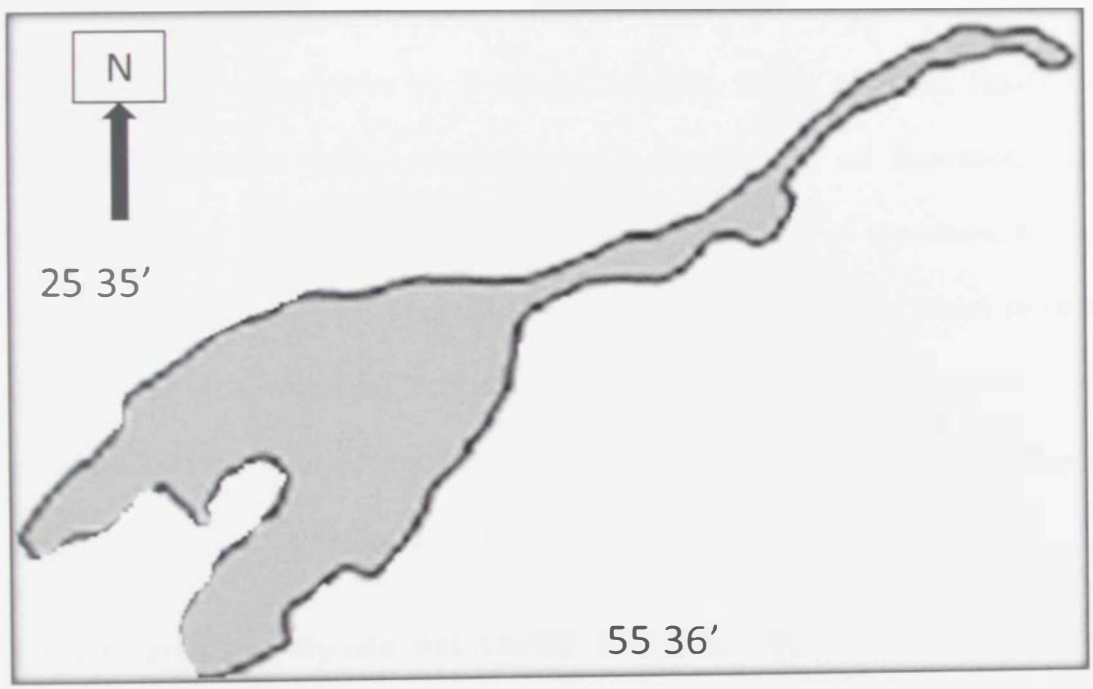

Figure 7: Siniya Island map, Umm Al Quwain

The ticks (adults and nymphs) used in the current study were collected in 2013 and 2016 as part of a project led by Sabir bin Muzaffar. The collected ticks were stored in

plastic tubes (1 tick per tube) in a -20°C freezer. The collected ticks included 150 individuals from each year (2013, 2016) were later subject to analysis (total n=300).

2.2 Tick Genomic DNA Extraction

Three different methods were used for tick genomic DNA extraction. Genomic DNA was extracted from individual whole ticks (n=300) using automated DNA extraction machine (Maxwell 16, Promega, Madison, USA) (135 samples) and an animal tissue extraction kit (133 samples) (Promega, Madison, USA). For the metagenomic study, DNA was extracted using QIAamp Tissue Kit (32 samples) (Qiagen, Hilden, Germany) to get the maximum amount of DNA. All extractions were conducted following the manufacturer's protocol. The extracted DNA was stored in the freezer at -20°C.

In the animal tissue extraction kit (Promega, Madison, USA), each tick separately was manually crushed with a tissue grinder in a sterile 1.5 ml Eppendorf tube containing 600 µl of chilled nuclei lysis buffer and 17.5 µl of proteinase K and incubated overnight at 55°C. Afterward, 3 µl of RNase solution was added to each tube and was gently mixed for incubated again at 37°C for 15 min. Then, 200 µl of protein precipitation solution was added to each tube; vortexed and chilled on ice for 5 min followed by centrifuging at 16000 x g (full speed) for 4 min.

Each supernatant in every tube was transferred to a fresh tube containing 600 µl of isopropanol which is mixed gently by inversion and was incubated again on ice for 10 min followed by centrifuging at 16000 x g (full speed) for 1 min. Each supernatant was removed and replaced with 600 µl of 70% ethanol and centrifuged once again at 16000 x g (full speed) for 1 min. The supernatant was again removed,

and the tubes were dried. The DNA from each tick specimen was eluted in 100 µl of DNA rehydration solution, and the extracted DNA was stored overnight at 4°C until the agarose gel electrophoresis.

In the QIAamp Tissue Kit (Qiagen, Hilden, Germany), each sample was crushed using tissue grinder in a sterile 1.5 ml Eppendorf tubes with the addition of 180 µl of Buffer ATL (animal tissue lysis) and 20 µl of Proteinase K to each tick sample. Samples then were incubated at 56°C overnight on a heating block. The next morning, samples were centrifuged at full speed (14,000 rpm) for 1 min, and 200 µl of a buffer AL (lysis buffer) was added to each sample and mixed by vortexing for 15 s. Subsequently, 200 µl of 70% ethanol was added, and samples were mixed in a vortex in 15 seconds. Thereafter, the mixture incubated for 5 minutes at room temperature to have centrifuged for separating and removing the liquid from the tube cap.

The samples were then loaded onto the Qiagen MinElute column and centrifuged at 8,000 rpm for 1 min. The flow-through was discarded, and the remaining sample was loaded onto the column. Successive washes with 500 µl of buffer AW1 and Buffer AW2 followed with centrifugation at 8,000 rpm for 1 min in each wash. The wash steps were carried out, and a fresh 2.0 ml collection tube was used in each step. The final product was eluted by adding 50 µl of AE elution buffer (Qiagen) to the column, incubating at room temperature for 5 min, and centrifuging for 1 min at 14,000 rpm.

Quantity and quality of the extracted DNA samples were determined with a spectrophotometer (NanoDrop ND-1000, Erlangen, Germany) and a Quantus Fluorometer (Promega, Madison, USA). The spectrophotometer was blanked with

sterile nano-pure water before reading, and DNA samples were stored at -20°C until use. Additionally, the quality of the extracted DNA was assessed by 1.5% agarose gel electrophoresis in TBE (Tris-Borate-EDTA) which was stained by ethidium bromide to enhance the visualization of DNA bands. A volume of 5 µl of each tick genomic DNA was loaded on to 1.5 % agarose gel and was visualized under UV light.

2.3 Detection of Bacteria in Ticks- PCR Analysis

In order to ensure the presence of bacterial DNA in the total genomic DNA extracted from each tick and to avoid having false negatives in disease detection, the 16S rDNA bacterial gene was detected using the following specific primers: FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rp2 (5' ACGGCTACCTTGTrACGACTT-3') (Noda *et al.*, 1997). All DNA samples (n=300) were individually screened for the presence of *Borrelia* spp., *Rickettsia* spp. and *Coxiella burnetii* using conventional PCR. Briefly, the conventional PCR experiment was performed in a total reaction volume of 25 µl, and each PCR reaction contained 12.5 µl Taq PCR master mix (Qiagen, Hilden, Germany), 1 µl of each primer, 5 µl of genomic DNA and 5.5 µl nuclease-free water. All PCR amplifications were performed on the Swift MaxPro thermo-cycler (ESCO, Singapore).

2.3.1 Detection of *Borrelia*

All DNA samples (n=300) were individually tested for the presence of *Borrelia* spp. using OspC1/OspC2 primer set (Fukunaga *et al.*, 1996), as shown below:

- OspC1: 5'-TAATGAAAAAGAATACATTAAGTG -3'
- OspC2: 5'- TTAAGGTTTTTTTGGACTTTCTGC-3'

Amplification was carried out for 35 cycles consisting of denaturation at 93°C for 1 min, annealing at 4°C below the denaturation temperature of the primer used for 1 min, and extension at 72°C for 1 min, and there was a final extension step consisting of 7 min at 72°C.

2.3.2 Detection of *Rickettsia*

Rickettsia pathogen was detected according to a published method (Blair *et al.*, 2004). Briefly, the detection involved conducting a nested PCR of the outer membrane protein (*ompA*) gene. In the first PCR, the amplification of a 590 bp fragment was obtained by using the forward primer (5'-ATGGCGAATATTTCTCCAAAA-3') and the reverse primer (5'-GTTCCGTTAATGGCAGCATCT- 3'). In the second PCR, the amplification of a 540 bp fragment was obtained by using the forward/reverse primers (5'-AAGCAATACAACAAGGTC-3') and (5'- TGACAGTTATTATACCTC -3'), respectively. Following initial denaturation for 1 min at 94°C, 35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 50°C, and extension for 4 min at 68°C were performed. A final extension step was done for 20 min at 72°C.

2.3.3 Detection of *Coxiella burnetii*

All DNA samples (n=300) were individually tested for the presence of *C. burnetii* using specific oligonucleotide primers (de Bruin *et al.*, 2011):

- ICDTRG_f (5'-CGGAGTTAACCGGAGTATCCA-3')
- ICDTRG_r (5'-CCGTGAATTTCAT-GATGTTACCTTT-3')

The primers were specific for the isocitrate dehydrogenase gene (*icd*). The thermocycling conditions were the following: 95°C for 15 min, 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s, followed by the last step at 72°C for 10 min.

2.4 Gel Electrophoretic Analysis

After the PCR cycles were complete, all reactions were analysed by agarose gel electrophoresis. The PCR products were loaded on 1.5% agarose gels stained with ethidium bromide to enhance the visualization of DNA bands. A 100bp ladder (Promega, Madison, USA) was used as a reference for determining the PCR product size. Following agarose gel electrophoresis, gels were examined under UV light.

2.5 DNA Preparation for Metagenomic Analysis

Samples from each of 2013 and 2016 were prepared as follows: a total of 28 DNA samples were collected from ticks and grouped into 5 pools for conducting the next generation sequencing. Each one of the first four pools contained DNA from 5 ticks and while the fifth pool contained DNA from 8 ticks. A volume of 5 µl of DNA was taken from each individual tick and combined to form one pool (25 µl). The DNA concentration of each pool was measured using a Quantus Fluorometer (Promega, Madison, USA) to prevent false negative results. Prior to testing, pools of DNA were created from the extracted samples as described above.

2.6 Next Generation Sequencing (NGS) Mi-Seq Workflow

The NGS was done entirely by Macrogen, South Korea and the report below describes the procedures from the company. The Illumina NGS workflows included

these necessary steps: i) sample preparation; ii) library construction; iii) sequencing; and iv) generation of raw data as detailed in Figure 8.

Figure 8: Next-generation sequencing workflow

(Source: https://dna.macrogen.com)

The analysis of data involved the following three steps: i) pre-processing and clustering; ii) taxonomic assignment; and iii) diversity statistics, as detailed in Figure 9.

Figure 9: Next-generation sequencing workflow

(Source: https://dna.macrogen.com)

2.6.1 Sample Preparation and Library Construction

Quality control of the DNA of each sample was performed to determine the concentration and quality. All sample must pass this step before proceeding to the library construction. The sequencing library was prepared by random fragmentation of the DNA sample, followed by 5' and 3' adapter ligation. Alternatively, "tagmentation" combines the fragmentation and ligation reactions into a single step that greatly increases the efficiency of the library preparation process. Adapter-ligated fragments are then PCR amplified and gel purified.

2.6.2 Sequencing Data and Results

For cluster generation, the library was loaded into a flow cell where fragments were captured on a lawn of surface-bound oligos complementary to the library adapters. Each fragment was then amplified into distinct, clonal clusters through bridge amplification. When cluster generation was complete, the templates were ready for sequencing. Raw data was produced through sequencing. The microbial communities were determined by Macrogen, South Korea, according to their established protocols.

2.7 Molecular Identification of *O. muesebecki*

Six *O. muesebecki* specimens from both 2013 and 2016 were first used for the genetic analysis. A molecular profile was established for ticks by using the following two primer pairs of (Ward *et al.*, 2005):

- Fish1F: 5'-TCAACCAACCACAAAGACATTGGCAC-3'
- Fish1R: 5'- TAGACTTCTGGGTGGCCAAAGAATCA-3'
- FishF2: 5′-TCGACTAATCATAAAGATATCGGCAC-3′
- FishR2: 5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′

These primers amplify a region in the cytochrome oxidase c subunit 1 (COI) of the mitochondrial DNA. Amplification was started with an initial denaturation for 2 min at 95°C followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 54°C, and extension for 90 s at 72°C. A final extension step was performed for 10 min at 72°C. The PCR products were visualized using an agarose gel and examined under UV light. Additionally, a universal COI primer was used (Folmer *et al.*, 1994):

- LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'
- HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'

Reactions were amplified through 35 cycles at the following parameters: 1 min at 95°C, 1 min at 40°C, and 1.5 min at 72°C, followed by a final extension step at 72°C for 7 min. A negative control was included in the test. Also, three primers were added to amplify the mitochondrial 16S rDNA and the nuclear 18S rDNA of the tick, to resolve the tick identification sequencing results in which CO1 primers were used. For 16S rDNA amplification, a specific primer which amplifies 460 bp product was used (Wolf *et al.*, 2016):

- 16S+1:5'-CCGGTCTGAACTCAGATCAAGT-3'
- 6S−1:5'-GCTCAATGATTTTTTAAATTGCTGT-3'

Amplification was carried under the following conditions: initial denaturation of 94°C for 5 minutes was followed by 32 cycles, each cycle consisting of a denaturation step of 1 min at 94°C, an annealing step of 1 min at 52.9°C and an extension step of 1 min at 72°C. Final extension was provided at 72°C for 15 min. A second primer set was also used to amplify 16S rDNA (Vial *et al.*, 2006b):

- Tm16S+1: 5'-CTGCTCAATGATTTTTTAAATTGC-3'
- Tm16S-1:5'-CCGGTCTGAACTCAGATCATGTA-3'

To obtain an amplicon of 475-pb, PCR conditions was followed by 10 cycles of 1 min at 92°C, 1.5 min at 48°C, and 1.5 min at 72°C and 32 cycles of 1 min at 92°C, 1.5 min of 54°C, and 1.5 min of 72°C. PCR detection of 18S rDNA of the tick was performed using the specific primers NS3 5'-GCAAGTCTGGTGCCAGCAGCC-3' and NS4 5'-CTTCCGTCAATTCCTTTAAG-3' (Vial *et al.*, 2006b), which amplify a 600 bp fragment of the 18S rDNA of tick species. The amplification protocol consisted of 30 cycles of 1 min at 92°C, 1.5 min at 54°C, and 1.5 min at 72°C. A final extension step was carried out for 10 min at 72°C.

The PCR products were verified by electrophoresis in 1.5% agarose gel stained with ethidium bromide and examined under UV light. The PCR purified amplicons were sent to Macrogen (Seoul, South Korea) for Sanger sequencing. To verify the identity of the sequences, they were analysed by BLAST (National Centre for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi) sequence analysis tool in the GenBank database. DNA sequences were compared to published sequences available in the NCBI GenBank. Sequences of PCR products and those obtained from GenBank were aligned using Muscle alignment tool performed by MEGA7 software (Kumar *et al.*, 2016). A concatenation of these alignments was subjected to phylogenetic analysis by using the neighbour-joining method in the MEGA7 software performing 1000 bootstrap replications. All primers used in this study are summarized in Table 1.

2.8 Scanning Electron Microscopy (SEM)

Prior to image capturing, ticks were cleaned with a soft brush submerged in distilled water to remove all dust and impurities. After that, the samples were gold coated in a sputter coater (Polaron-SC7620) and were examined under SEM (FEI-Quanta,

operated at 15kV). All scanning images were taken in the high voltage mode. Scanning electron microscopy was done in the Physics Department at the UAE University.

Table 1: Summary of all primers used in this study

Specificity	Target Gene	Primer Names	Primer Sequences	Ref.
	16S rDNA	FD1 rp2	5'-AGAGTTTGATCCTGGCTCAG-3' 5'-ACGGCTACCTTGTrACGACTT-3'	(Noda *et al.*, 1997)
Borrelia spp.	OspC	OspC1 OspC2	5'-TAATGAAAAAGAATACATTAAGTG-3' 5'- TTAAGGTTTTTTTGGACTTTCTGC-3'	(Fukunaga *et al.*, 1996)
Rickettsia spp.	ompA	RR190-70 RR190-701 190-FN1 190-RN1	5'- ATGGCGAATATTTCTCCAAAA-3' 5'- GTTCCGTTAATGGCAGCATCT- 3' 5'-AAGCAATACAACAAGGTC-3' 5'- TGACAGTTATTATACCTC -3'	(Blair *et al.*, 2004)
Coxiella	Icd	icdtrg_f icdtrg_r	5'-CGGAGTTAACCGGAGTATCCA-3' 5'-CCGTGAATTTCATGATGTTACCTTT-3'	(de Bruin *et al.*, 2011)
Tick DNA	COXI	Fish1F Fish1R FishF2 FishR2	5'-TCAACCAACCACAAAGACATTGGCAC-3' 5'- TAGACTTCTGGGTGGCCAAAGAATCA-3' 5'-TCGACTAATCATAAAGATATCGGCAC-3' 5'-ACTTCAGGGTGACCGAAGAATCAGAA-3'	(Ward *et al.*, 2005)
Tick DNA	COXI	LCO1490 HCO2198	5'-GGTCAACAAATCATAAAGATATTGG-3' 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	(Folmer *et al.*, 1994)
Tick mitochondria	16S rRNA	16S+1 16S−1	5'-CCGGTCTGAACTCAGATCAAGT-3' 5'-GCTCAATGATTTTTTAAATTGCTGT-3'	(Wolf *et al.*, 2016)
Tick mitochondria	16S rDNA	Tm16S+1 Tm16S-1	5'-CTGCTCAATGATTTTTTAAATTGC-3' 5'-CCGGTCTGAACTCAGATCATGTA-3'	(Vial, L. *et al.*, 2006)
Tick Nucleus	18S rDNA	NS3 NS4	5'-GCAAGTCTGGTGCCAGCAGCC-3' 5'-CTTCCGTCAATTCCTTTAAG-3'	(Vial, L. *et al.*, 2006)

Chapter 3: Results

3.1 PCR Amplification of Bacterial 16S rDNA

A total of 300 adults and nymphs were collected between 2013 and 2016 from Siniya Island (see Figure 7; p. 30). All ticks were identified morphologically as *O. muesebecki* (Ixodoidea: Argasidae). *O. muesebecki* ticks were analysed for the presence of tick-borne pathogens. They were collected from a Socotra cormorant colony on the Island. The primer set fD1 and rP2, which amplify the bacterial16S rDNA was tested against all DNA samples (n=300) to ensure each one of them contained bacterial DNA (16S rDNA gene). The primer was found to work in all tick DNA, as shown in Figure 10. The DNA fragment, which was produced by this primer set was about 1,425-bp long.

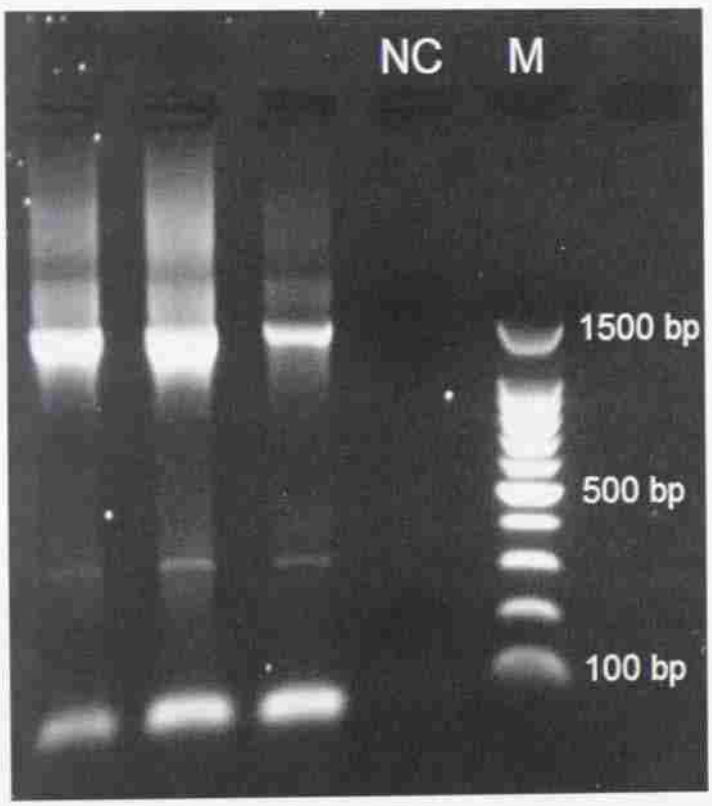

Figure 10: Bacterial 16S rDNA gene length per lane 100 bp ladder
(the gene in 1.5% agarose gel electrophoresis stained by ethidium bromide)

3.2 Bacterial Infection Prevalence

A total of 300 ticks collected from bird hosts were examined by PCR for the presence of *Rickettsia* spp., *Borrelia* spp., and *Coxiella burnetii*. Results were negative for *Rickettsia* spp. and *Borrelia* spp. On the other hand, 286 out of 300 specimens yielded PCR products for the *Coxiella* genes.

3.2.1 *Rickettsia* spp. Detection in *O. muesebecki*

The spotted fever group *Rickettsia* spp. in collected ticks using *ompA* gene-specific primers in a nested PCR was not detected from *O. muesebecki*. The results show that tick DNA extracts did not contain DNA from *Rickettsia* spp. and this is not related to the PCR because the PCR conditions and primers produced the right target PCR products (540-bp) in the positive control reactions (Figure 11). Additionally, based on the results of the 16S rDNA PCR reactions it can be concluded that all the tested DNA samples contained bacterial DNA. Thus the negative results reported here are not false positives, but are the result of zero prevalence of the *Rickettsia* spp.

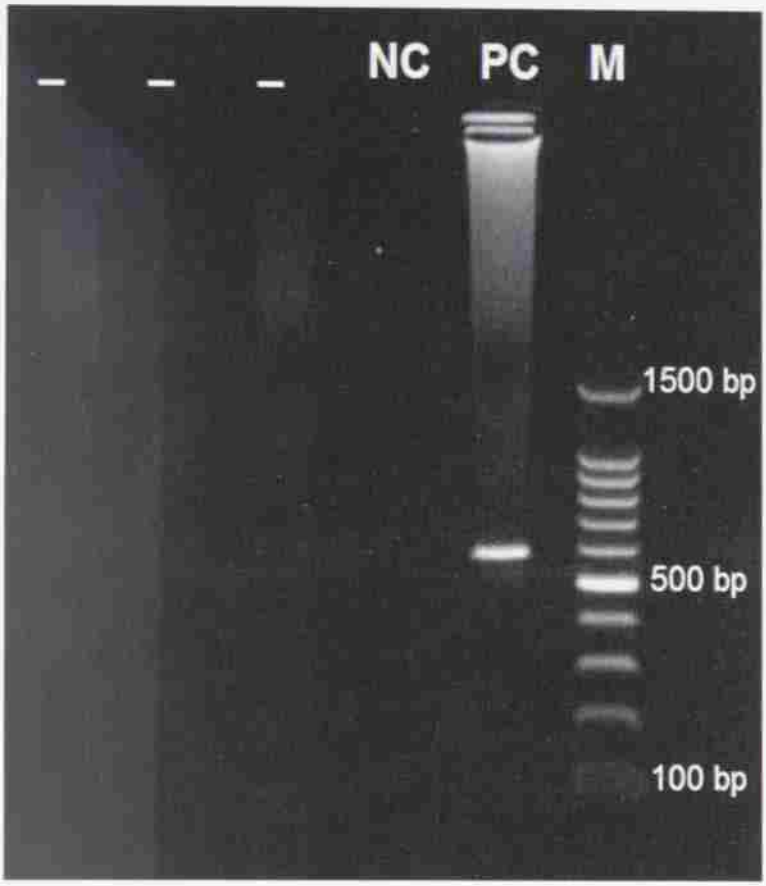

Figure 11: PCR amplification of the spotted fever group *Rickettsia* spp.

[PCR amplification was from *O. muesebecki* using the primer set RR190-70 /RR190-701 followed by 190-FN1/190-RN1 in 1.5% agarose gel stained with ethidium bromide. The gel is showing negative results of Rickettsia spp. NC is negative control; PC is positive control (*Rickettsia* endosymbiont of Amblyomma maculatum ompA, GenBank# JX134638); M is100-bp DNA ladder (Promega, Madison, USA)]

3.2.2 *Borrelia* spp. Detection in *O. muesebecki*

A total of 300 ticks were examined by PCR for the presence of the *Borrelia* spp., which was not detected by PCR in any of the specimens (Figure 12). Detection was carried out using specific primers targeting the *OspC* gene.

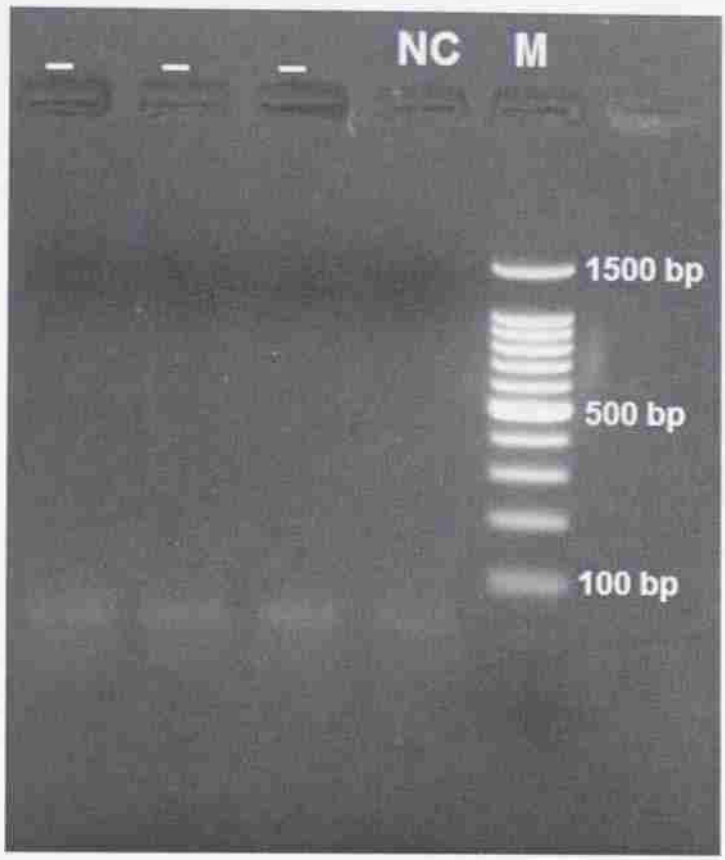

Figure 12: Amplification of *Borrelia* spp. from *O. muesebecki*

[PCR Amplification using the primer set OspC1 and OspC2 in 1.5% agarose gel stained with ethidium bromide. The gel is showing negative results of *Borrelia* spp. NC is negative control; M is 100-bp DNA ladder (Promega, Madison, USA)]

3.2.3 *Coxiella* Detection in *O. muesebecki*

In this work, *Coxiella* prevalence was studied for all specimens to check if these ticks on breeding colonies harbour this bacterium. In PCR the primer *icd* amplified the expected 738-bp region when tested with the nymph and adult ticks and accordingly bands were produced on an agarose gel (as shown in Figure 13). A total of 139 out of 150 samples from 2013 were positive compared to 147 positive samples out 150 from 2016. Figure 14 shows the percentage of *Coxiella* in 2013 and 2016.

Figure 13: *Coxiella* positive samples produced by *icdtrg*-r & *icdtrg*-f primers

[Agarose gel electrophoresis (1.5%) stained with ethidium bromide showing bands of *Coxiella* positive samples produced by the icdtrg-r and icdtrg-f primers and amplified by PCR. The band represents the expected 738-bp PCR product of the isocitrate dehydrogenase (icd); NC is negative control; PC is positive control (*C. burnetii*). M is 100-bp DNA ladder (Promega, Madison, USA)]

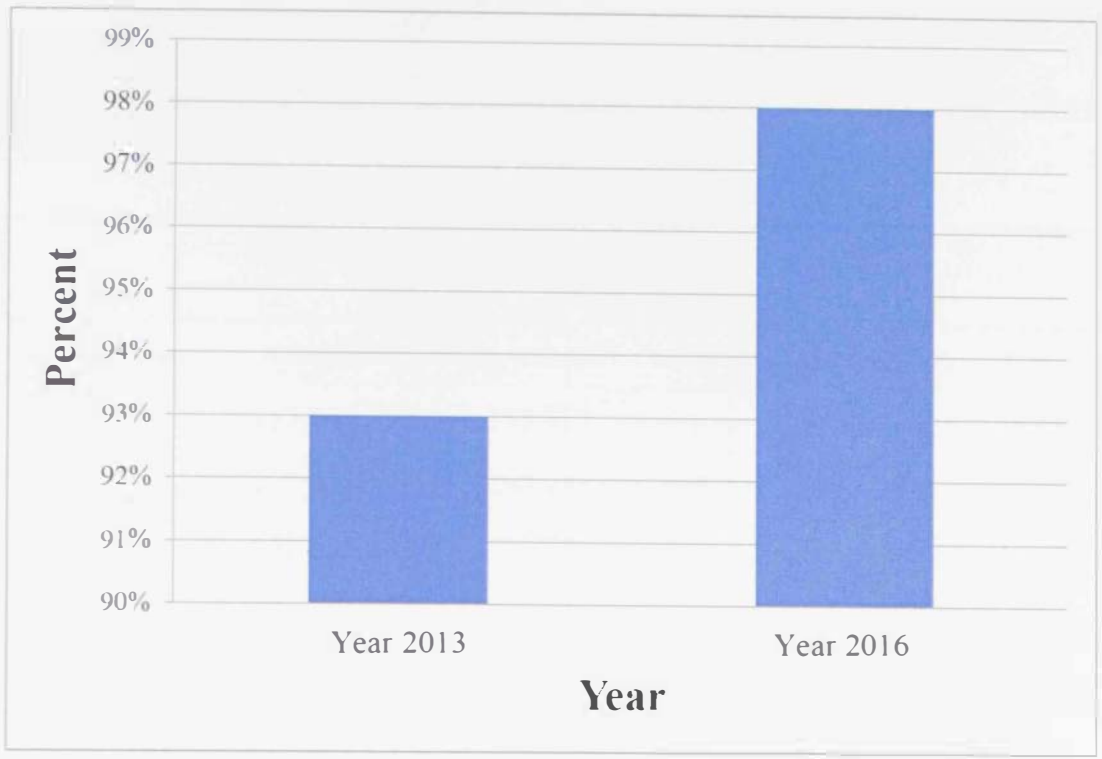

Figure 14: Difference of *Coxiella* presence in 2013 and 2016

3.3 Metagenomic Profile of Tick Microbial Communities

3.3.1 Sequencing Data Quality Analysis- Sample 2013

In this study, the total microbiota associated with the tick *O. muesebecki* was identified via 16S rRNA gene using Miseq system sequencer. Details on total numbers of bases, sequencing reads, and percentage of GC (%), Q20 (%), and Q30 (%) are provided in (Table 2). A total of 2,691,632 reads were produced from five pooled groups, and total read bases were 208.2 Mbp. The number of reads per sample ranged from 294,156 to 341,203. The GC content (%) was 55.176%, and Q30 was 74.003%.

Table 2: Total Nos of bases, reads, and GC (%), Q20 (%), and Q30 (%) calculated for the 5 samples in 2013

Read Quality by Sample						
Sample Name	**Total Bases**	**Read Count**	**N(%)**	**GC(%)**	**Q20(%)**	**Q30(%)**
01	152,947,377	332,117	0.0	54.53	97.75	93.06
02	135,405,149	294,156	0.0	54.39	97.76	93.07
03	152,506,182	331,830	0.0	54.31	97.73	92.93
04	144,942,961	314,993	0.0	54.19	97.68	92.81
05	156,833,055	341,203	0.0	53.63	97.71	92.86

* Total Bases: The total number of bases in reads identified
* Read Count: The total number of sequence reads
* N(%): The N percentage in sequence reads
* GC(%): The GC percentage sequence reads
* Q20(%): The percentage of bases in which the Phred score is above 20
* Q30(%): The percentage of bases in which the Phred score is above 30

* OTUs: Operational Taxonomic Unit is an operational definition of a species or group of species often used when only DNA sequence data is available
* Chao1: returns the Chao1 richness estimate for an OTU definition
* Shannon: The Shannon index takes into account the number and evenness of species.
* Simpson: The Simpson index represents the probability that two randomly selected individuals in the habitat will belong to the same species.
* Goods Coverage: Coverage is calculated as $C = 1-(s/n)$,
where s is the number of unique OTUs and n is the number of individuals in the sample.
*This index gives a relative measure of how well the sample represents the larger environment

Sequence quality trimming and filtering relied upon the perfect identity of paired-end read overlaps. This approach generates very high-quality reads by eliminating the majority of sequencing errors. Quality filtering was carried out using strict criteria of no ambiguous bases and no N bases. Chimeric sequences were detected and removed, leaving 50,756 unique sequences (Table 3). Reads were clustered into

operational taxonomic units (OTUs) at 97% sequence identity level. Cluster analysis at 97% identified 270,508 OTUs across the entire data.

Table 3: Pre-processing & Clustering (by CD-HIT-OTU)

	Sample Name	Read Count
Results of Clustering (cut-off: 97%)	01	60,927
	02	56,207
	03	53,756
	04	51,689
	05	47,929
Results of Pre-processing	Sample Count	5
	Read Count	270,508
	Gamma-diversity	323
Counts/sample summary	Min	47,929
	Max	60,927
	Median	53,756
	Mean	54,101
Filtered Read Count	Ambiguous	0
	Low-Quality	183,356
	Chimera	50,756
	Other	1,109,679

* Sample Count: The total number of sample
* Read Count: The total number of sequence reads
* Sample Count : The total number of sample
* Gamma-diversity represents the diversity across an entire landscape. (alpha + beta diversity)
* Alpha-diversity corresponds to species diversity in sites/habitats at a local scale
* Beta-diversity comprises species diversity among sites/habitats
* Min : Minimum number of sequence per samples
* Max : Maximum number of sequence per samples
* Median : The number separating the higher half of a data samples
* Mean : The average number of the sequence of samples
* Ambiguous : Filtered seqs with ambiguous base calls
* Low-Quality : Filtered seqs with low-quality bases (Quality score offset 33)

3.3.2 Taxonomic Assignment

The bacterial diversity associated with *O. muesebecki* is presented here at taxonomical levels as dominant bacterial phyla, genera, and species. Of the sixteenth total bacterial phyla present in the whole *O. muesebecki* tick samples, the *Firmicutes,*

Proteobacteria, and *Bacteroidetes* were found to be the most dominant (Table 4). The percent abundance was variable with an average 74.6% *Firmicutes*, 14.5% *Proteobacteria* and only 6.7 % *Bacteroidetes*.

There was a total of 105 bacterial families observed in the *O. muesebecki* whole tick microbiome. Only three bacterial families were detected at more than 15% abundance (Figure 15) and (Table 6), and the most dominant was *Bacillaceae 2* followed by Staphylococcaceae. Overall, there were 150 bacterial genera present in the whole *O. muesebecki* tick samples. Of these, *Salinicoccus* (17%), *Bacillus* and *Virgibacillus* (9.8%) were the dominant genera observed based on the number of reads (Figure 16) and (Table 6). The overall tick microbiota in this study was less diverse, with only one dominant bacterial species (Figure 17) and (Table 6). The most prevalent species observed, with greater than 1% abundance in the ticks, was the *Coxiella burnetii*.

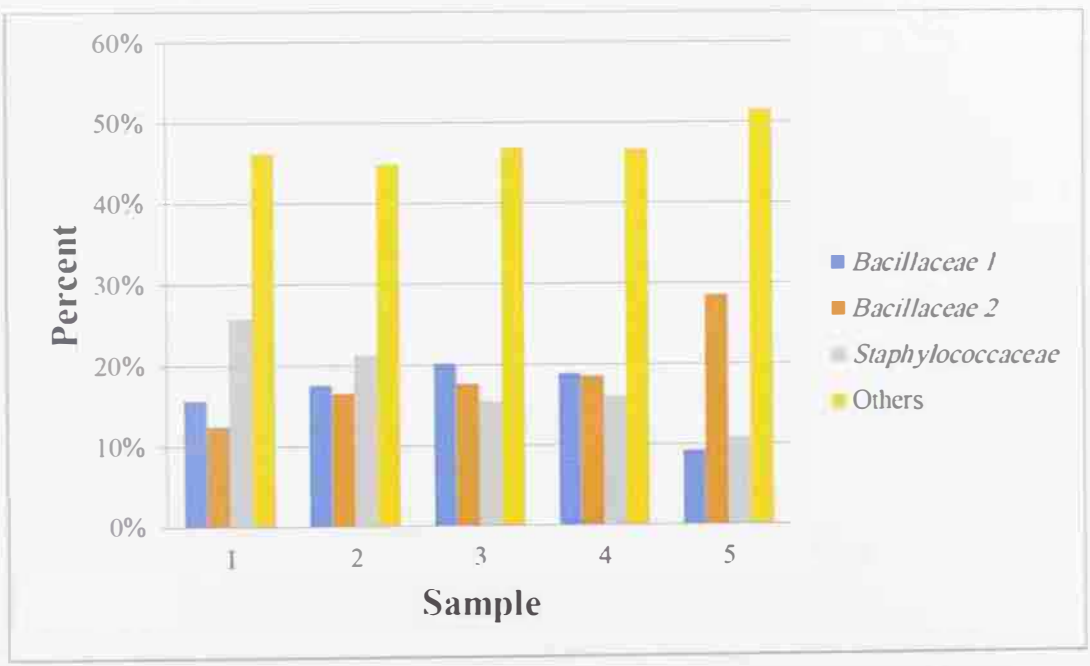

Figure 15: Bacterial diversity at the family level in *O. muesebecki* ticks

[The percentage of sequence reads of each bacterial family was presented from individual ticks. The bacterial families with less than 1% pooled together were presented as 'others']

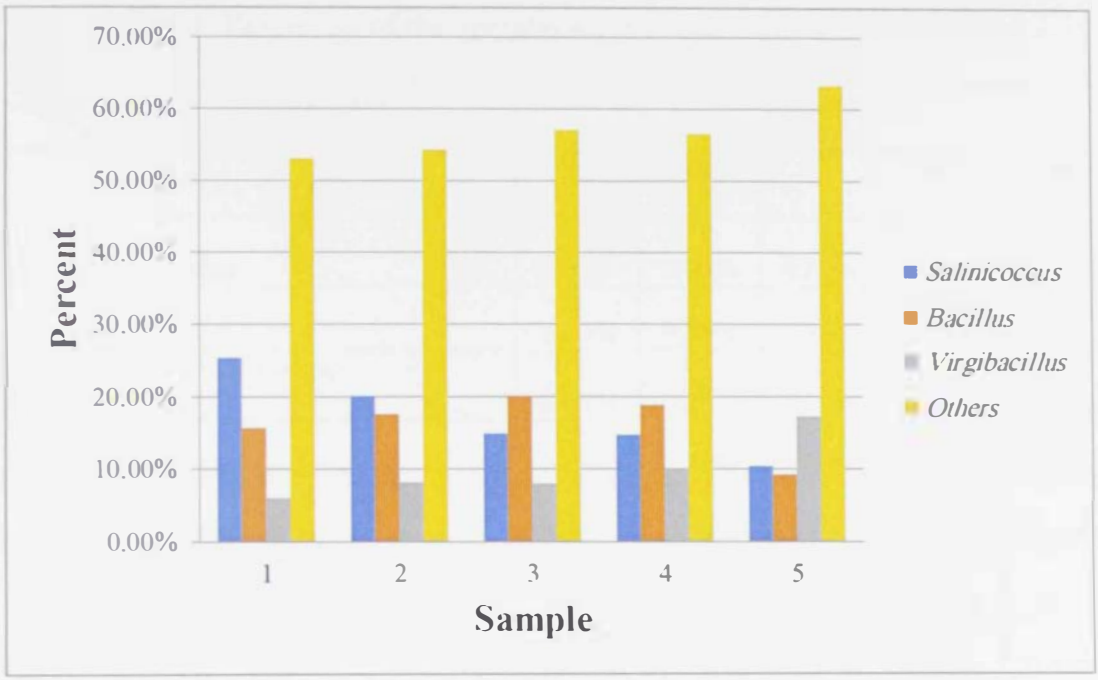

Figure 16: Bacterial diversity at genus level in *O. muesebecki* ticks

[The percentage of sequence reads of each bacterial genus were presented from individual ticks. The bacterial genera with less than 1% pooled together were presented as 'others']

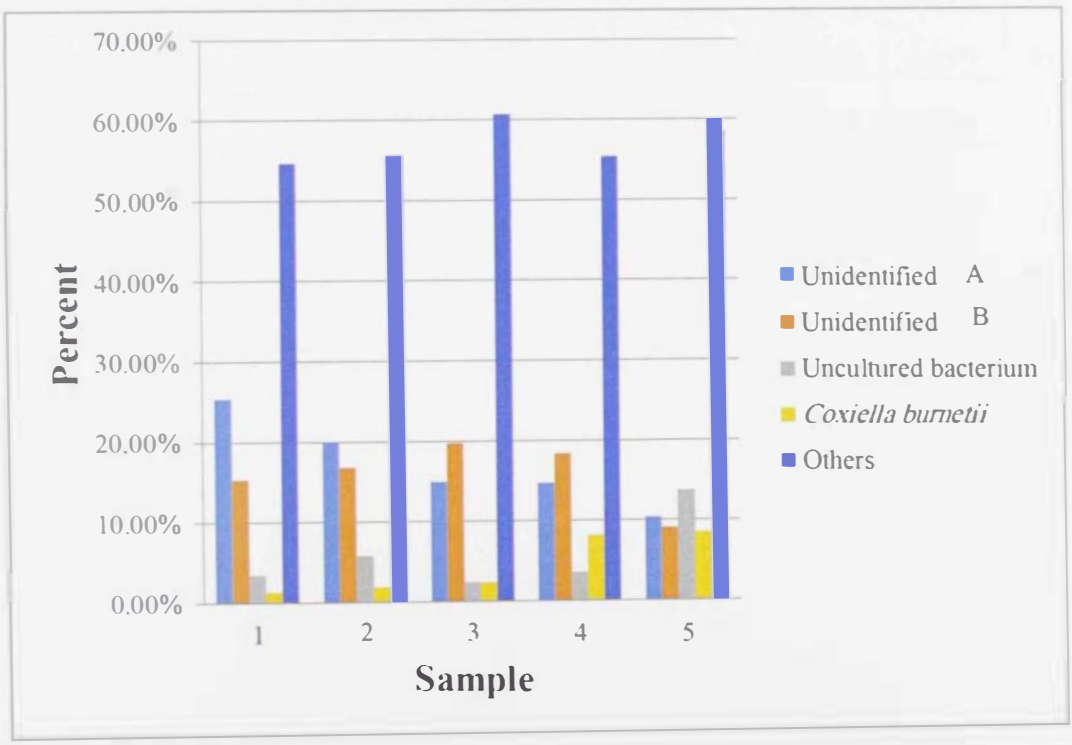

Figure 17: Bacterial diversity at the species level in *O. muesebecki* ticks

[The percentage of sequence reads of each bacterial species were presented from individual ticks. The bacterial species with less than 1% pooled together were presented as 'others']

Table 4: Taxonomy of the operational units (OTU) at the phylum level

Kingdom	Phylum	DNA Samples				
		01	02	03	04	05
Bacteria	Other	0.03%	0.00%	0.00%	0.06%	0.00%
Bacteria		1.26%	0.66%	0.76%	0.79%	0.62%
Bacteria	*Acidobacteria*	0.00%	0.29%	0.07%	0.00%	0.00%
Bacteria	*Actinobacteria*	3.05%	2.84%	1.75%	2.07%	1.47%
Bacteria	*Bacteroidetes*	3.32%	6.03%	5.07%	4.99%	14.23%
Bacteria	*Chloroflexi*	0.08%	0.38%	0.15%	0.10%	0.07%
Bacteria	*Fusobacteria*	0.14%	0.41%	0.39%	0.33%	0.48%
Bacteria	*Gemmatimonadetes*	0.02%	0.00%	0.08%	0.00%	0.03%
Bacteria	*Planctomycetes*	0.40%	0.17%	0.10%	0.05%	0.03%
Bacteria	*Proteobacteria*	13.34%	13.85%	11.21%	17.17%	17.06%
Bacteria	*Spirochaetes*	0.00%	0.00%	0.00%	0.07%	0.00%
Bacteria	*Tenericutes*	0.00%	0.00%	0.00%	0.06%	0.00%
Bacteria	*Verrucomicrobia*	0.00%	0.00%	0.06%	0.00%	0.03%
Bacteria	*Candidatus Saccharibacteria*	0.00%	0.00%	0.00%	0.13%	0.00%
Bacteria	*Firmicutes*	78.37%	75.15%	80.01%	74.13%	65.68%
Bacteria	*candidate div. WPS-1*	0.00%	0.00%	0.00%	0.00%	0.08%

Table 5: Abundance/taxonomy of dominant bacteria in 5 samples from 2013

[The top five taxa/groups are shown for each of these five ranks]

Level	OUT ID	DNA Samples				
		01	02	03	04	05
Class	*Bacilli*	76.56%	73.15%	78.53%	72.17%	62.99%
	Gammaproteobacteria	4.49%	5.92%	5.56%	12.07%	13.42%
	Flavobacteriia	1.40%	2.30%	2.45%	3.11%	9.42%
	Alphaproteobacteria	4.85%	3.30%	3.62%	3.22%	2.12%
	Cytophagia	1.80%	2.23%	2.01%	1.44%	4.22%
Order	*Bacillales*	71.65%	68.33%	73.56%	68.58%	59.75%
	Legionellales	1.29%	1.85%	2.30%	8.13%	8.42%
	Lactobacillales	4.91%	4.38%	4.97%	3.60%	3.24%
	Flavobacteriales	1.40%	2.30%	2.45%	3.11%	9.42%
	Cytophagales	1.80%	2.23%	2.01%	1.44%	4.22%
Family	*Bacillaceae 2*	12.43%	16.48%	17.62%	18.54%	28.50%
	Staphylococcaceae	25.72%	21.15%	15.44%	16.02%	10.79%
	Bacillaceae 1	15.65%	17.56%	20.11%	18.79%	9.18%
	Planococcaceae	12.40%	7.99%	12.23%	6.87%	8.23%
	Coxiellaceae	1.29%	1.85%	2.30%	8.13%	8.42%
Genus	*Salinicoccus*	25.37%	20.07%	14.89%	14.65%	10.36%
	Bacillus	15.65%	17.56%	20.11%	18.79%	9.18%
	Virgibacillus	5.99%	8.09%	7.91%	9.99%	17.22%
	Sporosarcina	10.81%	6.06%	10.24%	5.36%	7.08%
	Coxiella	1.29%	1.85%	2.30%	8.13%	8.42%
Species	Other A	25.37%	20.00%	14.89%	14.65%	10.36%
	Other B	15.29%	16.78%	19.75%	18.35%	9.06%
	Other C	10.81%	6.06%	9.67%	5.36%	6.86%
	uncultured bacterium	3.44%	5.73%	2.36%	3.52%	13.65%
	Coxiella burnetii	1.29%	1.85%	2.30%	8.13%	8.42%

3.3.3 Ecological Parameters: Richness and Diversity Indices

Rarefaction curves and alpha-diversity indices, based on the species richness, were calculated to obtain information on ecological parameters. The values obtained for the alpha-diversity indices are presented in (Table 6). The OTU number ranged from 138 to 183, with a total of 809 OTU detected at 97% sequence identity (cut-off level of 3%) (Table 6). The Chao 1, Shannon and Simpson indices measured of the richness and diversity percentage in the community (Table 6). Good's coverage was calculated to demonstrate the sample coverage.

As for Chao1, values were quite similar in all samples, whereas the highest and lowest values were reached in sample 5 and sample 1, respectively. Shannon–Wiener index values were between 4.73 (sample 1) and 5.09 (sample 2). The Simpson index values were near to 1 in all samples.

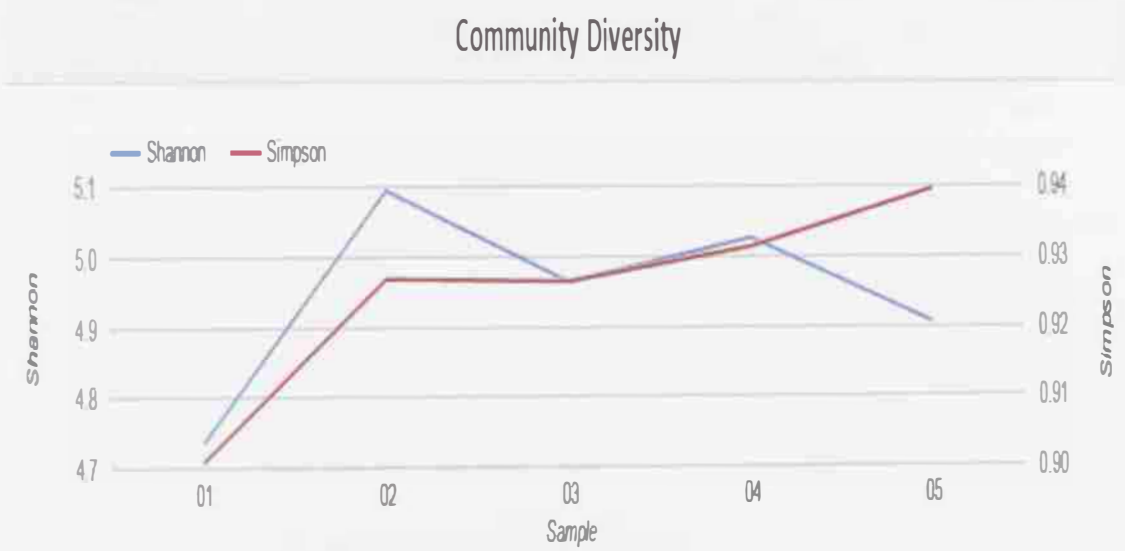

Figure 18: Differences in bacterial diversity within tick samples

[using Shannon and Simpson Index]

Table 6: Estimated OTU for five samples of *O. muesebecki* in 2013

[OUT regarding richness, diversity indices, and estimated sample coverage]

OTUs					
Community Diversity					
Sample Name	**OTUs**	**Chao1**	**Shannon**	**Simpson**	**Goods Coverage**
01	143.0	143.0	4.735005321	0.900834632812	0.999983586915
02	138.0	138.0	5.09541793019	0.926906173555	1.0
03	175.0	175.0	4.96402940365	0.926506901701	1.0
04	170.0	170.0	5.02667250994	0.931416830023	1.0
05	183.0	187.5	4.90566395913	0.939692059936	0.999812222245

* OTUs : Operational Taxonomic Unit is an operational definition of a species or group of species often used when only DNA sequence data is available
* Chao1 : returns the Chao1 richness estimate for an OTU definition
* Shannon : The Shannon index takes into account the number and evenness of species.
* Simpson : The Simpson index represents the probability that two randomly selected individuals in the habitat will belong to the same species.
*Goods Coverage: Coverage is calculated as $C=1-(s/n)$, where s is the number of unique OTUs and n is the number of individuals in the sample.
*This index gives a relative measure of how well the sample represents the broader environment.

3.3.4 Determination of Species Richness by Rarefaction Curve

Alpha rarefaction graph shows whether the number of reads used in the analysis was sufficient in identifying species/OTU. If the curve becomes flattered to the right, it indicates that a reasonable number of reads have been used in the analysis. Thus additional sequencing is not necessary. In contrast, if the graph does not plateau, the additional reads are likely to discover more OTUs for the sample (x-axis: read number; y-axis: number of OTUS).

In this thesis, rarefaction curves were calculated as shown in (Figure 19). All samples were rarefied to a depth of 53,750 sequence reads. The rarefaction curves for the first three samples (1-2-3) reached saturation at 53,750 sequence reads indicating different OTU levels; 143, 138, 175 respectively. In sample 4, however, rarefaction curve reached saturation at 48,376 sequence reads indicating that the sampling effort covered almost 170 different OTUs. The highest OTUs was observed in sample 5 which was about 183 OTUs at 43,002 sequences read. The rarefaction curves for all samples reached the saturation plateau, demonstrating that our sequencing depth was sufficient, and the sequence database was enough to capture the diversity of bacterial communities in the present study.

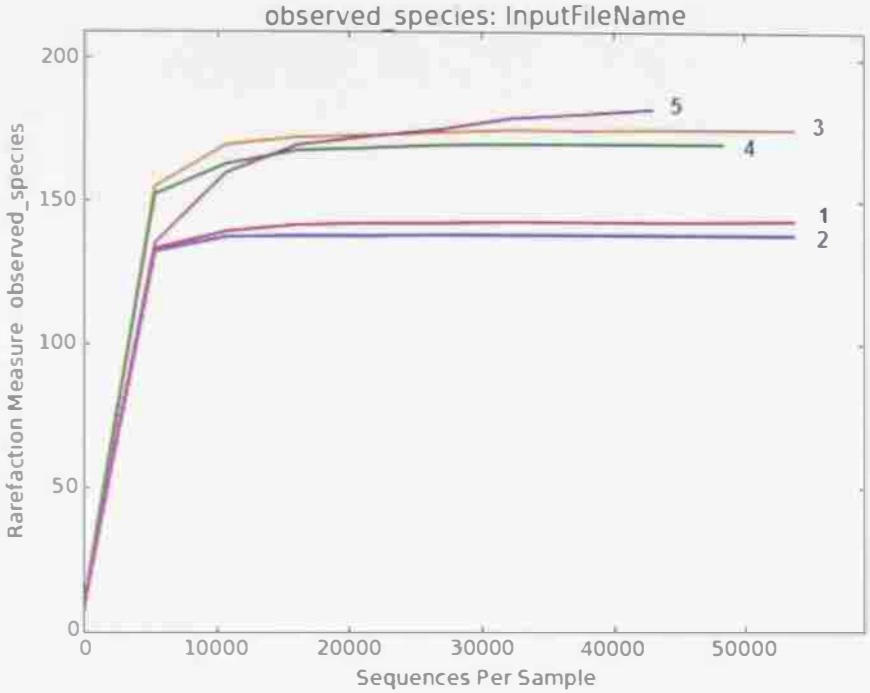

Figure 19: Rarefaction curves per number of observed species in tick samples

[Curves are horizontal with the x-axis indicating that additional sequencing would not yield additional novel data]

3.3.5 OTU Heatmap

The OTU heat map displayed raw OTU counts per sample, where the counts were coloured based on the contribution of each OTU to the total OTU count present in that sample (blue: contributes a low percentage of OTUs to sample; red: contributes a high percentage of OTUs). The heatmap in (Figure 20) shows the relative prevalence of the dominant tick bacterial species across the tick samples. Only pathogenic *Coxiella burnetii* was observed in a pooled sample of ticks as the highest dominant bacteria.

Figure 20: Rarefied abundances heatmap of most abundant spp. in each sample

[Species are arranged in order of increasing prevalence from top to bottom. Samples on the x-axis are ordered. The gap represents deleted low percentage OTUs (blue colour)]

3.3.6 Classification of Bacterial Taxa -RDP and NCBI

Two databases namely the Ribosomal Database Project (RDP) classifier and the National Center for Biotechnology Information (NCBI) taxonomy were used to assign taxonomic levels to the samples collected in 2013. Each OTU produced by the QIIME pipeline was classified to the bacterial kingdoms. Between 47,929 and 60,927 sequence reads of *O. meusbeci* tick, were assigned from phylum to the species level. There were no differences on microbial population analysis between RDP and NCBI databases in the genus (Figure 21, Figure 22) and species levels (Figure 23, Figure 24). A large number of bacteria was categorized into the phylum *Firmicutes* from the tick samples, therefore a phylum with relative abundance ≥ 5% was defined as a dominant phylum.

At the phylum level, these reads were classified as *Proteobacteria*, *Bacteroidetes*, *Actinobacteria, Acidobacteria, Chloroflexi, Fusobacteria, Gemmatimonadetes, Planctomycetes, Spirochaetes, Tenericutes, Verrucomicrobia, Candidatus Saccharibacteria* and *Candidate division WPS-1* by the RDP and NCBI databases. A high percentage of sequence reads in these samples were unclassified at the species level. *Coxiella burnetii*, however, accounted for most of the microbial population in all samples.

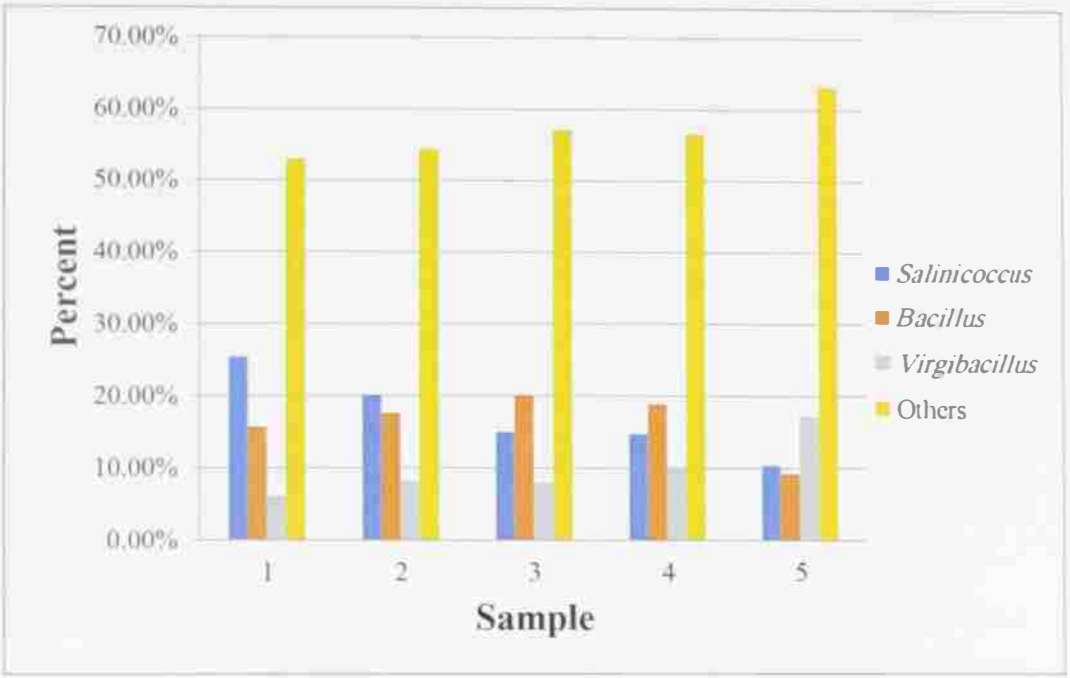

Figure 21: *O. muesebecki* tick classified at the geneus level used NCBI database [Prevalence of the bacteria was similar to RDP classifier]

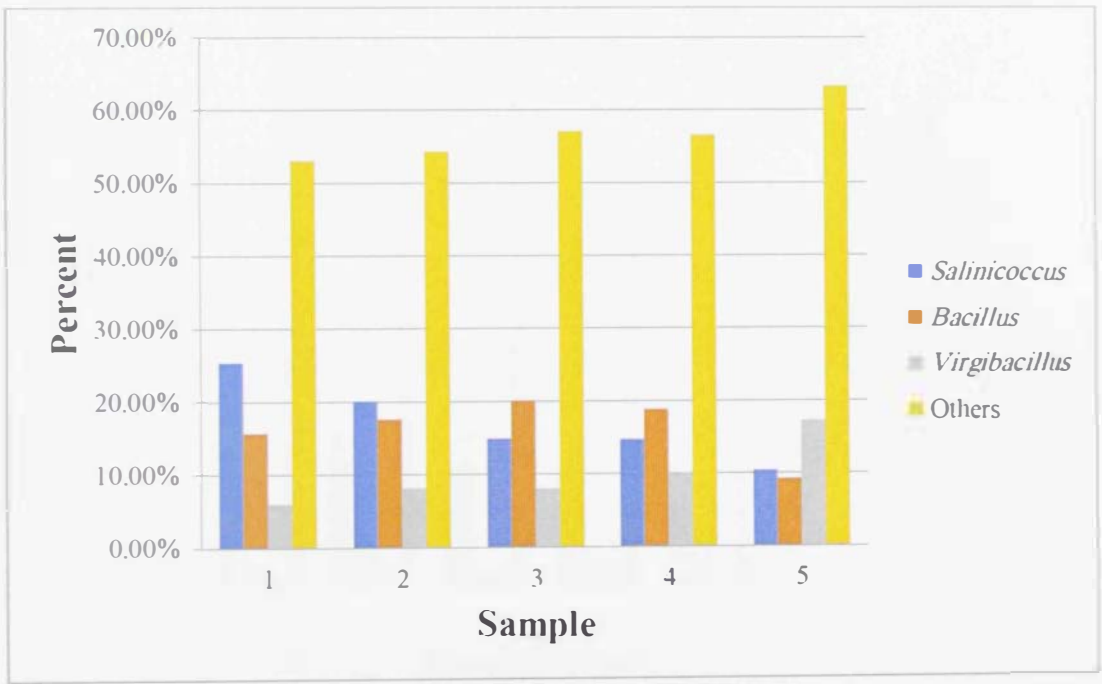

Figure 22: *O. muesebecki* tick classified at genus level used RDP classifier

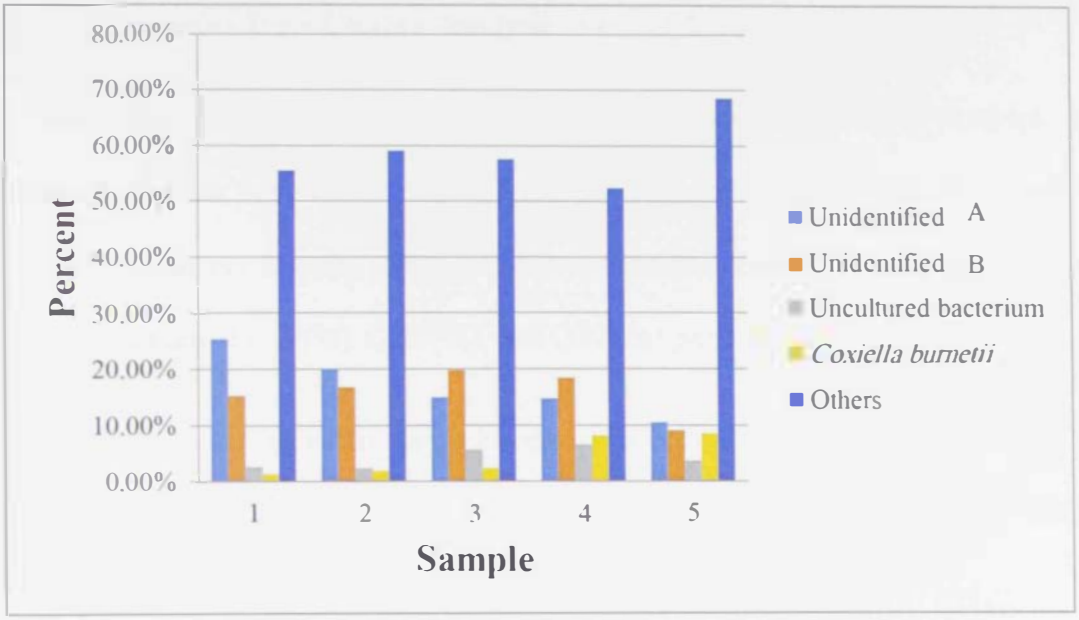

Figure 23: *O. muesebecki* tick classified at species level used NCBI database

[Prevalence of the bacteria was similar to RDP classifier]

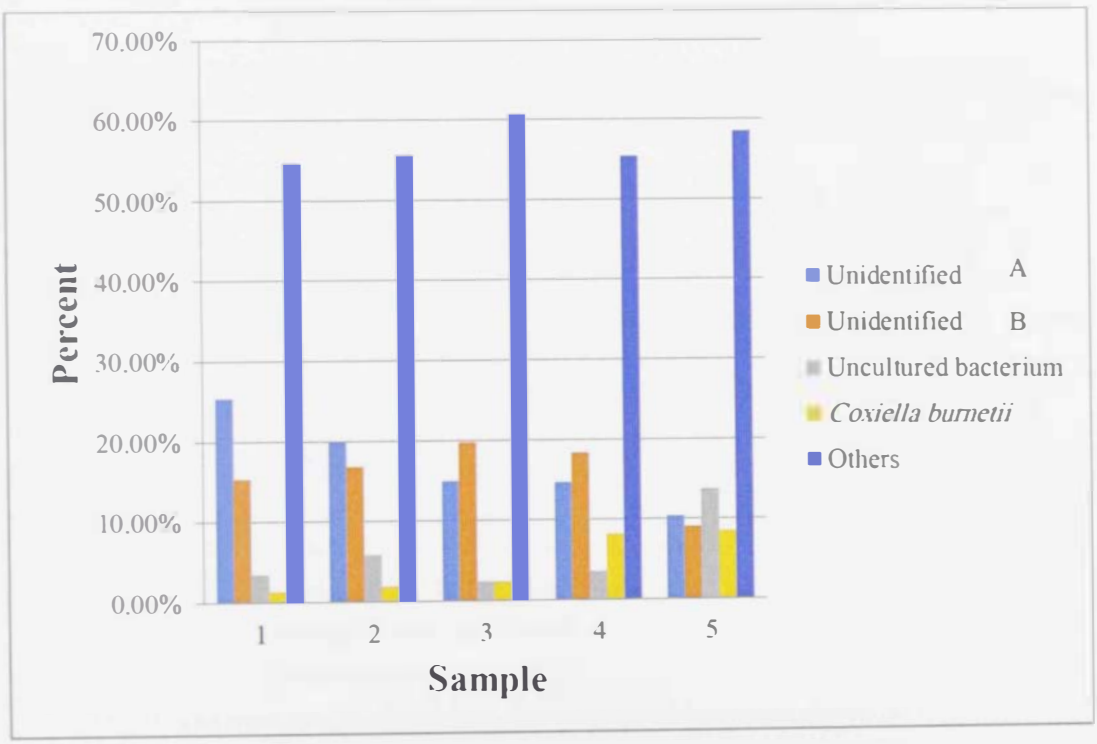

Figure 24: *O. muesebecki* tick classified at species level used RDP classifier

3.3.7 Sequencing Data Quality Analysis- Sample 2016

A total of 915,715 effective sequences were obtained from the 5 samples. The number of reads per sample ranged from 181,003 and 186,079 with an average of 183,143 reads per sample. Table 7 illustrates numbers of bases, sequencing reads, and percentage of GC (%), Q20 (%), and Q30 (%) per a group of ticks.

After quality filtering, reads were clustered into operational taxonomic units (OTUs) at 97% sequence identity level (Table 8). Through clustering, a total of 310,579 OTUs were recovered at the cutoff level of 3% across the entire data.

Table 7: Total Nos of bases, reads, GC (%), Q20 (%), and Q30 (%) calculated for the 5 samples in 2016

Sample Name	Total Bases	Read Count	N(%)	GC(%)	Q20(%)	Q30(%)
06	83,059,832	181,798	0.0	53.19	98.26	94.3
07	85,334,416	186,079	0.0	53.95	98.24	94.27
08	83,714,093	185,309	0.0	52.9	98.3	94.44
09	82,141,959	181,003	0.0	53.03	98.06	93.87
10	82,988,008	181,526	0.0	52.38	98.16	94.04

* Total Bases: The total number of bases in reads identified
* Read Count: The total number of sequence reads
* N(%): The N percentage in sequence reads
* GC(%): The GC percentage sequence reads
* Q20(%): The percentage of bases in which the phred score is above 20
* Q30(%): The percentage of bases in which the phred score is above 30

Table 8: Preprocessing & Clustering (by CD-HIT-OTU)

	Sample Name	Read Count
Results of Clustering (cut-off: 97%)	06	62,177
	07	67,852
	08	63,501
	09	56,718
	10	60,331
Results of Pre-processing	Sample Count	5
	Read Count	310,579
	Gamma-diversity	1920
Counts/sample summary	Min	56,718
	Max	67,852
	Median	62,177
	Mean	62,115
Filtered Read Count	Ambiguous	0
	Low-Quality	18,628
	Chimera	56,836
	Other	529,672

* Sample Count: The total number of sample
* Read Count: The total number of sequence reads
* Sample Count: The total number of sample
* Gamma-diversity represents the diversity across an entire landscape. (alpha + beta diversity)
* Alpha-diversity corresponds to species diversity in sites/habitats at a local scale
* Beta-diversity comprises species diversity among sites/habitats
* Min: Minimum number of sequence per samples
* Max: Maximum number of sequence per samples
* Median: The number separating the higher half of a data samples
* Mean: The average number of the sequence of samples
* Ambiguous: Filtered seqs with ambiguous base calls
* Low-Quality: Filtered seqs with low-quality bases (Quality score offset 33)
* Chimera: Filtered seqs with chimeric reads

3.3.8 Taxonomic Assignment

For 2016 specimens, the taxonomy-based analyses were performed using the RDP database, and all reads were classified to the Archaea and Bacterial kingdoms. The diversity of the bacteria in tick specimens indicated that the identified OTUs were classified into 38 phyla, 84 classes, 145 orders, 264 families, 539 genera and 836 species at a 97% sequence similarity level. Furthermore, four main phyla, six classes, five orders, five families, five genera and four species (>5% abundance) were found in the five samples (Table 9 and Table 10). In detail, at the phylum level, *Proteobacteria* and *Firmicutes* were the two main phyla, accounting for 37% of the total phyla (Table 9).

The most dominant phylum was *Proteobacteria* in samples 6, 8, 9 and 10; whereas *Firmicutes* was the most highly represented phylum in sample 7 (Table 9). Members of other phyla such as *Bacteroidetes* and *Cyanobacteria/Chloroplast* were also commonly presented in these samples at a relatively lower abundance (Table 9). Also, similar trends were observed in these samples at the class level, where *Gammaproteobacteria* and *Bacilli* were dominant with populations ranging from 11.36 to 58.77% (Table 10).

A total of 145 orders were detected, and about 41 of which were shared by the five samples, including *Acidimicrobiales, Bifidobacteriales, Coriobacteriales, Bacteroidales, Cytophagales, Clostridiales, Verrucomicrobiales, Pseudomonadales, Bacillales, Lactobacillales* and so on, and accounted for1.74% of all the sequences. Moreover, 104 orders appeared only in specific samples, such as *Mycoplasmatales* in sample 9 (1.84%), *Acholeplasmatales* in 10 (1.49%), *Gaiellales* in sample 7 (0.03%), and others.

There was a total of 264 bacterial families observed in the *O. muesebecki* whole tick microbiome. Only three bacterial families were detected at more than 2% abundance (Figure 25), and the most dominant was *Coxiellaceae* followed by Bacillaceae *1* and *Bacillaceae 2*. In terms of genus, the top five most abundant genera were *Coxiella, Bacillus, Virgibacillus, Sporosarcina* and *Lactobacillus* and all of them occupied more than 8.5% among all of the detected sequences (Figure 26). The overall tick bacterial species in this study was less diverse, with only one dominant bacterial species (Figure 27). *Coxiella burnetii* was the most prevalent species observed in the ticks with greater than 10% abundance.

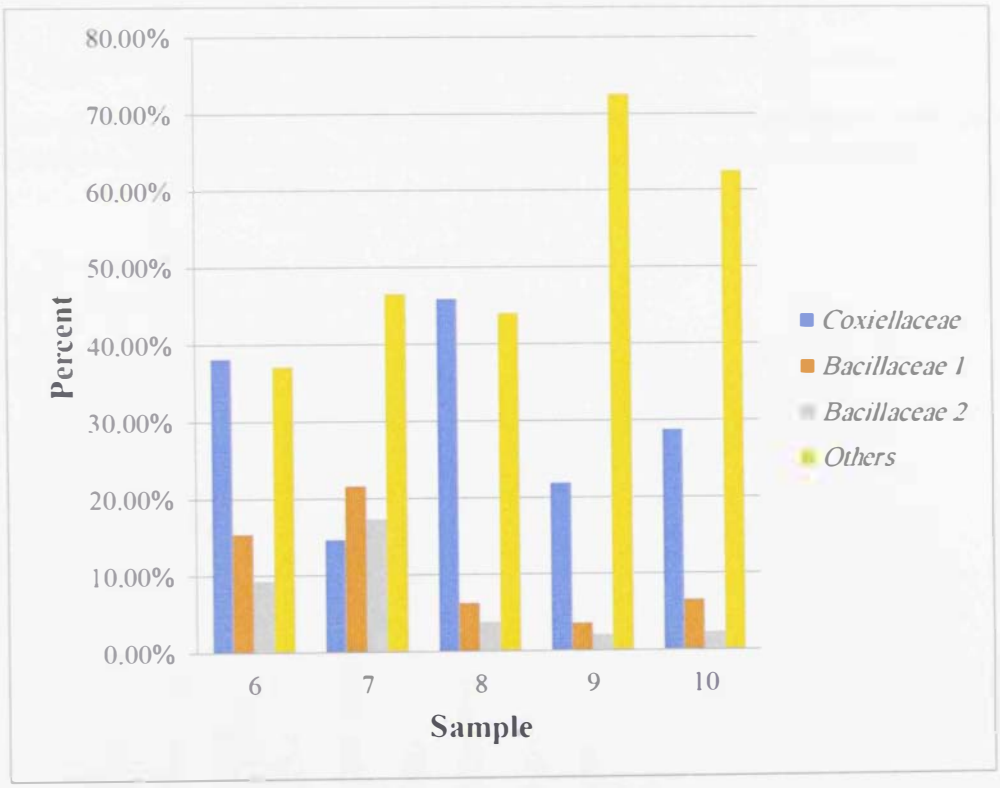

Figure 25: Bacterial diversity at the family level in *O. muesebecki* ticks

[The percentage of sequence reads of each bacterial family was presented from individual ticks. The bacterial families with less than 1% pooled together were presented as 'others']

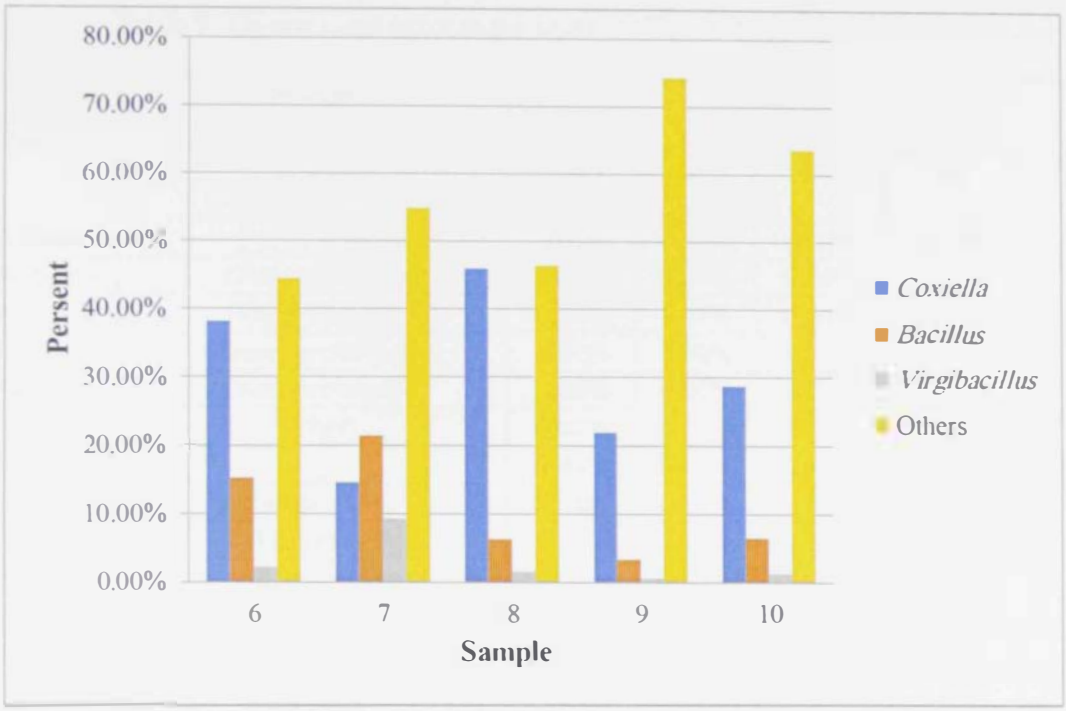

Figure 26: Bacterial diversity at genus level in *O. muesebecki* ticks

[The percentage of sequence reads of each bacterial genus was presented from individual ticks. The bacterial genus with less than 1% pooled together was presented as 'others']

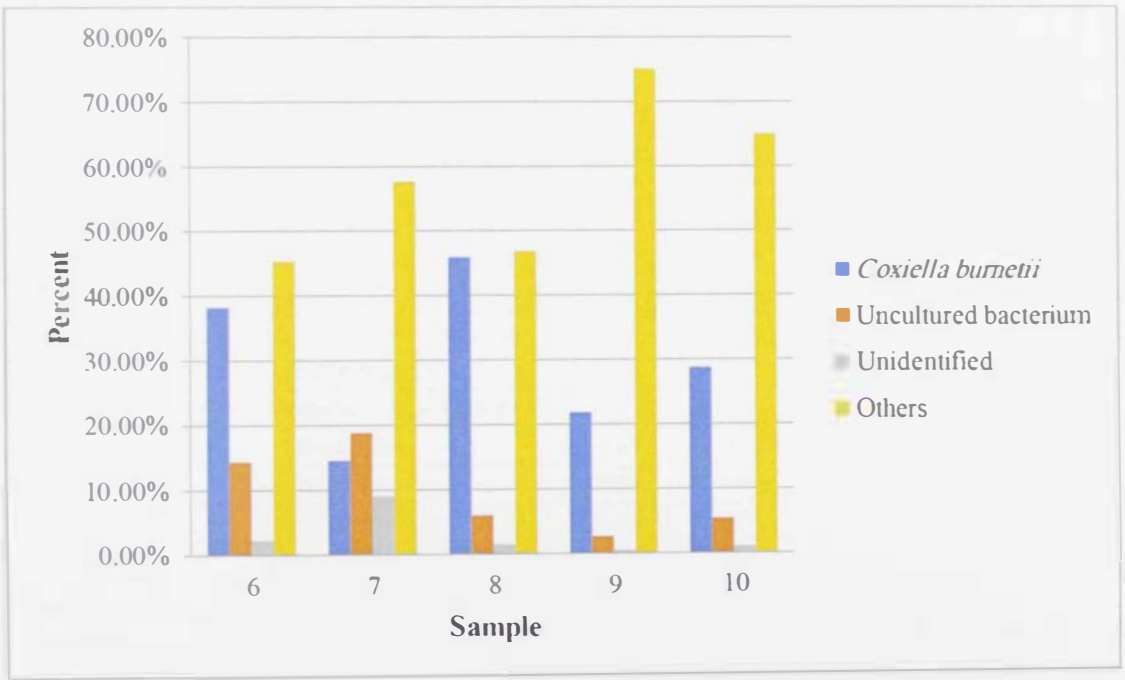

Figure 27: Bacterial diversity at the species level in *O. muesebecki* ticks

[The percentage of sequence reads of each bacterial species were presented from individual ticks. The bacterial species with less than 1% pooled together were presented as 'others']

Table 9: Operational taxonomic units (OTU) at the phylum level

Kingdom	Phylum	DNA Samples				
		06	07	08	09	10
Archaea	__	0.00%	0.00%	0.00%	0.04%	0.00%
Archaea	*Crenarchaeota*	0.00%	0.00%	0.00%	0.00%	0.00%
Archaea	*Euryarchaeota*	0.44%	0.02%	0.33%	0.22%	0.33%
Archaea	*Thaumarchaeota*	0.01%	0.00%	0.05%	0.01%	0.00%
Archaea	*Woesearchaeota*	0.00%	0.00%	0.00%	0.02%	0.00%
Bacteria	*Other*	0.02%	0.03%	0.02%	0.05%	0.39%
Bacteria	__	0.44%	0.51%	0.96%	2.81%	2.35%
Bacteria	*Acidobacteria*	0.16%	0.67%	0.07%	0.59%	0.01%
Bacteria	*Actinobacteria*	5.55%	3.91%	4.17%	10.54%	0.54%
Bacteria	*Armatimonadetes*	0.05%	0.02%	0.00%	0.44%	0.00%
Bacteria	*Bacteroidetes*	3.60%	2.38%	4.46%	10.27%	21.10%
Bacteria	*Chlamydiae*	0.00%	0.00%	0.00%	0.00%	0.02%
Bacteria	*Chloroflexi*	0.09%	0.08%	0.24%	0.22%	0.46%
Bacteria	*Deferribacteres*	0.09%	0.08%	0.02%	0.00%	1.42%
Bacteria	*Deinococcus-Thermus*	0.09%	0.22%	0.10%	0.08%	0.04%
Bacteria	*Elusimicrobia*	0.00%	0.00%	0.00%	0.00%	0.01%
Bacteria	*Fibrobacteres*	0.35%	0.00%	0.00%	0.00%	0.37%
Bacteria	*Fusobacteria*	0.01%	0.23%	0.11%	0.48%	0.46%
Bacteria	*Gemmatimonadetes*	0.01%	0.06%	0.01%	0.08%	0.00%
Bacteria	*Lentisphaerae*	0.01%	0.00%	0.05%	0.00%	0.02%
Bacteria	*Planctomycetes*	0.03%	0.36%	0.34%	0.56%	0.14%
Bacteria	*Proteobacteria*	43.56%	22.51%	58.18%	41.44%	36.47%
Bacteria	*Spirochaete*	0.02%	0.00%	0.03%	0.00%	1.21%
Bacteria	*Synergistetes*	0.00%	0.00%	0.07%	0.00%	1.21%
Bacteria	*Tenericutes*	0.05%	0.00%	0.00%	1.84%	1.49%
Bacteria	*Thermotogae*	0.00%	0.01%	0.00%	0.00%	0.15%
Bacteria	*Verrucomicrobia*	0.17%	1.35%	0.21%	5.49%	0.89%
Bacteria	*BRC1*	0.03%	0.00%	0.00%	0.00%	0.00%
Bacteria	*Candidatus Saccharibacteria*	0.17%	0.19%	0.05%	0.02%	1.17%
Bacteria	*Cloacimonetes*	0.02%	0.00%	0.00%	0.00%	0.23%
Bacteria	*Cyanobacteria/Chloroplast*	2.27%	4.82%	2.16%	7.07%	1.41%
Bacteria	*Firmicutes*	42.36%	61.97%	28.29%	15.09%	27.11%
Bacteria	*Ignavibacteriae*	0.00%	0.00%	0.00%	0.00%	0.00%
Bacteria	*Latescibacteria*	0.03%	0.03%	0.00%	0.04%	0.00%
Bacteria	*Nitrospirae*	0.04%	0.04%	0.00%	0.00%	0.00%
Bacteria	*Parcubacteria*	0.00%	0.09%	0.00%	0.56%	0.02%
Bacteria	*SR1*	0.16%	0.00%	0.00%	0.00%	0.02%
Bacteria	*candidate division WPS-2*	0.00%	0.07%	0.00%	0.00%	0.00%

Table 10: Abundance/taxonomy of dominant bacteria in 5 samples from 2016
[Taxonomy at class, order, family, genus, and species levels. The top five taxa/groups are shown for each of these five ranks]

Level	OTU ID	DNA Samples				
		06	07	08	09	10
Class	*Gammaproteobacteria*	40.77%	16.59%	49.84%	25.54%	31.15%
	Bacilli	37.30%	58.77%	15.44%	11.36%	18.16%
	Alphaproteobacteria	—	—	5.41%	6.61%	—
	Actinobacteria	5.55%	—	—	10.39%	—
	Bacteroidia	—	—	—	—	12.63%
	Clostridia	—	—	12.05%	—	8.04%
Order	*Bacillales*	29.38%	55.12%	13.19%	9.39%	17.54%
	Legionellales	38.16%	14.66%	45.89%	21.86%	28.71%
	Clostridiales	—	—	12.03%	—	8.03%
	Lactobacillales	7.92%	—	—	—	—
	Bacteroidales	—	—	—	—	12.63%
Family	*Coxiellaceae*	38.16%	14.65%	45.89%	21.86%	28.71%
	Bacillaceae 1	15.42%	21.58%	6.32%	—	6.54%
	Bacillaceae 2	9.28%	17.26%	—	—	—
	Planococcaceae	—	13.03%	—	—	6.94%
	Lactobacillaceae	5.26%	—	—	—	—
Genus	*Coxiella*	38.16%	14.54%	45.89%	21.86%	28.71%
	Bacillus	15.19%	21.39%	6.29%	—	6.47%
	Virgibacillus	—	9.23%	—	—	—
	Sporosarcina	—	9.72%	—	—	6.73%
	Lactobacillus	5.26%	—	—	—	—
Species	*Coxiella burnetii*	38.16%	14.54%	45.89%	21.86%	28.71%
	uncultured bacterium	14.33%	18.77%	5.91%	—	5.38%
	Sporosarcina newyorkensis	—	8.27%	—	—	—
	uncultured bacterium	—	—	—	—	5.09%

3.3.9 Diversity Statistics

The alpha diversity of the bacterial community in the different samples of ticks was calculated using Simpsons and Shannon-Wiener based on the amplicon sequencing data (Figure 28). The OTU number ranged from 404 to 651, with a total of 2686 OTU detected at 97% sequence identity (cut-off level of 3%) (Table 11) . The Chao 1 and Shannon–Wiener (H′) indices measured the richness and diversity present in the community, respectively. As for Chao1, values were entirely different in all

samples, whereas the highest and lowest values were reached in samples 08 and 10, respectively. Shannon–Wiener (H') index values were between 4.4 (06) and 6.6 (09), and only sample 08 showed a lower level of diversity. As for the Simpson index analyzed, it represented a measure of the evenness and values were near to 1 except for samples 06 and 08.

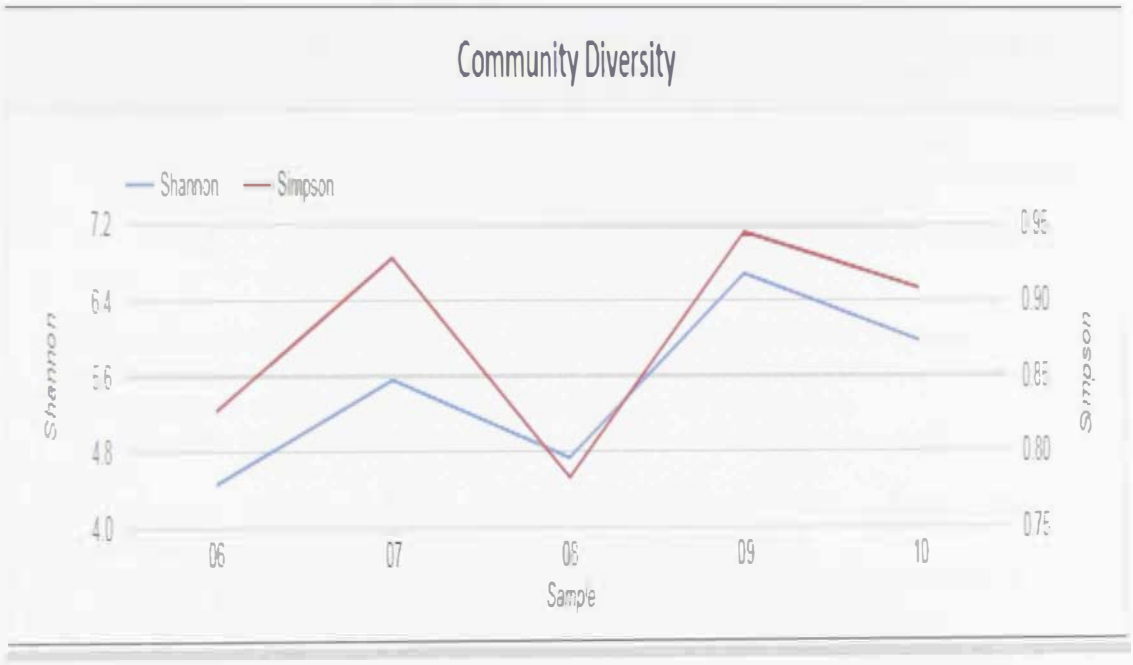

Figure 28: Difference of bacterial diversity within five tick samples

[Differences revealed by using Shannon and Simpson Index]

Table 11: Estimated OTU for five samples of *O. muesebecki* in 2016

[OUT regarding richness, diversity indices, and estimated sample coverage]

OTUS					
Community Diversity					
Sample Name	**OTUs**	**Chao1**	**Shannon**	**Simpson**	**Goods Coverage**
06	444.0	445.0	4.46336574611	0.826962189895	0.999919584412
07	573.0	573.857142857	5.55916880951	0.927437732284	0.999941048164
08	404.0	406.0	4.7382547422	0.782999468357	0.999937008866
09	614.0	614.142857143	6.66999171398	0.944334774096	0.999964737826
10	651.0	658.0	5.96759116485	0.9075257917	0.999867398187

* OTUs: Operational Taxonomic Unit is an operational definition of a species or group of species often used when only DNA sequence data is available
* Chao1: returns the Chao1 richness estimate for an OTU definition
* Shannon: The Shannon index takes into account the number and evenness of species.
* Simpson: The Simpson index represents the probability that two randomly selected individuals in the habitat will belong to the same species.
* Goods Coverage: Coverage is calculated as $C=1-(s/n)$, where s is the number of unique OTUs and n is the number of individuals in the sample.

*This index gives a relative measure of how well the sample represents the larger environment.

3.3.10 Rarefaction

Rarefaction curves were calculated to evaluate species richness within tick samples (Figure 29). All samples were rarefied to a depth of 62,170 sequence reads. The rarefaction curves for the first three samples (06-07-08) reached saturation at 62,170 sequence reads indicating at different OUT level; 444, 573, 404 respectively. In sample 09, however, rarefaction curve reached saturation at 55,954 sequence reads indicating that the sampling effort covered almost 614 different OTUs. The highest OTUs was observed in sample 10 which is about 651 OTUs at 55,954 sequence reads. Rarefaction curves demonstrated that the majority of curves plateaued, thus additional sequencing was unlikely to yield novel data in most cases.

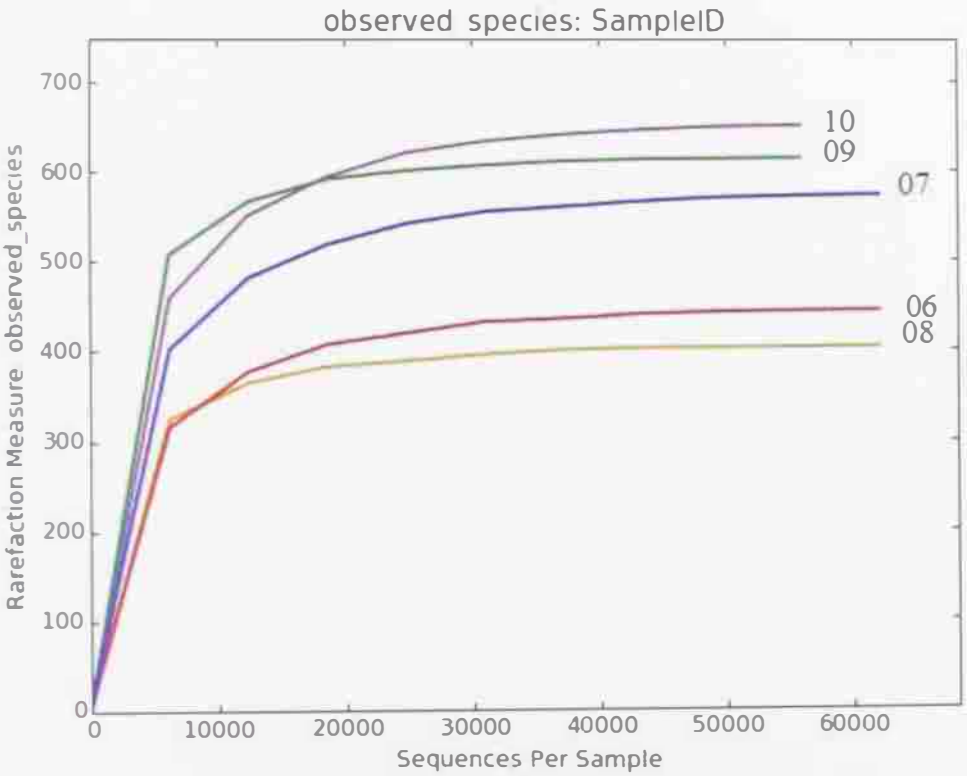

Figure 29: Rarefaction curves per number of observed species for tick samples

[Curves are horizontal with the x-axis indicating that additional sequencing would not yield additional novel data]

3.3.11 OTU Heatmap

The heatmap in (Figure 30) shows the relative prevalence of the dominant tick bacterial species across the tick samples. Only pathogenic *Coxiella burnetii* was observed in a pooled sample of the tick as the highest dominant bacteria.

Figure 30: Rarefied abundances heatmap of most abundant spp. in each sample

[Species are arranged in order of increasing prevalence from top to bottom. Samples on the x-axis are ordered. The gap represents deleted low percentage OTUs (blue colour)]

3.4 Molecular Identification of *O. muesebecki*

In the PCR, both primer sets of Fish1F/Fish1R and FishF2/FishR2 did not amplify any fragment and accordingly no bands were produced on an agarose gel (Figure 31) and (Figure 32). However, the LCO1490/HCO2198 primer amplified the target region and produced the expected 710-bp band on an agarose gel (Figure 33). The primer set 16s+1/16s-1 amplified 6 samples and produced bands at the expected size 460-bp (Figure 34). In contrast, none of the analyzed ticks yielded 475-bp sequences with the Tm16S+1/ Tm16S-1 primer set (Figure 35). In addition, the amplification of 18S rDNA was successful for 3 samples and as a result band appeared in the gel (Figure 36).

Based on the search in the BLAST database of the GenBank the *O. muesebecki* cytochrome c oxidase 1 partial gene sequence did not show high similarity with other tick sequences. The Neighbor-Joining homology tree revealed *O. muesebecki* in a separate group while the other analyzed sequences were clustered in two distinct groups namely *Carios vespertilionis* and *Heamaphysalis concinna* (Figure 37). The Bootstrap values which were generated from 1000 permutations showed strong support (values near or equal to 100) for the grouping of the sequences within the *C. vespertilionis* and *H. concinna* clusters, but this was not the case with *O. muesebecki*. Also, when the primer NS3 was used it successfully produced the target band in PCR, however, the results of DNA sequence analysis revealed that *O. muesebecki* was very close to *Carios* spp. as well as to some other *Ornithdoros* spp. (Figure 38). This finding was manifested by the Neighbor-Joining homology tree in which several *Carios* and *Ornithdoros* appeared in one group (clade). So, the scale of the tree was 0.0050, which

indicated very close homology among all of the analyzed sequences including *O. muesebecki*.

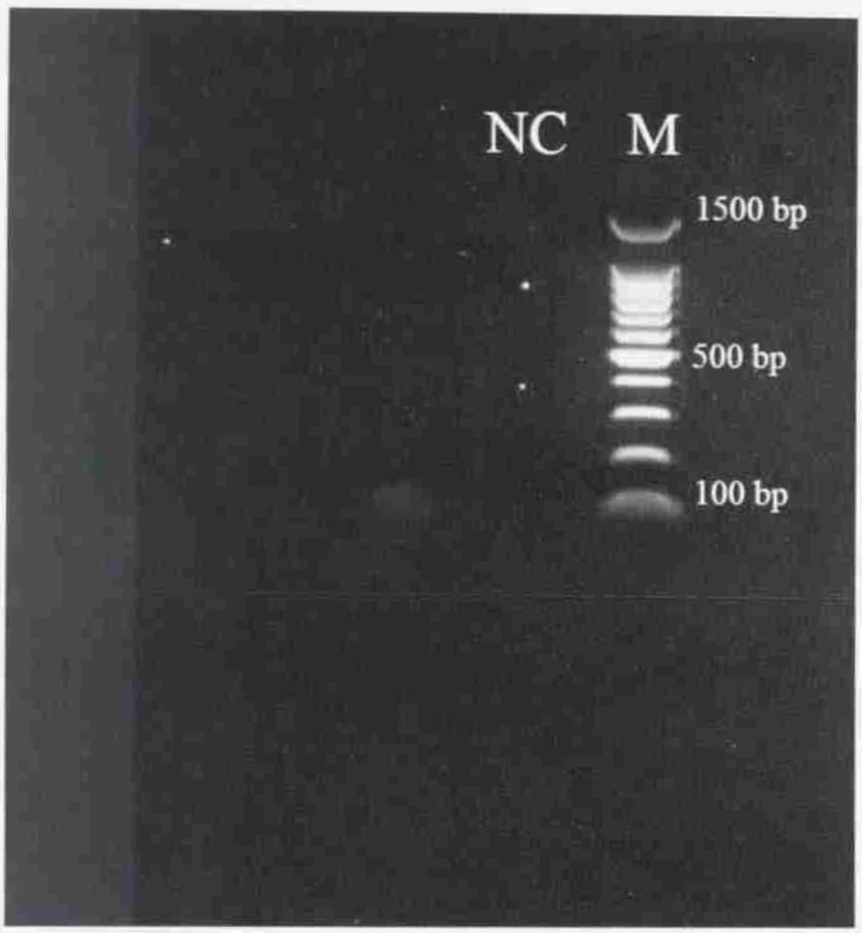

Figure 31: Agarose gel of PCR products of *O. muesebecki* samples with Fish1F/Fish1R primer set

[NC is negative control; M represents 100-bp DNA ladder (Promega, Madison, USA)]

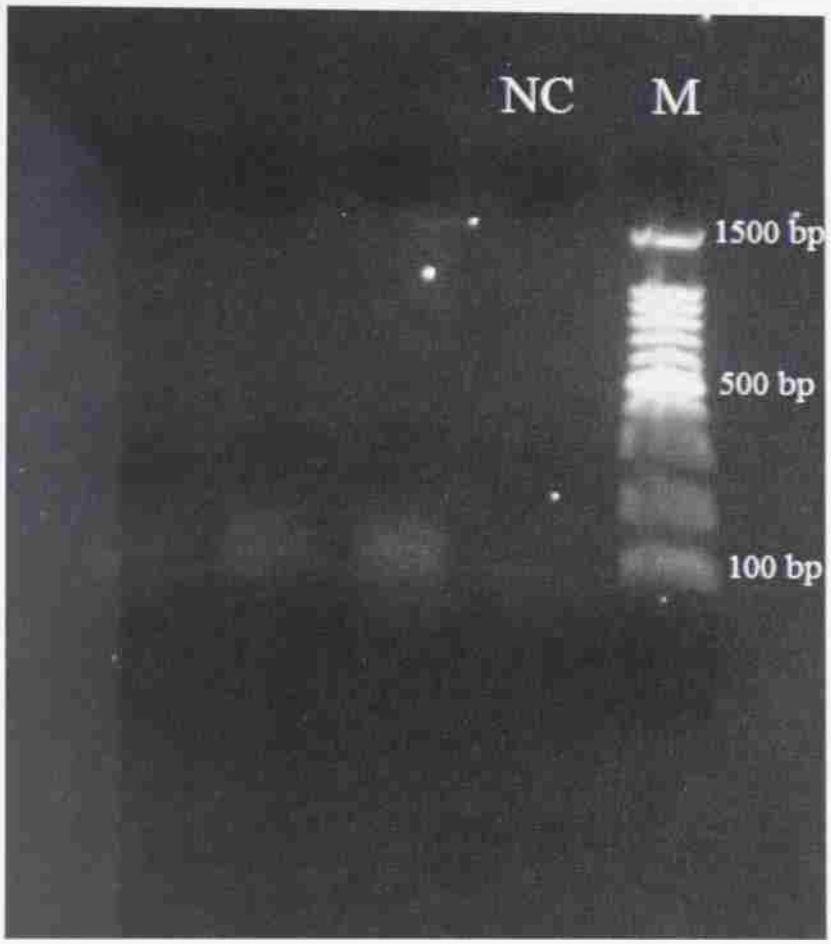

Figure 32: Agarose gel of PCR products of *O. muesebecki* samples with Fish F2/Fish R2 primer set

[NC is negative control; M represents 100-bp DNA ladder (Promega, Madison, USA)]

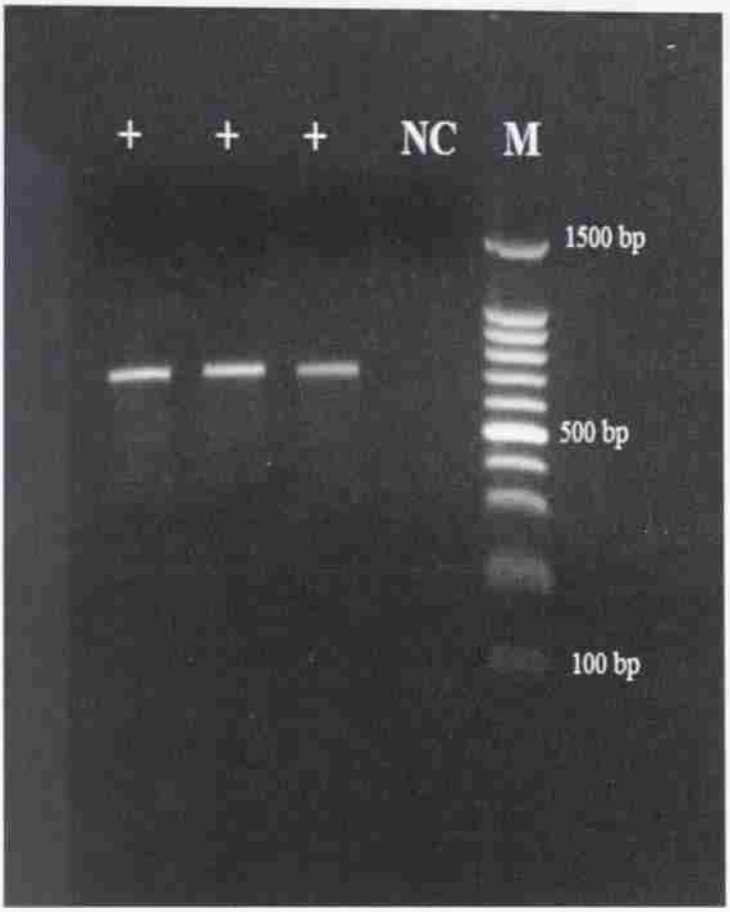

Figure 33: Agarose gel of PCR products of *O. muesebecki* samples with LCO/HCO primer set

[NC is negative control; M represents 100-bp DNA ladder (Promega, Madison, USA)]

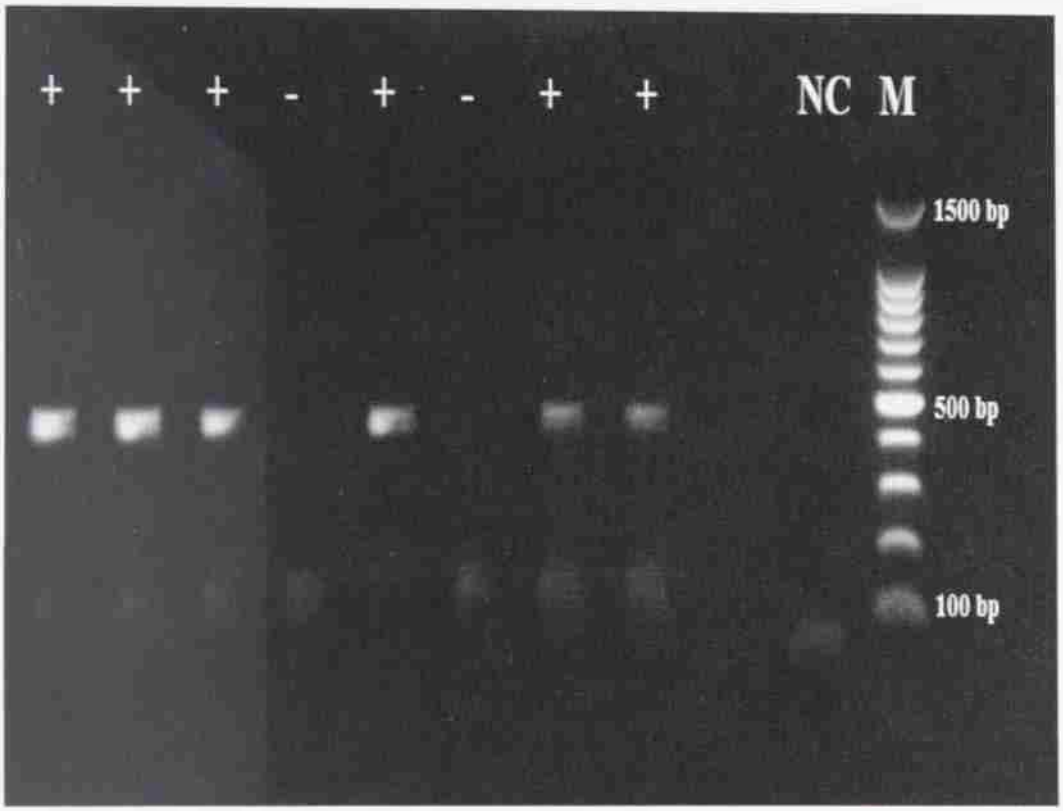

Figure 34: Agarose gel electrophoresis of PCR products of *O. muesebecki* samples with 16S+1/16S-1 primer set

[NC is negative control; M represents 100-bp DNA ladder (Promega, Madison, USA)]

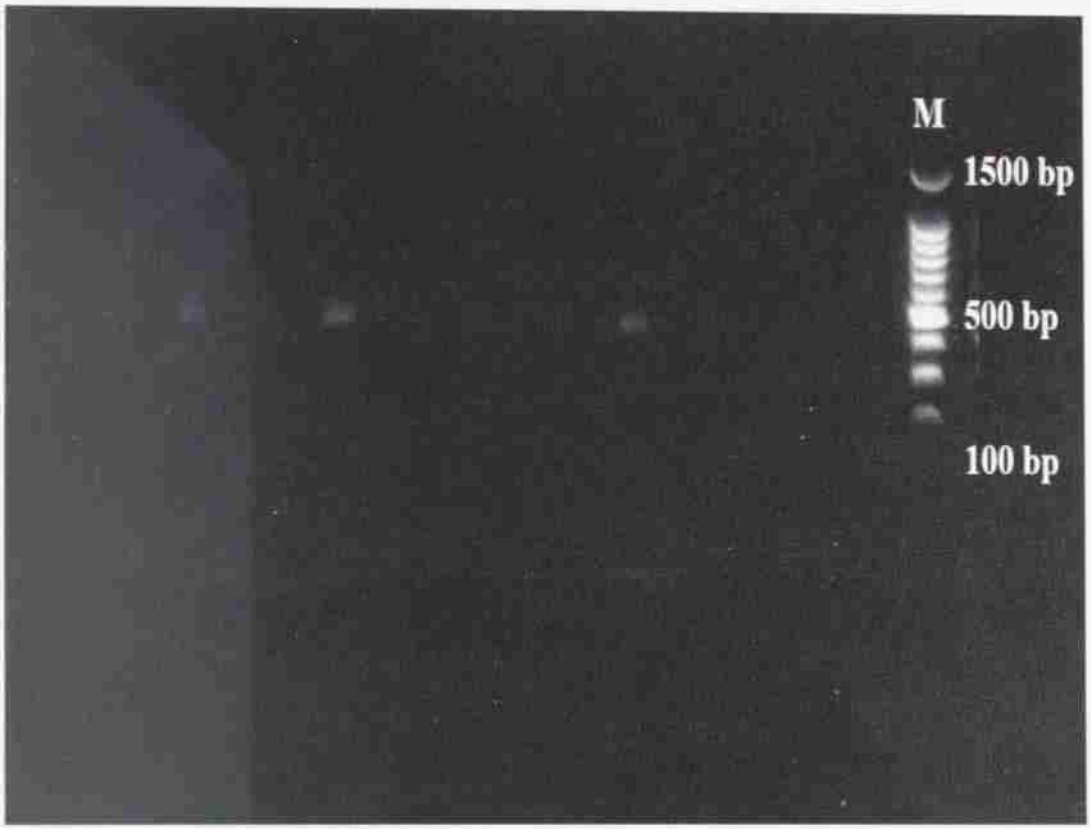

Figure 35: Agarose gel electrophoresis of PCR products of *O. muesebecki* samples with Tm16S+1/ Tm16S-1primer set

[M represents 100-bp DNA ladder (Promega, Madison, USA)]

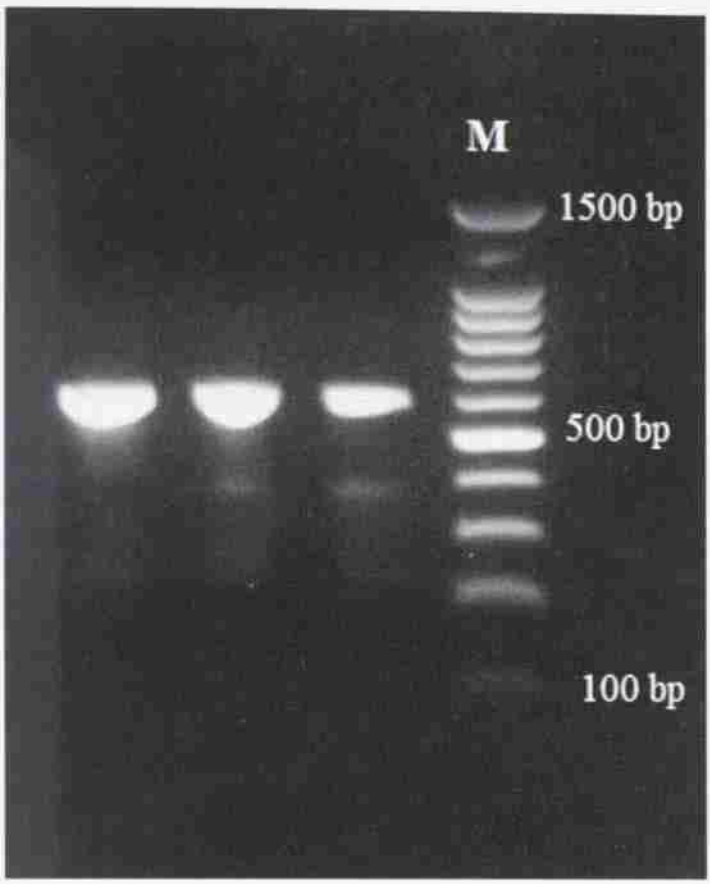

Figure 36: Agarose gel electrophoresis of PCR products of *O. muesebecki* samples with the NS3/ NS4 primer set

[M represents 100-bp DNA ladder (Promega, Madison, USA)]

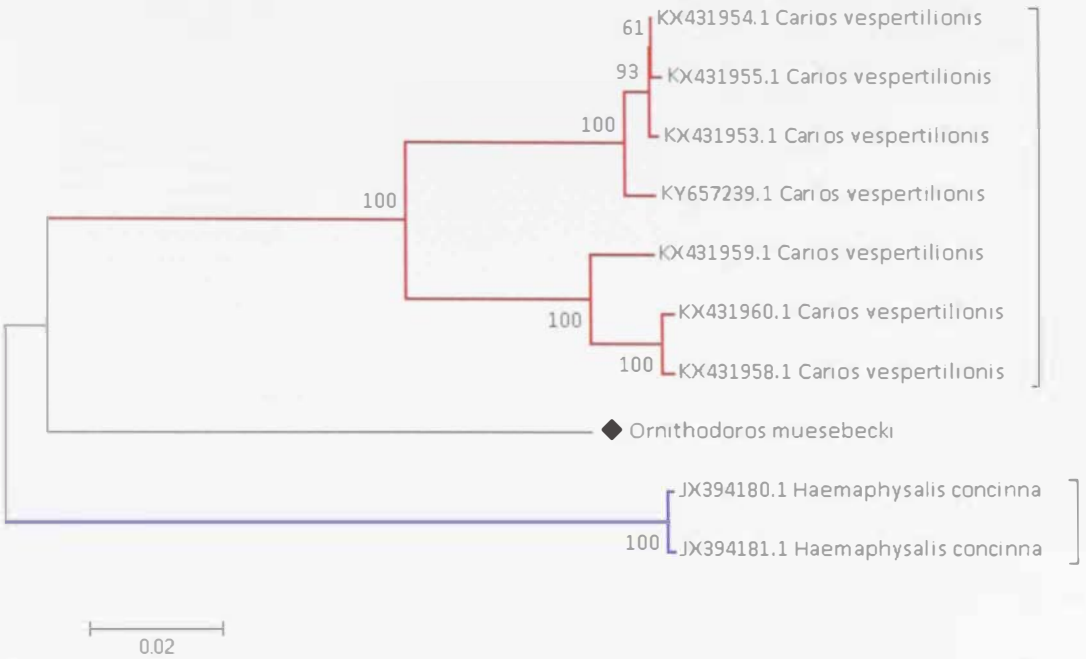

Figure 37: Neighbour-joining homology tree of the *O. muesebecki* cytochrome c-oxidase 1 gene compared with other sequences of the GenBank

[Nine sequences with the highest similarity values after a BLAST search were used for phylogenetic analysis. Bootstrap values (1000 replications) are indicated at each node]

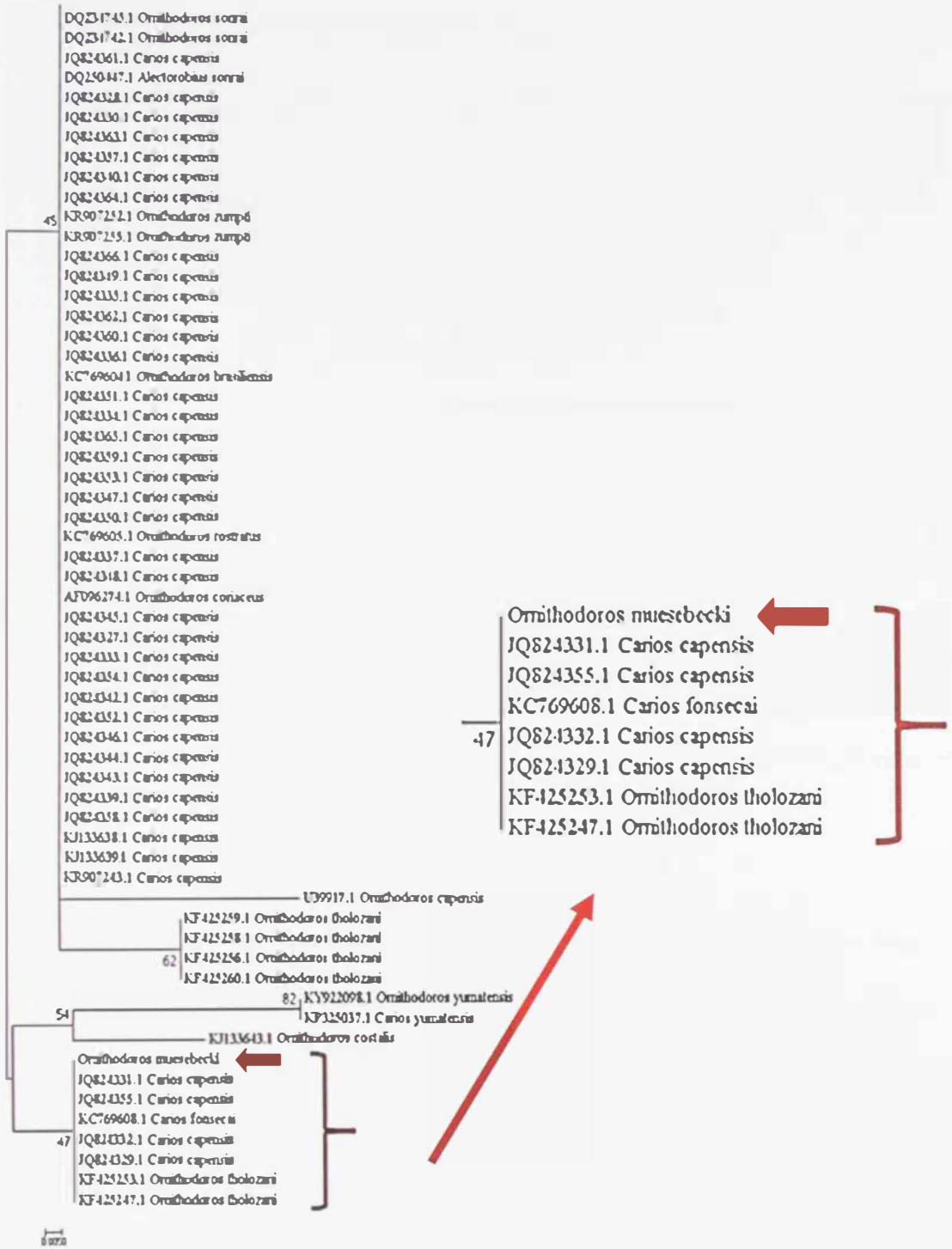

Figure 38: Neighbor-Joining homology tree of the *O. muesebecki* 18S rDNA gene compared with 84 sequences of the GenBank

[These are all the sequences produced after a BLAST search, and they were used for phylogenetic analysis. Bootstrap values (1000 replications) are indicated at each node]

3.5 Scanning Electron Microscopy (SEM) and Stereoscope

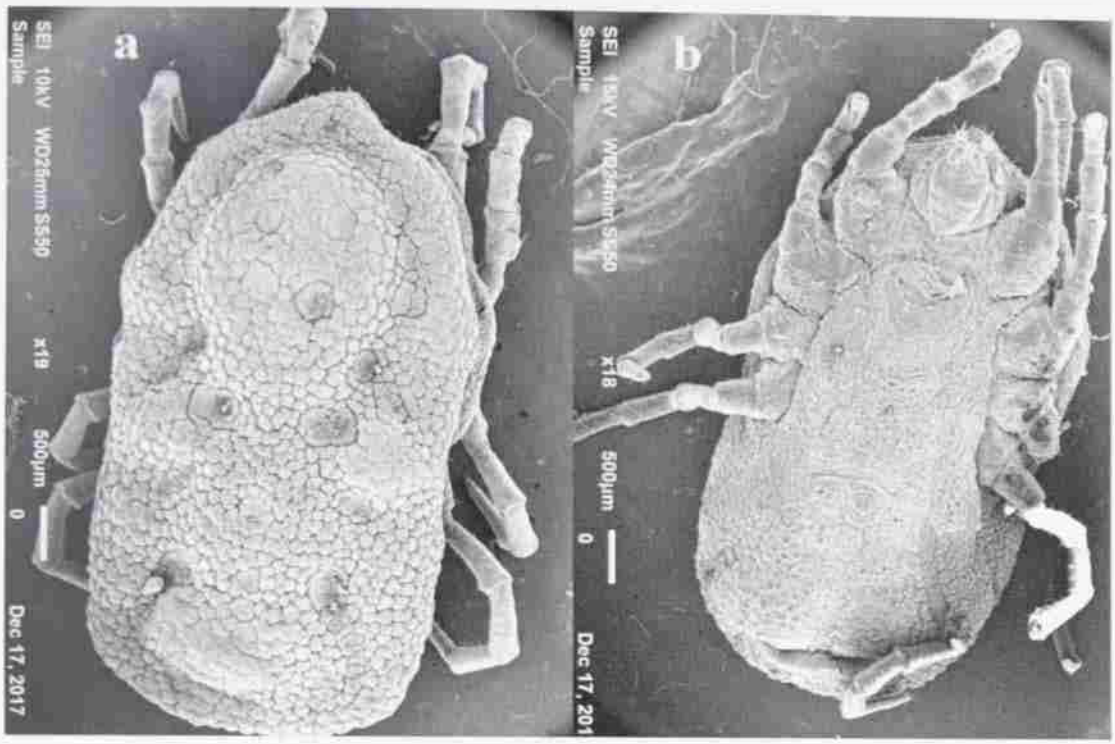

Figure 39: Scanning electron micrographs showing (a) dorsal and (b) ventral view of *O. muesebecki* collected in UAE

The dorsal side of *O. muesebecki*, dorsal (Figure 39 and Figure 41). The shape is oval or pear-shaped. The texture of integument has mammillae, and the surface is without a scutum. The ventral side (Figure 40) shows short mouthparts and absence of eyes. The legs end in a pair of claws but without a pulvillus between the claws.

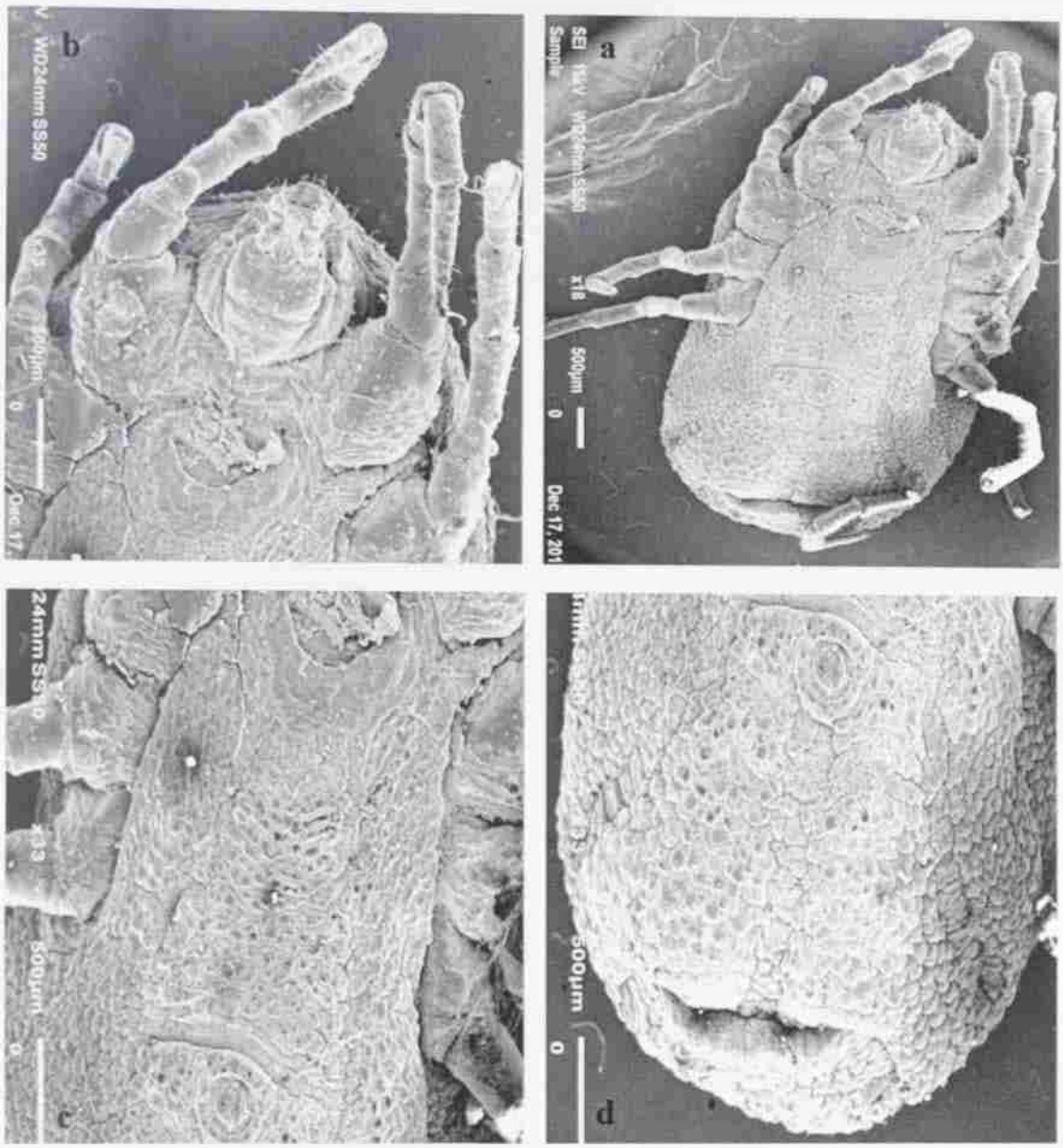

Figure 40: Scanning electron micrographs of *O. muesebecki*: A. ventral view; B. Capitulum, ventral view ; C. midgut; D. execratory system

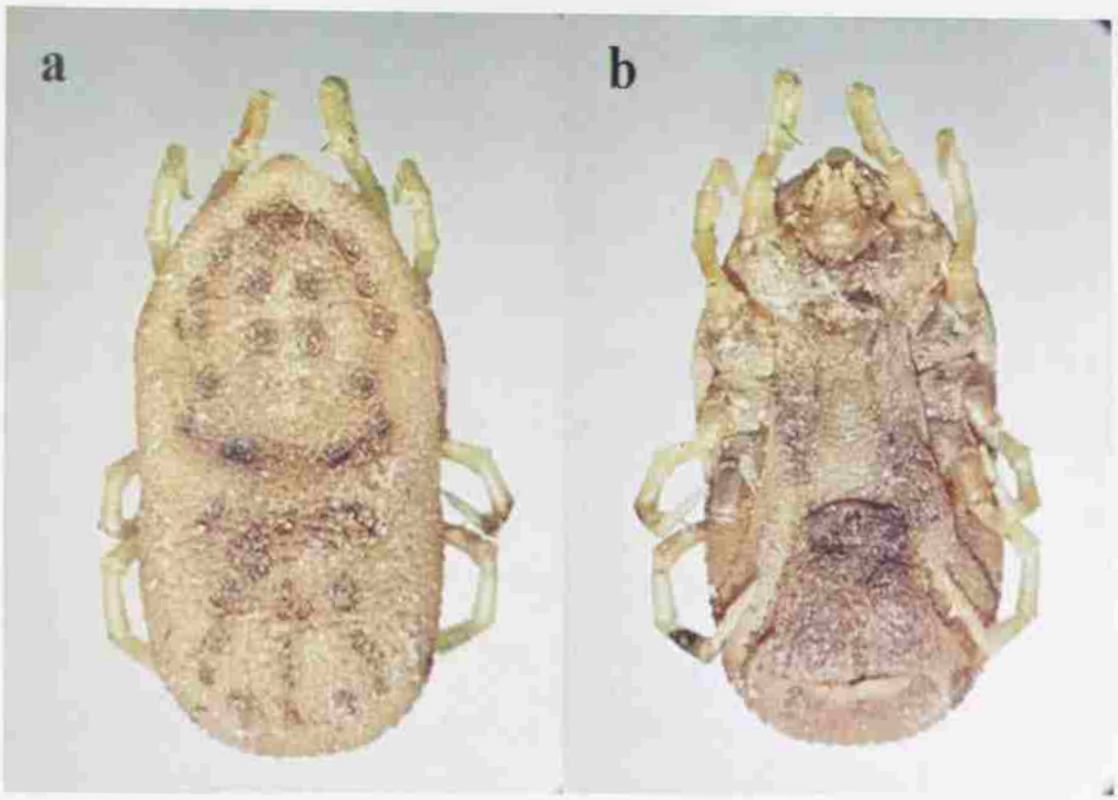

Figure 41: *O. muesebecki* adult showing (a) dorsal and (b) ventral surfaces

Chapter 4: Discussion

4.1 Molecular Detection and Prevalence of Tick-borne Diseases

The current study did not detect any of the targeted diseases using pathogen-specific primers. In this work as well as in previous studies this tick has been suspected to harbour infectious diseases. One objective of this research was to detect specific tick-borne diseases using PCR. Ticks (*O. muesebecki*) from a Socotra Cormorant colony were investigated for the presence of three genera of common tick-borne pathogens namely *Borrelia*, spotted fever group *Rickettsia* and *Coxiella burnetii*. Based on the results, *Borrelia* spp. and *Rickettsia* spp. were not detected in any tick specimen. In the UAE, data on tick distribution and their ability to vector pathogens causing disease in wildlife and domestic animals is limited. Similarly, limited information on the ticks associated with wildlife was documented in neighbouring countries compared to an extensive survey of tick fauna on domestic animals.

Borrelia spp. are well known to cause zoonotic disease is usually transmitted by soft ticks of the genus *Ornithodoros* (Humair, 2002). Several TBRF borreliosis have frequently been investigated in livestock transmitted by several *Ornithodoros* species including *O. sonrai* in countries like Morocco, Libya, Egypt, Iran, Syria, and Iraq (Rebaudet and Parola, 2006). Unlike several studies in which *Borrelia* species were identified in *Ornithodoros* ticks which vector them to mammals, limited data documenting their vectoring of these pathogens to seabird colonies. The dispersal of *O. muesebecki* in Siniya Island within Socotra cormorant colony raises expectations on the presence of some avian *Borrelia* species such as *B. anserine*, the avian borreliosis agent which is known to be transmitted by the soft tick *Arags persicus*

(Humair, 2002). Although this would happen very rarely in this region, the distribution of the Arabian Gazelle on islands could contribute to the life cycle of tick and to the maintenance of the pathogen in nature. For example, *B. burgdorferi,* the causative agent of Lyme disease available in every stage of tick life cycle was found to be present in different hosts (Humair, 2002). Birds were found to be parasitized by the immature ticks (larvae and nymphs). Large-sized mammals such as rodents and deer are the host for this agent and these mammals contribute to the life cycle and in the maintenance of *B. burgdorferi* in nature (Capligina *et al.*, 2014; Lee *et al.*, 2014; Pereira *et al.*, 2017). Several studies suggested that wild animals such as deer have been considered to be the potential reservoir for a large number of tick-borne pathogens including bacteria (Overzier *et al.*, 2013; Han *et al.*, 2017).

Spotted fever group *Rickettsia* were not detected in any of the ticks collected in this study. *Rickettsia* spp., however, has been well documented in Tunisia, Algeria, Morocco and Egypt (Parola *et al.*, 2013). Only one study has shown the presence of *R.andeanae* in *Hyalomma dromedarii* camel tick in the UAE (Al-Deeb *et al.*, 2015). In Morocco, four pathogenic *Rickettsiae* have been documented from domestic animals: *R. slovaca, R. helvetica, R. monacensis* and *R. raoultii.* These are transmitted via different ixodid tick species (Sarih *et al.*, 2008). Similarly, in Egypt, several ixodid ticks such as *Hyalomma*, *Boophilus*, and *Rhipicephalus* have been identified in camels and cows vectored of *Rickettsia* spp. including *R. africae* (Abdel-Shafy *et al.*, 2012). Studies on the prevalence of *Rickettsia* spp. in argasid ticks parasitizing birds are rare.

In Algeria, a study by Lafri *et al.*, (2015) detected novel *Rickettsia* species in four different *Ornithodoros* species from rodent burrows and seabird nests. The detection

of this non-pathogenic agent was on nymphal and adult stages. This and other studies did not present a full picture on maintenance and distribution of soft tick transmission of *Rickettsia* spp. in nature especially within wild animals (Hildebrandt *et al.*, 2010; Obiegala *et al.*, 2017). We can say that soft ticks generally play a role in the spread of vectored arboviruses and relapsing fever spirochetes (*Borrelia spp.*), but they are rarely found to transmit of rickettsial agents naturally (Lafri *et al.*, 2015).

Coxiella-like endosymbiont has already been detected in *O. muesebecki* ticks from Siniya Island using DNA-based techniques (Al-Deeb *et al.*, 2016). In this research, detection of *Coxiella* in the same species was also found in tick specimens in 2013 and 2016. The high prevalence of *Coxiella* endosymbiont was shown in samples 2016 (98%) compared to samples 2013 (93%) (Figure 14). This indicates that this microorganism is still present in the area and is widely distributed on the island.

The present study showed that the three above-mentioned tick-borne pathogens were not present in *O. muesebecki* ticks from UAE habitat, according to the screened tick sample. However, further investigations are recommended to detect other bacterial or viral communities using specific primers, which will be helpful in understanding tick-borne diseases in general.

4.2 Metagenomic Profile of Tick Microbial Communities

The work reported in this thesis represents the first study to characterize all bacteria in *O. muesebecki* ticks using next-generation sequencing. This method has not been applied yet in the identification of detailed microbiota profiles in any other tick study in this region. Tick fauna in the UAE is not well studied, and a comprehensive investigation of bacteria in *O. muesebecki* was needed. In Saudi Arabia and Yemen

(Hoogstral and Kaiser, 1959; Banaja and Roshdy, 1978; Banaja and Ghandour, 1994), the endemic tick fauna is well documented, however, our understanding of bacterial population and communities in those two countries still limited.

The data presented in the current study was collected from one habitat (Siniya Island) within two different time periods (2013 and 2016). Illumina- MiSeq based metagenomic analysis was successfully applied to detect and characterize the bacterial community residing in whole *O. muesebecki* ticks. The MiSeq sequencing platform used in this study demonstrated a higher resolution for bacterial diversity and structure analysis as evidenced by the 2,530,014 total sequences in all 10 samples and a total of 809 OTUs within 5 sample from 2013 and 2686 OTUs within 5 sample from 2016 (3% cutoff level). A significant difference in OTU number was shown (Table 5 and 10), where ticks from 2016 had a higher number of OTUs than 2013, indicating high diversity in 2016, which was further confirmed by the alpha diversity indices. The selected OTUs in the heatmap also confirmed the difference of bacterial diversity between two years. OTUs had appeared in higher numbers in 2016 samples but with a low proportion of cultured bacteria compared to the low number of OTUs in samples of 2013 but with a high proportion of defined bacteria.

Metagenomic analysis for the samples of 2013 identified 16 phyla, 33 classes, 59 orders,124 families,182 genera, and 229 species whereas for the 2016 samples were classified into 38 phyla, 84 classes, 145 orders, 264 families, 539 genera, and 836 species based on the identified OTUs. When characterizing bacteria in ticks, bacterial endosymbionts were predominant, and there was also a high percentage of uncultured bacteria. With respect to non-pathogenic bacteria, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were detected in pooled *O. muesebecki*, indicating

that these bacteria are part of the tick microbiome. Evidence of the presence of similar bacteria in ticks associated with migratory birds has been previously shown by the use of pyrosequencing (Budachetri *et al.*, 2017b). Regarding the unculturable bacteria in this study, further studies need to be conducted to identify whether these bacteria are pathogenic or primary endosymbionts that aid in this tick's survival.

Both the results of metagenomic and conventional PCR assay revealed that the ticks were infected with a member of the genus *Coxiella*. The metagenomic analysis in all pooled samples showed in both years that ticks were infected by *Coxiella burnetii*. However, results from the more specific PCR analysis in this study which is based on *and* gene-specific primer from 300 samples and on phylogenetic analysis from a previous study indicate the presence of *Coxiella* endosymbionts and not the pathogenic *C. burnetii*. This finding indicates that although NGS is an advanced and easy method to investigate bacterial communities and provides high sequence depth of data our results demonstrated that it might not be able to confirm the presence of some pathogenic species of bacteria such as *C. burnetii*.

In conclusion, this study showed the presence of high and diverse bacterial communities in *O. muesebecki*. Our study enhanced our understanding of bacteria communities in seabird colony and expanded our knowledge of microbial ecology. The analysis also indicated the existence of many unknown bacteria which means that more studies are required to investigate the undefined bacterial communities associated with *O. muesebecki*.

4.3 Molecular Identification of *O. muesebecki*

The tick on Socotra cormorant nesting sites was morphologically identified as *Ornithodoros muesebecki* (Acari: Argasidae). It was previously identified from seabirds breeding in a UAE island and studied morphologically (Hoogstraal *et al.*, 1970). This thesis provided the first molecular record of *O. muesebecki* in the GenBank. The BLAST search results and the Neighbor-Joining analysis provided molecular evidence that the tick in this study was not *Carios vespertilionis* or *Heamaphysalis concinna*, which was something already established based on the morphological identification.

The gained results also indicated that the universal primer pair LCO1490/HCO2198, which is used in the molecular identification of animals annealed successfully to the target gene but was not suitable for making a good comparison between the *O. muesebecki* and other organisms (mainly ticks) in the GenBank. The same can be said about the primer NS3. Although, these primers are mostly used in population studies and phylogenetic inferences (Nava *et al.*, 2009) but detecting phylogenetic relationship to other *Ornithodoros* species was not successful in the current study. Moreover, the current study proved that the Fish 1, Fish 2 and Tm16S primers did not work when used with *O. muesebecki*. Therefore, further molecular markers using specific primers such as those from mitochondrial genome will be needed to detect specific regions (or genes) of *Ornithodoros* (Burger *et al.*, 2014).

4.4 Scanning Electron Microscopy

SEM images revealed that the tick is a member of the *O. muesebecki*. The dorsal side of *O. muesebecki*, dorsal. The shape is oval or pear-shaped. The texture of integument has mammillae, and the surface is without a scutum. The ventral side shows short mouthparts and absence of eyes. The legs end in a pair of claws but without a pulvillus between the claws. These findings are in agreement with Estrada-Peña, 2015.

Chapter 5: Conclusion

In conclusion, the results of this study revealed the presence of *Coxiella* genus in ticks collected from a Socotra cormorant breeding colony and indicated that the prevalence of this bacteria appears to have increased between 2013 and 2016. In addition, this study did not find the tick-borne pathogens *Rickettsia* and *Borrelia*. It also involved the first application of the high-throughput sequencing method to investigate the diversity of bacterial communities associated with soft seabird ticks in the UAE. The analysis revealed the existence of a diverse array of bacterial communities in the tick samples, indicating a sizeable bacterial diversity in this area. The SEM images confirmed that the tick is a member of the *O. muesebecki*. Moreover, further studies are needed to identify the uncharacterized bacteria revealed as a result of the metagenomic analysis.

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