CHARACTERIZATION OF *RICKETTSIA RAOULTII* REPLICATION KINETICS IN DIFFERENT TICK CELL LINES

NURUL AINI BINTI HUSIN

INSTITUTE FOR ADVANCED STUDIES
UNIVERSITI MALAYA
KUALA LUMPUR

2022

CHARACTERIZATION OF *RICKETTSIA RAOULTII* REPLICATION KINETICS IN DIFFERENT TICK CELL LINES

NURUL AINI BINTI HUSIN

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

INSTITUTE FOR ADVANCED STUDIES
UNIVERSITI MALAYA
KUALA LUMPUR

2022

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **Nurul Aini Binti Husin**

Registration/Matric No: **17069171/3**

Name of Degree: **Master of Philosophy**

Title of Project Paper/Research Report/Dissertation/Thesis ("this work"):

Characterization of *Rickettsia raoultii* replication kinetics in different tick cell lines

Field of Study: **Life Science (Biology and Biochemistry)**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every right in the copyright to thus Work to the Universiti Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work, I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

Name:
Designation:

CHARACTERIZATION OF *RICKETTSIA RAOULTII* REPLICATION KINETICS IN DIFFERENT TICK CELL LINES

ABSTRACT

Rickettsia raoultii is a spotted fever group *rickettsia* and emerging pathogen that poses a threat to public health with *Dermacentor* species of ticks as the main vector and known as one of the causative agents of tick-borne lymphadenopathy (TIBOLA) in humans. This bacterium was previously isolated into and propagated in tick and mammalian cell lines. Although the growth characteristics of *R. raoultii* have been investigated in mammalian cells, the same has not been investigated in detail in tick cells. In this study, the growth and replication kinetics of *R. raoultii* in three tick cell lines derived from different tick genera, *Rhipicephalus microplus*-derived BME / CTVM23, *Rhipicephalus sanguineus*-derived RSE / PILS35 and *Ixodes scapularis*-derived IDE8 tick cell lines were presented. Tick cell cultures were infected in duplicate with cryopreserved *R. raoultii* prepared from the homologous cell lines. In all infected cell cultures, 100 % of the cells were infected by 12 to 14 days post infection as visualized in Giemsa-stained cytocentrifuge smears. A rickettsiae-specific citrate synthase (*gltA*) gene quantitative polymerase chain reaction (qPCR) assay was used to demonstrate the replication kinetics of *R. raoultii* in the infected cultures. The *R. raoultii* growth curves were exhibited with initial lag, exponential, stationary and death phases. Exponential phases between 4 and 12 days and generation times between 0.9 and 2.6 days were observed in the *R. raoultii*-infected tick cell cultures. The highest levels of multiplication of *R. raoultii* in BME / CTVM23 and RSE / PILS35 cultures were, respectively, 39.5 and 37.1-fold increases compared to the inoculum. In contrast, the highest level of multiplication of *R. raoultii* in IDE8 cultures was 110.1-fold greater than the inoculum. In addition, a 7 day stationary phase was observed in *R. raoultii*-infected IDE8 cultures. The findings here suggest that there is variation in the growth kinetics of *R. raoultii* in the

different tick cell lines tested, amongst which IDE8 cells could tolerate the highest levels of *R. raoultii* replication. Variations in genotypic and phenotypic characteristics, as well as species differences between tick cell lines, may have contributed to this finding. Further studies into the characterization of *R. raoultii* and its interaction with tick vectors are needed to better understand its survival within tick populations, and tick cell lines remain an important tool to this end.

Keywords: vector-borne disease; *Rickettsia raoultii*; infectious disease; tick cell line

CIRI-CIRI KINETIK REPLIKASI *RICKETTSIA RAOULTII* DALAM GARISAN SEL KUTU BERBEZA

ABSTRAK

Rickettsia raoultii, kumpulan *rickettsia* demam berbintik-bintik dan patogen yang menimbulkan ancaman kepada kesihatan awam dengan kutu daripada spesies *Dermacentor* sebagai vektor utama, dan dikenali sebagai salah satu agen penyebab limfadenopati (*TIBOLA*) pada manusia. Bakteria ini sebelumnya pernah diasingkan ke dalam dan dibiakkan dalam garis sel kutu dan mamalia. Walaupun ciri pertumbuhan *R. raoultii* telah diselidiki pada sel mamalia, yang sama belum diteliti secara terperinci dalam sel kutu. Dalam kajian ini, data menunjukkan pertumbuhan dan kinetik replikasi *R. raoultii* dalam tiga garis sel kutu yang berasal dari generik kutu yang berbeza, *Rhipicephalus microplus*-berasal BME / CTVM23, *Rh. sanguineus*-berasal RSE / PILS35 dan *Ixodes scapularis*-berasal IDE8 tandakan garis sel. Dua kultur sel kutu daripada garis yang sama dijangkiti dengan *R. raoultii* yang disimpan secara kryo dari garis sel homolog. Dalam semua kultur sel yang dijangkiti, 100 % sel telah dijangkiti pada 12 hingga 14 hari selepas jangkitan seperti yang digambarkan dalam dalam emparan sito pencelupan Giemsa. Ujian qPCR *sintase sitrat* gen (*gltA*) khusus rickettsiae digunakan untuk menunjukkan kinetik replikasi *R. raoultii* dalam kultur yang dijangkiti. Lengkuk pertumbuhan *R. raoultii* dipamerkan dengan fasa ketinggalan awal, eksponensial, pegun dan kematian. Fasa eksponensial di antara 4 hingga 12 hari dan masa penjanaan antara 0.9 hingga 2.6 hari diperhatikan dalam kultur sel kutu yang dijangkiti *R. raoultii*. Tahap pendaraban tertinggi *R. raoultii* dalam kultur BME / CTVM23 dan RSE / PILS35 masing-masing meningkat 39.5 dan 37.1 kali ganda berbanding dengan inokulum. Sebaliknya, tahap pendaraban tertinggi *R. raoultii* dalam kultur IDE8 adalah 110.1 kali ganda lebih besar daripada inokulum. Di samping itu, fasa pegun selama tujuh hari diperhatikan dalam kultur IDE8 yang dijangkiti *R. raoultii*. Hasil kajian di sini menunjukkan bahawa

terdapat variasi dalam kinetik pertumbuhan *R. raoultii* pada sel kutu berbeza yang diuji, di antaranya sel IDE8 dapat bertoleransi dengan tahap tertinggi replikasi *R. raoultii*. Variasi ciri genotipik dan fenotipik, serta perbezaan spesies antara garis sel kutu, mungkin menyumbang kepada penemuan ini. Kajian lebih lanjut mengenai pencirian *R. raoultii* dan interaksinya dengan vektor kutu diperlukan untuk pemahaman yang lebih baik mengenai kelangsungan hidupnya dalam populasi kutu, dan garis sel kutu tetap menjadi alat penting untuk tujuan ini.

Kata kunci: penyakit bawaan vector; *Rickettsia raoultii*; penyakit berjangkit; garis sel kutu

ACKNOWLEDGEMENTS

Almost two years have taken for this project to be completed, and it is always a pleasure to remind all the people that helped me a lot in completing this project successfully. Firstly, I want to express a deep sense of gratitude and gratefulness to the supervisors, Prof. Dr. Sazaly Bin Abu Bakar and Dr. Khoo Jing Jing, for trusting and giving me a chance to do this project. Secondly, I would like to thank the great people in Tick Cell Biobank, especially to Dr. Lesley Bell-Sakyi (TCB, University of Liverpool) and Mulya Mustika (TCB, Asia-Outpost, Universiti Malaya), for supporting me greatly. Their cordial support, exemplary guidance and constant encouragement kept me positive and determined to solve problems and knockdown obstacles I faced throughout the research. My dearest thanks to my mother, Sutipah Mohamad and my father, Husin Adom, for their continuous support and encouragement. Their blessing and guidance were a great inspiration to me. Thanks to all staff in TIDREC for providing facilities throughout the period. I want to apologize to any other unnamed individuals who helped in various ways to complete this project. Last but not least, this research was supported in parts by the Higher Institution Centre of Excellence (HICoE) program (Project MO002-2019) from the Ministry of Education, Malaysia, Research University grant (RU008-2018) from Universiti Malaya and Centre of Excellence Top 100 research grant (UM.00000188/HGA.GV). This research was also supported by an Institutional Links grant, ID 332192305, under the Newton-Ungku Omar Fund partnership. The grant is funded by the UK Department of Business, Energy and Industrial Strategy (BEIS), the Energy and Industrial Strategy and Malaysian Industry-Government Group for High Technology (MIGHT) and delivered by the British Council. I want to thank the Tick Cell Biobank, the University of Liverpool, for the provision of tick cell lines used in this work. In addition, we thank Professor Ulrike Munderloh, University of Minnesota, for permission to use the IDE8 cell line.

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

LIST OF SYMBOLS AND ABBREVIATIONS

AGE	:	Agarose Gel Electrophoresis
ANE58	:	*Dermacentor* (*Anocentor*) *nitens*
ATBF	:	African tick-bite fever
BLAST	:	Basic Local Alignment Search Tool
BLASTn	:	Nucleotide Basic Local Alignment Search Tool
BSL 2	:	Biosafety Level 2
BSL 3	:	Biosafety Level 3
bp	:	base pair
BME / CTVM23 cell	:	*Rhipicephalus* (*Boophilus*) *microplus* cells
CNS	:	Central nervous system
Ct	:	Cycle threshold
$CaCl_2$	:	Calcium chloride
°C	:	degree Celcius
d.p.i.	:	days post infection
DMSO	:	Dimethyl Sulfoxide
DEBONEL	:	*Dermacentor*-borne Necrosis Erythema and Lymphadenopathy
DNA	:	Deoxyribonucleic Acid
DIC	:	Disseminated intravascular coagulation

ELISA	:	Enzyme-linked immunosorbent assay
FBS	:	Fetal Bovine Serum
g	:	g-force
G	:	Gauge
gltA	:	Citrate Synthase gene
HL-60 cell	:	Human Leukemia cells
HMEC-I cell	:	Human mammary epithelial cell
IDE8	:	*Ixodes scapularis* cell line (8)
ISE6 cell	:	*Ixodes scapularis* cell line (6)
IFA	:	Immunofluorescence assay
JSF	:	Japanese spotted fever
kb	:	kilobase
L929 cell	:	Mouse fibroblast cell line
LB broth	:	Luria Bertani Broth
LB agar	:	Luria Bertani Agar
MALDI-TOF MS	:	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MSP1a	:	Major Surface Protein 1a
MSP1b	:	Major Surface Protein 1b
M	:	Molar
ml	:	millilitre

MSF	:	Mediterranean spotted fever
MIF	:	Microimmunofluorescence
NAAT	:	Nucleic acid amplification test
NCBI	:	National Center for Biotechnology Information
nt	:	nucleotide
OD	:	Optical density
PCR	:	Polymerase Chain Reaction
Putative GST	:	Putative Glutathione S-Transferase gene
pH	:	potential of hydrogen
qPCR	:	quantitative Polymerase Chain Reaction
RSE / PILS35 cell line	:	*Rhipicephalus sanguineus* cell line
rpl6	:	*Ixodes scapularis* ribosomal protein L6
R^2	:	Coefficient of determination
RG 3	:	Risk group 3
RML/RSE cell	:	*Rhipicephalus sanguineus* cell line
RNA	:	Ribonucleic acid
RPA	:	Recombinase polymerase amplification
RMSF	:	Rocky Mountain spotted fever
rOmpB	:	Outer membrane protein B
SFG	:	Spotted fever group
STG	:	Scrub typhus group

SENLAT	:	Neck lymphadenopathy after tick bite
TG	:	Typhus group
TIBOLA	:	Tick-borne Lymphadenopathy
TPB	:	Tryptose Phosphate Buffer
TBP	:	Tick-borne pathogen
µm	:	micrometer
µl	:	microliter
Vero cell	:	African green monkey kidney cells
%	:	Percent
≈	:	approximately

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Rickettsia bacteria is a strictly intracellular vector-borne bacteria that fall in the alphaproteobacterial family, which causes mild to severe diseases in humans and several animals (Merhej, V. et al., 2011). Some of the rickettsial diseases in humans include Mediterranean spotted fever (*R. conorii*), cat flea rickettsiosis (caused by *R. felis*), epidemic typhus (*R. prowazekii*), Rocky Mountain spotted fever (RMSF) (*R. rickettsii*), murine typhus (*Rickettsia typhi*) and around ten other rickettsial diseases have been identified worldwide (Brown, L. D. et al., 2016; Raoult, D. et al., 1997b).

Owing to the characteristic of *Rickettsia* as an obligate intracellular bacterium, *in vitro* studies of its mechanism of infection, transmission, host-pathogen interaction, vector-pathogen interaction and also antibiotic susceptibilities necessitate the application of a cell culture system. However, compared to other tick-borne bacteria like *Ehrlichia* and *Anaplasma*, the use of cell culture systems based on tick cells in *Rickettsia* research is not as common (Blouin, E. F. et al., 2002; Woldehiwet, Z. et al., 2002). Up to now, some *Rickettsia* studies have been done in mammalian cell culture (Eremeeva, M. E. et al., 2006; Martinez, J. J. et al., 2004), but only a few studies have been conducted using tick cells. The use of tick cells for growing rickettsial agents is a useful tool as ticks are natural reservoirs and vectors of some *Rickettsia* bacteria. However, the information on the interaction between *Rickettsia* and their vector is still very limited. Therefore, to understand the transmission of *Rickettsia* in the population of ticks and the ecology of tick-borne rickettsial diseases, research about the interaction of *Rickettsia* and the tick vector is important.

Furthermore, *Rickettsia* falls into the risk group 3 (RG 3) pathogens, which requires only trained personnel and biosafety level (BSL) 3 facilities to safely handle the pathogen, which can be expensive (Blacksell, S. D. et al., 2019). Compared to other *Rickettsia* species within the spotted fever group (SFG), *Rickettsia*, *R. raoultii* was shown to be less pathogenic than other *Rickettsia* species (Parola et al., 2009) thus can be possibly used as a model for *Rickettsia* studies in BSL 2 facilities. So far, there is no evidence for the airborne transmission of *R. raoultii*. Therefore, together with the use of the tick cell lines, *R. raoultii* may be a valuable model organism used for investigating the vector-pathogen (tick-*Rickettsia*) interaction at the cellular, molecular and genomic levels.

Until now, there are only several tick cells lines (*Anocentor nitens*-derived ANE58, *Rh. microplus*-derived BME / CTVM2, *Rh. microplus*-derived BME / CTVM23 and *Rh. sanguineus*-derived RML / RSE) known to be permissive to the infection of *R. raoultii* and are able to support the propagation (Alberdi, M Pilar et al., 2012b; Santibáñez, S. et al., 2015). However, further characterization of the *R. raoultii* and its interaction with tick cells has not been investigated. In addition, not every researcher has access to all tick cell lines; therefore, it will be useful to identify additional tick cell lines that are susceptible to the infection and propagation of *R. raoultii*.

Besides, to monitor the growth of bacteria in the tick cell lines, a reliable method for quantifying rickettsial bacteria such as qPCR in tick cell cultures is essential. The monitoring of rickettsial bacteria growth in cell cultures is often achieved by Giemsa or Giminez-staining of cytocentrifuge preparations of infected cell cultures (Sayler, K. A. et al., 2014). The staining method is easy to perform, but the enumeration of infected cells can be laborious and time-consuming. Up till now, qPCR assays have only been utilized to detect the presence of *Rickettsia* bacteria in the tick cell cultures (Sayler, K. A. et al., 2014) and have been designed primarily to detect rickettsial DNA in clinical and

environmental samples (Jiang, J. et al., 2012; Znazen, A. et al., 2015). However, the qPCR assays have been developed and applied successfully in detecting *Rickettsia* agents with high specificity and sensitivity, with the potential in quantifying the bacterial deoxyribonucleic acid (DNA) copy numbers in real time (Stenos, J. et al., 2005). Hence, it will be useful to adapt published qPCR protocols and develop appropriate DNA standards for monitoring the growth kinetics of *Rickettsia,* and bacterial quantification in tick cell cultures will aid in research into *Rickettsia* using tick cells.

1.1 Study objectives

Therefore, the overall objective of this study was to investigate the replication kinetics of the spotted fever group *Rickettsia*, *R. raoultii*, in *Rh. microplus*-derived BME / CTVM23, *I. scapularis*-derived IDE8 and *Rh. sanguineus*-derived RSE / PILS35 tick cell cultures. The main objective was pursued through three specific aims; (i) inoculation of IDE8 and RSE / PILS35 cell cultures with semi-purified *R. raoultii* bacteria cultures, (ii) determination of the infection rates of *R. raoultii* in BME / CTVM23, IDE8 and RSE / PILS35 tick cell lines, and (iii) establishment of the replication kinetic curves of *R. raoultii* in BME / CTVM23, IDE8 and RSE / PILS35 tick cell lines.

CHAPTER 2: LITERATURE REVIEW

2.1 *Rickettsia*

Rickettsia is a group of small, gram-negative pleomorphic rods and obligate intracellular bacteria that cause zoonotic diseases around the world that are populated by humans and animals (Labruna, M. B. et al., 2004). Owing to the characteristic of an intracellular bacterium, they strictly require a host cell to survive and are naturally transmitted to humans by arthropod vectors, including fleas, ticks, mites or lice. *Rickettsia* species are commonly recognized as human pathogens vectored primarily by hematophagous arthropods that raised public health concerns in numerous countries all around the world. Several species of *Rickettsia* bacteria are identified as the causative agents of some diseases in humans, such as *R. typhi*, the causative agent of murine typhus, *R. prowazekii*, the causative agent of epidemic typhus and *R. rickettsii*, the causative agent of RMSF (Fournier, P.-E. et al., 2009b). The transmission of these *Rickettsia* agents to humans or animals can occur via bites of the infected ectoparasites or by exposure to an infected animal reservoir host. *Rickettsia* bacteria will infect their target cells via the bloodstream after entering from the portal of entry in the skin and subsequently multiply in the cytosol by binary fission and destroy host cells that are heavily parasitized (Walker, D. H., 1996).

2.1.1 *Rickettsia* classification

The name of the genus *Rickettsia* was taken after Howard Taylor Ricketts, who became the first person to discover the causative agent and the mode of transmission of Epidemic typhus and RMSF (Walker, D. H., 2004). The typhus group (TG), the SFG, and the scrub typhus group (STG) were traditionally the three main groups classified for the *Rickettsia* genus, which were distinguished based on phenotypic characteristics. However, the advanced use of molecular tools in recent years has led to reorganizations of rickettsiae taxonomy. As a result, studies into *Rickettsia* phylogenetics have revealed that the genus can be divided further into four different phylogenetic groups. These four groups include (i) the SFG, which is vectored primarily by ticks and less commonly by mites and fleas, (ii) the TG, which consists of the agents of murine and epidemic typhus that are vectored by fleas and lice, (iii) the translational group and lastly, (iv) the ancestral group that consists of *Rickettsia belli* and *Rickettsia canadensis* (Murray, G. G. et al., 2016; Sekeyová, Z. et al., 2019). This classification has been confirmed based on clinical, ecological, phenotypic, and through analysis of phylogenetic sequence data from a number of *Rickettsia* genes and the complete genome of *Rickettsia* species (Fournier, P.-E. et al., 2009b; Gillespie, J. J. et al., 2007; Ngwamidiba, M. et al., 2006; Roux, V. et al., 1997). Both SFG and TG *Rickettsia* have been classified by the US National Institute of Allergy and Infectious Diseases (NIAID) and the Centre for Disease Control (CDC) as 'select agents' for bioterrorism (Martinez, J. J. et al., 2004).

At present, the genus *Rickettsia* consists of 31 recognized species and a number of uncharacterized strains that cause diseases in both domestic and wild animals and humans (Chisu, V. et al., 2017; Merhej, V. et al., 2014). The TG consists of *R. typhi* and *R. prowazekii*. The SFG consists of *R. africae*, *R. amblyommatis*, *R. conorii*, *R. fournierii*, *R. heilongjiangensis*, *R. honei*, *R. japonica, R. sibirica*, *R. slovaca*, *R. parkeri*, *R. peacockii*, *R. aeschlimannii*, *R. gravesii*, *R. massiliae*, *R. montanensis*, *R. rhipicephali*, *R. buchneri*, *R. asiatica*, *R. helvetica*, *R. tamurae*, *R. rickettsii* and *R. raoultii*. The translational group contains *R. akari*, *R. asembonensis*, *R. australis*, *R. hoogstraalii* and *R. felis* (Fournier, P.-E. et al., 2003; Fournier, P.-E. et al., 2009b; Sekeyová, Z. et al., 2019). However, there are a lot of rickettsial isolates that still have not yet been characterized fully, which may possibly represent new species.

2.1.2 Transmission of *Rickettsia*

Life cycles of *Rickettsia* are complex as they commonly involve a wide range of vectors, vertebrate hosts and modes of transmission (Azad, A. F. et al., 1998). Ticks are known as the primary vector and constitute a risk of rickettsial infection despite their developmental stages (Sekeyová, Z. et al., 2019). Other arthropod vectors that are known to transmit *Rickettsia* bacteria include fleas, lice and mites. Apart from being transmitted by bites of the infected ectoparasites, inoculation of faeces or fluids originating from the infected ectoparasites into the skin is one of the modes of *Rickettsia* transmission (Azad, A. F. et al., 1998). The horizontal transmission of *Rickettsia* bacteria to vertebrates is achieved through a number of arthropod vectors that feed on various species of animals and humans.

In the horizontal transmission, infected ticks at different development stages will transmit the *Rickettsia* bacteria to animals or human hosts. Animals commonly served as the amplifying agent that resulted in the survival and persistence of the *Rickettsia* bacteria without causing damage to the host. Whereas humans usually act as the dead-end host that results in pathogenesis and damage to the host (Narra, H. P. et al., 2020). In the transovarial transmission, infected adults transmitted the *Rickettsia* bacteria during oviposition, causing the eggs to be infected. While in transstadial transmission, the *Rickettsia* bacteria are carried from one developmental stage to another (from infected nymphs to infected adult ticks) leading to survival and persistence of infection (Narra, H. P. et al., 2020).

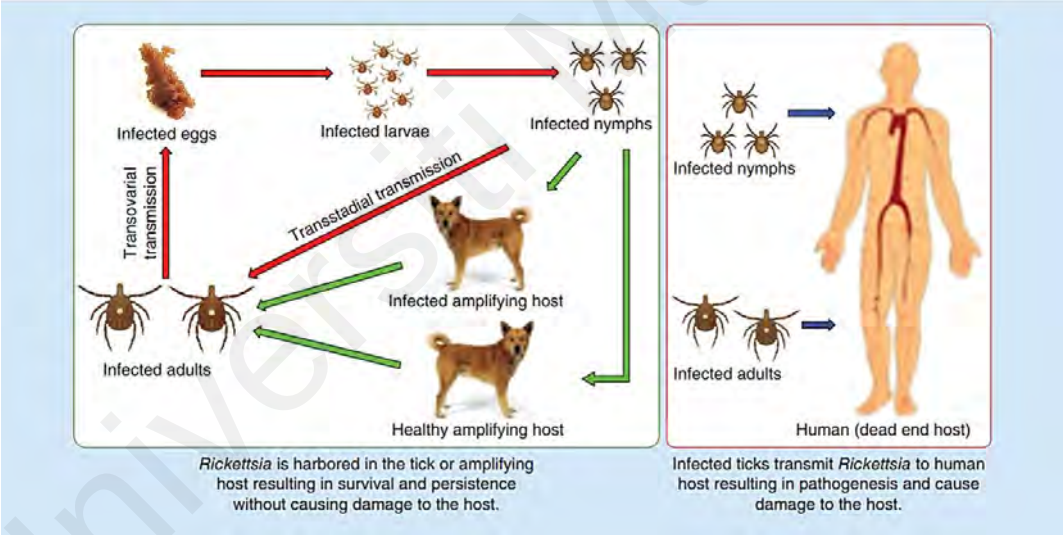

Figure 2. 1: Transmission of tick-borne *Rickettsia*. The major steps in the vector life cycle are indicated by red arrows. Infected nymphs feed on healthy or amplifying hosts, leading to the spread and persistent of infection (green arrows). The transmission of *Rickettsia* to humans via infected nymph or adult tick bite is indicated by blue arrows. Retrieved from (Narra, H. P. et al., 2020).

2.1.3 Ticks as a vector for *Rickettsia* transmission

Ectoparasites, for instance, ticks and fleas, show an important part in the transmission of *Rickettsia* disease. Because of this characteristic, *Rickettsia* bacteria are often called arthropod vectored pathogens of vertebrate hosts (Raoult, D. et al., 1997b). Ticks and fleas are obligate blood-feeders that can attach strongly to their hosts for relatively long periods of time, thus, allowing a sufficient opportunity to transfer *Rickettsia* bacteria into the hosts (Cupp, E. W., 1991). The majority of *Rickettsia* species appear to have no detrimental effect on their respective arthropod hosts, as they commonly multiply in fluids and most organs of the host. That eventually leads to the vertical transovarial transmission from adult arthropods to the offspring and horizontal transmission during feeding (Kenny, M. J. et al., 2003). Continuously transmitted to animals or humans via the bite of the infected arthropod vector in the horizontal transmission. Transstadial passage, which transfers *Rickettsia* from one stage to another, is an essential element to maintain the vectorial competence of the ticks. When *Rickettsia* is transmitted efficiently in both transovarial and transstadial in a tick, the tick will become the reservoir for the bacteria and the distribution of rickettsial diseases will be the same as the distribution of the tick host (Parola, P. et al., 2005).

The presence of pathogens in a specific area can be determined through the detection of hematophagous vectors as they may contain the pathogen and infected host blood (Irwin, P. J. et al., 2004). Besides that, cats and dogs can also be known as close companions of people and serve as the intermediate host that can carry the vector of the disease, thus, leading to infection in humans (Lempereur, L. et al., 2011).

Rickettsia species known to be transmitted by tick vector include *R. aeschlimannii*, *R. africae*, *R. australis*, *R. conorii*, *R. heilongjiangensis*, *R. helvetica*, *R. honei*, *R. japonica*, *R. massiliae*, *R. montanensis*, *R. raoultii*, *R. rickettsii*, *R. sibirica* and

R. slovaca. *Rickettsia* species known to be transmitted by flea vector include *R. felis* and *R. typhi*, while *R. prowazekii* is transmitted by lice vector and *R. akari* mite vector (Parola, P. et al., 2005).

2.1.4 Geographic distribution of *Rickettsia*

The geographic distribution of *Rickettsia* bacteria is not only restricted to certain countries but distributed almost all across the world. All continents, excluding Antarctica, have reported the detection of *Rickettsia* bacteria (Figure 2.2) (Abdad, M. Y. et al., 2018). Climatic conditions, together with vectors and natural hosts constraints, lead to the region-locked distribution of most *Rickettsia* species. However, *R. felis* and *R. typhi* are globally distributed as these two known species of *Rickettsia* bacteria are transmitted commonly by fleas. Thus, different from most *Rickettsia* species that are dependent on tick vectors that tend to be restricted to the specific geographical distribution of the ticks (Abdad, M. Y. et al., 2018).

Tick-borne spotted fever group *Rickettsia* is the most commonly reported in traveller-associated *Rickettsia* infections (Rolain, J.-M. et al., 2004). People of all ages are at risk to get infected with *Rickettsia* during travelling, especially in the disease-endemic areas, and the risk of exposure is greater when they engage in outdoor activities (Chapman, A. S., 2006). Because the incubation period for most *Rickettsia* diseases is around 5 to 14 days (Biggs, H. M. et al., 2016), tourists normally do not acquire any symptoms while on vacation, and disease development may occur after they return home or within a week of their return.

RMSF, also known as Brazilian spotted fever, is reported throughout much of the Western Hemisphere, including Mexico, Canada, the United States and several other countries in Central and South America, including Brazil, Argentina, Colombia, Panama and Costa Rica (Dantas-Torres, F., 2007). In addition, involvement in outdoor activities, such as camping, hunting, hiking and contact with dogs from rural or urban areas increases the chance of getting a rickettsial infection (Chapman, A. S., 2006).

R. felis and *R. typhi* are broadly distributed by fleas, commonly detected throughout tropics and subtropics areas (Boostrom, A. et al., 2002). Therefore, when travelling in endemic areas, people exposed to flea-infested cats or dogs and domestic animals have the highest risk of being infected with flea-borne *Rickettsia*. In addition, Southeast Asia and Africa are among countries where the incident of Murine typhus has been reported previously (Parola, P. et al., 1998).

The infection of *R. prowazekii* causes epidemic typhus is seldom detected from tourists but may occur in refugee populations and poor communities where body lice are prevalent. Some parts of Africa and the Andes regions of South America are regions where epidemic typhus is commonly detected, with body lice as the main vector that transmits *R. prowazekii*. Whereas Brazil, Ethiopia and Mexico areas of tick-associated reservoirs of *R. prowazekii* (Azad, A. F. et al., 1998).

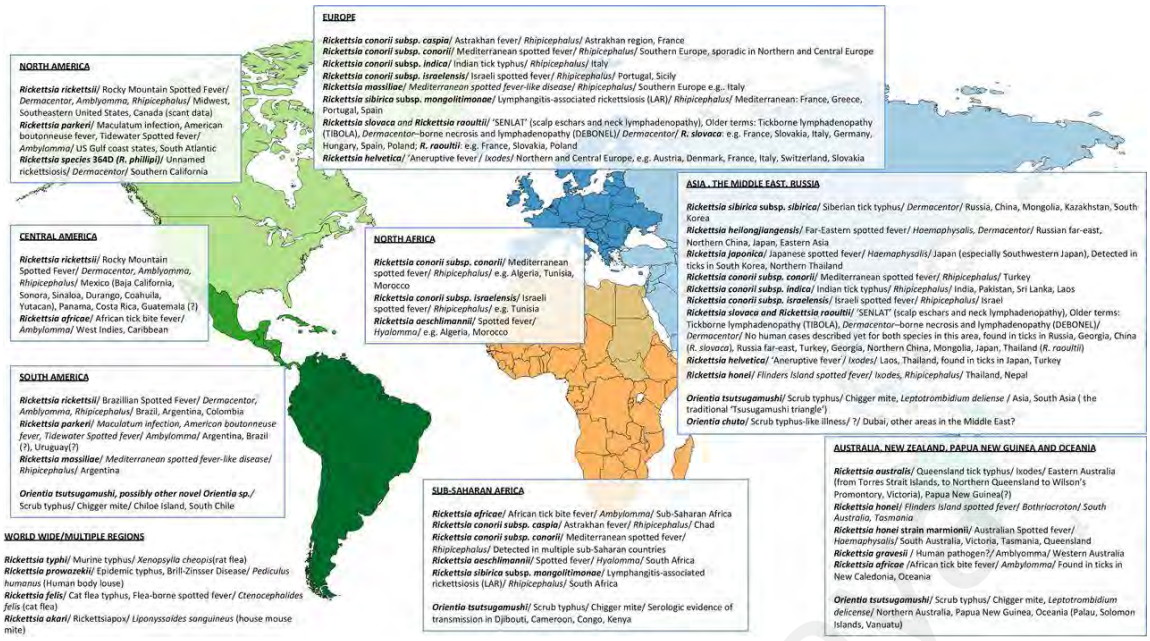

Figure 2. 2: *Rickettsia* species distribution. Retrieved from (Abdad et al., 2018).

2.1.5 Pathogenesis of *Rickettsia*

Rickettsia is inoculated into the dermis of the skin through scratched skin from infectious faeces or fluids from infected ectoparasites or via bites of infected ectoparasites. *Rickettsia* starts the pathogenesis by entering from the skin, infecting the endothelium by spreading through the bloodstream and, in some cases infecting the vascular smooth muscle cells (Walker, D. H. et al., 2003). The first step of the pathogenesis of *Rickettsia* bacteria is adherence to the host cell. Once attached to the cell membrane, *Rickettsia* will be phagocytosed by the host cell. After being phagocytosed by the host cell, *Rickettsia* is seen to escape from the phagosome membrane and start entering the cytoplasm (Walker, D. H. et al., 2008).

The mechanisms of intracellular movement of *Rickettsia* and the destruction of the host cells after infection were observed to be different between the TG and SFG *Rickettsia* (Figure 2.3) (Parthasarathy, A., 2013). The TG escaped from the host cells by lysis mechanism. For instance, after *R. prowazekii* or *R. typhi* infection, the *Rickettsia*

continues to multiply in number until the cell becomes packed with the bacteria and leads to burst. In contrast, SFG does not lyse the infected host cells. Instead, they are often released from the host cell by triggering the polymerization of host cell-derived actin tails. Eventually leads to the emergence of the bacteria through tips of membranous extrusions that propel the bacteria from the cytoplasm (Parthasarathy, A., 2013).

The release of *Rickettsia* will cause infection in other cells, leading to the progression of vasculitis that eventually develops to become maculopapular skin rashes, thrombosis and perivascular tissue necrosis. Subsequently, the disseminated endothelial lesions cause an increase in capillary permeability, haemorrhage, hypotensive shock and oedema. In addition, the endothelial damage may cause disseminated intravascular coagulation (DIC) through the activation of the clotting system (Parthasarathy, A., 2013).

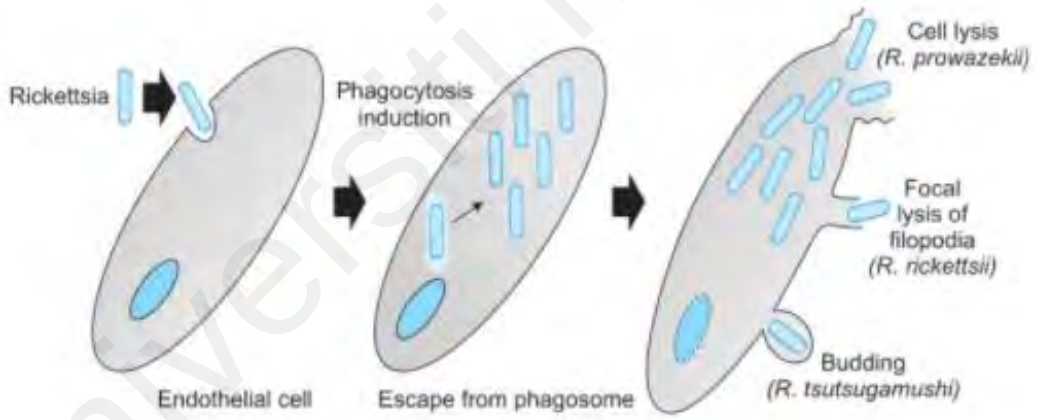

Figure 2. 3: Movement of *Rickettsia* inside host cells. Retrieved from (Parthasarathy, 2013).

2.1.6 Diseases and clinical presentation caused by *Rickettsia* infection

Rickettsia species and clinical diseases associated with infection by *Rickettsia* bacteria often vary based on the geographical area (Eremeeva, M. E. et al., 2015; Parola, P. et al., 2013; Weinert, L. A. et al., 2009). *Rickettsia* disease is an emerging infectious disease that is frequently being ignored in many countries in Southeast Asia. In Asia, tick-borne SFG such as *R. conorii*, *R. heilongjiangensis*, *R. honei, R. japonica*, *R. raoultii*, *R. sibirica* and *R. tamurae* have been reported. While, flea-borne rickettsiosis is regularly caused by *R. felis* and *R. typhi* (Kho, K. L. et al., 2017).

Besides that, few reported cases of *Rickettsia* infection in humans have previously been detected in some Asia countries (Fournier, P.-E. et al., 2005). For instance, in China, there were two reported cases of humans infected with *R. raoultii* (Jia, N. et al., 2014). In addition, human infection with *R. honei* (Jiang, J. et al., 2005) and *R. felis* (Edouard, S. et al., 2014) in Thailand and the first three cases of murine typhus in travellers coming back from Indonesia. The emergence of these *Rickettsia* infections among travellers suggests that murine typhus must be included in the consideration for the diagnosis associated with febrile illness, especially in travellers coming back from disease-endemic areas such as Indonesia (Parola, P. et al., 1998).

Fever, generalized cutaneous rash, and inoculation eschar are the most typical symptoms of rickettsial diseases. However, other possible symptoms that may develop in infected patients include nonspecific flu-like symptoms, cough, fever, generalized lymphadenopathy, abdominal pain, myalgia and some neurological symptoms (Sekeyová, Z. et al., 2019). Frequently, patients will recover without complication. Nevertheless, the clinical symptoms showed by individuals infected with rickettsial diseases may vary from mild to severe, with 2 % to 30 % of the fatality rate for highly virulent rickettsiae (Azad, A. F., 2007). The severity of rickettsial disease is commonly

associated with host-related factors and differences in pathogen virulence (Walker, D. H. et al., 2003). Host-related factors include age, renal dysfunction and hepatic, central nervous system (CNS), involvement of lungs and delay in diagnosis (Walker, D. H. et al., 2003). In addition, studies have suggested that pathogen virulence that is varied between different species of *Rickettsia* may be associated with the degree of genomic degradation (Diop, A. et al., 2019).

RMSF is caused by *R. rickettsii* and is known as one of the rickettsial diseases with the presence of neurological symptoms (Horney, L. F. et al., 1988). The onset of symptoms is often sudden, with severe headaches, chills, fever, arthralgias, prostration and myalgias. Common symptoms include headaches, insomnia, restlessness and back stiffness. During the peak of fever accompanied by delirium or coma, alternating restlessness (Helmick, C. G. et al., 1984; Horney, L. F. et al., 1988; Kulkarni, A., 2011). Delayed diagnosis and treatment frequently lead to encephalitis, which may be fatal (Baganz, M. D. et al., 1995). In addition, patients who survived the infection of *R. rickettsii* may still carry symptoms that include deafness, hemiplegia, slurred speech, visual disturbances, cranial neuropathies and mental disorientation for a few weeks after recovery (Archibald, L. K. et al., 1995; Kirk, J. L. et al., 1990; Massey, E. et al., 1985; Sexton, D. J. et al., 1998; Steinfeld, H. et al., 1988). According to histological investigations, in RMSF, the most severe pathologic changes have been reported in the skin; however, the lungs, heart and CNS are all affected (Sekeyová, Z. et al., 2019). One *in vitro* study on the involvement of neuronal cells during rickettsial infection showed *R. rickettsii* could infect neurons and cause significant neuronal apoptotic cell death (Joshi, S. G. et al., 2007). Besides, small round nodules composed of elongated microglia, lymphocytes, and endothelial cells were observed from the microscopic lesions (Pai, H. et al., 1997).

R. conorii is the causative agent of Mediterranean spotted fever (MSF), also known as Marseilles fever and Boutonneuse fever. Despite being commonly reported as a self-limiting febrile illness and benign (Dolado, M. et al., 1994; Raoult, D. et al., 1986; Walker, D. H. et al., 1987), the MSF has been recorded between 2 % to 7 % fatality rate among hospitalized patients (Duque, V. et al., 2012). Furthermore, patients that showed severe symptoms and fatal forms of MSF had complications such as thrombocytopenia, acute renal failure, myocarditis, gastric haemorrhage, multiple organ failure, and pneumonitis have been reported in severe MSF case series from Spain and France (Amaro, M. et al., 2003; Dolado, M. et al., 1994; Raoult, D. et al., 1986; Walker, D. H. et al., 1987). Additionally, CNS involvement has been recorded in the course of MSF, manifesting as myelitis (Ezpeleta, D. et al., 1999), meningitis (Ezpeleta, D. et al., 1999; Tzavella, K. et al., 2006), encephalitis (Amaro, M. et al., 2003; Benhammou, B. et al., 1991; Dolado, M. et al., 1994; Ezpeleta, D. et al., 1999; Texier, P. et al., 1984; Walker, D. H. et al., 1987) or meningoencephalitis (Bougteba, A. et al., 2010; Dolado, M. et al., 1994; Tikare, N. V. et al., 2010) in a few reports in the literature.

R. japonica causes Japanese spotted fever (JSF), which was originally reported in 1984 (Mahara, F. et al., 1985). JSF is characterized by symptoms such as rash, high fever and inoculation eschar often found at the tick bite site (Mahara, F. et al., 1985). The clinical presentation of JSF and scrub typhus (caused by *Orientia tsutsugamushi*) is similar, yet with a more severe course (Mahara, F. et al., 1985). CNS involvement is uncommon, but complicated incidences of meningoencephalitis or pneumonia have been reported (Honda, E. et al., 2003; Kodama, K. et al., 2000; Kodama, K. et al., 2001). The first fatal case of JSF was reported with all patients having fever, aseptic meningitis and disturbance in their mental health, in which three patients had seizures and three patients had nuchal rigidity (Kodama, K. et al., 2002; Nakata, R. et al., 2012).

TIBOLA also called *Dermacentor*-borne necrosis erythema and lymphadenopathy (DEBONEL) and scalp eschar and neck lymphadenopathy after the tick bite (SENLAT), is caused by *R. raoultii and R. slovaca* (Angelakis, E. et al., 2010; Lakos, A., 2002; Lakos, A. et al., 2012; Raoult, D. et al., 1997b; Selmi, M. et al., 2008). TIBOLA is currently known as the most dominant tick-borne rickettsiosis found in Europe after MSF (Oteo, J. A. et al., 2012). This syndrome is associated with a tick bite and inoculation eschar frequently present on the scalp encircled with circular erythema and accompanied by painful regional lymphadenopathy (Raoult, D. et al., 2002). The *R. slovaca* was initially considered pathogenic, with the first isolate cultivated from a field of *D. marginatus* tick in central Slovakia (Brezina, R. et al., 1969; Lakos, A., 1997; Raoult, D. et al., 1997a). Later in 1997, the *R. slovaca* was recognized as the source of clinical symptoms associated with neck lymphadenopathy and scalp eschar subsequently after a tick bite (Raoult, D. et al., 1997a). The DNA of *R. raoultii* was later detected from a tick removed from the scalp of a TIBOLA patient in 2008 (Mediannikov, O. et al., 2008). Six more cases of *R. raoultii* infection were reported in 2009 (Parola, P. et al., 2009). Thus far, *R. raoultii* has been defined to cause a milder form of TIBOLA compared to the infection by *R. slovaca* (Parola, P. et al., 2009).

African tick-bite fever (ATBF) is caused by *R. africae* and is recognized as the most common rickettsial infection reported in travellers who visit sub-Saharan Africa (Fujisawa, T. et al., 2012; Harrison, N. et al., 2016; Zammarchi, L. et al., 2014). The symptoms presented in ATBF are often mild, self-limiting, and absence of any sequelae (Sekeyová, Z. et al., 2019). Nevertheless, in 2006, long-lasting subacute neuropathy resulting from ATBF was reported in six patients after safari visits in southern Africa (Jensenius, M. et al., 2006).

R. prowazekii is the agent of epidemic typhus, which normally causes neurological complications that include confusion, seizures and coma (Sekeyová, Z. et al., 2019). Besides, it also may lead to death in untreated agitated delirium. In addition, meningoencephalitis with tinnitus, meningism and hyperacusis may develop in severe patients, followed by dysphoria, agitation and deafness (Sekeyová, Z. et al., 2019). The recurrent form of epidemic typhus, known as Brill-Zinsser, may arise after a month or even years, subsequently after the first infection. The symptoms of Brill-Zinsser are commonly milder and similar to the primary infection (Marrie, T. J. et al., 1992).

Murine typhus, or endemic typhus, is caused by *R. typhi* (Pether, J. et al., 1994). Rat flea *Xenopsylla cheopis* is the main vector in transmitting *R. typhi* to humans (Pether, J. et al., 1994). Murine typhus is frequently associated with milder symptoms; however, severe symptoms were previously described in refugee camps. In addition, death cases have been reported in the United Kingdom but were originally infected in Spain (Pether, J. et al., 1994). The frequency in the association of murine typhus with neurological manifestation ranges from 2 % to 20 % of cases (Allen, A. C. et al., 1945; Bitsori, M. et al., 2002). Common symptoms include headache, but encephalitis and meningitis are infrequently reported (Silpapojakul, K. et al., 1991). In the past, no neurological complications were reported (Cowan, G., 2000); however, recently *R. typhi* has been linked to meningitis and abducens nerve palsy (Moy, W. L. et al., 2015).

R. akari is the agent of rickettsialpox and was initially described in New York City in 1946 (Huebner, R. J. et al., 1946). *Liponyssoides sanguineus* mite is the main known vector in transmitting *R. akari* (Brouqui, P. et al., 2007). Rickettsialpox cases were reported in Croatia (Radulovic, S. et al., 1996), Ukraine (Eremeeva, M. et al., 1995), Northern Europe (Renvoisé, A. et al., 2012), and still occurring in New York (Brettman, L. et al., 1981; Kass, E. M. et al., 1994). Clinical signs of rickettsialpox develop between 7 to 10 days after the bite of the infected mite. Vesicle appears at the bite site that will

dry up and leave a scar. A generalized popular rash may develop to be vesicular and looks like chickenpox. Common symptoms include a sudden high fever accompanied by severe headaches and myalgias. Additional signs of rickettsialpox include nausea and regional lymphadenopathy. Neurological symptoms associated with rickettsialpox include neck stiffness, dizziness, meningitis and photophobia. Rickettsialpox is self-limiting and commonly without complications, however, headaches and myalgias might continue about two additional weeks after the rash (Raoult, D. et al., 1997b).

R. felis is the agent for flea-borne spotted fever and is transmitted to humans by flea (*Ctenocephalides felis*). Patients often exhibit cutaneous rash, elevated fever, myalgia and headaches. Other clinical manifestations include eschar, nausea, abdominal pain, vomiting and photophobia (Lindblom, A. et al., 2010; Renvoisé, A. et al., 2009).

2.1.7 Diagnosis of rickettsial diseases

Without using the right tools available, the difficulty for the attending physician to diagnose rickettsial infections increases. The diagnosis of rickettsial diseases is tricky because their symptoms are often confused with other diseases that cause febrile illness (Parola, P. et al., 2005). Additionally, the tests that physicians can perform are limited by the type of tissue collected; hence, knowledge of the available assays is necessary (Abdad, M. Y. et al., 2018). A comprehensive clinical history describing a potential exposure to rickettsial infection or source of the disease, as well as laboratory tests, is usually the primary step to diagnosing rickettsial infection. In some cases, if available, arthropods present at the bite site during inspection or an eschar should be collected for the detection of rickettsial materials. Thrombocytopenia, hyponatremia and transaminitis are common laboratory findings associated with rickettsial infection (Bechah, Y. et al., 2011). To make it more difficult, in many endemic areas around the world, physicians may only examine for rickettsial infection if the initial diagnostics are unable to determine the cause of the disease (Robinson, M. T. et al., 2019).

Serology is the commonly used test for the primary diagnostic of rickettsial diseases applied in the national public health laboratories and large medical reference laboratories in developed countries (Abdad, M. Y. et al., 2018). Early detections methods for *Rickettsia* species relied heavily on the use of the Weil-Felix test and followed by Gimenez staining (Cruickshank, R., 1927; Giménez, D. F., 1964). Before the widespread acceptance and use of molecular testing, the use of immunofluorescence as an adaptation from modern serological techniques for the diagnosis of rickettsial infection significantly increased diagnostic accuracy and was regarded as the gold standard (Philip, R. et al., 1976). However, because of the short turnaround time needed to produce the result and the minimum preparation for the sample, serological testing is still being used in a lot of laboratories throughout the world. Some examples of serological testing available for the

detection of rickettsial infection include dipsticks, enzyme-linked immunosorbent assay (ELISA) kits and immunofluorescence assays (IFA) (Husin, N. A. et al., 2021a). For several rickettsial infections, serological assays, for instance, IFA, are still the most often utilized techniques for rickettsial diagnosis (Luce-Fedrow, A. et al., 2015). The disadvantage in employing serological assays includes the limitation in the availability of diagnostic assays or antigens that are commonly only available in reference laboratories from national or regional. Furthermore, the generation of the kinetics antibody in the course of the disease and an unreliable baseline value especially in those endemic areas, limiting serological applicability by affecting their detection accuracy.

Western blotting is a technique that can help to identify the causative agent with a higher specificity; however, it requires a large collection of rickettsial antigens, which is only accessible in a few reference labs. Because of their dependence on a few well-established rickettsial species, including *R. conorii* and *R. rickettsii*, a lot of kits available on the market are low in specificity and sensitivity (Abdad, M. Y. et al., 2018). However, reference laboratories for rickettsial diagnosis are commonly equipped with in-house IFA and microimmunofluorescence (MIF) protocols, as well as a greater range of rickettsial species that offer more reliable outcome results and diagnoses for the rickettsial infection that are targeted to be different from those used (Abdad, M. Y. et al., 2018). The MIF is similar to IFA, but wells in MIF are marked with numerous rickettsial antigens at the same time to allow for simultaneous detection. Rickettsial antigen has been reported to elicit a 4-fold higher dilution of antibody titer compared to antigens from other species in the MIF setting, suggesting the causative agent (Brouqui, P. et al., 2007). However, this may not be the case due to cross-reactivity hence paired (acute and convalescent) are recommended (Paris, D. H. et al., 2016). Other mitigation measures, for instance, the cross-adsorption assays, are difficult, expensive and limited to only reference and research laboratories. Moreover, IgM detection may not be useful for diagnosing acute

disease due to their cross-reactivity with other species of *Rickettsia* and the possibility of persistence of IgM antibodies after acute illness. Serologic tests for *Rickettsia* species usually become positive between 7 until 10 days of infection, and for some species, such as *R. africae*, this can take up to 25 days or longer (Fournier, P.-E. et al., 2002).

Polymerase chain reaction (PCR) based on nucleic acid amplification tests (NAATs) may be helpful for the diagnosis of rickettsial infection. Together with well-characterized genomes of many *Rickettsia* species, the use of PCR techniques to identify different rickettsial genes, including 16S rRNA, outer membrane protein A (*ompA*), outer membrane protein B (*ompB*), gene D (*sca4*) and *gltA* is a useful method (Luce-Fedrow, A. et al., 2015; Portillo, A. et al., 2017). The rapid turnaround time eliminates the necessity to wait for the process of seroconversion or growth time in a culture that can be lengthy, which can take anywhere from 10 to 4 weeks. Moreover, buffy coats, whole blood or material from eschar (swabs, crust or biopsy samples) can all be used for PCR (Fournier, P.-E. et al., 2004; Mouffok, N. et al., 2011). In nested conventional PCR, more data able to be collected from sequencing due to the longer outcome amplicon accompanied by high sensitivity. However, nested PCR is more likely to get contamination in the resulting amplicon. Therefore, to address this problem, suicide PCR (single-use in nested conventional PCR protocol) has been proposed (Fournier, P.-E. et al., 2004). Nevertheless, the fact that NAAT is more sensitive during acute illness is one of its other limitations (Paris, D. H. et al., 2016). The use of molecular-based tests like nested or qPCR could broaden the screening window to include infection from an early stage. The molecular assays have the upper hand in identifying the specific rickettsiae species that is infecting the host, especially when combined with DNA sequencing.

Next-generation sequencing (NGS) and whole-genome sequences have been useful in identifying novel and known species of *Rickettsia* detected from the vectors or the hosts (Vayssier-Taussat, M. et al., 2013), clarifying phylogeny (Abdad, M. Y. et al.,

2017), and studying pathogenicity (Fournier, P.-E. et al., 2009a). Although it has not been reported, for organisms not discovered with routine cultures, whole-genome next-generation sequencing done straight from human clinical samples has been utilized to produce clinically good data, and it is likely to be effective for the diagnosis of the infection caused by *Rickettsia* bacteria (Abdad, M. Y. et al., 2018). A new study explained the application of a cell-free NGS technique to help in diagnosing *R. typhi* in pregnant patients, suggesting the potential utility of NGS technologies in rickettsiosis diagnosis (Stafford, I. A. et al., 2020). More study and development of NGS-based diagnostic techniques are thus required, especially as NGS technology becomes more cost-effective and accessible. As a result, all new assays must take into account the cost needed to complete the assay as well as the accessibility of the equipment required to execute the assays. It will be critical in ensuring increased access to good diagnostic techniques, particularly in resource-poor nations where rickettsial illnesses are most prevalent.

In addition, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been shown to be effective to be used to identifying the species of the ticks and determining whether they are infected with *Rickettsia* and other tick-borne diseases (Vayssier-Taussat, M. et al., 2013; Yssouf, A. et al., 2015; Yssouf, A. et al., 2013). Moreover, the advance of novel tests from the adaptation of new technologies, for instance, recombinase polymerase amplification (RPA) assays, could lead to high assays sensitivity that is also faster and easier to complete. These assays will aid in the development of point-of-care quick testing for improved patient management in healthcare settings. Although the NGS has been used to monitor pathogens in arthropods, it has limited use in identifying rickettsial infections in people.

2.1.8 Treatment of rickettsial diseases

The non-specific clinical manifestations of rickettsial diseases caused the diagnosis of *Rickettsia* infection to be tricky and difficult. The administration of early treatment with proper antibiotic intake, as well as patient management, is the primary treatment measure for rickettsial infection. Additionally, careful clinical monitoring from the physician, accompanied with the accessibility to reliable diagnostic techniques, is also important. Tetracycline derivatives, primarily doxycycline, are the most effective antibiotics for rickettsial diseases. The daily dose of 200 mg is taken orally and usually, is enough to cure rickettsial diseases (Sekeyová, Z. et al., 2019). The treatment period can take up to 7 days or after 2 days later when the fever is gone (Rolain, J.-M. et al., 2002; Rolain, J. et al., 1998). Intravenous doxycycline may be used in cases of severe infection. Alternatively, rickettsial infections can be treated with chloramphenicol and macrolides (clarithromycin, roxithromycin and azithromycin) (Cascio, A. et al., 2001; Colomba, C. et al., 2006). Antibiotics active against rickettsioses are rarely used as part of empirical treatment for CNS infections (Sekeyová, Z. et al., 2019).

2.2 *R. raoultii*

R. raoultii falls in SFG rickettsial species, which is transmitted by the *Dermacentor* species of tick and is the etiological agent of SENLAT. The initial name of this rickettsial disease is TIBOLA or DEBONEL.

2.2.1 Distribution of *R. raoultii*

R. raoultii was initially isolated in *D. nutalli* (previously reported as genotypes DnS14 and DnS28) and *Rh. pumilio* (previously reported as RpA4) ticks collected from Siberia (Rydkina, E. et al., 1999). It was later detected from *Dermacentor* species ticks (*D. reticulatus*, *D. silvarum*, *D. nutalli* and *D. marginatus*) from several countries in Europe (Duscher, G. G. et al., 2016; Klitgaard, K. et al., 2017; Mediannikov, O. et al., 2008; Selmi, M. et al., 2009), Russia (Mediannikov, O. et al., 2008) and Asia (Seo, M.-G. et al., 2020; Speck, S. et al., 2012; Tian, Z.-C. et al., 2012; Wen, J. et al., 2014). In addition, *R. raoultii* has also been detected in *Haemaphysalis erinacei* (Guo, L.-P. et al., 2015) and *I. ricinus* (Chmielewski, T. et al., 2009) ticks, and also in other different species of arthropod, for instance, *Melophagus ovinus* (Liu, D. et al., 2016). This suggests a possible wider host range in addition to *Dermacentor* species ticks (Husin, N. A. et al., 2021b). Moreover, *Rickettsia* species genetically related to *R. raoultii* (>98% identity in target genes) were also described from *Dermacentor* species ticks collected from Southeast Asian countries (Lim, F. S. et al., 2020; Nooroong, P. et al., 2018).

2.2.2 Clinical manifestation of *R. raoultii* infection

R. raoultii, and *R. slovaca*, are known as the causative agents of TIBOLA, also known as DEBONEL in humans (Parola, P. et al., 2009). TIBOLA / DEBONEL is common in Europe (Hungary, France, Germany and Spain) and occurs more often during colder months, especially in women and children. The syndrome is associated with a tick bite, an eschar on the scalp at the site of a tick bite and cervical lymphadenopathies (Parola, P. et al., 2009). *R. raoultii* infections with erythematous rash and fever, however, without lymphadenopathy, were also reported in patients from China (Jia, N. et al., 2014). Other clinical manifestations of *R. raoultii* infections reported include meningeal syndrome (Igolkina, Y. et al., 2018) and neurological abnormalities such as eyelid droop and high cerebrospinal pressure (Dong, Z. et al., 2019). Although normally associated with mild infections, more severe infections with leukopenia, thrombocytopenia and septic parameters were also reported, suggesting varying degrees of virulence for (Switaj, K. et al., 2012) or susceptibility to *R. raoultii*. Although TIBOLA/DEBONEL is attributed to both *R. slovaca* and *R. raoultii*, studies have suggested that *R. raoultii* is more highly prevalent among *Dermacentor* sp. ticks (Parola, P. et al., 2009). However, there have been more reports of *R. slovaca* infection, implying that *R. raoultii* is less pathogenic (Parola, P. et al., 2009) or less frequently transmitted to humans. The clinical description of *R. raoultii* and *R. slovaca* is similar. However, alopecia last for several months has been reported in more than 59 % of the infection from *R. slovaca* and not from *R. raoultii*. Despite its widespread presence in Europe, Russia and Asia, information related to the virulence and pathogenicity of *R. raoutlii* and *R. slovaca* is still scarce.

2.2.3 *Dermacentor* tick and *R. raoultii*

D. reticulatus (ornate dog tick) is a dark-coloured tick with a light-coloured pattern presence on its scutum. *D. reticulatus* has been found in a variety of animals such as wild and domesticated carnivores, horses, sheep and cattle (Estrada-Peña, A. et al., 2004; Jongejan, F. et al., 2015); but the human infestation is rare (Földvári, G. et al., 2013; Jongejan, F. et al., 2015; Parola, P. et al., 2009). Compared to *I. ricinus* tick, which prefers big shelter within coniferous and deciduous woodland areas (Estrada-Peña, A. et al., 2004), *D. reticulatus* prefers open areas including meadows, floodplains and dune valleys (Jongejan, F. et al., 2015) with higher humidity (Estrada-Peña, A. et al., 2004). Furthermore, *D. reticulatus* have a different seasonal activity compared to other European tick species. The larvae and nymphs are active during spring and summer and are followed by an adult activity that begins during early autumn that lasts well into winter. When environmental conditions become too harsh, the activity will have a brief pause and resume thereafter, causing the additional activity that peaks in spring to be reported in several geographical areas (Duscher, G. G. et al., 2013; Estrada-Peña, A. et al., 2004; Jongejan, F. et al., 2015; Randolph, S. et al., 1999). *D. reticulatus* is active practically for the entire year due to its pattern activity. Furthermore, *D. reticulatus* has been spreading to new areas in recent years, increasing its presence in Europe (Dautel, H. et al., 2006; Giangaspero, A. et al., 2015; Hodžić, A. et al., 2017; Hofmeester, T. R. et al., 2016; Jongejan, F. et al., 2015) and increasing human and animal exposure to this species of tick and their associated pathogens. As a result, the spread of *D. reticulatus* to the new areas accompanied by the seasonal activity in northern regions and the potential transfer of zoonotic infections create a major threat (Wijnveld, M. et al., 2016). The hazards of *D. reticulatus* bites must be made more widely known. *R. raoultii* is the most common pathogen transmitted by *D. reticulatus*, with infection rates as high as 20 % in questing

ticks (Reye, A. L. et al., 2013) and even higher rates (50 – 58 %) in some locations (Szekeres, S. et al., 2016; Wójcik-Fatla, A. et al., 2013).

2.2.4 *R. raoultii* in tick cell line

R. raoultii was primarily isolated from *Dermacentor* species ticks into Vero and L929 cells (Mediannikov, O. et al., 2008). Later, *R. raoultii* was also isolated into embryo-derived tick cell lines originating from *Rh. microplus* (Alberdi, M Pilar et al., 2012b; Munderloh, U. G. et al., 1996; Palomar, A. M. et al., 2019) and *Rh. sanguineus* (Santibáñez, S. et al., 2015). The bacterium was also found to be able to infect cell lines derived from *D. albipictus* and *D. nitens* (Alberdi, M Pilar et al., 2012b). Since ticks are natural reservoirs and vectors of some *Rickettsia* species, tick cell lines are a useful system for the isolation and propagation of *R. raoultii* from ticks or clinical samples for further investigation of its virulence and pathogenicity (Alberdi, M Pilar et al., 2012b; Santibáñez, S. et al., 2015). In addition, continuous cultivation of the tick cell lines has shown to be a great tool to isolate and propagate a variety of vector-borne infections, allowing researchers to investigate these microorganisms in the laboratory setting and develop serological assays that will improve public health.

2.3 Ticks

Ticks are obligate blood-feeding ectoparasites that are able to transmit pathogens to animals and even humans. Nowadays, ticks are known to be second after mosquitoes as vectors of human diseases and are the most crucial vectors that cause diseases in domestic and wild animals (de la Fuente, J. et al., 2008). Besides, ticks are also able to harbour non-pathogenic microorganisms, including *Wolbachia* and *Midichloria mitochondrii* (Bonnet, S. I. et al., 2017; Khoo, J. J. et al., 2021).

2.3.1 Taxonomy of ticks

Ticks fall in the suborder Ixodida (Metastigmata) from the subclass Acari, class Arachnida and phylum Arthropoda. There are three families under the suborder Ixodida; (i) Ixodidae (hard ticks), (ii) Argasidae (soft ticks) and (iii) Nutalliellidae (Estrada-Peña, 2015). The hard tick comprises nearly 80 % of the world's tick population, and around 683 species of ticks have been recognized. The most important genera in this family include *Haemaphysalis*, *Dermacentor*, *Ixodes*, *Rhipicephalus*, *Amblyomma* and *Hyalomma*. The soft ticks consist of 210 known species that are classified into four genera, namely *Obtobius*, *Carios*, *Ornithodorus*, and *Argas* (Muñoz-Leal, S. et al., 2017).

2.3.2 Life cycle of Ixodidae ticks

Egg, larva, nymph and adult are the four stages encompassed in the life cycle of Ixoxidae ticks (hard ticks). First, the female and male adult ticks will be mating exclusively on their host. Following that, the female will fall from their hosts that they attach securely for the feeding process and begin oviposition in areas with a shady environment. Next, the female will lay thousands of eggs in one complete cycle and eventually, the eggs will hatch into larvae with six legs. Finally, the larvae will moult into nymphs, and the nymphs will mould into adults.

However, the members in the family of hard ticks can undergo different life cycles depending on the number of hosts they engage. Generally, they can undergo either one, two or three-host life cycles. In the one-host life cycle, all three stages of tick's larvae, nymph and adult, will happen on the same host. The engorged adult females will only leave the host once in the one-host life cycle, which is right before they start laying eggs. In the two-host cycle, the ticks will leave the first host between the nymph and adult stages. The ticks will stay on the first host and undergo moulting from larvae to nymph stages.

The first and second hosts can be from the same individuals, the same species or a different species. Next, in the three-host life cycle, the ticks will leave the host right after each stage of larvae, nymph and adult. Depending on the host availability, the three hosts can be from different species, also can be from the same species, or even from the same individual. Most species of ticks that are considered as public health importance will undergo the pattern of the three-host life cycle. This includes the members of the genera *Amblyomma* (caused ehrlichiosis and RMSF), *Dermacentor* (RMSF and tularemia), *Ixodes* (babesiosis, human granulocytic ehrlichiosis and Lyme borreliosis), and *Rhipicephalus* (RMSF and boutonneuse fever).

2.3.3 Ixodidae ticks as a vector for tick-borne diseases in animals and humans

Ticks are the most important ectoparasite of livestock globally that is responsible for severe economic losses in livestock. The ability of the ticks to transmit rickettsial, protozoan and viral diseases to the livestock contributes to the big impact on economic loss worldwide. The economically most important ixodid ticks of livestock belong to the genera of *Amblyomma*, *Hyalomma* and *Rhipicephalus* (Alemu, G. et al., 2014; Jongejan, F. et al., 2004b; Lynen, G. et al., 2007). Anaplasmosis, babesiosis, ehrlichiosis and theileriosis are some of the important diseases known to be transmitted to livestock by ticks, predominantly in domestic ruminants (Jongejan, F. et al., 2004b). Besides, dogs are recognized as accompanying human animals and could be infected with *Ehrlichia* species and *Babesia* species, in which *E. canis* infection commonly causes fatality (Jongejan, F. et al., 2004b).

Human tick-borne diseases started to be more known when Lyme borreliosis was discovered in 1982 (Burgdorfer, W. et al., 1982). A number of viral infections and pathogenic bacteria are known to be transmitted by ticks to humans and livestock. The tick-borne viruses that are recognized as a threat to human health include Crimean-Congo hemorrhagic fever (transmitted by *Hyalomma* spp.) and tick-borne encephalitis that is transmitted by *Ixodes* tick species. In addition, tick-borne viruses known in livestock, such as the Nairobi sheep disease virus (transmitted by *Rh. appendiculatus*) and the African swine fever virus (transmitted by *Ornithodoros* species) (Labuda, M. et al., 2004). Other than that, humans may also be infected with other pathogens that are transmitted by ticks, such as *Babesia* species and *Rickettsia* species (Jongejan, F. et al., 2004a).

2.4 Tick cell line

Ticks are known to be the most important vectors in transmitting pathogenic organisms that cause diseases in wild and domestic animals and are considered to be worldwide vectors in causing human diseases, second after mosquitoes (de la Fuente, J. et al., 2008). However, the maintenance of obligate hematophagous arthropod colonies for research purposes is often costly due to the need to maintain animal hosts for blood-feeding and is difficult as highly specialized skills are necessary to handle the colonies. *In vitro* culture systems, especially continuous cell lines that are derived from biological materials of the arthropod vectors, play an irreplaceable and precious part in many aspects of research into arthropod-borne, particularly tick-borne diseases, including host-vector-pathogen relationships, basic parasite biology and disease control (Bell-Sakyi, L. et al., 2007). Research into the host-vector-pathogen relationship of rickettsial diseases is important in revealing potential treatment or prevention measures.

2.4.1 History of tick cell line

Tick cell cultivation has been attempted for over 50 years (Bell-Sakyi, L. et al., 2007). Tick cells, together with tissue culture, have come a long way, starting from the early short-term cultures established in the 1950s and 1960s (Yunker, C., 1987). Nowadays, a lot of the economically significant tick species found in Africa, America and Europe have been established as a continuous cell line. Besides, a number of new young scientists in several less developed, lower and middle-income countries have demonstrated their potential to make primary cell cultures with the aim to establish cell lines originating from their local tick species (Bell-Sakyi, L. et al., 2018). Tick cell lines and their outstanding outputs are being used by a growing number of academics and research centres throughout the world.

Early research showed that the primary cultures of tick cells were able to survive *in vitro* for up to six months with their application for bacteria and viruses propagation has been broadly studied (Kurtti, T. et al., 1988; Varma, M., 1989). After more than two decades, the developments in protocols and techniques have helped to establish the first continuous tick cell lines derived from *Rh. appendiculatus* ticks (Varma, M. et al., 1975). The variety of microorganisms that could be cultured in tick cell lines has been expanded to include various bacterial diseases with significant medicinal and veterinary value around the world. Some tick species such as *Rh. microplus* and *Rh. appendiculatus* has been successfully used to establish multiple cell lines, whilst cells from other species have been challenging to produce a continuous culture (Bell-Sakyi, L. et al., 2007), despite relatively consistent procedures for the preparation of primary culture. For instance, a cell line derived from the *D. andersoni* tick took more than 30 years in cell culture research to yield a cell line (Yunker, C., 1987). Likewise, the first soft (Argasid) ticks cell lines took 30 years to establish, especially due to the less attention given to them compared to hard ticks (Kurtti, T. et al., 1988; Yunker, C., 1987). The two most important factors in establishing tick cell lines are (i) patience, as continuous cultivation requires around five years starting when the primary culture is initiated with a low success rate, and (ii) personnel experience (Bell-Sakyi, L. et al., 2007).

2.4.2 Establishment of primary tick cell line

Currently, there are over 63 cell lines from 18 species of Ixodid and three species of Argasid tick that are known to exist (Bell-Sakyi, L. et al., 2018). Ticks' embryos, nymphs or moulting larvae are the usually used material to establish cultures called tick cell lines. The Tissue Culture Association Terminology Committee (1987) declares that the term "line" was defined as the cell line that was established from the primary culture when the first subculture was started (Yunker, C. et al., 1981). The establishment of a tick cell line may take between 1 to 5 years and be accompanied by a low success rate (Bell-Sakyi, L. et al., 2007). Embryonic cells from tick eggs are the most commonly used source of material to initiate the tick cell lines due to their abundant source of undifferentiated cells and the most easily handled developmental stage (Bell-Sakyi, L. et al., 2018). Furthermore, the ability of tick cells to stay survive for a long period of time, up to months or even years, without the need to subculture, is the key element that is useful to attempt isolation and the research involving slow-growing microorganisms *in vitro* (Bell-Sakyi, L. et al., 2007). Because of the continuous exploration of tick cell lines, a numerous number of bacteria have been successfully propagated and isolated for instance, bacteria such as *Rickettsia* spp. (Alberdi, M. Pilar et al., 2012; Pornwiroon, W. et al., 2006; Simser, J. A. et al., 2001) *Anaplasma* spp. (Blouin, E. F. et al., 2002; Zweygarth, E. et al., 2006) and *Ehrlichia* spp. (Woldehiwet, Z. et al., 2002).

In short, engorged female ticks are surface-sterilized and placed in a sterile humid environment to lay eggs. The eggs are surface-sterilized again when developing embryos already visible in almost all of the egg's batches. Then the eggshells are crushed in a culture medium or balanced salt solution to free the embryos. The resultant suspension of crashed eggs and ticks tissue is placed in an incubator with a temperature between 28 ℃ to 34 ℃ and monitored weekly for any growth or contamination (Bell-Sakyi, L. et al., 2018).

Compared to cell lines established from other insects, tick cell lines demonstrated a longer adaptation period. The time taken for the adjustment in the cultural conditions is distinguished based on the long duration of the unsettled subculture intervals. The cell line can be called 'continuous' once a consistent subculture rate has already been achieved (Kurtti, T. J. et al., 1982). For instance, the RU TAE 12 V line (established from embryos of the mosquito *Toxorhynchites amboinensis*) and RA 243 line (established from developing adult tissues of *Rh. appendiculatus*) were subcultured 50 times and 14 times, respectively, within their first year in culture (Munderloh, U. et al., 1982; Varma, M. et al., 1975).

Despite the fact that more than 60 continuous cell lines have already been established from 18 species of ixodid and three species of Argasid tick (Bell-Sakyi, L. et al., 2018), tick and pathogen control research is still largely focused on cell lines that are derived from *Rh. microplus*. For instance, other tick cell lines that have been used in a smaller number of studies include tick cell lines derived from *I. ricinus*, which is a European tick that attacks humans as well as feeds on a large range of domestic and wild animals and *I. scapularis* that considered as a North American species of tick that cause a medical concern (Bell-Sakyi, L. et al., 2007).

2.4.2.1 *Rh. microplus*-derived BME / CTVM23 cell line

The BME / CTVM23 cell line was established from the embryo's eggs of *Rh. microplus* ticks were collected from cattle in Mozambique, South Africa (Nijhof, A. M. et al., 2007) using standard techniques explained in Section 2.4.2 (Bell-Sakyi, L., 1991). The *Rh. microplus* was formerly called *Boophilus microplus* and is the most well-known blood-feeding ectoparasite of livestock. *Rh. microplus* is most commonly seen in cattle, deer, and buffalo, but it also infects horses, goats, sheep, donkeys, dogs, pigs, and several wild species. (Low, V. L. et al., 2015). Additionally, this tick species is recognized to transmit babesiosis (caused by the protozoal parasites *Babesia bigemina* and *B. bovis*) and anaplasmosis (caused by *A. marginale*) (Low, V. L. et al., 2015). The BME / CTVM23 cell line was reported to be susceptible to infection with *R. raoultii* (Alberdi, M Pilar et al., 2012b) following sub-inoculation from *D. reticulatus* primary culture.

2.4.2.2 *Rh. sanguineus*-derived RSE / PILS35 cell line

The RSE / PILS35 cell line was maintained in L-15 medium and incubated at 28 °C from embryos eggs that were laid by one individual female *Rh. sanguineus* tick that was incompletely engorged. The tick was given by Dr. Oleg Mediannikov and Dr. Cristina Socolovschi from URMITE, Marseille, France (Koh-Tan, H. C. et al., 2016). The *Rh. sanguineus* tick, also recognized as the brown dog tick, is the most common tick in the world and a well-known vector of a variety of infections that affect dogs and people (Dantas-Torres, F., 2010). Besides, this tick can be detected in infesting dogs in both rural and urban environments, as it has evolved to stay within human residences and is active all year in tropical and subtropical climates, as well as some temperate places. Furthermore, *Rh. sanguineus* is a vector for a variety of disease agents, some of which

are zoonotic in nature (e.g., *R. conorii*, *R. rickettsii*, *E. canis* and *Coxiella burnetii*) (Dantas-Torres, F., 2008).

2.4.2.3 *I. scapularis*-derived IDE8 cell line

The IDE8 cell line was established in L-15B medium at 31 ℃ from a single egg mass laid by engorged female *I. scapularis* ticks (black-legged ticks) taken from *Odocoileus virginianus* deer (hunter-killed white-tailed deer) present in Minnesota close to the St. Croix River and in Polk County, Wisconsin (Munderloh, U. G. et al., 1994) by using standard techniques described previously (Bell-Sakyi, L., 1991). In the United States, the *I. scapularis* tick, also known as a deer tick, spreads a variety of disease agents, including the bacterium that causes Lyme borreliosis, the viruses like Powassan and protozoan agent of babesiosis (Stewart, P. E. et al., 2020). Numerous reported publications have utilized the IDE8 cell line in their experiments. Previously, the IDE8 cell line was successfully infected with the *E. ruminantium* Welgevonden strain (Tjale, M. A. et al., 2018) that caused heartwater disease in domestic and wild ruminants such as cattle, goats, sheep, buffalo and antelope. Besides, the IDE8 cell line has also been used to study the characterization of the St Croix River virus from the complete sequence of the genome (Alberdi, M Pilar et al., 2012a; Attoui, H. et al., 2001).

2.4.3 Tick cells morphology

Tick cells in cultures display different shapes and sizes of the cells compared to those commonly observed in tick tissues. Every tick cell lines consist of two or more cell morphotypes (Bell-Sakyi, L. et al., 2007). Several types of cell morphologies have been described, such as round, fibroblast-like and epithelial-like. The variety of cell types in culture will eventually decrease to one or two cell types throughout subculturing (Munderloh, U. G. et al., 1994). The size and shape of the cells are affected by the chemical environment that includes the ingredients of the media, the pH and the temperature (Pudney, M. et al., 1973). For instance, domination of epithelial-like and fibroblast-like cells was observed in storage temperature at 32 ℃ and small rounded cells at 28 ℃ (Holman, P. et al., 1980). However, with the increasing passage numbers, the morphological heterogeneity of tick cells started to decrease, and the cell populations of cell lines will tend to be more homogenous (Kurtti, T. J. et al., 1982).

2.4.4 The growth rate of tick cells

Tick cells in culture are known to grow slowly with the ability to survive for a quite long period of time, with the medium being changed regularly (once a week) and subcultures when the cell becomes too dense (Bell-Sakyi, L. et al., 2007). This practice correspondingly reflects the ability of ixodid ticks to stay alive for a long period in nature in between blood meals. Besides, the temperature may affect the multiplication rate, as faster multiplication was observed when there was a rise in incubation temperature (Yunker, C. et al., 1981).

2.4.5 The growth requirement of tick cells

Almost all tick cell cultures that have been established were maintained in media with components formulated for mammalian cells. The most commonly used constituents used in media to grow a variety of tick cells cultures involved the Leibovitz's L-15 medium supplemented with tryptose phosphate broth (TPB) and fetal bovine serum (FBS) or mixture with Eagle's MEM medium in Hanks' (Kurtti, T. J. et al., 1982).

FBS and TPB have commonly used supplements in culture media. The FBS was found among several sera that contained a concentrated amount of glutathione mixed disulfide that gave affecting the redox potential control in culture, which is needed to support the growth of cell lines (Bump, E. A. et al., 1977). However, the concentration of FBS that is needed by the cells was found to differ among tick species. For example, 5 % of FBS was found to be the ideal concentration needed to support the growth of *Rh. microplus* cell line, while *D. variabilis* and *Rh. appendiculatus* cell lines required 10 % of FBS (Kurtti, T. J. et al., 1982). TPB help in improving the buffering capacity of the medium and providing glucose which is not present in L-15 media. In addition, it also contains peptones and sodium chloride (Kurtti, T. J. et al., 1982). Other supplements such as lactalbumin hydrolysate (0.5 %) (Bell-Sakyi, L., 2004) or bovine plasma albumin (0.1 %) (Bhat, U. et al., 1977; Yunker, C. et al., 1981) also have been used. Antibiotics such as streptomycin (between 0.1 to 1.0 mg/ml) and penicillin (between 100 to 1000 units/ml) are commonly used in the medium.

As the growth environments and conditions required by the ticks are varied for different tick species, there were no defined optimal or ideal conditions known for culturing tick cell lines. Nevertheless, most cell lines and primary cultures have been maintained and isolated in a range temperature between 28 ℃ to 34 ℃. Different response to the change in incubation temperature was observed. For example, *Rh. microplus* was

observed to grow better at 32 ℃ and died when incubated at 28 ℃ (Yunker, C., 1987). Up till now, no study has been done to test the effect of different medium pH on the tick cell lines. The medium pH between 6.5 and 7.2 was commonly used to isolate and maintain most of the cell cultures.

2.4.6 The maintenance and storage of tick cells

Tick cell lines have commonly been stored in liquid nitrogen together with dimethyl sulfoxide (DMSO) or glycerol as a cryoprotectant in the growth medium, particularly for long-term cryopreservation. However, better recovery and growth rates of the cells stored in a medium with DMSO were observed in the previous study compared to the cells stored in a medium with glycerol (Bastos, C. V. et al., 2006). Most of the cryopreserved protocol has typically been done by using 10 % DMSO in the medium with gradual freezing techniques (Palomar, A. M. et al., 2019). Better resuscitation result was observed when the gradual freezing procedure was adapted rather than a direct introduction to liquid nitrogen (Bastos, C. V. et al., 2006).

2.4.7 The importance of tick cell lines to be used as research tools

Many elements of tick and tick-borne pathogen research can be studied using *in vitro* culture techniques, especially continuous cell lines (Bell-Sakyi, L. et al., 2007). The use of tick cell lines to isolate and propagate pathogens has created a useful model for studying their biologies, such as pathogen metabolism *in vitro* and their interactions with cultured cells, including mechanisms of cell invasion. Their use also aids in the creation of vaccines and diagnostic testing. Consequently, from the recent substantial breakthroughs in molecular biological investigations, tick cell lines can also be employed

in research on tick biology, pathogen genomes, and proteomics (Bell-Sakyi, L. et al., 2018).

2.4.7.1 Tick cell line in bacteriology

Nowadays, the application of tick cell lines in numerous fields of tick and tick-borne disease study is becoming interestingly useful. Numerous studies have been published on the use of tick cell lines in studying tick-borne bacteria, for example, *A. phagocytophilum* (Zweygarth, E. et al., 2006). Different strains of *A. phagocytophilum* were isolated from different animals and cultivated in tick cell cultures. Additionally, *E. phagocytophila* was isolated and propagated from a European ovine for the first time in *I. scapularis*-derived ISE6 and *I. scapularis*-derived IDE8 tick cell lines (Woldehiwet, Z. et al., 2002). Similarly, *A. phagocytophilum* was also successfully isolated from roe deer and cultivated in the *I. scapularis*-derived IDE8 tick cell lines (Silaghi, C., 2008). Furthermore, *A. marginale* was isolated from cattle and propagated in the *I. scapularis*-derived IDE8 cell line (Munderloh, U. G. et al., 1996). *A. marginale* isolated from cell culture showed to be infective to cattle and a potent antigen that can be used for serological tests and vaccine preparation (de la Fuente, J. et al., 2002; Saliki, J. T. et al., 1998).

Other than that, this model culture system for studying the infection of *A. marginale* in a controlled environment has provided new information on the bacterial invasion of cells, including the evaluation of MSP1 complex (MSP1a and MSP1b) in tick cell culture using recombinant proteins as it was shown to show a part in adhesion to host cells. The results of the adhesion assays suggested that MSP1a contributes to bacterial adhesion onto tick cells, while MSP1b was not (Blouin, E. F. et al., 2002). Moreover, the limited role of *A. marginale* MSP1-specific antibodies from bovine sera in preventing the

adhesion of the bacteria on tick cells was also demonstrated using tick cell culture system, suggesting that the immune response from naturally infected cattle may not suppress the transmission of the bacteria to its ticks (Blouin, E. F. et al., 2003).

In addition, functional genomic studies using RNA interference of differentially regulated tick genes in response against *A. marginale* infections in *I. scapularis*-derived IDE8 cell line allow for the identification of bacterial genes (vATPase, salivary selenoprotein M, ubiquitin and putative GST) that may have important functions during infection, such as bacterial trafficking and multiplication (de la Fuente, J. et al., 2007b). Other studies have also been conducted to compare the *A. phagocytophilum* gene transcription using tilling array analysis between *I. scapularis*-derived ISE6 tick cell line and human cell lines (HL-60 and HMEC-I). The result demonstrated an extensive differential transcription, suggesting different mechanisms are involved during *A. phagocytophilum* infections in ticks and human hosts (Nelson, C. M. et al., 2008). Altogether, these studies illustrate the utilities of tick cell lines in enhancing the research into the interaction between *Anaplasma*, its tick vectors and the animal or human hosts. On the other hand, the application of tick cell lines in the research involving *Rickettsia* infections in ticks is not as extensive.

Most available studies of *Rickettsia* are focused on investigating host-pathogen interactions using mammalian cells. These include the studies on the entry of *R. conorii* into mammalian cells that take place during the early signalling stage (Martinez, J. J. et al., 2004), investigating the function of SFG *Rickettsia* rOmpB protein during the adherence and the invasion of Vero cells (Uchiyama, T. et al., 2006), and also the host cell response to *R. typhi* and *R. akari* (Radulovic, S. et al., 2002). The interaction between SFG *Rickettsia* and the vector is less understood. There are only a handful of studies using tick cell lines in investigating the interaction of SFG *Rickettsia* with ticks. One study identified the tick proteins, such as Src family PTK, Rho GTPase RacI, actin-related

protein 2/3 complex phosphatidylinositol-3-kinase, neural Wiskott-Aldrich syndrome protein, protein tyrosine kinases (PTKs), actin filaments and focal adhesion kinases that play an important role in the invasion of *Rickettsia* bacteria in tick cells (Petchampai, N. et al., 2015). Another study explored the role of histone H2B in facilitating the internalization of *R. felis* in a tick cell line (Thepparit, C. et al., 2010).

2.4.7.2 Tick cell line in virology

For more than 35 years, the tick cell lines have been utilized to propagate arboviruses (Varma, M. et al., 1975; Yunker, C. et al., 1981). The growth of a pathogenic strain of tick-borne encephalitis in the *Rh. appendiculatus*-derived cell line demonstrated that this cell line was persistently infected with an attenuated strain resulting in partial inhibition of the growth of the pathogenic strain (Kopecky, J. et al., 1998). This type of *In vitro* study is an effective way to study arbovirus pathogenicity. Tick cell lines from *A. variegatum*, *I. ricinus*, and *Rh. appendiculatus* were also found to be susceptible to the infection with the tick-borne flaviviruses tick-borne encephalitis virus (TBEV), louping ill virus (LIV), Langat virus (LGTV), Negishi virus (NGV) and powassan virus (POWV) (Bell-Sakyi, L. et al., 2012). Additionally, mosquito-borne alphavirus Venezuelan equine encephalitis virus (VEEV) was capable of infecting *Rh. appendiculatus*-derived RAE / CTVM1 (Lawrie, C. et al., 2004). Besides, the Crimean-Congo hemorrhagic fever virus (CCHFV) has been reproduced in seven tick cell lines (Bell-Sakyi, L. et al., 2012)

2.4.7.3 Tick cell line in parasitology

The use of tick cell lines in research involving tick-borne protozoa was not common because primary cell culture was believed to be more suitable for this purpose

(Mosqueda, J. et al., 2008). An attempt to infect *Rh. microplus* tick cell culture with *B. bovis* was not successful (Droleskey, R. et al., 1981). However, in 1983, the first successful cultivation of *B. caballi* in tick cell cultures was reported (Kurtti, T. et al., 1983). Similarly, *B. bigemina* sporokinetes were grown in an *I. scapularis* cell line for up to eight days (Ribeiro, M. F. et al., 2009). In addition, *Leishmania* species could also be propagated in the tick cell lines (Nyindo, M. et al., 1987). Aside from that, the morphology of *Besnoitia besnoiti* was investigated by using *D. variabilis* and *Rh. microplus*-derived tick cell lines (Samish, M. et al., 1988). Furthermore, the bovine nematode *Onchocerca lienalis* was maintained by using the *Rh. appendiculatus* cell line that acts as a feeder layer (Litchfield, T. et al., 1991) suggests that nematode development and maintenance can be achieved using a cell culture system.

2.4.7.4 Tick cell line in the biology of tick and their associated pathogens

Tick cell lines have proven to be a valuable technique to be used to study the biology of several diseases and the biology of ticks. Tick cell cultures are a helpful method to understand the developmental cycles of the tick-borne pathogen as it occurs within the tick. In cell culture, the invasion and development of *A. marginale* were shown to be similar as in infected ticks (Blouin, E. F. et al., 1998). The intracellular growth of the human granulocytic ehrlichiosis (HGE) pathogen revealed that the HGE pathogen might infect tick cells during their blood stages (Munderloh, U. G. et al., 1999). The mechanism of SFG *Rickettsia* and *Borrelia burgdorferi* invading and movement in tick cells was explored using tick cell lines derived from a different tick species (Kurtti, T. J. et al., 1988; Munderloh, U. G. et al., 1998). Moreover, tick cell cultures were also used to investigate the effects of tetracycline antibiotics on cultured *A. marginale*. The result showed that tetracycline able to kill *A. marginale* by disrupting the ability of the bacteria

to complete its replicative cycle while in the cytoplasm of the host cell (Blouin, E. F. et al., 2002).

2.4.7.5 Tick cell line in the study of pathogen genomics and proteomics

Tick cells are a valuable resource for research into protein expression and stage-specific gene transcription. The understanding of the molecular survival and the adaptability strategies in the hosts has led to the development of novel prophylactic techniques and therapeutic targets that are able to stop the transmission and infection (Najm, N. A., 2012). The expression of the temperature-dependent protein was observed in the infected *D. albipictus* derived-DALBE3 and *I. scapularis* derived-IDE2 cells with *R. rickettsii* at both 28 °C and 34 °C (Policastro, P. F. et al., 1997). However, no changes were observed in protein expression at 34 °C in the mammalian and tick cells. In addition, *E. chaffeensis* was found to be transcriptionally less active in human cells compared to tick cells (Kuriakose, J. A. et al., 2011). Tick cells were found to express high levels of genes involved in protein modification, conversion, nutrition delivery and energy, whereas the majority of genes expressed in human cells were found at moderate levels (Najm, N. A., 2012).

2.4.7.6 Tick cell line in the study of acaricide resistance

Acaricides are pesticides that kill ticks and mites. Acaricides are one component of a tick-control strategy that should be paired with measures to decrease tick habitat. Chemical acaricides such as pyrethroids, carbamates, organophosphates, chlorinated hydrocarbons, and arsenic have been used extensively in tick control programs. These compounds, however, resulted in the development of resistance in the targeted vector (Chen, A. C. et al., 2007; Reck, J. et al., 2014).

Tick cell lines have been studied to see if they can be used to investigate acaricide resistance due to the potential as cheap and ethical techniques available. Some research focused on creating drug-resistant cell lines, which are beneficial for studying and understanding resistance processes. Besides, it is also a crucial step in improving the detection and prevention of the development of tick resistance against acaricides. Additionally, tick cell lines can also be used to find genes that cause acaricide resistance (Al-Rofaai, A. et al., 2020), suggesting that tick cell lines provide an excellent model to study the issue of the ticks' acaricide resistance.

The three *Rh. microplus* cell lines that were resistant to organophosphate were generated by exposing the BmVll-SCC cell line (Holman, P. J., 1981) to the gradual increase of the acaricide Coumaphos (Cossio-Bayugar, R. et al., 2002). Other studies have used a different approach by using a cell line that was suspected to be susceptible to ivermectin in order to assess the expression of selected ABC transporter subfamily B genes encoding P-glycoproteins (PgPs) available in the *I. ricinus* embryo-derived IRE / CTVM19 cell line after exposure to ivermectin (Mangia, C. et al., 2016). The scientists found that ivermectin had no effect on ABC pump expression and that expression of one subfamily gene was not identified. However, The *I. ricinus* embryo-derived IRE / CTVM19 cell line was found to be able to tolerate a higher concentration of ivermectin

(30 mg/ml) compared to the *Rh. microplus*-derived line BME26, which died after being exposed to only 12.5 mg/ml of ivermectin concentration (Pohl, P. C. et al., 2014). This observation could happen due to the biological variations between the two tick species used, variances in the cell lines' phenotypic composition, and/or discrepancies in the culture conditions utilized (Al-Rofaai, A. et al., 2020).

Tick cell lines have been used to identify genes that are involved in acaricide resistance (Koh-Tan, H. C. et al., 2016). Koh-Tan et al. (2016) have looked at the expression of the b-adrenergic octopamine receptor (bAOR) gene (linked to amitraz resistance) and the ATP-binding cassette B10 (ABCB10) gene (linked to macrocyclic lactone resistance) in cell lines derived from *Rh. evertsi*, *Rh. sanguineus*, *Rh. appendiculatus* and *Rh. microplus* tick species. These findings propose that *Rh. microplus*-derived BME / CTVM6 cells might be a viable model system to test *Rh. microplus* resistance to several acaricide groups; however, more research is needed to see if other genes in this cell line have been altered (Koh-Tan, H. C. et al., 2016).

2.4.7.7 Tick cell line in the novel control method

Tick cell lines have also been applied in the research of tick cell metabolism, which could aid in the development of new tick control approaches. By utilizing the *Rh. microplus*-derived BME26 cell line, the antagonistic part of glycogen synthase kinase 3 (GSK3) and protein kinase B (AKT) in cell viability and glycogen metabolism was revealed (de Abreu, L. A. et al., 2013). GSK3 inhibition resulted in an increase in glycogen content, whereas AKT inhibition resulted in a reduction in glycogen content, changes in cell membrane permeability, and cell viability.

Da Silva et al. (2015) utilizes the *Rh. microplus*-derived BME26 cell line to analyze various genes associated with glycolysis, gluconeogenesis and glycogen

metabolism processes which are the primary processes for carbohydrate anabolism and catabolism. The aim was to understand better the energy metabolism involved during embryonic development that occurs in ticks. Their findings revealed that numerous genes exhibit reciprocal regulation in response to the administration of glucose and that GSK3 regulates the transcription of the gluconeogenic genes in tick cells (Da Silva, R. M. et al., 2015). In addition, the *Rh. microplus*-derived BME26 cell line was also crucial in the primary publication describing the characterization of a cell cycle protein in arachnids, as well as the reversal of its roles with an inhibitor (Gomes, H. et al., 2013).

The *I. scapularis* embryo-derived ISE6 cell line (Kurtti, T. J. et al., 1996) is recognized as one of the most extensively utilized tick cell lines because of its susceptibility to a wide spectrum of Tick-borne pathogens (TBPs) (Oliver, J. D. et al., 2015). A previous study by (Cabezas-Cruz, A. et al., 2017) employed *I. scapularis* embryo-derived ISE6 cell line to investigate how boosting phosphoenolpyruvate production from tyrosine could help reduce infection with the obligatory intracellular bacterium, *A. phagocytophilum* which causes sickness in people, dogs, ruminants and horses. Ferritins are iron-storage proteins that bind and oxidize ferrous ions and generate ferric ions in the FER core cavity to store unused iron accessible in the cellular iron pool (Galay, R. L. et al., 2015). However, by revealing *I. scapularis* embryo-derived ISE6 cell line to varying doses of ferrous sulfate, the intracellular ferritin (FER1) protein expression was able to be induced (Hernandez, E. P. et al., 2018). They discovered that inhibiting FER1 expression by RNA interference reduced the proliferation of *I. scapularis* embryo-derived ISE6 cells and increased mortality, implying that *I. scapularis* embryo-derived ISE6 cell line might be a useful tool for learning more about FER1 action.

2.4.7.8 Tick cell line for the genetic manipulation study

Research into genetic modification of ticks and tick cell lines could substantially aid the development of novel tick control techniques. For instance, Kurtti et al. (2006) have demonstrated a stable transfection of the *I. scapularis*-derived ISE6 cell line with a gene expressing a fluorescent protein utilizing the technique called Sleeping Beauty transposon (Kurtti, T. et al., 2006). Other studies by Cassio and Miranda (2007) used a different strategy by retrovirally infecting the primary cultures initiated from *Rh. microplus* with green fluorescent protein demonstrated that the fluorescence was detected up to two weeks after retroviral transfection (Cossio-Bayugar, R. et al., 2007)

Another approach was made by Machado-Ferreira et al. (2015), who used *Agrobacterium tumefaciens* T-DNA constructs to achieve transgene expression in *Rh. microplus* ticks and the *Rh. microplus*-derived BME / CTVM2 cell line (Machado-Ferreira, E. et al., 2015). These findings showed that genetic editing techniques employed in other animal cell culture systems could be used in tick cell lines as well, therefore, raising the possibility of genetically altering ticks to prevent them from gaining resistance against acaricide (Al-Rofaai, A. et al., 2020).

2.4.7.9 Tick cell line in the development of the anti-tick vaccine

The tick control strategy through vaccination by using recombinant tick antigens is a promising method. Vaccines developed from recombinant tick antigens showed benefits such as cost-effectiveness, reduction in the use of acaricide and the reduction in the occurrence of tick-borne diseases by minimizing the exposure of cattle to infected ticks. However, the efficacy of these vaccines will vary greatly depending on the tick type and geographic area (de la Fuente, J. et al., 2007a). Tick cell lines have been crucial in finding tick protective antigens that have been used to develop a number of vaccinations

that help prevent and manage tick infestations and also infections associated with TBPs. Research undertaken by Antunes et al. (2014) demonstrated the discovery of tick proteins implicated in the interaction of the tick and the pathogen by utilizing three embryo-derived tick cell lines (*I. scapularis*-derived ISE6, *I. scapularis*-derived IDE8 and *Rh. microplus*-derived BME / CTVM2 (Antunes, S. et al., 2014; Munderloh, U. G. et al., 1994). The goal was to find potential protective antigens to be used as vaccines to prevent *Rh. microplus* tick infestations in cattle, as well as infection with the TBPs *B. bigemina* and *A. marginale* transmitted by *Rh. microplus* ticks. Antibodies made against the *Rh. microplus* protein subolesin were examined by using immunofluorescence and the result showed positive reactions in all three cell lines, but then no effect was reported on DNA levels of the pathogen (Al-Rofaai, A. et al., 2020). In contrast, Contreras and team (2017) demonstrated that *I. scapularis* lipocalin protein, lectin pathway inhibitor protein and their *I. ricinus* homologs are candidate protective antigens to be used for tick infestation control by utilizing *I. ricinus*-derived ISE6 and *I. ricinus*-derived IRE / CTVM20 cell lines (Bell-Sakyi, L. et al., 2007). Anti *I. scapularis* lipocalin protein and lectin pathway inhibitor IgG greatly enhanced the proportion of apoptotic tick cells and lowered the pathogen infection, suggesting that they might aid in the establishment of efficient vaccines for anti-tick and anti-pathogen (Contreras, M. et al., 2017).

2.4.8 The Tick Cell Biobank

The Tick Cell Biobank was originally founded in 2009, with three years of funding from the Wellcome Trust as a unit of collaboration between the Universities of Edinburgh. The centre was established to form a repository for the existing tick cell lines, generating novel cell cultures from various species of ticks not readily available in the collection and as a resource to help in their worldwide distribution by providing cell lines and maintenance to scientists all around the world (Bell-Sakyi, L. et al., 2018). An important component of Tick Cell Biobank was to give training for the maintenance of tick cell lines and the establishment of the primary tick cell and organ culture with the aim to assist an effective transfer and application in the recipient laboratories. The Tick Cell Biobank was transferred to The Pirbright Institute in 2012 and subsequently to the University of Liverpool in 2017. The Tick Cell Biobank, which is now part of the University of Liverpool's Institute of Infection and Global Health, has begun a new phase of research that focuses on problems caused by ticks, a number of other arthropods and the diseases that can transmit them in the least developed, middle and lower-income countries (Bell-Sakyi, L. et al., 2018).

2.4.8.1 The Tick Cell Biobank outpost

Tick and tick-borne diseases are reported throughout the world, but the economic loss they cause in the least developed, low and middle-income countries is worrisome. To address this problem, Tick Cell Biobank has established the outpost of the parent Tick Cell Biobank in Malaysia, Brazil and Kenya. The goal is to make tick cell lines more accessible to scientists from least developed, low and middle-income countries and increase the use of tick cell lines in their research (Bell-Sakyi, L. et al., 2018). In addition, it helps to distribute materials, expertise and knowledge in the neighbouring countries of

South-East Asia, South America and Africa. Each Tick Cell Biobank Outpost will be equipped with a panel of the most used cell lines, as well as others within their regional relevance and will disseminate cell lines and regionally specialized training for the maintenance.

2.5 Polymerase chain reaction

2.5.1 Principle of PCR

The PCR was invented by Kary Mullis in 1983 (Mullis, K. B., 1990), which is a technique where the DNA in the sample is copied rapidly and repeatedly in order to produce a sufficient concentration that is able to be analysed by conventional laboratory procedures. This technique employs repetitive cycle stages that comprise three main steps; (i) denaturing, (ii) annealing and (iii) extension. In the denaturation step, the double-stranded DNA which is made up of two complementary strands are separated by heating the sample solution to 95 ℃, which includes the DNA molecules, polymerases and primers. Next, in an annealing step, the temperature is reduced to 55 ℃ to stimulate the primers to attach to single-strand DNA. If the DNA segment and the primers that are attached are complimentary, the bonds between them are stable. The polymerases will then bind complementary nucleotides, enhancing the bonding forms between the primers and the DNA. Lastly, in the extension stage, the temperature is raised to 72 ℃, which is known as the ideal temperature for the polymerases to work. Subsequently, new nucleotides are added to the new DNA strand by the polymerases and at the same time, all other bonds that are non-complementary are broken. These steps are typical for conventional PCR, in which the amplicons or PCR products generated at the end of the steps will be visualized using an agarose gel electrophoresis as a final step.

2.5.2 Quantitative polymerase chain reaction

Quantitative PCR, also known as real-time PCR, is a technique where the data are obtained in real-time during the PCR process, in which the detection and amplification are complete together in a single step. Different fluorescent chemistries that convert the product concentration directly to fluorescence intensity are used to make the qPCR products visible (Wong, M. L. et al., 2005). The higher the concentration of the qPCR product, the stronger the fluorescence intensity produced. This technique encompassed four major phases; (i) early ground phase, (ii) exponential growth phase, (iii) linear growth phase and (iv) plateau phase (Tichopad, A. et al., 2003). Fluorescence emission will not rise above the background during the early ground phase. Subsequently, amplification will be detected when the fluorescence emission is higher than background levels during the exponential growth phase. The term of the cycle where this happens is known as cycle threshold (Ct), and it can be used to quantify the starting target number (Heid, C. A. et al., 1996). When the qPCR product amplifies after each cycle, the linear growth phase provides the optimal amplification duration. During the plateau phase, the decrease in the reaction components leads to insufficient fluorescence intensity for the data calculation (Wong, M. L. et al., 2005). qPCR tests are extremely sensitive as it is capable of detecting just one copy of a given transcript (Palmer, S. et al., 2003). The qPCR techniques do not necessitate post-PCR product handling, therefore, resulting in faster throughput assays and reducing potential contamination.

In qPCR assays, two types of fluorescent chemistries are used; single-stranded DNA intercalators and double-stranded DNA intercalators. SYBR® Green is an excellent example of double-stranded DNA intercalators, as it fluoresces strongly once they bind to double-stranded DNA. SYBR® Green is often used in qPCR because it requires less cost and applicability in a number of PCR protocols. The non-specific binding of dsDNA, such as primer dimers or other non-specific bindings, is the common

drawback when using SYBR® Green. However, the melting curve product, which is a key characteristic of SYBR® Green, can reveal the formation of non-specific amplicons (Bell, A. S. et al., 2002). Multiple DNA species are able to be detected and quantified by using different beacons or reporter probes in single-stranded DNA intercalators.

The hydrolysis probes, also recognized as TaqMan® probes, is the sequence-specific probe that is labelled by a quencher at the 3' end and a reporter dye at the 5'. The 5' – 3' exonuclease activity degrades the probe when it anneals to the target sequence allowing the quencher and the reporter to be separated and resulting in fluorescence (Bell, A. S. et al., 2002; Wong, M. L. et al., 2005). Next is the hybridization probe. This probe is labelled with an acceptor dye on the upstream probe (3' end) and a donor dye on the downstream probe (5' end). The fluorescence resonance energy transfer from the donor to the accepter will rise once the probes are bound. The transferred energy is then emitted as fluorescence by the acceptor dye. The approach is classified as very specific due to the requirement that the two probes be bound in order to produce fluorescence (Bell, A. S. et al., 2002; Wong, M. L. et al., 2005). Subsequently, the molecular beacon is made up of a specific region (loop) sequence linked together by complementary stem sequences. A quencher on the other end blocks the reporter on one end. Fluorescence emission is reduced due to the closeness of quencher and reporter when the beacon is found free in the reaction solution. The quenching effect is abolished after the beacon attaches to the target, resulting in emission by the reporter (Bell, A. S. et al., 2002; Wong, M. L. et al., 2005).

2.5.3 Molecular detection and quantification of *Rickettsia*

qPCR assays have been developed and applied successfully for the detection of *Rickettsia* agents with high specificity and sensitivity, with the potential in quantifying the bacterial DNA copy numbers in real-time (Stenos, J. et al., 2005). At present, qPCR assays are broadly used for molecular detection of *Orientiae* and *Rickettsiae* bacteria in both clinical and vector specimens (Henry, K. M. et al., 2007; Jiang, J. et al., 2004; Jiang, J. et al., 2012; Stenos, J. et al., 2005), with some protocols capable of detecting at a low level of 1-10 genomic equivalents per reaction (Richards, A. L., 2012). The initial use of qPCR assay in detecting *Orientiae* and *Rickettsiae* was evidenced in the reported detection of *R. prowazekii* (Jiang, J. et al., 2003). Afterwards, a number of genus, group and species-specific based on qPCR assays have been established to detect *Rickettsia* and *Orientiae* bacteria (Jiang, J. et al., 2004; Luce-Fedrow, A. et al., 2015; Paris, D. et al., 2009).

So far, qPCR methods have only been utilized to detect and confirm the presence of *Rickettsia* bacteria in tick cell cultures (Sayler, K. A. et al., 2014), and they have been designed primarily to detect DNA of *Rickettsia* from clinical and environmental samples (Luce-Fedrow, A. et al., 2015). The qPCR assay is a laboratory method used to monitor the amplification and quantification of the targeted DNA molecule in real-time. Hence, it will be useful to adapt the published qPCR methods for *Rickettsia* detection for investigating the growth kinetics and bacteria quantification in tick cell cultures.

The development of a cell culture system utilizing *R. raoultii* and a variety of tick cell lines where the growth of the bacteria can be monitored will enhance and increase the ease of the study of tick-*Rickettsia* interactions, especially for pathogenic SFG *Rickettsia*. The cell culture system can also be used for studies into the development of anti-microbials or novel therapeutics. The application of qPCR assay in quantifying

Rickettsia and monitoring bacterial growth kinetics in tick cell culture will aid in investigating the anti-microbials that can be used to inhibit *Rickettsia* infection in tick cell culture. Since *R. raoultii* infections are also becoming a public health concern, a cell culture system for *R. raoultii* will also allow for further characterization of this emerging rickettsial pathogen.

CHAPTER 3: METHODOLOGY

The methodologies involved in this study were performed according to the three specific aims listed in the Study objective chapter 1.1 (Figure 3.1). First, in Specific aim 1, the *Rh. sanguineus*-derived RSE / PILS35 and *I. scapularis*-derived IDE8 tick cell lines were inoculated with semi-purified *R. raoultii* to establish the bacteria stocks in respective cell lines for infection studies in Specific aim 2. Inoculation in Specific aim 1 referred to the introduction of *R. raoultii*-infected BME / CTVM23 cell suspension into healthy RSE / PILS35 and IDE8 cell lines to produce bacteria stock from respective cells lines with only homologous cells present. In the infection study (Specific aim 2), all cell lines were infected with cryopreserved bacteria stocks from the homologous cell line prepared and the infection rate was determined. Then in Specific aim 3, the growth of *R. raoultii* in the respective cell line was determined by amplifying rickettsiae-specific *gltA* qPCR.

In Specific aim 1, all the tick cells used in the study were maintained prior to inoculation. Next, the presence and the strain of *R. raoultii* were originally maintained in *Rh. microplus*-derived BME / CTVM23 culture was confirmed molecularly. Following that, the *R. raoultii* bacteria in heavily BME / CTVM23 cultures were semi-purified and inoculated into other cell lines. Once the infection was established, the bacterial cultures in respective inoculated tick cell lines were cryopreserved to be used in infection studies as bacteria stock. Cryopreserved bacteria stocks were produced from the homologous cell line. Then, the establishment of *R. raoultii* infection in the inoculated cell lines was confirmed by amplifying tick-specific 16S rRNA.

The bacteria stocks prepared in Specific aim 1 were continuously used to infect tick cell lines for infection rate (Specific aim 2) and replication kinetics of *R. raoultii* studies (Specific aim 3). Next, the infected tick cells were collected at intervals of two to four days post-infection for the smear preparation using a cytocentrifuge, and the

infection rate was calculated based on the percentage of infected cells counted in Giemsa-stained cytocentrifuge smears.

In the study of the replication kinetics of *R. raoultii* (Specific aim 3), firstly, the plasmid containing the rickettsiae-specific *gltA* gene target was transformed into *Escherichia coli* TOP10F´ to produce a sufficient quantity of the gene target to optimise the qPCR DNA standard curve. Next, the quantification of rickettsiae *gltA* (*R. raoultii* copy number) and tick ribosomal protein L6, *rpl6* (tick cell copy number) gene targets was performed by qPCR protocols adapted from the previous publication. Lastly, the generation time of *R. raoultii* was calculated based on the exponential phase in the bacterial growth curve.

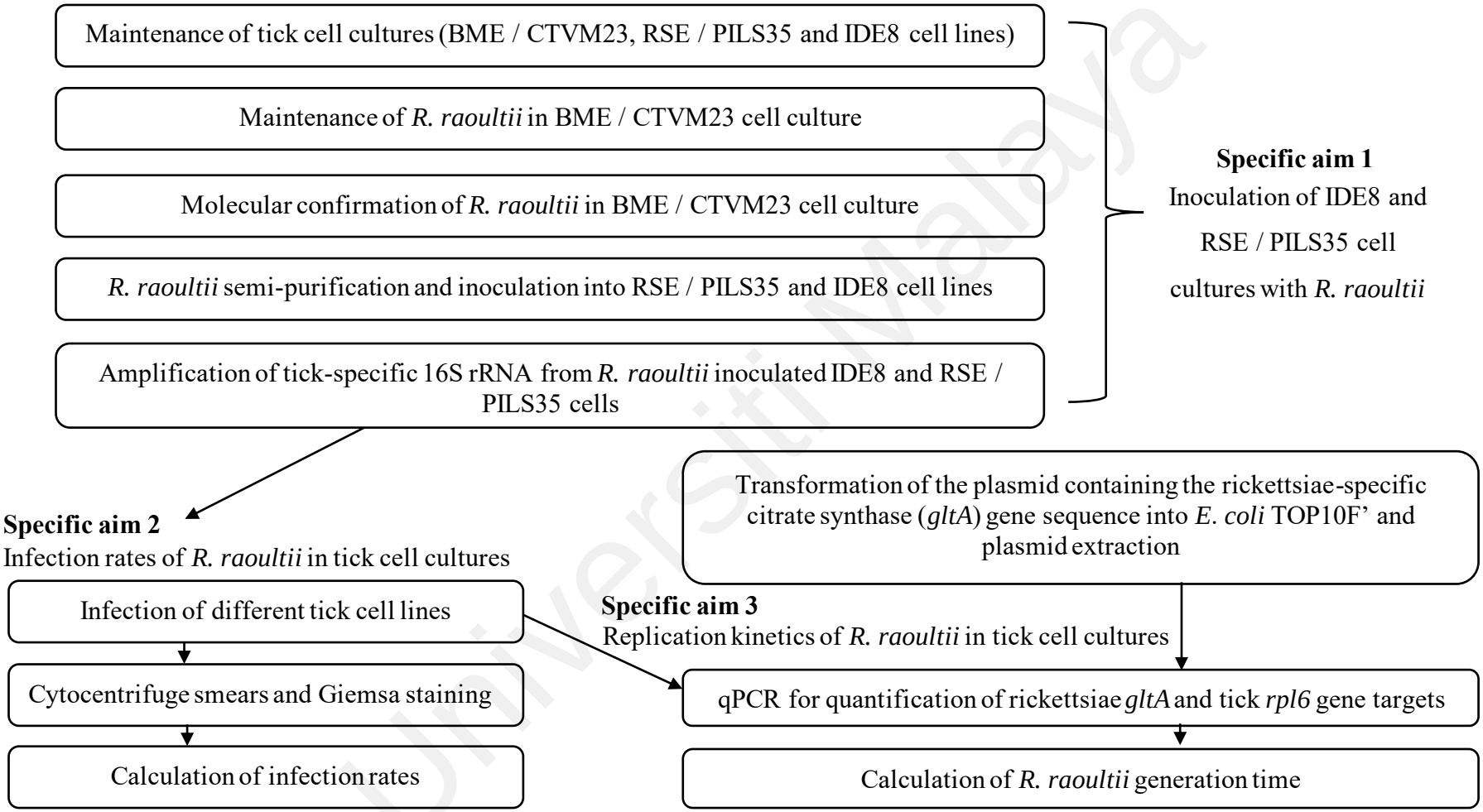

Figure 3. 12: The overview of the methodologies involved in this study.

3.1 Inoculation of IDE8 and RSE / PILS35 cell cultures with *R. raoultii*

3.1.1 Maintenance of tick cell cultures

The *Rh. microplus*-derived BME / CTVM23 cell line was maintained at 32 °C in L-15 (Leibovitz) medium (Gibco, USA) supplemented with 10 % TPB, 20 % FBS, 2 mM L-glutamine and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) (Munderloh, U. G. et al., 1989). The *Rh. sanguineus*-derived RSE / PILS35 cell line was incubated at 28 °C in a cell culture medium consisting of equal parts of L-15 (Leibovitz) medium, and Minimum Essential Medium (MEM) with Hank's salt (Gibco, USA) supplemented with 10 % TPB, 20 % FBS, 2 mM L-glutamine and antibiotics (Munderloh, U. G. et al., 1989). The *I. scapularis*-derived IDE8 cell line was incubated at 32 °C in L-15B medium supplemented with 10 % TPB, 5 % FBS, 0.1 % bovine lipoprotein (MP Biomedicals, USA), 2 mM L-glutamine and antibiotics (Munderloh, U. G. et al., 1989). All cell lines were maintained in flat-sided culture tubes (Nunc, USA), with ¾ of the media replaced weekly and sub-culture made at one to three months intervals.

3.1.2 Maintenance of *R. raoultii* in BME / CTVM23 cell culture

The *R. raoultii* (strain Białystok-1) used in this study was given in a culture of the BME / CTVM23 cell line (Bell-Sakyi, L. et al., 2018). The culture was received from the Tick Cell Biobank, University of Liverpool, United Kingdom, and has been approved for use in BSL 2 facilities in the biobank. All procedures involving the manipulation of live bacteria were performed in the biosafety cabinet (BSC) with approval from the Universiti Malaya Institutional Biosafety and Biosecurity Committee (IBBC). The bacterial culture was maintained following conditions for maintaining the BME / CTVM23 cell line described in Section 3.1.1 but with an incubation temperature at 28 °C instead of 32 °C. Cytopathic effects in the infected cells were observed using the inverted microscope (Biobase, China). The Dino-Eye Edge Digital Eye-Piece Camera (Dino-Lite, Taiwan) was attached to the inverted microscope, and DinoCapture 2.0 software was used to capture and analyze the images of the cells.

Cryopreserved bacteria stocks for *R. raoultii* from BME / CTVM23 cell line were prepared before the infection study in Section 3.2.1. Firstly, the bacteria stocks were prepared by resuspending heavily infected BME / CTVM23 cells in the culture tube on ice. Following that, 10 % DMSO was added and mixed properly. Next, 1 ml of the mixture was aliquoted into individual pre-chilled cryotubes and stored in liquid nitrogen until further use.

3.1.3 Molecular confirmation of *R. raoultii* in cell culture

Prior to the experiments, the presence of *R. raoultii* in the culture was confirmed by amplifying the rickettsiae-specific partial *gltA* gene using a previously published conventional PCR protocol (Roux, V. et al., 1997). Firstly, the DNA was extracted from the *R. raoultii* infected BME / CTVM23 culture using a commercially available NucleoSpin® Tissue kit (Macherey-Nagel, Germany) as described in Section 3.1.4. Then rickettsiae-specific partial *gltA* gene was amplified by using CS1d primer and CS890r primer (Table 1).

PCR reaction was completed following conditions of 2 minutes at 95 °C for activation, 40 cycles at 95 °C for 30 seconds, 45 °C for 30 seconds and 65 °C for 55 seconds. Followed by a final extension at 72 °C for 3 minutes. All reactions were made in a total of 50 µl reaction volumes, comprising a final concentration of 1 × MyTaq Mix (Bioline, USA), 0.4 µM of each primer and 2 µl of DNA template. The PCR reaction was performed using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA).

Next, the amplified PCR products were observed on 1 % AGE with an estimated size of 870 base pairs (bp). After that, the amplicon was purified using a commercial kit, Nucleospin® Gel and PCR Clean-up (Macherey-Nagel, Germany) before being sent for Sanger sequencing at First BASE Laboratories Sdn. Bhd. (Malaysia).

In the purification step, the DNA band with the correct size of the target gene as viewed on agarose gel electrophoresis (AGE) was sliced and transferred into an Eppendorf tube that contained 500 µl, NT1 Buffer. The mixture of sliced gel and NT1 Buffer was incubated for 5 to 10 minutes at 50 °C or up until the gel was solubilized. Next, the supernatant was transferred to a Nucleospin® Gel and PCR Clean-up column and centrifuged at 11 000 × g for 30 seconds. The flow-through was removed, and the column was put back in the collection tube. The centrifugation and discarding flow-

through steps were repeated for the subsequent washing steps using 600 µl NT3 Buffer. An additional centrifugation step for 1 minute at 11 000 × g was added, and the Nucleospin® tissue column was placed into a new 1.5 ml microcentrifuge tube. Next, 30 µl of the elution buffer was then added and centrifuged for 1 minute at 11 000 × g.

The purified DNA suspension was stored at 4 °C until collection by First BASE Laboratories Sdn. Bhd. (Malaysia). The sequencing result was analysed by Geneious v7.1.5 software and BLAST with the GenBank database from the National Center for Biotechnology Information (NCBI).

3.1.4 DNA extraction

For DNA extraction, 200 µl of cell suspension was collected from each culture and DNA was extracted using the commercial nucleic acid extraction kit NucleoSpin® Tissue (Macherey-Nagel, Germany) following the manufacturer's protocol.

First, 180 µl T1 Buffer and 20 µl of Proteinase K were added to pre lysed the tick cells, following 1-hour incubation at 56 °C. Then, 200 µl of B3 Buffer was added to the tube and incubated at 70 °C to ensure the tick cells were lysed completely. Next, 210 µl of absolute ethanol was added to adjust the DNA binding conditions. Following that, the supernatant was transferred to a Nucleospin® tissue column in a collection tube and centrifuged at 11 000 × g for 1 minute. Next, the flow-through was removed, and the column was put back in the collection tube. The centrifugation and discarding flow-through steps were repeated for the subsequent washing steps using 500 µl BW Buffer and 600 µl of B5 Buffer. After the additional centrifugation step for 1 minute at 11 000 × g, the Nucleospin® tissue column was put into a new 1.5 ml microcentrifuge tube. Next, 60 µl of the elution buffer was then added and centrifuged for 1 minute at 11 000 × g. The DNA suspension was stored at - 20 °C until further use.

3.1.5 *R. raoultii* semi-purification and inoculation into RSE / PILS35 and IDE8 cell lines

In this section, *R. raoultii* was semi-purified from the infected BME / CTVM23 cells for inoculation into IDE8 and RSE / PILS35 tick cell lines. Once the infection was established, the inoculated cultures were cryopreserved to be used in infection studies as bacteria stock to determine infection rates (Section 3.2) and replication kinetics of *R. raoultii* bacteria in each of the cell lines (Section 3.3).

Firstly, severely infected BME / CTVM23 were resuspended carefully in the culture tube. Next, all the cells in the culture tube were collected and transferred into six-well plates. Next, the *R. raoultii* was semi-purified by forcibly passing the cell suspension through a 25 G needle five times to release the bacteria from the cells. Finally, the resulting supernatant was filtered through a sterile 2.0 µm filter membrane to get rid of cell debris, and an equal volume of the filtrate was used to inoculate previously prepared RSE / PILS35 and IDE8 tick cell cultures.

The inoculated cells were incubated at 28 °C to allow the bacteria to multiply and were observed every day for any signs of cytopathic effects by using an inverted microscope (Biobase, China). The Dino-Eye Edge Digital Eye-Piece Camera (Dino-Lite, Taiwan) was attached to the inverted microscope, and DinoCapture 2.0 software was used to capture and analyze the images of the cells. Once severe cytopathic effects were observed (i.e., many cells detached from the bottom of the culture tube at approximately between 7 days to 2 weeks post inoculation), the bacterial cultures were immediately cryopreserved in aliquots in liquid nitrogen with the presence of 10 % DMSO, to be used as bacteria stock for infection studies.

3.1.6 Amplification of tick-specific partial 16S rRNA from inoculated tick cell lines

Amplification and sequencing of the tick-specific partial 16S rRNA gene (Black, W., 4th et al., 1994) from the cell suspension of the inoculated tick cell lines were performed to determine if there were any carryover of live BME / CTVM23 cells into the inoculated IDE8 and RSE / PILS35 cultures. 200 µl of the cell suspension was collected from each culture when severe cytopathic effects were observed, which was Day 7 for IDE8 and Day 13 for RSE / PILS35.

Next, 200 µl from the aliquot of the semi-purify *R. raoultii* used to inoculate IDE8 and RSE / PILS35 cultures were collected. The collected cell suspension was subjected to DNA extraction as described in section 3.1.4. In addition, 200 µl of *R. raoultii* inoculum semi-purified from infected BME / CTVM23 cultures was also subjected to DNA extraction.

PCR amplification of the tick-specific partial 16S rRNA sequence was conducted using previously published protocols utilizing primer pairs of 16S + 1 and 16S - 1, targeting around 460 bp product (Black, W., 4th et al., 1994). Amplification was performed following conditions of 2 minutes at 94 °C for activation, 10 cycles at 92 °C for 1 minute, 48 °C for 1 minute and 72 °C for 1 minute. Followed by 32 cycles at 94 °C for 1 minute, 54 °C for 1 minute and 72 °C for 1 minute. A final extension was carried out at 72 °C for 7 minutes. Each reaction contained a final concentration of 1 × Dream Taq Buffer (ThermoFisher Scientific, US), 0.4 µM of each primer, 0.2 mM dNTP mix (ThermoFisher Scientific, US), 0.1 U / µl DreamTaq DNA polymerase (ThermoFisher Scientific, US) and 2 µl DNA to give a total reaction volume of 50 µl.

The PCR reaction was performed using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA). The amplified PCR products were observed on 1 % AGE with an estimated size of 460 bp. Following that, the amplicon was purified using a

commercial kit, Nucleospin® Gel and PCR Clean-up (Macherey-Nagel, Germany), as described in Section 3.1.3. Finally, the purified amplicons were sent for Sanger sequencing by First BASE Laboratories Sdn. Bhd. (Malaysia). The sequencing result was analysed by Geneious v7.1.5 software and BLAST with the GenBank database from the NCBI.

3.2 Infection rates of *R. raoultii* in BME / CTVM23, IDE8 and RSE / PILS35 tick cell cultures

3.2.1 Infection of tick cell cultures to study infection rates and replication kinetics of *R. raoultii*

Two separate cultures (n=2) were infected with *R. raoultii* for each of the different tick cell lines tested, including BME / CTVM23, RSE / PILS35 and IDE8 tick cell lines. A day prior to infection, duplicate cultures were set up with equal cell densities respectively for each cell line. BME / CTVM23 cells were seeded at 6 x 10^6 cells / ml. RSE / PILS35 cell line were seeded at 2 x 10^6 cells / ml. IDE8 cells were seeded at 5 x 10^6 cells / ml. Seeding cell densities in each cell line were determined based on the highest achieved cell densities after two weeks of incubation.

Next, 200 µl of the cryopreserved bacterial stock from BME / CTVM23 cells prepared in Section 3.1.2 was used to infect the BME / CTVM23 cultures. For IDE8 and RSE / PILS35 cell lines, the cryopreserved bacterial stock prepared in Section 3.1.4 was diluted at 1: 20 for IDE8 and 1: 10 for RSE / PILS35 by using culture media. A 200 µl aliquot from the diluted bacterial stock was then used to infect IDE8 and RSE / PILS35 cultures, respectively. The amount of starting bacteria stock used to infect different cell lines was determined based on a previous series of infections to ensure the same infection time frame was obtained. Next, a 200 µl aliquot from the diluted bacterial stock used to initiate infection was collected for DNA extraction in Section 3.1.4. This DNA preparation served as the representative for the number of DNA copies present at time point 0 days post-infection (d.p.i.) in the infection time course. Subsequently, 50 µl of cell suspension was collected from each culture at the selected time points for the preparation of cytocentrifuge smears, and Giemsa staining (Section 3.2.2) and 200 µl was

collected for DNA extraction and bacterial quantification by qPCR (Section 3.3.3). A fresh medium was used to replace the volume that was removed when the cell suspension was taken from the culture tube.

3.2.2 Preparation of cytocentrifuge smears and Giemsa staining for the visualization of bacteria in infected cells

The *R. raoultii* infection rates in each cell culture were determined by counting the percentage of infected cells visualized by Giemsa staining in cytocentrifuge smears. The percentage of infected cells was determined by calculating the number of infected cells divided by the total number of cells (200 to 300 cells). At intervals of two to four days post-infection, the infected tick cells were collected for the smear preparation using a cytocentrifuge. The cytocentrifugation step allowed the cells in suspension to be deposited onto the glass microscope slide as a smear for cytological staining and microscopic observation.

The infected cells were resuspended by pipetting the content of the tube using a serological pipette in the BSC. Next, 50 µl of the cell suspension was transferred into a cytology funnel attached to the metal stage with a filter card and microscope glass slide sandwiched in between (Figure 3.2). Assembled cytology funnel on the metal stage was arranged on the rotor in the BSC and the rotor was sealed with a lid before transferring to the cytocentrifuge (Shandon, USA) outside the BSC (Figure 3.3). The rotor was placed gently on the Cytospin 3 cytocentrifuge (Shandon, USA) and centrifuged for 5 min at 1,000 r.p.m. Once finished, the sealed rotor was transferred back into the BSC and the lid was removed.

Next, the metal holder was removed from the rotor and the clip on the holder was unfastened. The filter card was lifted gently and discarded into the waste bag. Following that, the glass slide was lifted and laid flat on the surface inside the BSC to air dry the smear for a few minutes. The slides were then fixed in methanol for 3 minutes and stained with a Giemsa working solution for 30 minutes. The Giemsa working solution was prepared fresh by filtering 1 part of the Giemsa stock solution (Merck, Germany) into three parts of buffered water at pH 7.2.

After 30 minutes, the slides were washed with buffered water (pH 7.2) thrice to flush out the stain. Next, the slides were air-dried and examined under a compound microscope (GX microscope, UK) at 1000 X magnification for the presence of bacteria. The GXCAM digital camera and GXCapture software were used to capture and image the cells.

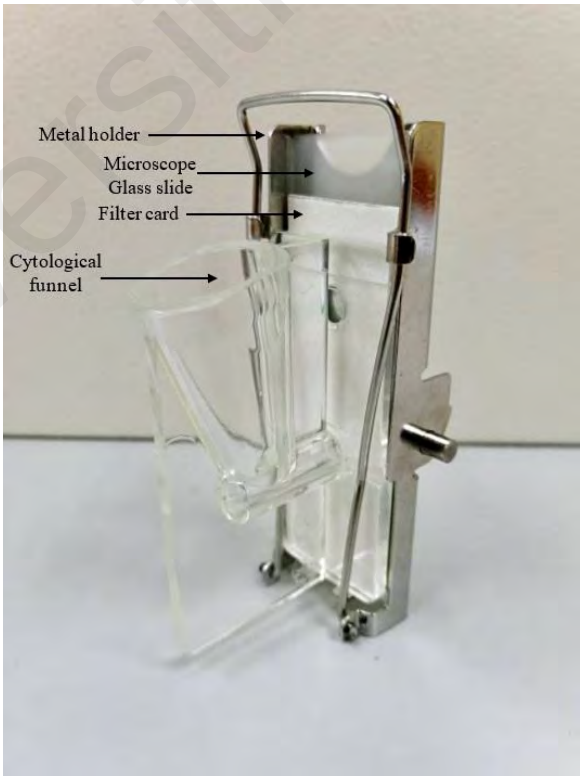

Figure 3. 2: Assembled cytological funnel on the metal stage holder with filter card and microscope glass slide placed in between.

Figure 3. 3: Assembled cytology funnels on the metal stages arranged on the sealed rotor.

3.3 Replication kinetics of *R. raoultii* in BME / CTVM23, IDE8 and RSE / PILS35 tick cell cultures

3.3.1 Transformation of the plasmid containing the rickettsiae-specific *gltA* gene sequence into *E. coli* TOP10F'

A portion of the rickettsiae-specific *gltA* gene sequence previously designed as a qPCR amplification target to detect spotted fever and typhus group rickettsiae (Stenos, J. et al., 2005) was used in this study to determine the replication kinetics of *R. raoultii* in different tick cell lines (Method Section 3.3.3). The transformation of the plasmid containing the rickettsiae-specific *gltA* gene target into *E. coli* TOP10F´ (Invitrogen, USA) was performed to produce sufficient quantities of the gene target to optimise the qPCR DNA standard curve and the replication kinetics study of *R. raoultii*.

The rickettsiae-specific *gltA* gene target sequence with 74 base pair fragments (5' TCGCAAATGTTCACGGTACTTTTTGCAATAGCAAGAACCGTAGGCTGGATGC ACAATGGAAAGAAATGCACGA 3') adapted from (Stenos, J. et al., 2005) was MiniGene synthesized and blunt-end ligated into the pIDTSmart Amp vector by a third-party service provider (Integrated DNA Technologies, Singapore). Customized Synthetic Genes by Integrated DNA Technologies (IDT) was constructed with Ultramer® DNA Oligos and was sequence verified by the Sanger system. Customized synthetic gene product from IDT was provided in pIDTSmart Amp vector that was ready to be transformed into *E. coli*.

Next, the customized gene readily provided with the plasmid vector was transformed into tetracycline-resistant *E. coli* TOP10F´ (Invitrogen, USA). When preparing the competent cells, a single colony of *E. coli* TOP10F´ was inoculated into 5

ml of Luria-Bertani (LB) broth and incubated overnight at 37 °C until the bacteria grew to the early exponential phase (Optical density, OD_{600} = 0.6 - 0.8) The growth of bacteria was measured by using NanoPhotometer™ UV/VIS Spectrophotometer (Implen, Germany). Subsequently, 1 ml of the culture was transferred into an Eppendorf tube and centrifuged at 5 000 × g for 3 minutes. After removing the supernatant, the pellet was resuspended gently in 500 µl of cold 0.1 M calcium chloride ($CaCl_2$) by using the cut-off tips at 4 °C. Following that, 1 ml of cold 0.1 M $CaCl_2$ was added and the mixture was incubated on ice for 2 hours.

During transformation, the competent cells were centrifuged at 2,000 × g for 3 minutes at 4 °C. After removing the supernatant, 80 µl of cold 0.1 M $CaCl_2$ and 4 µl of 1 × 10^5 ng/ml recombinant plasmid were added to the tube and mixed gently by using cut-off tips at 4 °C. Next, the mixture was incubated on ice for 1 hour. Following that, the bacterial suspension was heat-shocked at 42 °C for 1 minute and immediately incubated on ice for 3 to 5 minutes. Next, 1 ml of LB broth was added and mixed by inversion. Lastly, the cells were grown at 37 °C for 1 hour to allow the bacteria to recover. After centrifuging at 2 000 × g for 3 minutes and discarding the supernatant, the remaining pellet was finally plated on the LB agar supplemented with 50 µg / ml ampicillin concentration for antibiotic selection.

A single colony of transformed *E. coli* TOP10F´ was inoculated into 5 ml of LB broth supplemented with 50 µg/ml Ampicillin and grown at 37 °C overnight. The bacteria culture was used for subsequent recombinant plasmid extraction in Section 3.2. In addition, approximately 750 µl of bacteria cells containing the recombinant plasmid were stored in 250 µl of 80 % pre-sterilized glycerol in the screw-cap vial at - 80 °C as bacteria glycerol stock.

3.3.2 Plasmid extraction

The extraction of recombinant plasmid DNA from *E. coli* TOP10F′ was performed by using the FavorPrep Plasmid Extraction Mini Kit (Favorgen Biotech Corporation, Taiwan) following the manufacturer's protocol.

Firstly, 1 to 3 ml of the bacteria culture was transferred to the centrifuge tube. Next, the bacteria culture was centrifuged at 11 000 × g for 1 minute and the supernatant was discarded. Subsequently, 200 µl of FAPD1 buffer (RNase A added) was added to the mixture and resuspended completely by pipetting. Subsequently, 200 µl of FAPD2 buffer was added to the tube and gently inverted 10 times until the lysate became clear. The sample mixture was then incubated at room temperature for 2-5 minutes to lyse the cells. The 300 µl of FAPD3 buffer was added and the tube was gently inverted 10 times to neutralize the lysate. After neutralization, the sample mixture was centrifuged at full speed (17 000 × g) for 5 minutes. Then the supernatant was transferred to a FAPD Column in a collection tube and centrifuged at 11 000 × g for 30 seconds. The flow-through was discarded and the column was placed back in the collection tube. Next, the steps of centrifugation and discarding flow-through were repeated for the subsequent washing steps using 400 µl of W1 buffer and 700 µl of wash buffer. After an additional centrifugation step at full speed (17 000 × g) for 3 minutes, the FAPD Column was placed into a new 1.5 ml microcentrifuge tube. Next, 100 µl of the elution buffer was then added and centrifuged at 17 000 × g for 1 minute.

Lastly, the concentration of the extracted recombinant plasmid DNA was measured using Nanodrop™ 2000 / 2000c spectrophotometer (ThermoFisher Scientific, US) and the corresponding copy number was calculated using the following equation (Equation 1). Besides, the extracted recombinant plasmid DNA was also analysed by 1.2 % AGE to confirm the size of the plasmid. Next, the recombinant plasmid DNA was

stored at - 20 °C until further use. This recombinant plasmid will be used to build the standard curves for the rickettsiae-specific *gltA* qPCR (Methods Section 3.3.3) in the replication kinetic studies. The determination of the appropriate range of DNA copy numbers used for the standard curve was described in the Results Section 4.3.3.

Equation 3. 1:The equation for copy number calculation.

$$\text{Number of DNA copies/ml} = \frac{\text{Amount of DNA (ng/ml)} * 6.0221 \times 10^{23}\ \text{(molecule/mole)}}{\text{Length of dsDNA (bp)} * 660\ \text{(g/mole)} * 1 \times 10^{9}\ \text{(ng/g)}}$$

6.0221×10^{23} (molecule/mole) = Avogadro's constant

660 (g/mole) = Average mass of 1 bp dsDNA

1×10^{9} (ng/g) = Conversion factor

3.3.3 qPCR for quantification of rickettsiae *gltA* and tick *rpl6* gene targets

A previously-published qPCR protocol targeting 74 bp fragments (5' TCGCAAATGTTCACGGTACTTTTTGCAATAGCAAGAACCGTAGGCTGGATGC ACAATGGAAAGAAATGCACGA 3') of the rickettsiae-specific *gltA* gene (Table 1) was used to quantitate *R. raoultii* copy number in infected cells (Stenos, J. et al., 2005). The PCR cycling condition was as follows: Initial holding temperature at 50 °C for 3 minutes, followed by 95 °C for 5 minutes and 40 cycles of 95 °C for 20 seconds and 60 °C for 40 seconds. All reactions were performed in a total of 25 µl reaction volumes, comprising final concentrations of 1 × TaqMan Fast Advanced Master Mix, *gltA* primers and probe at 200 nM each, and 1 µl DNA template. The qPCR reactions were performed using a CFX96 Touch Real-Time PCR Detection System (BioRad, UK) with a 6-carboxyfluorescein (FAM) and Black-Hole Quencher (BHQ1-) labelled TaqMan probe (synthesized by Integrated DNA Technologies, Singapore). For every amplification reaction, molecular biological grade water was included as a negative control.

As a measure of tick DNA copy numbers, a qPCR assay targeting 77 bp fragments (5' CCGGTCCAAGATGTTCCACAAGCGGGGCCTGTTTAAGGTGAAGCACGCC CCTCCGACCAAGGAGAAGAGGAAGCGCA 3') of a tick single-copy nuclear gene, *rpl6* (Table 1), was performed as described previously (Al-Khafaji, A. M. et al., 2019). Each reaction contained final concentrations of 1 × SensiFast SYBR No-ROX master mix (Bioline, USA), 200 nM of *rpl6* primers and 1 µl DNA to give a total reaction volume of 20 µl. A DNA standard curve was made by using a long oligonucleotide (synthesized by Sigma-Aldrich) obtained from Dr. Benjamin Makepeace, University of Liverpool. Serial dilutions of synthetic standards ranging from 5×10^{-1} to 5×10^{6} copies (Al-Khafaji, A. M. et al., 2019) were prepared in duplicate for use on each plate as the standard curve. The reactions were conducted with an initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of denaturation at 95 °C for 15 seconds, annealing at 55 °C for 30

seconds and extension at 72 °C for 15 seconds. Following amplification, a melt curve was produced from 55 °C to 95 °C with increasing increments of 0.5 °C per cycle. The qPCR reactions were also performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, UK).

Table 3. 1: Primers and gene targets used for amplification and sequencing.

Primers	Sequence	Gene target	Fragment size (bp)	Reference
CS1d (forward)	5′ ATGACTAATGGCAATAAT AA 3′	Rickettsiae-specific partial *gltA* gene	870	(Roux, V. et al., 1997)
CS890r (reverse)	5′ GCTTTIAGCTACATATTTA GG 3′			
Black 16S+1 (forward)	5′ CTGCTCAATGATTTTTTA AATTGCTGTGG 3′	Tick-specific partial 16S rRNA	460	(Black, W., 4th et al., 1994)
Black 16S-1 (reverse)	5′ CCGGTCTGAACTCAGATC AAGT 3′			
CS-F (forward)	5’ TCGCAAATGTTCACGGTA CTTT 3’	Rickettsiae-specific *gltA* gene	74	(Stenos, J. et al., 2005)
CS-R (reverse)	5’CACAATGGAAAGAAAT GCACGA 3’			
CS-P (probe)	5’-6-FAM-TGC AAT AGC AAG AAC CGT AGG CTG GAT G-BHQ-1-3’			

rpl6 (forward)	5' CCGGTCCAAGATGTTCCACA 3'	*Ixodes* ribosomal protein L6 nuclear gene	77	(Al-Khafaji, A. M. et al., 2019)
rpl6 (reverse)	5' TGCGCTTCCTCTTCTCCTTG 3'			

3.3.4 Generation time of *R. raoultii*

The generation time for *R. raoultii* to double up in the cultures was calculated once the replication kinetics curve of *R. raoultii* was established based on the quantification of the rickettsiae-specific *gltA* gene (Section 3.3.3). The generation time, also known as doubling time, is the period of time required by the bacteria or cell to double in size. Hence, the time needed by *R. raoultii* to double up in different tick cell lines was calculated using the following equation shown in Equation 2 (Prescott, H., 2002).

Equation 3. 2: The equation for generation time calculation.

$$\text{Generation time} = \frac{0.301t}{\log_{10} N_t - \log_{10} N_0}$$

where N_0 = number of bacteria at the beginning of the log phase

N_t = number of bacteria at the end of the log phase

t = time in between N_t and N_0

CHAPTER 4: RESULTS

4.1 Inoculation of IDE8 and RSE / PILS35 cell cultures with *R. raoultii*

4.1.1 Microscopic observation of *R. raoultii*-infected BME / CTVM23 cells

BME / CTVM23 cell cultures infected with *R. raoultii* were monitored periodically for the presence of cytopathic effects. Non-infected BME / CTVM23 cells consisted of a mixture of abundant epithelial-like cells with elongated shapes and a few rounded cells (Figures 4.1 B and 4.1 C). In addition, BME / CTVM23 cells grew closely attached to each other forming clumps (Figure 4.1 A).

Heavily infected BME / CTVM23 cells appeared more rounded (Figures 4.1 E and 4.1 F). Moreover, many of the cells were floating in the culture medium, with fewer cells attached to the bottom of the culture vessel (Figure 4.1 D).

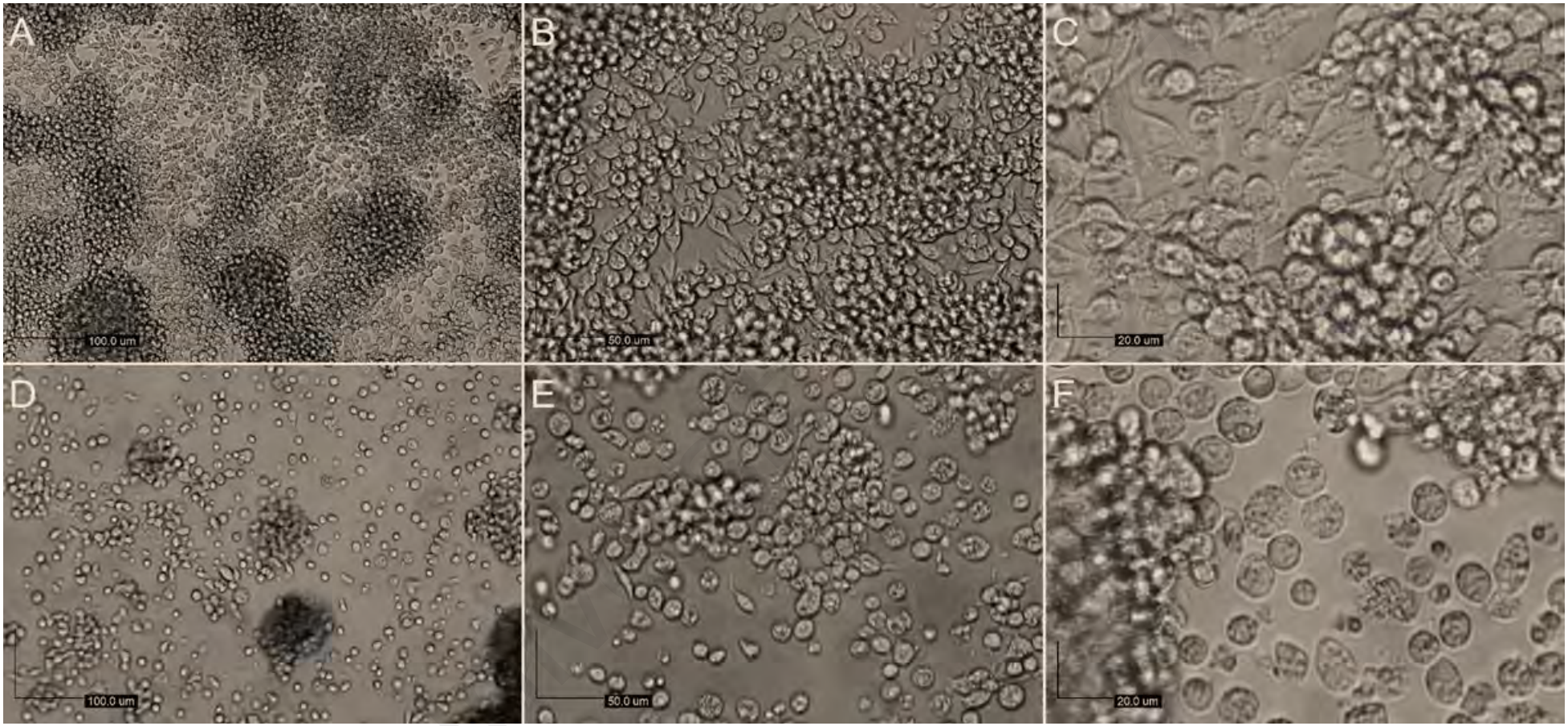

Figure 4. 14: Microscopic image of non-infected BME / CTVM23 cells (A, B, and C) and BME / CTVM23 heavily infected with *R. raoultii* (D, E and F) at different magnifications. A and D: 10 X magnification, B and E: 20 X magnification, C and F: 40 X magnification.

4.1.2 Molecular confirmation of *R. raoultii* infection in BME / CTVM23 cell cultures

The presence of *R. raoultii* originally provided in the BME / CTVM23 cell culture was first confirmed by PCR amplification and sequencing of the *gltA* gene. The rickettsiae-specific partial *gltA* gene was amplified from the DNA extracted from *R. raoultii*-infected BME / CTVM23 cells and visualized by AGE. The PCR amplification of the rickettsiae-specific partial *gltA* gene was performed with the extracted DNA from two different cultures of *R. raoultii*-infected BME / CTVM23 cells (culture 10.8.4 and 11.8.4). The amplification of the rickettsiae-specific partial *gltA* gene with an estimated size of around 870 bp was seen in 1 % AGE for both cultures of *R. raoultii*-infected BME / CTVM23 cells (Figure 4.2).

Lane 4 was *R. raoultii*-infected BME / CTVM23 cells (culture 10.8.4) and Lane 5 was *R. raoultii*-infected BME / CTVM23 cells (culture 11.8.4). In continuous, only *R. raoultii*-infected BME / CTVM23 cells (culture 10.8.4) were purified and subjected for sequencing since both cultures showed a single bright band with the same estimated size of product in AGE. Negative control was loaded in Lane 2 and no amplicon was loaded on Lane 3. The chromatogram of the amplified rickettsiae-specific partial *gltA* gene sequence showed evenly-spaced single-nucleotide peaks with no baseline noise (Appendix A). BLAST analysis of the *gltA* amplicon sequence revealed 100 % similarity to the existing *R. raoultii* strain reported in NCBI Genbank (Table 4.1).

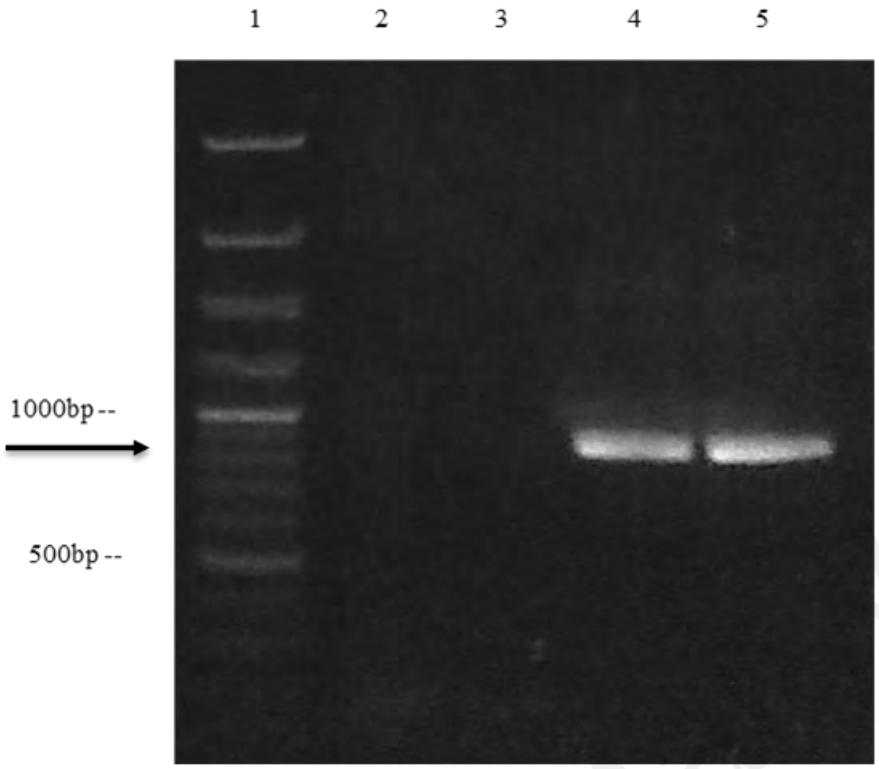

Figure 4. 2: AGE (1 %) illustrates the PCR product of rickettsiae-specific partial *gltA* gene target with an estimated size of ≈ 870 bp, amplified from *R. raoultii*-infected BME / CTVM23 cells DNA. Lane 1: 100 bp DNA ladder, Lane 2: Negative control 1, Lane 3: N/A, Lane 4: *R. raoultii*-infected BME / CTVM23 cells (culture 10.8.4), Lane 5: *R. raoultii*-infected BME / CTVM23 cells (culture 11.8.4).

Table 4. 1: Blastn results of rickettsiae-specific partial *gltA* gene amplicon (710 nt) from *R. raoultii* in infected *Rh. microplus*-derived BME / CTVM23 cells (culture 10.8.4).

Rickettsia strain	Max Score	Total Score	Query Coverage	E-value	Percentage Identity	Accession number
R. raoultii isolate Tomsk	1474	1474	100 %	0.0	100 %	MK304547.1
R. raoultii isolate Xinjiang	1474	1474	100 %	0.0	100 %	MF002517.1
R. raoultii isolate Xinjiang-EM	1474	1474	100 %	0.0	100 %	MF002516.1
R. raoultii	1474	1474	100 %	0.0	100 %	KX258621.1
R. raoultii isolate Crimea-1 type ll	1474	1474	100 %	0.0	100 %	KU961537.1

nt: nucleotide

4.1.3 Microscopic observation of IDE8 and RSE / PILS35 cells after *R. raoultii* inoculation

To produce cryopreserved bacterial stock in IDE8 and RSE / PILS35 cell lines, the bacteria was semi-purified from heavily infected BME / CTVM23 cultures and inoculated into IDE8 and RSE / PILS35 cultures.

Tick cell cultures from different cell lines were observed right before inoculation with *R. raoultii* under phase-contrast microscopy to ensure the cells were healthy. In addition, the cellular morphologies, including the cytopathic effect of the cells post inoculation with *R. raoultii* were also observed every day.

Healthy, non-inoculated RSE / PILS35 and IDE8 cells generally grow as a combination of multi-layer and suspension cultures comprising two or more cell morphotypes (Figures 4.3 A - C and 4.4 A - C). Cytopathic effects for *R. raoultii* infection were observed when the cells started to become more rounded in shape and started to detach from the wall of the cell-culture tube. Cytopathic effects were observed starting on Day 4 post inoculation for RSE / PILS35 cells and Day 3 post inoculation for IDE8 cells.

Severe cytopathic effects were observed when many of the cells were detached from the bottom of the cell-culture tube (Figures 4.3 D - F and 4.4 D - F). Severe cytopathic effects were seen on Day 13 post inoculation for RSE/PILS35 cell cultures (Figures 4.3 D - F) and Day 7 post inoculation for IDE8 cell cultures (Figures 4.4 D - F).

Non-inoculated RSE / PILS35 cells culture was morphologically comprised of a mixture of epithelial-like or fibroblast-like cells and a few round cells (Figures 4.3 B and 4.3 C). Many RSE / PILS35 cells grew closely attached together in discrete patches or clumps (Figure 4.3 A). On Day 13 post inoculation, most of the cells in the RSE / PILS35

culture were floating singly with the domination of round cells with a lot of debris in the culture (Figures 4.3 D - F).

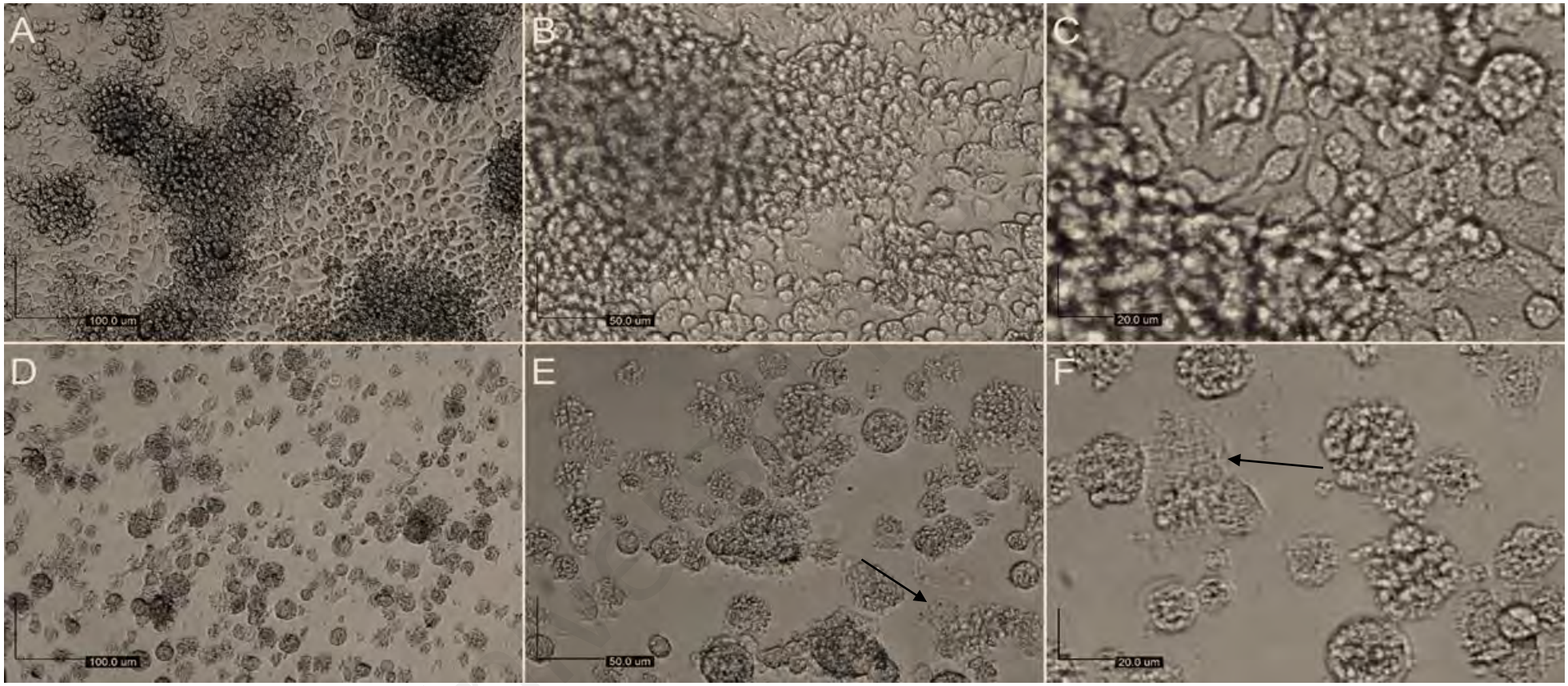

Figure 4. 3: Microscopic image of non-inoculated RSE / PILS35 cells (A, B, and C) and *R. raoultii*-inoculated RSE / PILS35 on Day 13 post inoculation (D, E and F) at different magnifications. A and D: 10 X magnification, B and E: 20 X magnification, C and F: 40 X magnification. Arrow indicated debris.

Non-inoculated IDE8 cells culture morphologically consists of a mixture of large and small round cells (Figures 4.4 B and 4.4 C) that grew abundantly by adhering to the cell-culture tube in a multi-layer pattern. At 10 X magnification of non-inoculated IDE8 cells culture, the abundant layer of round cells with a few dense clumps can be seen (Figure 4.4 A). On Day 7 post inoculation, almost all IDE8 cells were floating in the culture medium (Figure 4.4 D). Besides, a lot of cell debris can be seen in IDE8 cell cultures on Day 7 post inoculation (Figures 4.4 E and 4.4 F). No difference in the size of the inoculated cells was observed.

The RSE / PILS35 and IDE cultures showing severe cytopathic effects (Day 13 and Day 7 post inoculation, respectively) were then cryopreserved and used as the bacterial stock for infection studies to determine infection rates (Section 4.2) and replication kinetics (Section 4.3).

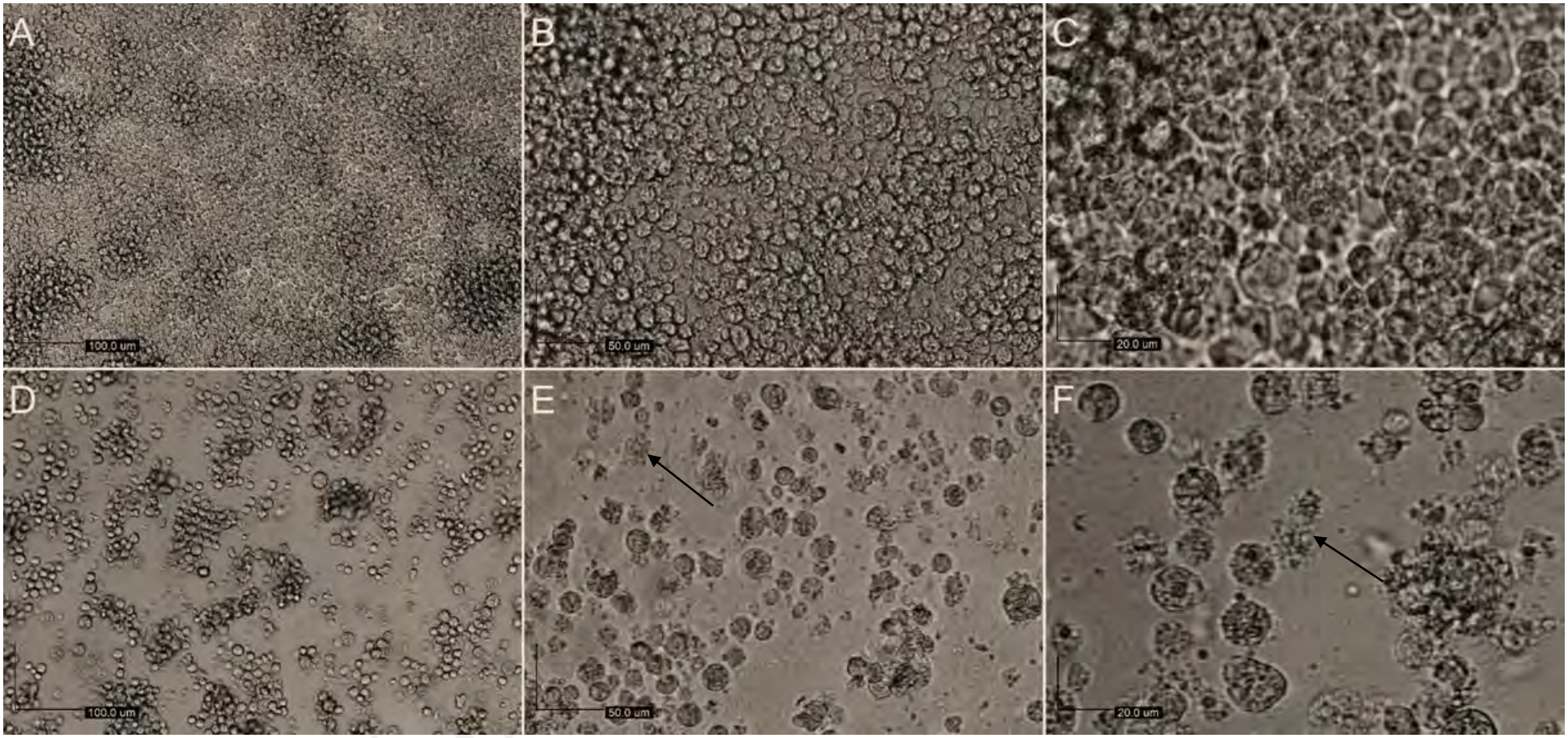

Figure 4. 4: Microscopic image of non-inoculated IDE8 cells (A, B, and C) and *R. raoultii*-inoculated IDE8 on Day 7 post inoculation (D, E and F) at different magnifications. A and D: 10 X magnification, B and E: 20 X magnification, C and F: 40 X magnification. Arrow indicated debris.

4.1.4 Tick-specific 16S rRNA PCR for *R. raoultii* inoculated IDE8 and RSE / PILS35 cells

To ensure that there was no carryover of *Rh. microplus*-derived BME / CTVM23 cells into *I. scapularis*-derived IDE8 and *Rh. sanguineus*-derived RSE / PILS35 cell cultures after inoculation with the semi-purified *R. raoultii*, an aliquot of the inoculated cells was collected on Day 7 (IDE8) and Day 13 (RSE / PILS35) for DNA extraction and PCR amplification of the tick-specific partial 16S rRNA gene.

A single band with the expected size of 460 bp was seen in AGE (Figure 4.5) for *R. raoultii* inoculum semi-purified from infected BME / CTVM23 cultures (Lane 2 and Lane 3), IDE8 on Day 7 after *R. raoultii* inoculation (Lane 4), and RSE / PILS35 on Day 13 after *R. raoultii* inoculation (Lane 5). The chromatogram of the amplified tick-specific partial 16S rRNA sequence from *R. raoultii* inoculum semi-purified from infected BME / CTVM23 cultures, Day 7 post-inoculated IDE8 and from Day 13 post-inoculated RSE / PILS35 culture showed a single nucleotide peak with no baseline noise observed (Appendix B, C and D).

From BLAST analyses, DNA sequences matching to *Rh. microplus* were detected in *R. raoultii* inoculum semi-purified from infected BME / CTVM23 cultures, indicating that DNA materials from the BME / CTVM23 cells may be present in the inoculum. However, on Day 7 post inoculation, DNA matched to *Rh. microplus* were no longer detected in the IDE8 culture. The same was observed for *R. raoultii*-inoculated RSE / PILS35 cells on Day 13 (Table 4.2). This observation indicated that the inoculated IDE8 and RSE / PILS35 cultures should be free of DNA material and hence viable cells from the *Rh. microplus*-derived BME / CTVM23 culture at Day 7 and Day 13 post inoculation, respectively.

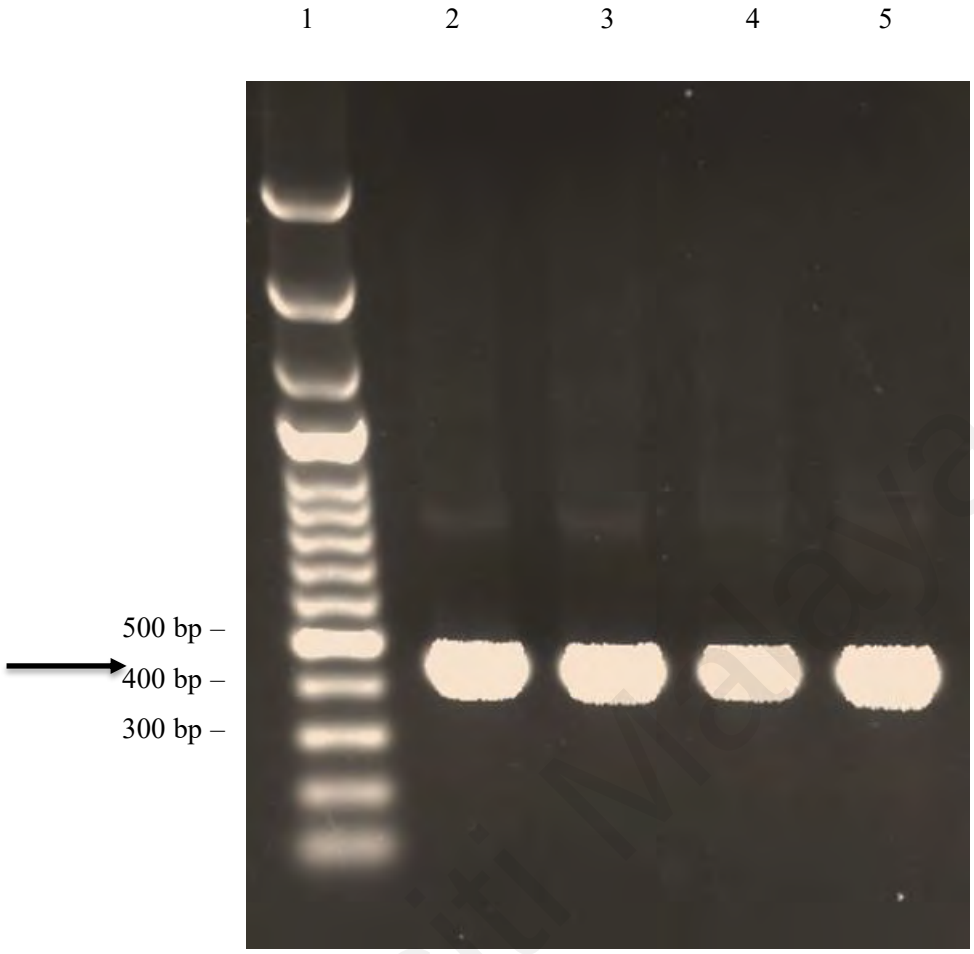

Figure 4. 5: AGE (1 %) of PCR amplicons of tick-specific 16S rRNA gene target with an estimated size ≈ 460 bp. PCR amplification was performed on DNA prepared from the cell cultures at the indicated time-points post inoculation with *R. raoultii*. Lane 1: 100 bp DNA ladder, Lane 2 & 3: *R. raoutlii* inoculum, Lane 4: IDE8 Day 7, Lane 5: RSE / PILS35 Day 13.

Table 4. 2: Blastn results of tick-specific 16S rRNA gene amplicon (350 nt) from DNA extracts of *I. scapularis*-derived IDE8 and *Rh. sanguineus*-derived RSE / PILS35 cell cultures post inoculation with semi-purified *R. raoultii*.

Sample	Tick species strain / % nucleotide identity with tick-specific 16S rRNA gene	GenBank accession number
Semi-purified *R. raoultii* inoculum (Prepared from infected BME / CTVM23 cultures)	*Rh. microplus* isolate PB012 / 100 %	MN650726.1
	Rh. microplus isolate DM024 / 100 %	MN650725.1
	Rh. microplus isolate 16S_Rm_CayoCoco_Cuba / 100 %	MT462222.1
	Rh. microplus clone TickCU610MRm / 100 %	MN880401.1
IDE8 Day 7 Post Inoculation	*I. scapularis* strain Is-63 / 100 %	HG916788.1
	I. scapularis voucher 20170605-176 / 100 %	MG242325.1
	I. scapularis cell-line IDE12 / 100 %	EF636465.1
	I. scapularis cell-line IDE8 / 100 %	EF636464.1
RSE / PILS35 Day 13 Post Inoculation	*Rh. sanguineus* / 100 %	MF805004.1
	Rh. sanguineus isolate C2 / 100 %	KX553961.1
	Rh. sanguineus sensu lato Rs3 / 100 %	KU498301.1
	Rh. sanguineus isolate Fra / 100 %	KT382452.1

4.2 Infection rates of *R. raoultii* in tick cell cultures

Two cultures for each cell line were infected with the bacterial stock produced from Section 4.1.3. Giemsa-stained cytocentrifuge smears from two separate *R. raoultii*-infected cultures for each cell line were observed respectively at the indicated time points from 0 to 18 d.p.i. (Section 4.2.1). The infection rates of *R. raoultii* in BME / CTVM23, IDE8 and RSE / PILS35 cell lines were then determined by calculating the [the number of infected cells x 100] divided by the total number of cells (at least 200 cells examined for each sample) on Giemsa-stained cytocentrifuge smears (Section 4.2.2).

4.2.1 Microscopic observation of Giemsa-stained cytocentrifuge smears of *R. raoultii*-infected BME / CTVM23, IDE8 and RSE / PILS cells

The size of uninfected BME / CTVM23 and IDE8 cells on Giemsa-stained cytocentrifuge smears ranged from 10 – 20 µm (Figures 4.6 A, 4.7 A, 4.10 A and 4.11 A). However, uninfected RSE / PILS35 cells appeared to have a larger range in sizes, from 10 – 50 µm (Figures 4.8 A and 4.9 A). There was no observable difference in the size of the cells before and after infection with *R. raoultii*. Pleiomorphic rickettsiae-like, with a mixture of short rods and cocci, were observed in the cytoplasm of the tick cells after infection with *R. raoultii* (Figures 4.6 B - G – 4.7 B - G and 4.8 B - D – 4.11 B - D). No bacteria were observed in the cells before the infection with *R. raoultii* (Figures 4.6 A – 4.11 A).

Rickettsiae-like bacteria started to be observed on both cultures of *R. raoultii*-infected BME / CTVM23 (cultures 1 and 2) on 3 d.p.i. (Figures 4.6 B and 4.7 B). However, at this point, not many cells were infected with most of the bacteria were still extracellular (Figures 4.6 B and 4.7 B). There appeared to be more infected cells and

bacteria observed in the cell cytoplasm of both cultures when observed in the periods between 5 d.p.i. to 12 d.p.i. (Figures 4.6 C - F and 4.7 C - F). All the cells in both cultures were infected by 15 d.p.i. (Figures 4.6 G and 4.7 G). Large numbers of bacteria can be found outside the cells together with cell cytoplasm degradation by 18 d.p.i. (Figures 4.6 H and 4.7 H).

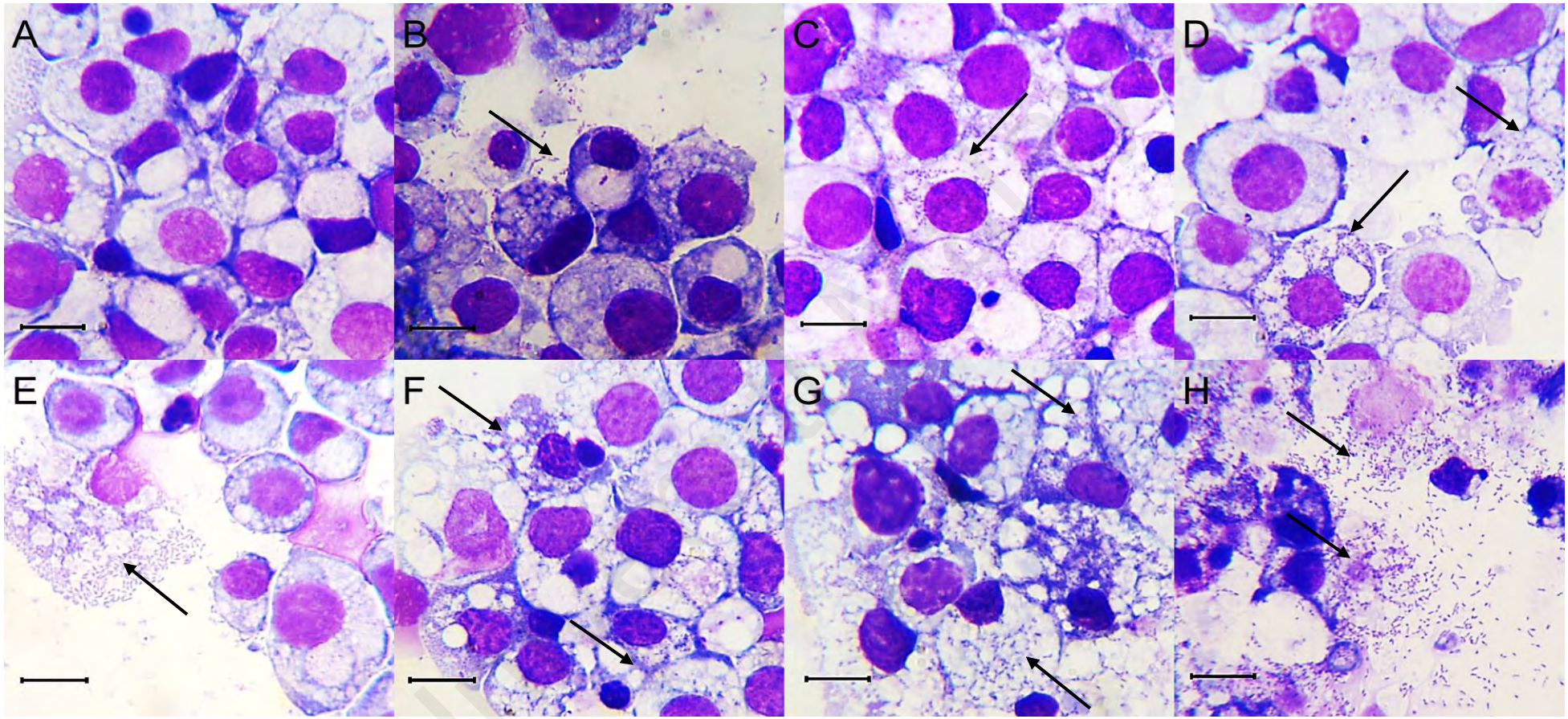

Figure 4. 6: Giemsa-stained cytocentrifuge smears of *R. raoultii*-infected BME / CTVM23 cells (culture 1). A: before *R. raoultii* infection, B: 3 d.p.i., C: 5 d.p.i., D: 7 d.p.i., E: 10 d.p.i., F: 12 d.p.i., G: 15 d.p.i., H: 18 d.p.i. Arrows indicate the presence of bacteria. Scale bars = 10 µm.

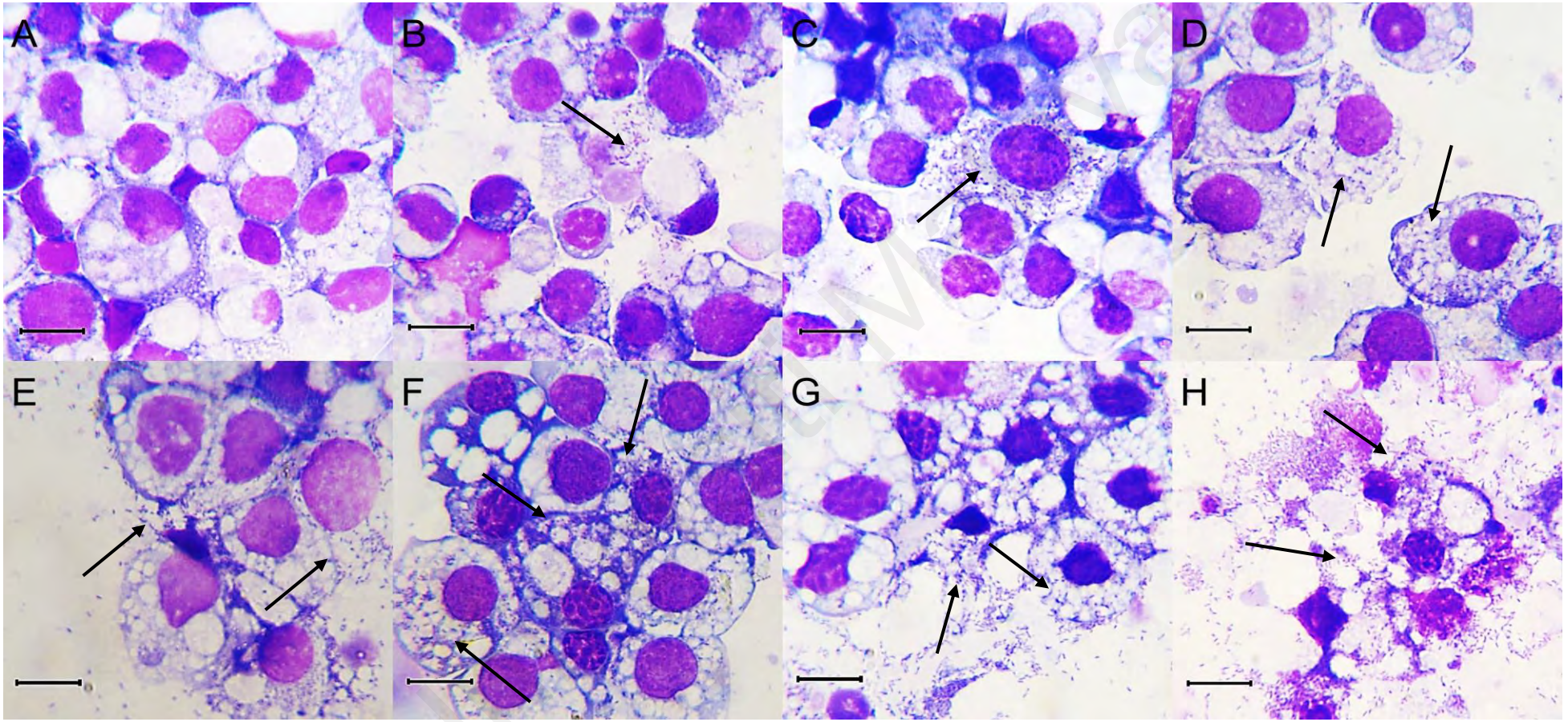

Figure 4. 7: Giemsa-stained cytocentrifuge smears of *R. raoultii*-infected BME / CTVM23 cells (culture 2). A: before *R. raoultii* infection, B: 3 d.p.i., C: 5 d.p.i., D: 7 d.p.i., E: 10 d.p.i., F: 12 d.p.i., G: 15 d.p.i., H: 18 d.p.i. Arrows indicate the presence of bacteria. Scale bars = 10 µm.

For *R. raoultii*-infected RSE / PILS35, the rickettsiae-like bacteria were readily observed inside the cell cytoplasm on 3 d.p.i. in both cultures (cultures 1 and 2) (Figures 4.8 B and 4.9 B). More infected cells and bacteria inside the cell cytoplasm were seen between 3 d.p.i. (Figures 14 B and 15 B) and 7 d.p.i. (Figures 4.8 C and 4.9 C). A lot of infected cells were lysed and a large number of bacteria were seen outside the cells after 10 d.p.i. and 14 d.p.i. for both cultures (Figures 4.8 D - E and 4.9 D - E).

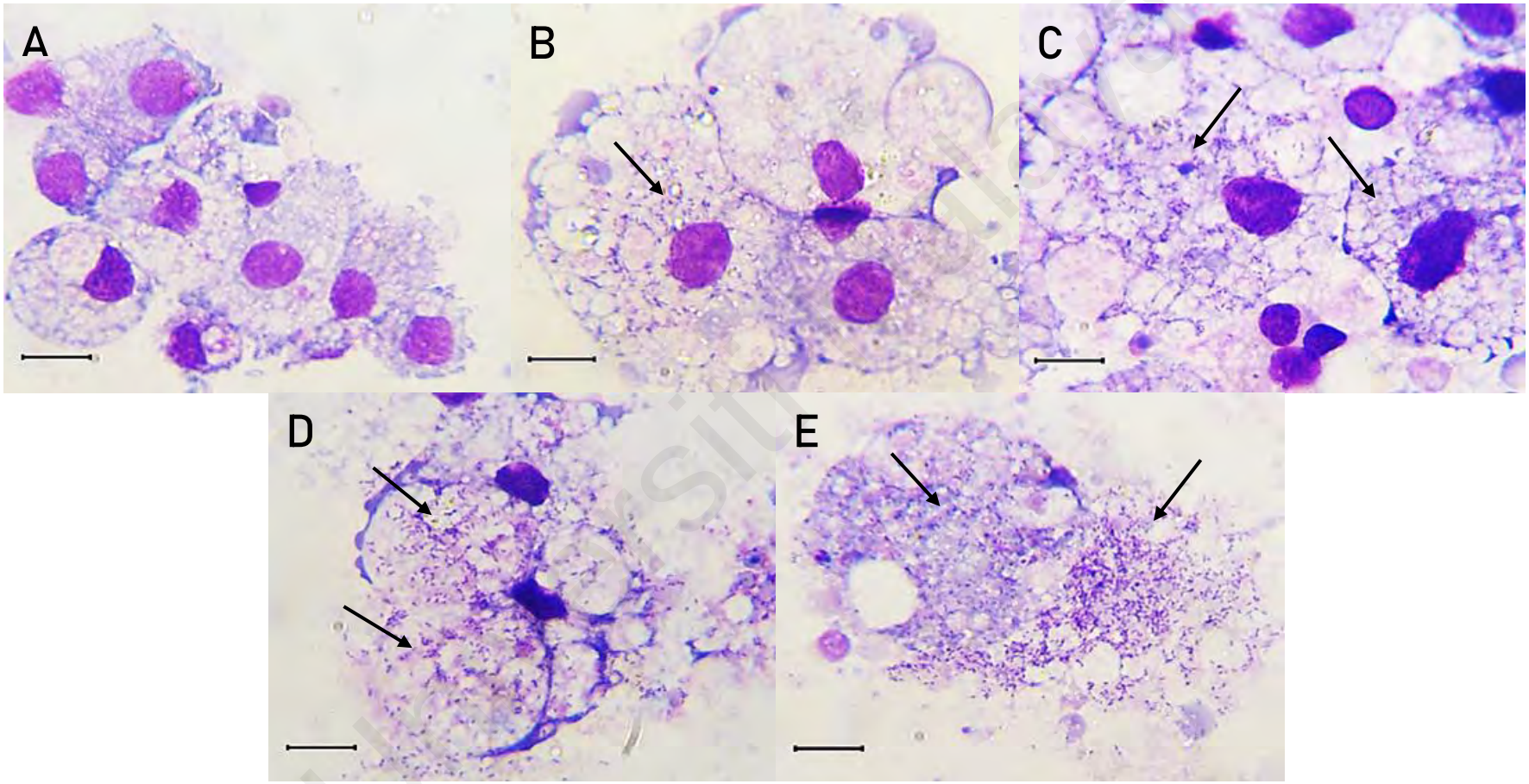

Figure 4. 8: Giemsa-stained cytocentrifuge smears of *R. raoultii*-infected RSE / PILS35 cells (culture 1). A: before *R. raoultii* infection, B: 3 d.p.i., C: 7 d.p.i., D: 10 d.p.i., E: 14 d.p.i. Arrows indicate the presence of bacteria. Scale bars = 10 µm.

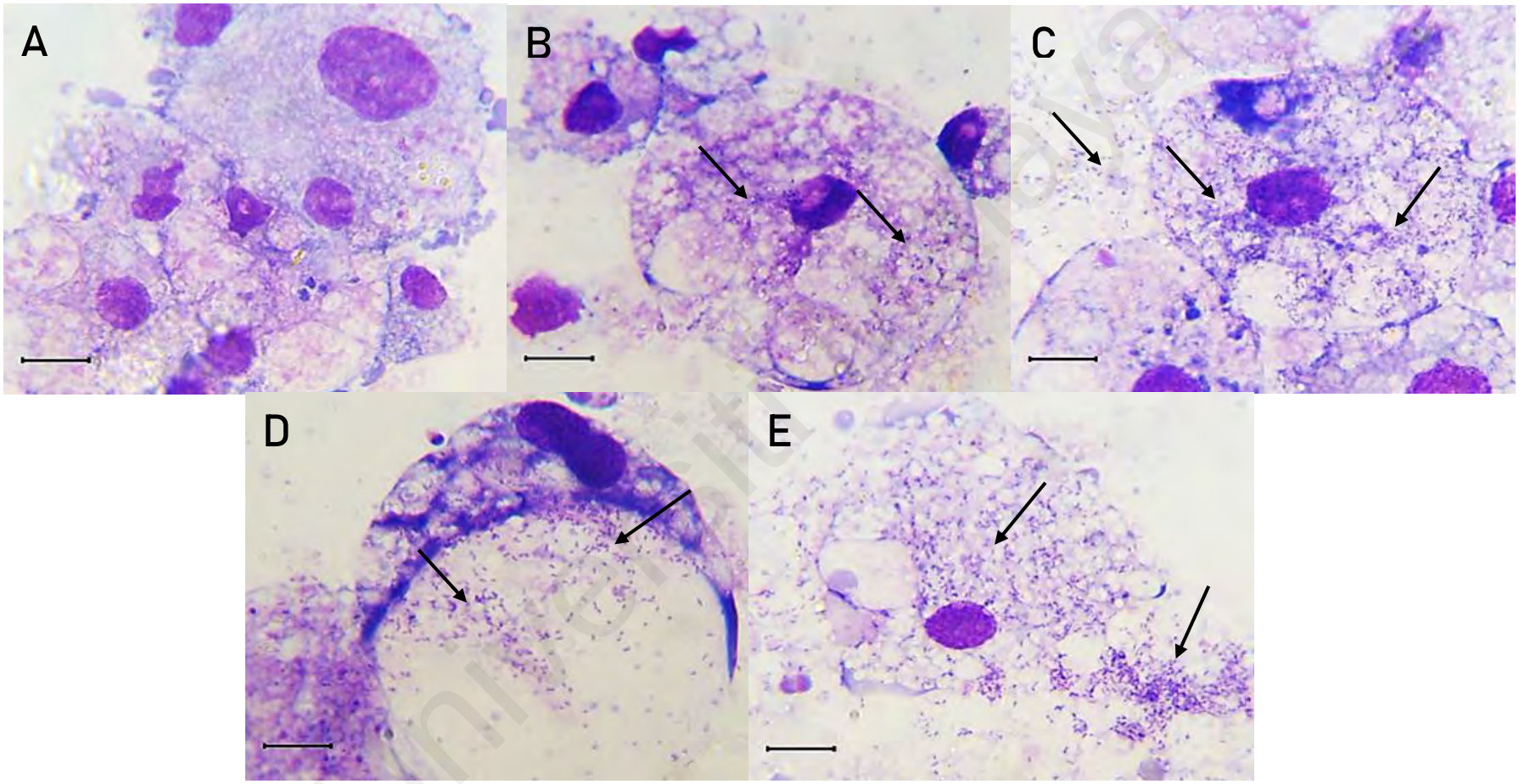

Figure 4. 9: Giemsa-stained cytocentrifuge smears of *R. raoultii*-infected RSE / PILS35 cells (culture 2). A: before *R. raoultii* infection, B: 3 d.p.i., C: 7 d.p.i., D: 10 d.p.i., E: 14 d.p.i. Arrows indicate the presence of bacteria. Scale bars = 10 µm.

For *R. raoultii*-infected IDE8 cells, the rickettsiae-like bacteria were started to be seen inside the cell cytoplasm on 3 d.p.i. (cultures 1 and 2) (Figures 4.10 B and 4.11 B). More infected cells and bacteria inside the cell cytoplasm were also observed on 7 d.p.i. (Figures 4.10 C and 4.11 C) in comparison to 3 d.p.i. (Figures 4.10 B and 4.11 B). A lot of bacteria were found outside the cells as many of the infected cells were already lysed after 10 d.p.i. and 14 d.p.i. for both cultures (Figures 4.10 D - E and 4.11 D - E).

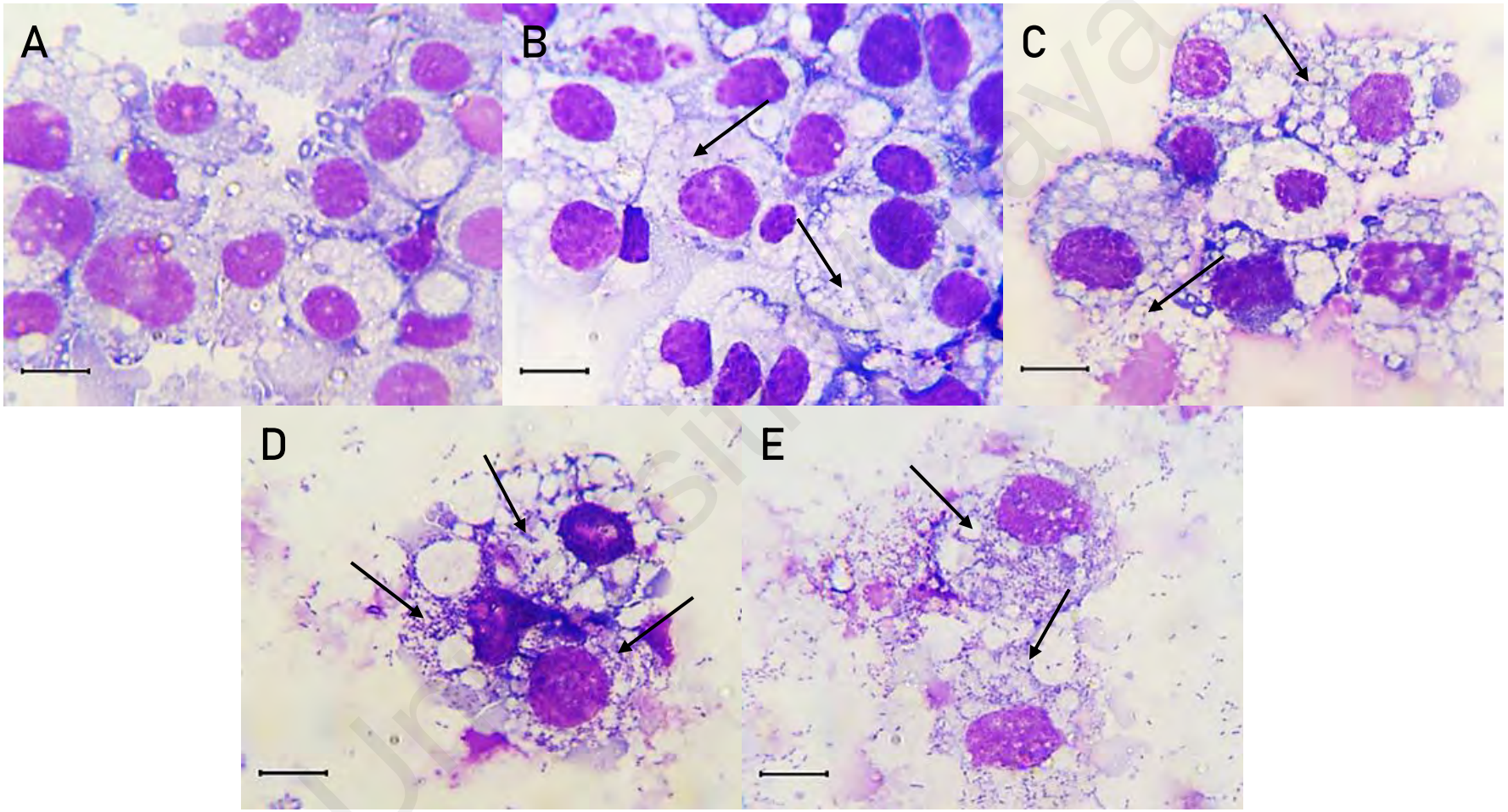

Figure 4. 10: Giemsa-stained cytocentrifuge smears of *R. raoultii*-infected IDE8 cells (culture 1). A: before *R. raoultii* infection, B: 3 d.p.i., C: 7 d.p.i., D: 10 d.p.i., E: 14 d.p.i. Arrows indicate the presence of bacteria. Scale bars = 10 µm.

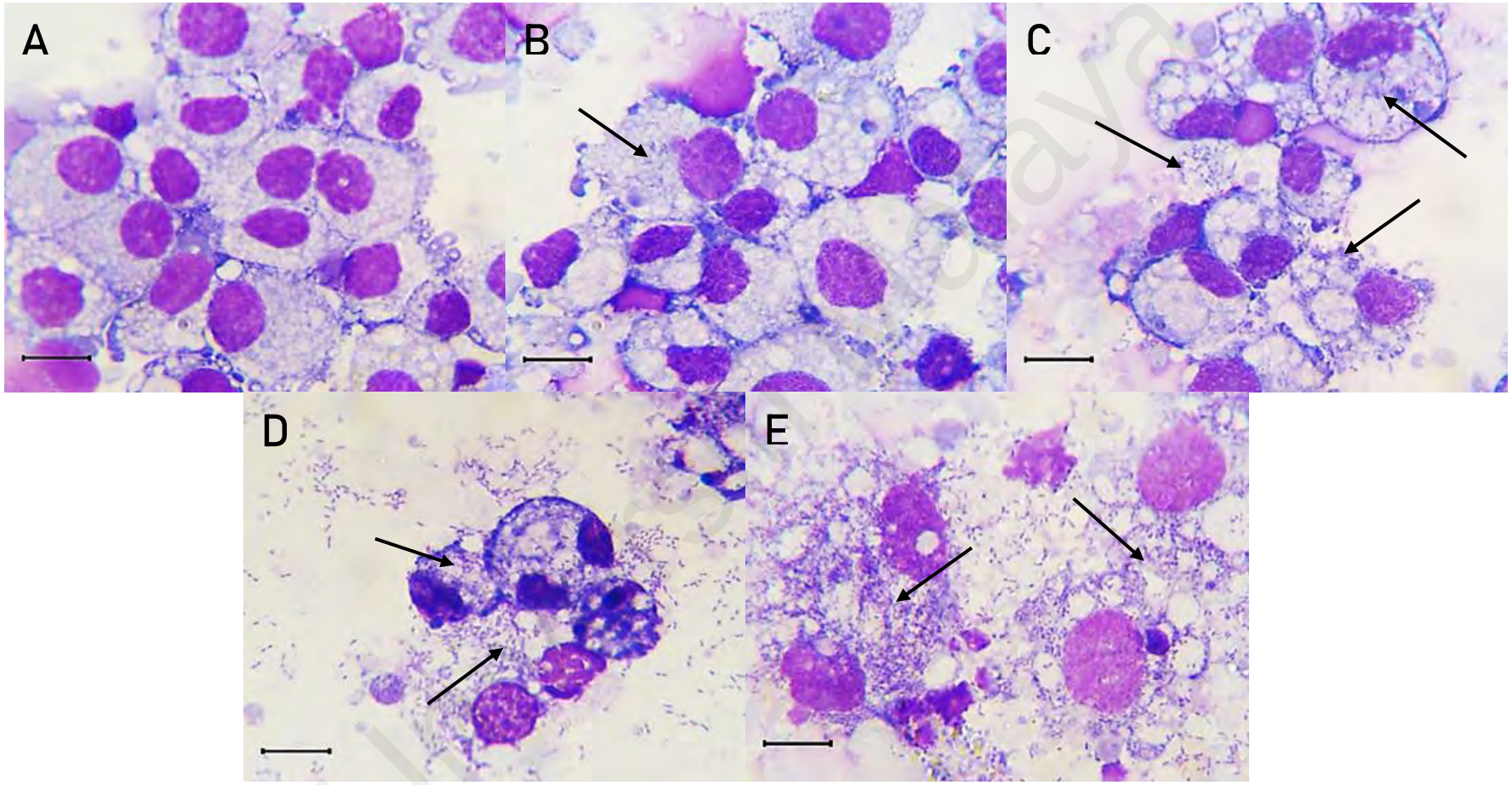

Figure 4. 11: Giemsa-stained cytocentrifuge smears of *R. raoultii*-infected IDE8 cells (culture 2). A: before *R. raoultii* infection, B: 3 d.p.i., C: 7 d.p.i., D: 10 d.p.i., E: 14 d.p.i. Arrows indicate the presence of bacteria. Scale bars = 10 µm.

4.2.2 Infection rate curves for *R. raoultii* in BME / CTVM23, IDE8 and RSE / PILS cell cultures

The percentage of infected cells in BME / CTVM23 cell cultures (cultures 1 and 2) increased exponentially from 0 d.p.i. to 12 d.p.i.. By 5 d.p.i., 56.9 % and 65 % of the BME / CTVM23 in culture 1 and culture 2 already been infected. Followed by on 10 d.p.i., 84.5 % of the cells in BME / CTVM23 (culture 1) and 94.5 % of the cells in BME / CTVM23 (culture 2) were observed to be infected. By 12 d.p.i., 100 % of BME / CTVM23 cells from culture 2 and 98 % from culture 1 were infected. By 15 d.p.i., both cultures were already 100 % infected (Figure 4.12).

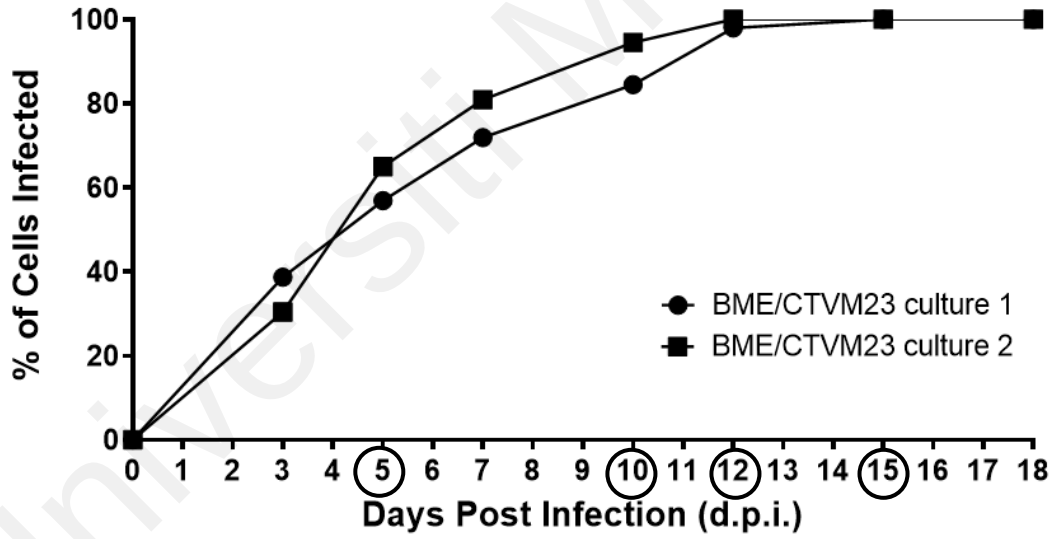

Figure 4. 12: Infection rate curve of *R. raoultii*-infected BME / CTVM23 cells for two different cultures (cultures 1 and 2). The infection rates were established based on the percentage of infected cells out of 200 to 300 cells counted at the indicated days after infection as visualized in Giemsa-stained cytocentrifuge smears.

The percentage of infected cells in both RSE / PILS35 cell cultures (cultures 1 and 2) increased exponentially from 0 d.p.i. until 10 d.p.i. On 3 d.p.i., 24 % and 33 % of the RSE / PILS35 cells observed in Giemsa-stained cytocentrifuge smears from culture 1 and culture 2 were infected. By 10 d.p.i., the number of infected cells in RSE / PILS35 from culture 1 and culture 2 were increased to 96.9 % and 98.6 %, respectively. In continuous, 100 % of the cells in both cultures were observed to be infected by 14 d.p.i. (Figure 4.13).

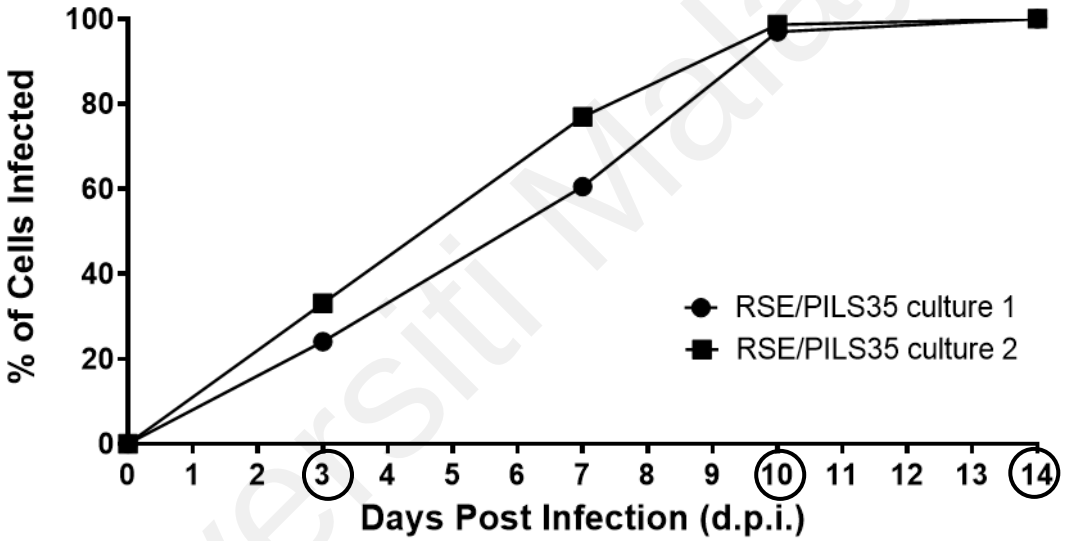

Figure 4. 13: Infection rate curve of *R. raoultii*-infected RSE / PILS35 cells for two different cultures (cultures 1 and 2). The infection rates were established based on the percentage of infected cells out of 200 to 300 cells counted at the indicated days after infection as visualized in Giemsa-stained cytocentrifuge smears.

A lag phase pattern of the infection rate was observed from 0 d.p.i. to 3 d.p.i. in both IDE8 cell cultures (cultures 1 and 2). Followed by an exponential increase in the percentage of the infected cells from 3 d.p.i. to 10 d.p.i. By 10 d.p.i., 93 % and 96 % of the IDE8 cells from culture 1 and 2 was already infected. Subsequently, 100 % of the cells in both cultures were observed to be infected on 14 d.p.i. (Figure 4.14).

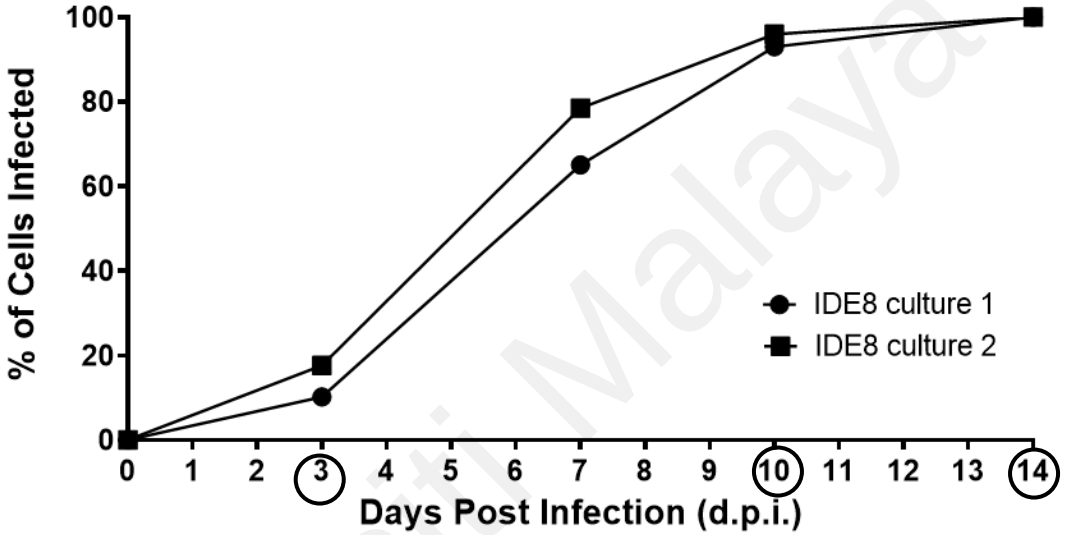

Figure 4. 14: Infection rate curve of *R. raoultii*-infected IDE8 cells for two different cultures (cultures 1 and 2). The infection rates were established based on the percentage of infected cells out of 200 to 300 cells counted at the indicated days after infection visualized in Giemsa-stained cytocentrifuge smears.

4.3 Replication kinetics of *R. raoultii* in tick cell cultures

To better understand the replication kinetics of *R. raoultii* in the separate tick cell cultures, the copy number of *R. raoultii* at the indicated time points, d.p.i. was determined by qPCR based on the rickettsiae-specific *gltA* gene. Besides, the copy number of tick-specific *rpl6* gene at the different time points post infection was also quantified to monitor the number of tick cells in the cultures.

4.3.1 Confirmation of recombinant plasmid DNA containing rickettsiae *gltA* gene after *E. coli* transformation

To quantify the rickettsiae *gltA* target gene in the infected cultures, the target gene sequence was synthesized in a recombinant plasmid DNA. The presence and the size of the recombinant plasmid DNA (pIDTSmart Amp vector and partial rickettsiae *gltA* gene sequence) extracted from three different *E. coli* TOP10F′ colonies after transformation were visualized on a 1.2 % AGE (Figure 4.15). The total size of the recombinant plasmid DNA was estimated to be around 2133 bp (2056 bp of pIDTSmart Amp vector plus 74 bp of rickettsiae-specific *gltA* gene). All three bands from the extracted recombinant plasmid DNA were at the correct size, as shown in Figure 4.15. This result indicated the successful transformation of recombinant plasmid DNA into *E. coli* TOP10F′.

The higher molecular weight bands observed in the AGE (Red arrow) suggested the presence of nicked plasmid DNAs that formed when one of the double-stranded plasmid DNA was broken, creating large floppy circular plasmid DNA that migrates the slowest in an AGE (Figure 4.15). Conversely, the faint bands observed at the lower molecular weight (Green arrow) in the AGE indicated the presence of permanently denatured plasmid DNA (Figure 4.15).

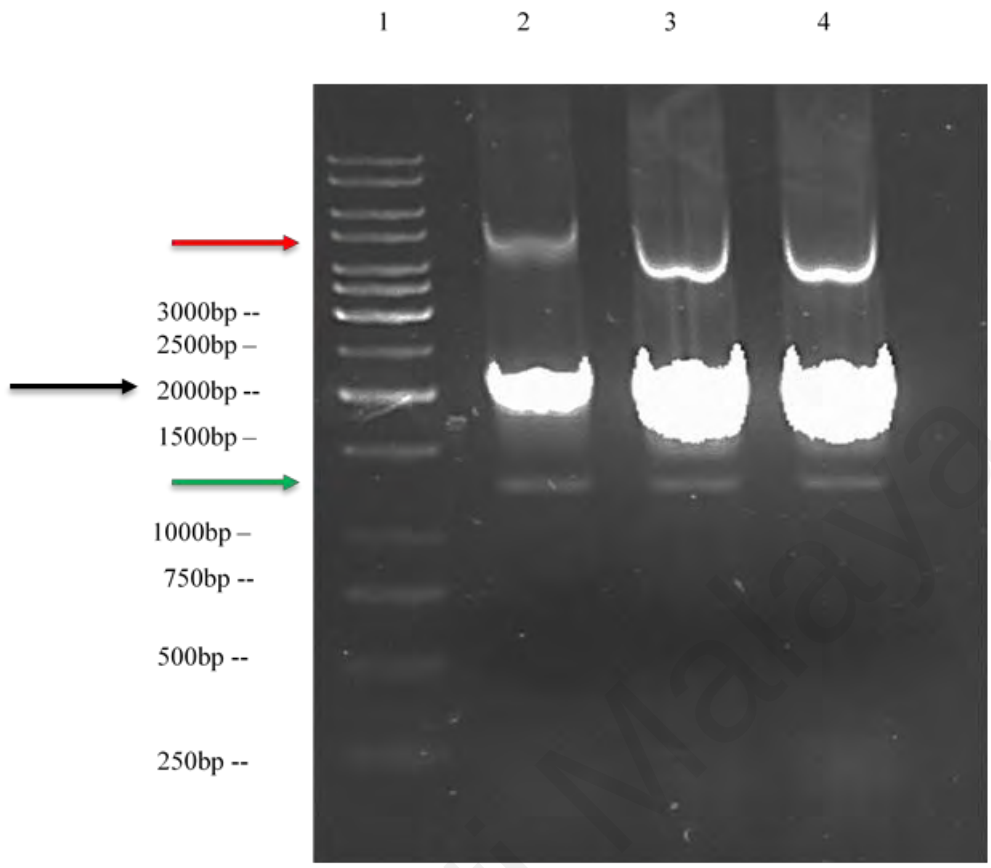

Figure 4. 15: AGE (1.2 %) illustrating uncut extracted recombinant plasmid DNA (pIDT-Smart Amp vector and rickettsiae *gltA* gene) with an estimated size of 2133 bp (Black arrow), nicked plasmid DNA with higher molecular weight (Red arrow) and circular, single-stranded plasmid DNA (Green arrow). The recombinant plasmid was extracted from three different single *E. coli* TOP10F´ colonies. Lane 1: 1 kb DNA ladder, Lane 2: Colony 1, Lane 3: Colony 2, Lane 4: Colony 3.

4.3.2 Concentration of the recombinant plasmid DNA and copy number calculation

The pIDTSmart Amp *gltA* recombinant plasmid preparations were checked for concentration and purity using Nanodrop™ 2000 / 2000c spectrophotometer. The concentration of the extracted recombinant plasmid DNA ranged from 7.32×10^4 to 9.34×10^4 ng/ml, and the quantification of the rickettsiae *gltA* gene copy number in the plasmid preparation was within 1.22×10^{13} to 1.70×10^{13} copies/ml (Table 4.3). The A_{260} / A_{280} ratio of the plasmid DNA extracted from all three colonies ranged from 1.76 to 1.91, suggesting the plasmid DNA preparations were pure from contamination. The recombinant plasmid DNA was then used to construct the standard curve by qPCR in the next section.

Table 4. 3: Concentration, purity and copy number of extracted recombinant plasmid DNA.

Colony	Concentration (ng/ml)	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio	Copy no (gene copies/ml)
1	8.16×10^4	1.87	1.90	1.69×10^{13}
2	7.32×10^4	1.89	1.76	1.22×10^{13}
3	9.34×10^4	1.90	1.91	1.70×10^{13}

4.3.3 Establishing the copy number range for the rickettsiae *gltA* qPCR standard curve

The appropriate range of rickettsiae *gltA* DNA copy numbers for constructing the standard curve was determined in multiple qPCR runs by trying different possible ranges of DNA copies, as shown in Table 4.4. In the 1ST Run, the copy number ranging from 1 × 10^3 to 1 × 10^8 copies/ml was used; however, no amplification was detected at 1 × 10^5 copies/ml and below. In the 2ND Run, the copy number ranging from 1 × 10^8 to 1 × 10^{13} copies/ml was used to determine the upper limit for the DNA copy number that could be detected. The result showed that the amplification could be detected up to 1 × 10^{13} rickettsiae copies/ml with a Ct value of 9.10.

Next, in the 3RD Run, the qPCR with the low copy numbers was repeated to confirm the lower limit. The copy number range from 1 × 10^3 to 1 × 10^{13} copies/ml was used to determine. However, no amplification was detected in 1 × 10^3 copies/ml, and the lower limit was detected at 1 × 10^5 rickettsiae copies/ml with a Ct value of 40.91.

In the 4TH Run, the copy number ranging from 1 × 10^5 to 1 × 10^{13} copies/ml was used. The lowest Ct value of 8.60 was detected for 1 × 10^{13} rickettsiae copies/ml in the 4TH run, which was consistent with the 2ND and 3RD Run result. A standard curve was then plotted using the results from the 4th Run, with the copy numbers ranging from 1 x 10^5 to 1 x 10^{13} copies/ml (Figure 4.16). The coefficient of determination (R^2) for the standard curve was 0.9948. The high R^2 value showed a good correlation between the *gltA* target copy number and Ct values. Therefore, the range of 1 x 10^5 to 1 x 10^{13} copies/ml was for the standard curves in the qPCR for determining the replication kinetics of *R. raoultii* infection in the separate tick cell cultures.

Table 4. 4: Range determination of rickettsiae DNA standard calibration curve.

DNA copies/ml	Ct value			
	1ST RUN	2ND RUN	3RD RUN	4TH RUN
1×10^{13}	-	9.10	8.70	8.60
1×10^{12}	-	13.68	-	13.48
1×10^{11}	-	17.61	17.20	17.40
1×10^{10}	-	21.73	-	21.13
1×10^{9}	-	23.90	23.15	23.82
1×10^{8}	27.06	27.98	-	28.02
1×10^{7}	31.28	-	30.87	30.57
1×10^{6}	36.42	-	-	36.12
1×10^{5}	N/A	-	40.91	40.51
1×10^{4}	N/A	-	-	-
1×10^{3}	N/A	-	N/A	-

N/A: No amplification
-: Not included in the Run

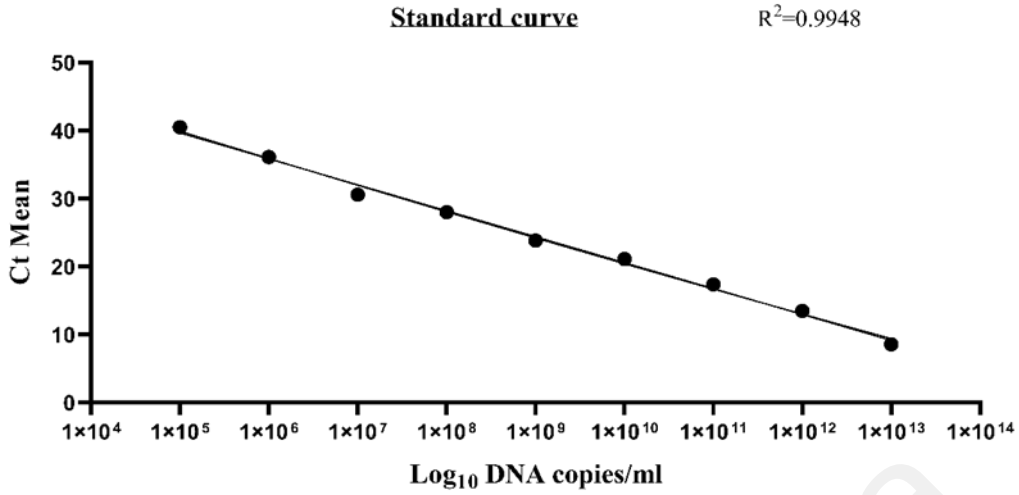

Figure 4. 16: Rickettsiae-specific *gltA* gene target standard curve for quantification of rickettsiae-specific *gltA* gene by qPCR. A pIDTSmart (Amp) plasmid vector containing the *gltA* gene sequence was serially diluted (from 10^5 to 10^{13} copies/ml) and served as the standard reference. R^2 value represents the coefficient of determination. Each data point represents the mean of 2 technical replicates.

4.3.4 Tick *rpl6* gene target qPCR standard curve

For the quantification of tick single-copy nuclear *rpl6* copy numbers on the different days' post infection, qPCR was performed using a standard curve with a tenfold dilution series of the gene target copy number ranging from 5 x 10^{-1} to 5 x 10^{6} copies / µl. The coefficient of determination (R^2) for the standard curve was 0.9963.

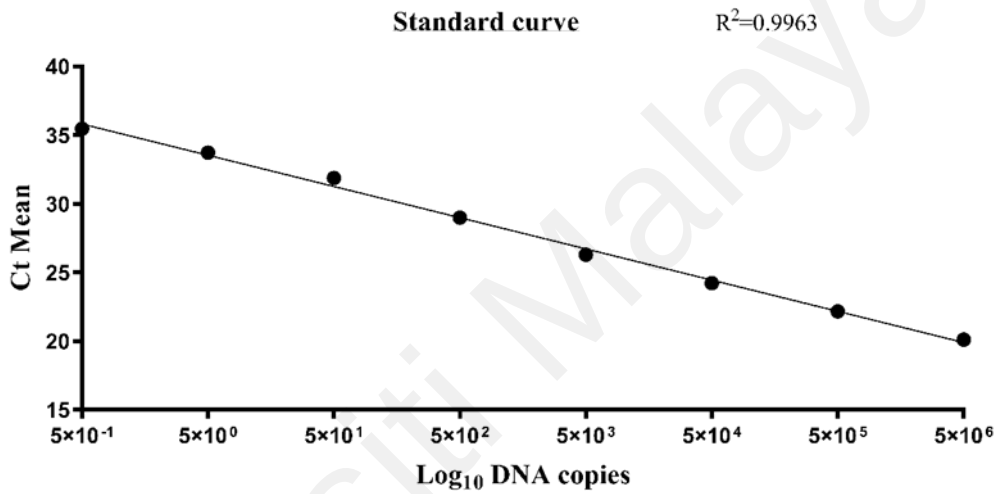

Figure 4. 17: Standard curve for the quantification of tick-specific *rpl6* gene by qPCR. The synthetic oligonucleotide based on the *rpl6* gene sequence was serially diluted (from 5 x 10^{-1} to 5 x 10^{6} copies / µl) and served as the standard reference. R^2 value represents the coefficient of determination. Each data point represents the mean of 2 technical replicates.

4.3.5 Rickettsiae *gltA* and tick *rpl6* qPCRs for *R. raoultii*-infected BME / CTVM23 cell cultures

The number of *R. raoultii*, as represented by the copy numbers of the rickettsiae-specific *gltA* gene target, in each of the BME / CTVM23 cultures (cultures 1 and 2) are shown in Figure 4.18 A. Approximately 2.63 x 10^9 DNA copies of *R. raoultii* were used to initiate infections in both BME / CTVM23 cell cultures (cultures 1 and 2), as indicated on 0 d.p.i. in Figure 4.18 A. The curve for both BME / CTVM23 cell cultures demonstrates an exponential increase from 0 to 15 d.p.i., a subsequent declining phase from 15 to 18 d.p.i. and remained low until the end of the experiment (Figure 4.18 A).

The mean generation time for *R. raoultii* in BME / CTVM23 cells was 2.2 days. On 15 d.p.i., the highest mean copy number was recorded, at 1.04 × 10^{11} copies/ml representing an approximately 39.5 fold increase compared to the inoculum.

Concurrently, a gradual decrease in the tick cell numbers, as represented by the *rpl6* target copy numbers was observed in both *R. raoultii*-infected BME / CTVM23 cell cultures (cultures 1 and 2) from 3 d.p.i. to 15 d.p.i. (Figure 4.18 B). Then followed by a steeper decline as infected cells began to die (Figure 4.18 B).

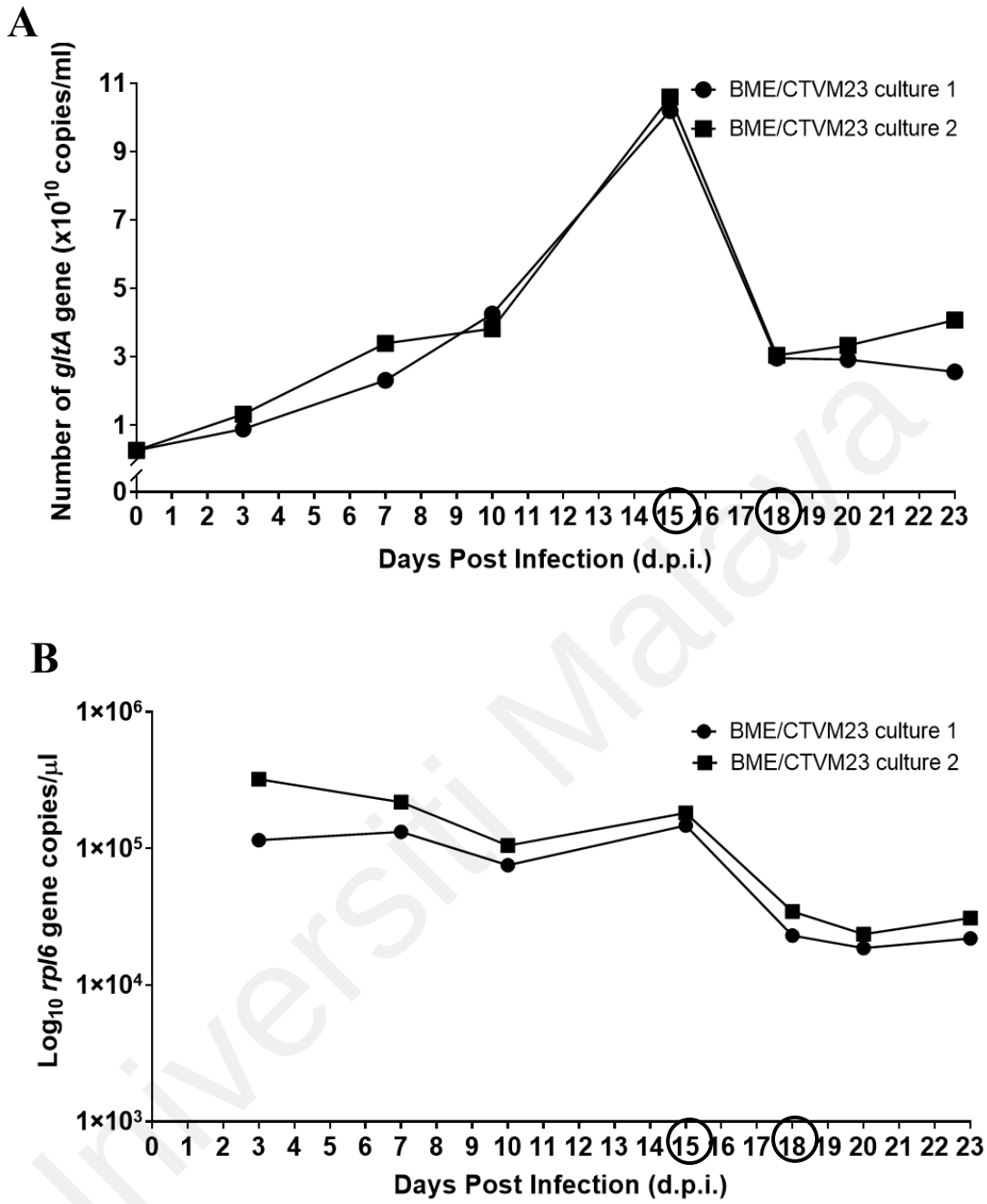

Figure 4. 18: (A) Replication kinetics of *R. raoultii* in two separate BME / CTVM23 cell cultures (cultures 1 and 2) based on the copy number of rickettsiae-specific *gltA* gene. (B) Tick cells copy numbers at different time-point of infection based on the amplification of the tick-specific *rpl6* gene in two different BME / CTVM23 cell cultures (cultures 1 and 2).

4.3.6 Rickettsiae *gltA* and tick *rpl6* qPCRs for *R. raoultii*-infected RSE / PILS35 cell cultures

The number of *R. raoultii* as represented by the *gltA* target copy numbers in two separate cultures of RSE / PILS35 cells (cultures 1 and 2) are shown in Figure 4.19 A. Approximately 6.85 x 10^7 DNA copies/ml of *R. raoultii* were used to initiate infections in both RSE / PILS35 cell cultures, as indicated at 0 d.p.i. in Figure 4.19 A.

The curve for both RSE / PILS35 cell cultures demonstrates an initial lag phase from 0 to 3 d.p.i. Followed by an increase in the *gltA* target copies from 3 d.p.i. to 14 d.p.i. and a subsequent decrease from 14 d.p.i. to 17 d.p.i. in culture 1. On the other hand, in culture 2, the exponential increase in the *gltA* target copies from 3 to 10 d.p.i. was followed by a stationary phase between 10 to 14 d.p.i. and a subsequent decrease from 14 d.p.i. to 17 d.p.i. The *R. raoultii* generation times were calculated to be 2.6 and 1.6 days in culture 1 and 2, respectively.

On 14 d.p.i., the highest mean copy number was recorded, at 2.54 × 10^9 copies/ml representing an approximately 37.1-fold increase compared to the inoculum. In parallel, tick cell numbers remained relatively stable from 3 to 7 d.p.i. in both *R. raoultii*-infected RSE / PILS35 cell cultures and followed by a subsequent decline until 21 d.p.i. (Figure 4.19 B). However, in RSE /PILS35 culture 1, an increase in the copy number of the *rpl6* gene was observed on day 14 d.p.i. (Figure 4.19 B).

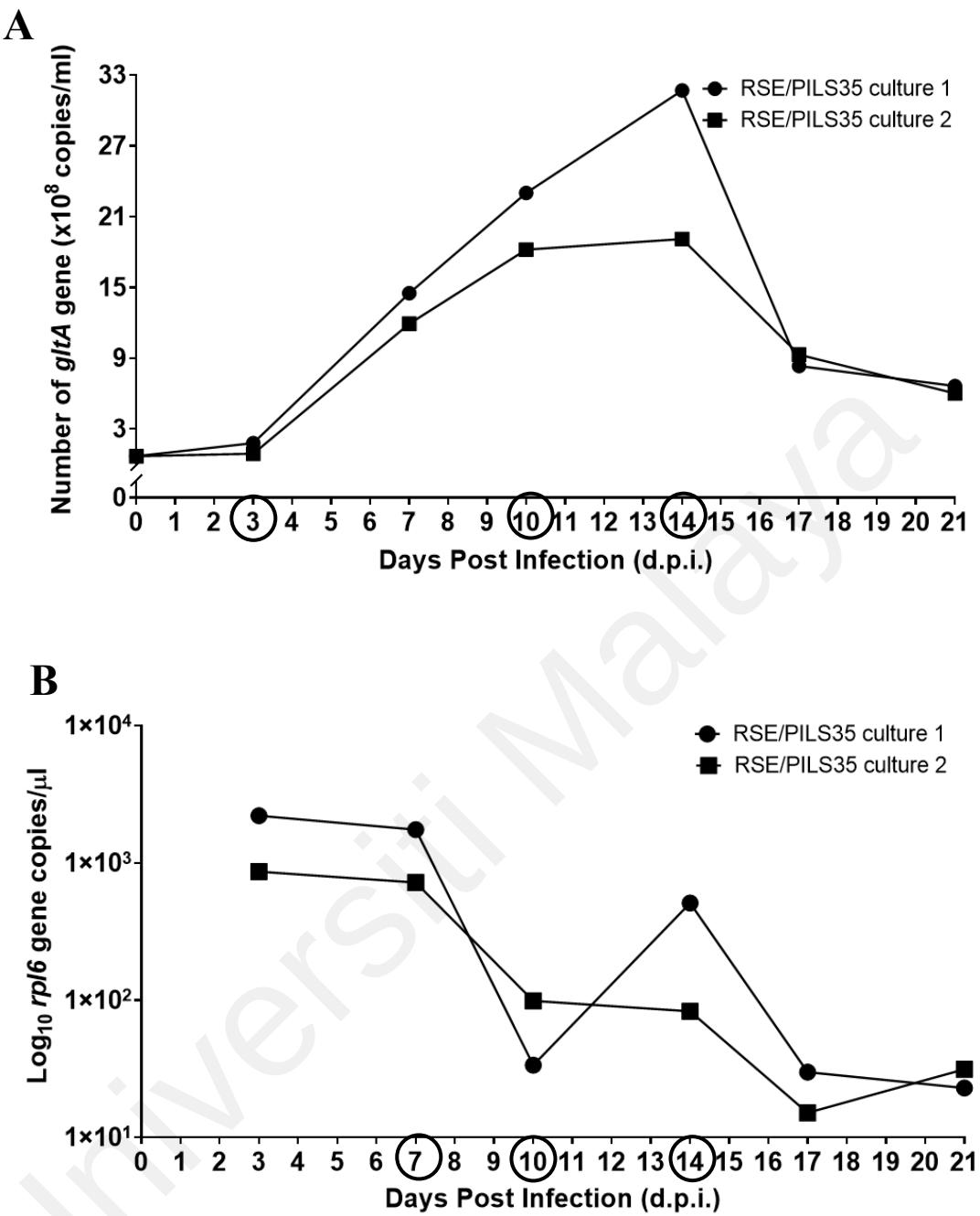

Figure 4. 19: (A) Replication kinetics of *R. raoultii* in two separate RSE / PILS35 cell cultures (cultures 1 and 2) based on the copy number of rickettsiae-specific *gltA* gene. (B) Tick cells copy numbers at different time-point of infection based on the amplification of the tick-specific *rpl6* gene in two different RSE / PILS35 cell cultures (cultures 1 and 2).

4.3.7 Rickettsiae *gltA* and tick *rpl6* qPCRs for *R. raoultii*-infected IDE8 cell culture

R. raoultii numbers in two separate cultures of IDE8 cells (cultures 1 and 2), as represented by the rickettsiae-specific *gtlA* target copy numbers are shown in Figure 4.20 A. Approximately 2.46 x 10^8 DNA copies of *R. raoultii* were used to initiate infections in both IDE8 cell cultures, as indicated on 0 d.p.i. in Figure 4.20 A.

R. raoultii grew rapidly in both IDE8 cell cultures, and the curve demonstrates an initial short lag phase from 0 d.p.i. to 3 d.p.i. Followed by an exponential increase in *gltA* copy numbers from 3 d.p.i. to 7 d.p.i., a subsequent stationary phase from 7 to 14 d.p.i., and a declining phase from 14 d.p.i. to 17 d.p.i. that remained low until 21 d.p.i. in both cultures (Figure 4.20 A). The mean generation time for *R. raoultii* in IDE8 cells was 0.9 day. On 14 d.p.i., the highest mean copy number was recorded, at 2.71 × 10^{10} copies/ml representing an approximately 110.1-fold increase compared to the inoculum. Simultaneously, a gradual decrease in the tick cell numbers as represented by the *rpl6* target copy number was observed in both *R. raoultii*-infected IDE8 cell cultures from 3 to 14 d.p.i., followed by a steeper decline until 21 d.p.i. as infected cells began to die (Figure 4.20 B).

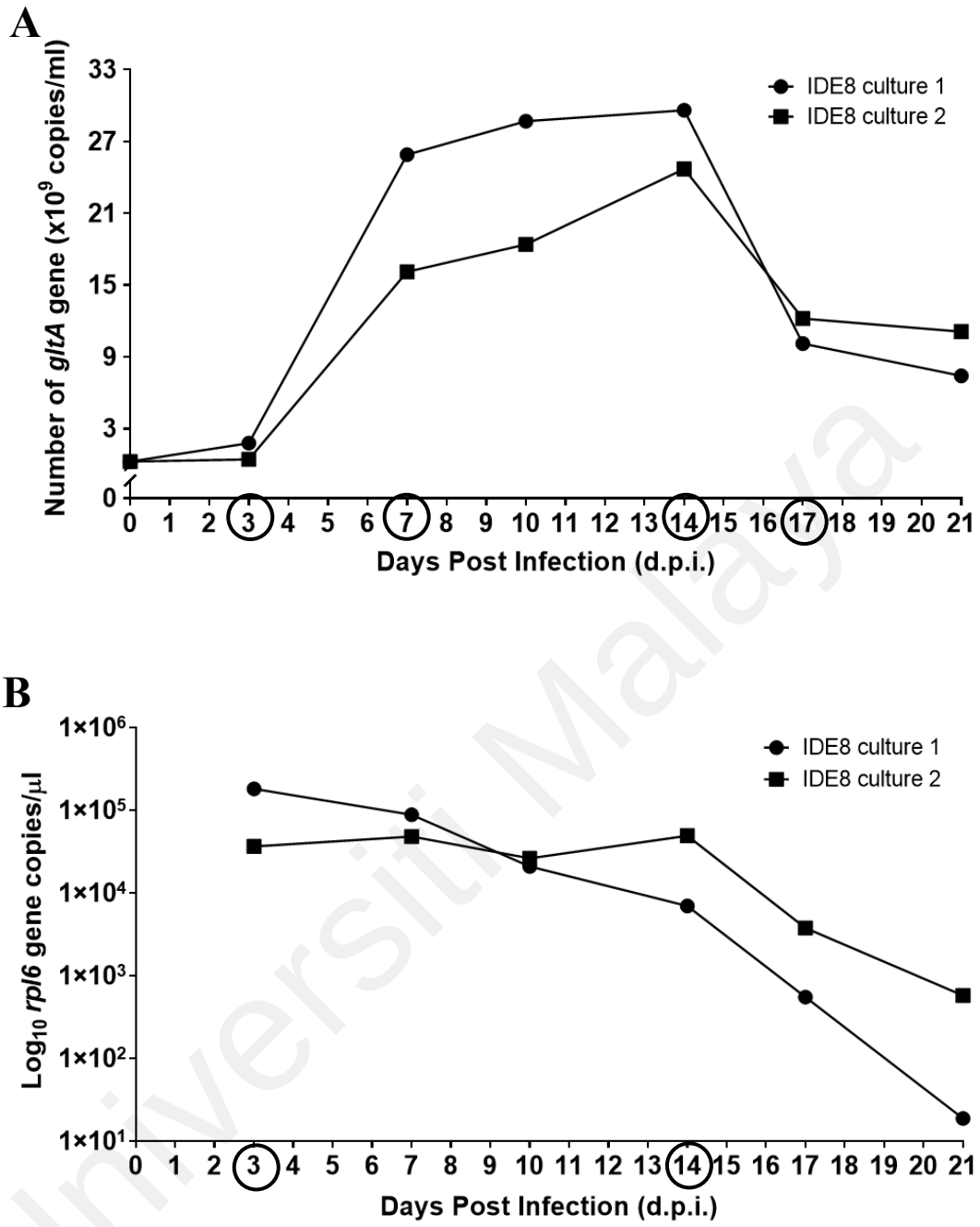

Figure 4. 20: (A) Replication kinetics of *R. raoultii* in two different IDE8 cell cultures (cultures 1 and 2) based on the copy number of rickettsiae-specific *gltA* gene. (B) Tick cells copy numbers at different time-point of infection based on the amplification of the tick-specific *rpl6* gene in two different IDE8 cell cultures (cultures 1 and 2).

CHAPTER 5: DISCUSSION

The data presented in this study showed that *R. raoultii* is able to infect and propagate in the *Rh. microplus*-derived BME / CTVM23, *Rh. sanguineus*-derived RSE / PILS35 and *I. scapularis*-derived IDE8 cell lines. The BME / CTVM23 cell line was chosen for this study for the reason that the *R. raoultii* strain used was primarily isolated in this line (Palomar, A. M. et al., 2019). The RSE / PILS35 cells were used because *R. raoultii* has been reported previously to be isolated in another cell line derived from *Rh. sanguineus* tick species (Santibáñez, S. et al., 2015). The *I. scapularis* cell lines are identified to be permissive to infection with North American *Rickettsia* species (Kurtti, T. J. et al., 2015; Kurtti, T. J. et al., 2005; Munderloh, U. G. et al., 1998; Policastro, P. F. et al., 1997; Pornwiroon, W. et al., 2006); the IDE8 line was used to study whether *I. scapularis* cells were able to support replication of the Eurasian species *R. raoultii*. The presence of noticeable cytopathic effect was observed in all infected tick cells in this study. However, a previous study by (Palomar, A. M. et al., 2019) has shown that *R. raoultii* caused almost no cytopathic effects in primary *D. marginatus* cell cultures, despite this species being one of the main arthropod vectors of the bacterium.

The infection rate curves showed that the percentage of infected cells in all three cell lines increased steadily from less than 40 % at 3 d.p.i. to 100 % of the cells at the end of the observation period. Since the quantification of bacteria from Giemsa-stained cytocentrifuge smear is difficult due to the variable number of bacteria infecting a single cell, it is necessary to perform qPCR alongside to determine the growth of bacteria in the infected cells.

Admittedly, the Giemsa stain is not well suited for identifying minor changes in morphology. However, there is no indication that rickettsiae are released into the extracellular as a result of mass host cell death. Rather than that, the morphological

observations support the concept that *R. raoultii* has a strong tendency to escape from the cytoplasm of infected cells into the extracellular environment and to enter other cells, at least in the first 3 to 5 days of incubation. A similar observation of *R. rickettsii* bacteria was observed in chicken embryos and L929 cells, in which cell destruction was observed together with an increasing number of extracellular *Rickettsia* (Wisseman, C. et al., 1976).

Generally, the replication kinetics for bacteria can be represented by four common phases; lag phase, exponential phase, stationary phase and death phase (Fenollar, F. et al., 2003). These growth phases could be seen in both infected IDE8 cultures and one of the infected RSE / PILS35 cultures. Similar replication kinetics of *R. raoultii* and *R. rickettsii* bacteria were observed in Vero cells, in which all four phases were observed (Eremeeva, M. E. et al., 2003; Špitalská, E. et al., 2012). In contrast, the stationary phase was not detected in either of the infected BME / CTVM23 cultures and one of the RSE / PILS35 cultures. For intracellular bacteria such as *R. raoultii*, the death phase may occur when all cells in the culture are infected and begin to die. This could be observed in the decline of the tick cell copy numbers and the onset of the bacterial death phase after 100 % of the cells were infected in all three cell lines. The high bacterial numbers appear to have promoted cell death very quickly in these cells and prevented the occurrence of a stationary phase in the BME / CTVM23 and RSE / PILS35 cultures.

An initial lag phase in *R. raoultii* copy numbers early in the infection was observed in both infected RSE / PILS35 and IDE8 cultures. This observation may represent the adaptation of the bacteria to the host cell during the lag phase. The presence of a lag phase after the introduction of the bacterial inoculum was consistent with other studies of replication kinetics for different *Rickettsia* spp. in mammalian cell lines. These include lag phases of seven days for *R. raoultii* (Špitalská, E. et al., 2012), two days for *R. helvetica* (Elfving, K. et al., 2012) and one day for *R. rickettsii* (Eremeeva, M. E. et al., 2003) during infection of Vero cells, six days for *R. raoultii* during infection of L929 cells

(Špitalská, E. et al., 2012), and 7.5 hours for *R. prowazekii* during infection of embryonic chicken cells (Wisseman, C. et al., 1976). The variable lengths of the lag phase for the different rickettsial species may be influenced by how the bacteria adapt to the different cell lines and the culture conditions. In addition, the length of the lag phase may also be dependent on the bacterial growth phase from which they were isolated for use in the infection (Marcelino, I. et al., 2005). On the other hand, the lag phase was not detected in either infected BME / CTVM23 culture. The absence of a lag phase may be because the bacteria was originally propagated in BME / CTVM23 cells, hence it was already accustomed to growth in this cell line.

The mean lengths of the exponential phase for *R. raoultii* infection in tick cells, ranging from four to twelve days, were observed to be longer than the reported lengths of the exponential phase during *R. raoultii* and *R. slovaca* infections in mammalian cells (Boldiš, V. et al., 2010; Špitalská, E. et al., 2012). Apart from IDE8, the generation times for *R. raoultii* in the infected tick cultures ranged from 1.6 to 2.2 days, which are also greater than the generation times reported for *R. raoultii* and *R. slovaca* in mammalian cells, which ranged from 20 to 22 hours (Boldiš, V. et al., 2010; Elfving, K. et al., 2012). The discrepancies observed in the growth rates of the *Rickettsia* species could be due to two possible explanations. Firstly, the infected tick cell cultures were maintained at a lower temperature compared to the usual incubation temperatures for the infected mammalian cells in previous studies, which could have influenced the growth rate of the bacteria. A recent report showed that *Candidatus* Rickettsia vini induced cell death in tick and Vero cells at approximately the same rate at incubation temperatures of, respectively, 28 °C and 32 °C (Al-Khafaji, A. M. et al., 2020). Secondly, there may be a possible variation in the interaction between the rickettsiae and the different cell lines.

The increase of *R. raoultii* copies observed during infection also varied between the tick cell lines tested in our study. Compared to the starting inoculum in IDE8 cultures, the overall increase in bacterial numbers appeared to be greater than in BME / CTVM23 and RSE / PILS35 cultures (i.e. 101.1 times vs 39.5 and 37.1 times). This observation suggests that *R. raoultii* bacteria were able to multiply to high numbers in IDE8 cells. Furthermore, a stationary phase lasting up to seven days could be observed in the infected IDE8 cultures, and there was only a marginal decline in the tick cell copy numbers during this phase. This suggests that IDE8 cells may be more tolerant than the other two cell lines of heavy bacterial burden before the onset of cell death in the culture conditions used.

However, differences in seeding cell densities between different cell lines, ranging from 2 to 6 × 10^6 cells / ml, might cause the variation in the growth of *R. raoultii* in the different cell lines. However, the same number of optimum cell densities after two weeks of incubation was unable to be achieved due to different behaviour in cell lines seeded on a cell culture tube. The seeding cell densities of the IDE8 cells used in this study were between seeding cell densities of IDE8 cells used to study the growth of TBEV (5 × 10^5 cells / ml of IDE8 cells) *C. burnetii* (1 × 10^7 cells / ml of IDE8 cells) (Herrin, B. et al., 2011; Weisheit, S. et al., 2015). Until now, no seeding cell densities for BME / CTVM23 and RSE / PILS35 cell lines have been reported for bacteria or virus growth studies.

One of this study's limitations is the difference in the number of starting bacteria used to start the infection in different cell lines. Bacteria stock in respective cell line were prepared by directly cryopreserved the infected cell culture suspension with DMSO in liquid nitrogen. The number of bacteria in the cryopreserved bacteria stock was then quantitated by qPCR targeting the rickettsiae *glta* gene. Hence, it was impossible to know the number of bacteria prior to infection. Nevertheless, this study determined the number of starting bacteria used for infection in different cell lines based on the infection time

frame. The infection time frame for all tick cell lines (from day 0 to terminal infection) was recorded within 15 days. Therefore, the cryopreserved bacterial stock was diluted using culture media at 1: 20 for IDE8 and 1: 10 for RSE / PILS35.

CHAPTER 6: CONCLUSION

In conclusion, this study has shown that *R. raoultii* is able to infect and propagate in *Rh. microplus*-derived BME / CTVM23, *Rh. sanguineus*-derived RSE / PILS35 and *I. scapularis*-derived IDE8 cell lines represent three species of prostriate and metastriate ticks not recognized to harbour this bacterium in nature. Besides, this study has further shown the growth kinetics of *R. raoultii* in these cell lines. The observations include longer generation times and exponential phase, as well as a higher level of bacterial multiplication in tick cell lines in comparison to the reported observations in mammalian cells from previous studies.

However, more experiments are still necessary to study the effect of the variation of incubation temperatures against bacterial growth. Following the same culture conditions in all infected tick cell cultures, IDE8 cells showed to be able to tolerate higher *R. raoultii* multiplication levels and infection burden compared to the two metastriate tick cell lines. Further studies will be needed to determine if the same observations are made under variable culture conditions and to study if the phenotypic and genotypic differences between the tick cell lines affect the *R. raoultii* growth rates.

It is also necessary to note that the tick cell lines utilized in this research did not establish from the natural vector for *R. raoultii* and thus do not represent the natural host for this bacteria. However, these cell lines will still be valuable for studying arthropod-pathogen interactions, particularly when the continuous cell lines from any of the natural arthropod vectors are absent. Further study into the interaction between *R. raoultii* and the vector arthropod at the molecular and cellular level is important in understanding the maintenance of *R. raoultii* among the tick population and the ecology of tick-borne rickettsial diseases.

REFERENCES

Abdad, M. Y., Abou Abdallah, R., El Karkouri, K., Beye, M., Stenos, J., Owen, H., . . . Nguyen, T.-T. (2017). *Rickettsia gravesii* sp. nov.: a novel spotted fever group *rickettsia* in Western Australian *Amblyomma triguttatum triguttatum* ticks. *International Journal of Systematic and Evolutionary Microbiology, 67*(9), 3156-3161. doi:https://doi.org/10.1099/ijsem.0.001865.

Abdad, M. Y., Abou Abdallah, R., Fournier, P.-E., Stenos, J., & Vasoo, S. (2018). A concise review of the epidemiology and diagnostics of rickettsioses: *Rickettsia* and *Orientia* spp. *Journal of Clinical Microbiology, 56*(8), e01728-01717. doi:https://doi.org/10.1128/JCM.01728-17

Al-Khafaji, A. M., Bell-Sakyi, L., Fracasso, G., Luu, L., Heylen, D., Matthysen, E., . . . Palomar, A. M. (2020). Isolation of *Candidatus* Rickettsia vini from Belgian *Ixodes arboricola* ticks and propagation in tick cell lines. *Ticks and Tick-Borne Diseases, 11*(6), 101511. doi:https://doi.org/10.1016/j.ttbdis.2020.101511

Al-Khafaji, A. M., Clegg, S. R., Pinder, A. C., Luu, L., Hansford, K. M., Seelig, F., . . . Feil, E. J. (2019). Multi-locus sequence typing of *Ixodes ricinus* and its symbiont *Candidatus* Midichloria mitochondrii across Europe reveals evidence of local co-cladogenesis in Scotland. *Ticks and Tick-Borne Diseases, 10*(1), 52-62. doi:https://doi.org/10.1016/j.ttbdis.2018.08.016

Al-Rofaai, A., & Bell-Sakyi, L. (2020). Tick cell Lines in research on tick control. *Frontiers in Physiology, 11*, 152. doi:https://doi.org/10.3389/fphys.2020.00152

Alberdi, M. P., Dalby, M. J., Rodriguez-Andres, J., Fazakerley, J. K., Kohl, A., & Bell-Sakyi, L. (2012a). Detection and identification of putative bacterial

endosymbionts and endogenous viruses in tick cell lines. *Ticks and Tick-Borne Diseases, 3*(3), 137-146. doi:https://doi.org/10.1016/j.ttbdis.2012.05.002

Alberdi, M. P., Nijhof, A. M., Jongejan, F., & Bell-Sakyi, L. (2012). Tick cell culture isolation and growth of *Rickettsia raoultii* from Dutch *Dermacentor reticulatus* ticks. *Ticks and Tick-borne Diseases, 3*(5), 349-354. doi:10.1016/j.ttbdis.2012.10.020

Alberdi, M. P., Nijhof, A. M., Jongejan, F., & Bell-Sakyi, L. (2012b). Tick cell culture isolation and growth of *Rickettsia raoultii* from Dutch *Dermacentor reticulatus* ticks. *Ticks and Tick-Borne Diseases, 3*(5-6), 349-354. doi:https://doi.org/10.1016/j.ttbdis.2012.10.020

Alemu, G., Chanie, M., Mengesha, D., & Bogale, B. (2014). Prevalence of Ixodid ticks on cattle in Northwest Ethiopia. *Acta Parasitologica Globalis, 5*(2), 139-145. doi:https://doi.org/10.5829/idosi.apg.2014.5.2.84292

Allen, A. C., & Spitz, S. (1945). A comparative study of the pathology of scrub typhus (tsutsugamushi disease) and other rickettsial diseases. *The American journal of pathology, 21*(4), 603.

Amaro, M., Bacellar, F., & Franca, A. (2003). Report of eight cases of fatal and severe Mediterranean spotted fever in Portugal. *Annals of the New York Academy of Sciences, 990*(1), 331-343. doi:https://doi.org/10.1111/j.1749-6632.2003.tb07384.x

Angelakis, E., Pulcini, C., Waton, J., Imbert, P., Socolovschi, C., Edouard, S., . . . Raoult, D. (2010). Scalp eschar and neck lymphadenopathy caused by *Bartonella henselae* after tick bite. *Clinical Infectious Diseases, 50*(4), 549-551. doi:https://doi.org/10.1086/650172

Antunes, S., Merino, O., Mosqueda, J., Moreno-Cid, J. A., Bell-Sakyi, L., Fragkoudis, R., . . . Domingos, A. (2014). Tick capillary feeding for the study of proteins involved in tick-pathogen interactions as potential antigens for the control of tick infestation and pathogen infection. *Parasites & Vectors, 7*(1), 1-12. doi:https://doi.org/10.1186/1756-3305-7-42

Archibald, L. K., & Sexton, D. J. (1995). Long-term sequelae of Rocky Mountain spotted fever. *Clinical Infectious Diseases, 20*(5), 1122-1125. doi:https://doi.org/10.1093/clinids/20.5.1122

Attoui, H., Stirling, J. M., Munderloh, U. G., Billoir, F., Brookes, S. M., Burroughs, J. N., . . . de Lamballerie, X. (2001). Complete sequence characterization of the genome of the St Croix River virus, a new orbivirus isolated from cells of *Ixodes scapularis*. *Journal of General Virology, 82*(4), 795-804. doi:https://doi.org/10.1099/0022-1317-82-4-795

Azad, A. F. (2007). Pathogenic rickettsiae as bioterrorism agents. *Clinical Infectious Diseases, 45*(Supplement_1), S52-S55. doi:https://doi.org/10.1086/518147

Azad, A. F., & Beard, C. B. (1998). Rickettsial pathogens and their arthropod vectors. *Emerging Infectious Diseases, 4*(2), 179. doi:https://doi.org/10.3201/eid0402.980205

Baganz, M. D., Dross, P. E., & Reinhardt, J. A. (1995). Rocky Mountain spotted fever encephalitis: MR findings. *American Journal of Neuroradiology, 16*(4), 919-922.

Bastos, C. V., das Vasconcelos, M. M. C., Ribeiro, M. F. B., & Passos, L. M. F. (2006). Use of refrigeration as a practical means to preserve viability of *in vitro*-cultured IDE8 tick cells. *Experimental and Applied Acarology, 39*(3), 347-352. doi:https://doi.org/10.1007/s10493-006-9006-5

Bechah, Y., Socolovschi, C., & Raoult, D. (2011). Identification of rickettsial infections by using cutaneous swab specimens and PCR. *Emerging Infectious Diseases, 17*(1), 83. doi:https://doi.org/10.3201/eid1701.100854

Bell-Sakyi, L. (1991). Continuous cell lines from the tick *Hyalomma anatolicum anatolicum*. *The Journal of Parasitology*, 1006-1008. doi:https://doi.org/10.2307/3282757

Bell-Sakyi, L. (2004). *Ehrlichia ruminantium* grows in cell lines from four Ixodid tick genera. *Journal of Comparative Pathology, 130*(4), 285-293. doi:https://doi.org/10.1016/j.jcpa.2003.12.002

Bell-Sakyi, L., Darby, A., Baylis, M., & Makepeace, B. L. (2018). The Tick Cell Biobank: A global resource for *in vitro* research on ticks, other arthropods and the pathogens they transmit. *Ticks and Tick-Borne Diseases, 9*(5), 1364-1371. doi:https://doi.org/10.1016/j.ttbdis.2018.05.015

Bell-Sakyi, L., Kohl, A., Bente, D. A., & Fazakerley, J. K. (2012). Tick cell lines for study of Crimean-Congo hemorrhagic fever virus and other arboviruses. *Vector-Borne and Zoonotic Diseases, 12*(9), 769-781. doi:https://doi.org/10.1089/vbz.2011.0766

Bell-Sakyi, L., Zweygarth, E., Blouin, E. F., Gould, E. A., & Jongejan, F. (2007). Tick cell lines: tools for tick and tick-borne disease research. *Trends in Parasitology, 23*(9), 450-457. doi:https://doi.org/10.1016/j.pt.2007.07.009

Bell, A. S., & Ranford-Cartwright, L. C. (2002). Real-time quantitative PCR in parasitology. *Trends in Parasitology, 18*(8), 338-342. doi:https://doi.org/10.1016/S1471-4922(02)02331-0

Benhammou, B., Balafrej, A., & Mikou, N. (1991). Mediterranean boutonneuse fever disclosed by severe neurological involvement. *Archives Francaises de Pediatrie, 48*(9), 635-636.

Bhat, U., & Yunker, C. (1977). Establishment and characterization of a diploid cell line from the tick, *Dermacentor parumapertus Neumann* (Acarina: Ixodidae). *The Journal of Parasitology*, 1092-1098. doi:https://doi.org/10.2307/3279853

Biggs, H. M., Behravesh, C. B., Bradley, K. K., Dahlgren, F. S., Drexler, N. A., Dumler, J. S., . . . Reports. (2016). Diagnosis and management of tickborne rickettsial diseases: Rocky Mountain spotted fever and other spotted fever group rickettsioses, ehrlichioses, and anaplasmosis—United States: A practical guide for health care and public health professionals. *65*(2), 1-44.

Bitsori, M., Galanakis, E., Gikas, A., Scoulica, E., & Sbyrakis, S. (2002). *Rickettsia typhi* infection in childhood. *Acta Paediatrica, 91*(1), 59-61. doi:https://doi.org/10.1111/j.1651-2227.2002.tb01641.x

Black, W., 4th, & Piesman, J. (1994). Phylogeny of hard-and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. *Proceedings of the National Academy of Sciences, 91*(21), 10034-10038. doi:https://doi.org/10.1073/pnas.91.21.10034

Blacksell, S. D., Robinson, M. T., Newton, P. N., Ruanchaimun, S., Salje, J., Wangrangsimakul, T., . . . Richards, A. L. (2019). Biosafety and biosecurity requirements for *Orientia* spp. diagnosis and research: recommendations for risk-based biocontainment, work practices and the case for reclassification to risk group 2. *BMC Infectious Diseases, 19*(1), 1044. doi:https://doi.org/10.1186/s12879-019-4653-4

Blouin, E. F., de la Fuente, J., Garcia-Garcia, J. C., Sauer, J. R., Saliki, J. T., & Kocan, K. M. (2002). Applications of a cell culture system for studying the interaction of *Anaplasma marginale* with tick cells. *Animal Health Research Reviews, 3*(2), 57. doi:https://doi.org/10.1079/AHRR200241

Blouin, E. F., & Kocan, K. M. (1998). Morphology and development of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in cultured *Ixodes scapularis* (Acari: Ixodidae) cells. *Journal of Medical Entomology, 35*(5), 788-797. doi:https://doi.org/10.1093/jmedent/35.5.788

Blouin, E. F., Saliki, J. T., de la Fuente, J., Garcia-Garcia, J. C., & Kocan, K. M. (2003). Antibodies to *Anaplasma marginale* major surface proteins 1a and 1b inhibit infectivity for cultured tick cells. *Veterinary Parasitology, 111*(2-3), 247-260. doi:https://doi.org/10.1016/S0304-4017(02)00378-3

Boldiš, V., & Špitalská, E. (2010). *Dermacentor marginatus* and *Ixodes ricinus* ticks versus L929 and Vero cell lines in *Rickettsia slovaca* life cycle evaluated by quantitative real time PCR. *Experimental and Applied Acarology, 50*(4), 353-359. doi:https://doi.org/10.1007/s10493-009-9322-7

Bonnet, S. I., Binetruy, F., Hernández-Jarguín, A. M., & Duron, O. (2017). The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. *Frontiers in Cellular and Infection Microbiology, 7*, 236. doi:https://doi.org/10.3389/fcimb.2017.00236

Boostrom, A., Beier, M. S., Macaluso, J. A., Macaluso, K. R., Sprenger, D., Hayes, J., . . . Azad, A. F. (2002). Geographic association of *Rickettsia felis*-infected opossums with human murine typhus, Texas. *Emerging Infectious Diseases, 8*(6), 549. doi:https://doi.org/10.3201/eid0806.010350

Bougteba, A., Basir, A., & Charradi, N. (2010). Meningoencephalitis caused by *Rickettsia conorii* in a young infant. *Revue Neurologique, 167*(2), 173-176. doi:https://doi.org/10.1016/j.neurol.2010.07.010

Brettman, L., Lewin, S., Holzman, R. S., Goldman, W. D., Marr, J. S., Kechijian, P., & Schinella, R. (1981). Rickettsialpox: report of an outbreak and a contemporary review. *Medicine, 60*(5), 363-372. doi:https://doi.org/10.1097/00005792-198109000-00004

Brezina, R., Rehácer, J., Ac, P., & Majerska, M. (1969). Two strains of rickettsiae of Rocky Mountain spotted fever group recovered from *Dermacentor marginatus* ticks in Czechoslovakia. Results of preliminary serological identification. *Acta Virologica, 13*(2), 142-145.

Brouqui, P., Parola, P., Fournier, P. E., & Raoult, D. (2007). Spotted fever rickettsioses in southern and eastern Europe. *FEMS Immunology & Medical Microbiology, 49*(1), 2-12. doi:https://doi.org/10.1111/j.1574-695X.2006.00138.x

Brown, L. D., & Macaluso, K. R. (2016). *Rickettsia felis*, an emerging flea-borne rickettsiosis. *Current Tropical Medicine Reports, 3*(2), 27-39. doi:https://doi.org/10.1007/s40475-016-0070-6

Bump, E. A., & Reed, D. J. (1977). A unique property of fetal bovine serum: high levels of protein-glutathione mixed disulfides. *In Vitro, 13*(2), 115-118. doi:https://doi.org/10.1007/BF02615075

Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E., & Davis, J. (1982). Lyme disease-a tick-borne spirochetosis? *Science, 216*(4552), 1317-1319. doi:https://doi.org/10.1126/science.7043737

Cabezas-Cruz, A., Espinosa, P. J., Obregón, D. A., Alberdi, P., & de la Fuente, J. (2017). *Ixodes scapularis* tick cells control *Anaplasma phagocytophilum* infection by increasing the synthesis of phosphoenolpyruvate from tyrosine. *Frontiers in Cellular and Infection Microbiology, 7*, 375. doi:https://doi.org/10.3389/fcimb.2017.00375

Cascio, A., Colomba, C., Di Rosa, D., Salsa, L., di Martino, L., & Titone, L. (2001). Efficacy and safety of clarithromycin as treatment for Mediterranean spotted fever in children: a randomized controlled trial. *Clinical Infectious Diseases, 33*(3), 409-411. doi:https://doi.org/10.1086/321864

Chapman, A. S. (2006). Diagnosis and management of tickborne rickettsial diseases; Rocky Mountain spotted fever, ehrlichioses, and anaplasmosis--United States: a practical guide for physicians and other health-care and public health professional.

Chen, A. C., He, H., & Davey, R. B. (2007). Mutations in a putative octopamine receptor gene in amitraz-resistant cattle ticks. *Veterinary Parasitology, 148*(3-4), 379-383. doi:https://doi.org/10.1016/j.vetpar.2007.06.026

Chisu, V., Leulmi, H., Masala, G., Piredda, M., Foxi, C., & Parola, P. (2017). Detection of *Rickettsia hoogstraalii*, *Rickettsia helvetica*, *Rickettsia massiliae*, *Rickettsia slovaca* and *Rickettsia aeschlimannii* in ticks from Sardinia, Italy. *Ticks and Tick-Borne Diseases, 8*(3), 347-352. doi:https://doi.org/10.1016/j.ttbdis.2016.12.007

Chmielewski, T., Podsiadly, E., Karbowiak, G., & Tylewska-Wierzbanowska, S. (2009). *Rickettsia* spp. in ticks, Poland. *Emerging Infectious Diseases, 15*(3), 486. doi:https://doi.org/10.3201/eid1503.080711

Colomba, C., Saporito, L., Polara, V. F., Rubino, R., & Titone, L. (2006). Mediterranean spotted fever: clinical and laboratory characteristics of 415 Sicilian children. *BMC Infectious Diseases, 6*(1), 1-5. doi:https://doi.org/10.1186/1471-2334-6-60

Contreras, M., Alberdi, P., Fernandez De Mera, I. G., Krull, C., Nijhof, A., Villar, M., & De La Fuente, J. (2017). Vaccinomics approach to the identification of candidate protective antigens for the control of tick vector infestations and *Anaplasma phagocytophilum* infection. *Frontiers in Cellular and Infection Microbiology, 7*, 360. doi:https://doi.org/10.3389/fcimb.2017.00360

Cossio-Bayugar, R., & Miranda-Miranda, E. (2007). Heterologus gene expression in a cattle tick *Rhipicephalus microplus* embryonic cell culture. *Journal of Animal and Veterinary Advances, 6*(10), 1214-1218.

Cossio-Bayugar, R., Wagner, G. G., & Holman, P. J. (2002). *In vitro* generation of organophosphate resistant *Boophilus microplus* (Acari: Ixodidae) cell lines. *Journal of Medical Entomology, 39*(2), 278-284. doi:https://doi.org/10.1603/0022-2585-39.2.278

Cowan, G. (2000). Rickettsial diseases: the typhus group of fevers—a review. *Postgraduate Medical Journal, 76*(895), 269-272. doi:http://dx.doi.org/10.1136/pmj.76.895.269

Cruickshank, R. (1927). The Weil-Felix reaction in typhus fever. *Journal of Hygiene*, 27(1), 64-69. doi:https://doi.org/10.1017/S0022172400031818

Cupp, E. W. (1991). Biology of ticks. *Veterinary Clinics of North America: Small Animal Practice, 21*(1), 1-26. doi:https://doi.org/10.1016/S0195-5616(91)50001-2

Da Silva, R. M., Della Noce, B., Waltero, C. F., Costa, E. P., De Abreu, L. A., Githaka, N. W. o., . . . Da Silva Vaz, I. (2015). Non-classical gluconeogenesis-dependent glucose metabolism in *Rhipicephalus microplus* embryonic cell line BME26. *International Journal of Molecular Sciences, 16*(1), 1821-1839. doi:https://doi.org/10.3390/ijms16011821

Dantas-Torres, F. (2007). Rocky Mountain spotted fever. *The Lancet Infectious Diseases, 7*(11), 724-732. doi:https://doi.org/10.1016/S1473-3099(07)70261-X

Dantas-Torres, F. (2008). The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806)(Acari: Ixodidae): from taxonomy to control. *Veterinary Parasitology, 152*(3-4), 173-185. doi:https://doi.org/10.1016/j.vetpar.2007.12.030

Dantas-Torres, F. (2010). Biology and ecology of the brown dog tick, *Rhipicephalus sanguineus*. *Parasites & Vectors, 3*(1), 1-11. doi:https://doi.org/10.1186/1756-3305-3-26

Dautel, H., Dippel, C., Oehme, R., Hartelt, K., & Schettler, E. (2006). Evidence for an increased geographical distribution of *Dermacentor reticulatus* in Germany and detection of *Rickettsia* sp. *RpA4*. *International Journal of Medical Microbiology, 296*, 149-156. doi:https://doi.org/10.1016/j.ijmm.2006.01.013

de Abreu, L. A., Calixto, C., Waltero, C. F., Della Noce, B. P., Githaka, N. W. o., Seixas, A., . . . Ohashi, K. (2013). The conserved role of the AKT/GSK3 axis in cell survival and glycogen metabolism in *Rhipicephalus (Boophilus) microplus* embryo tick cell line BME26. *Biochimica et Biophysica Acta -General Subjects, 1830*(3), 2574-2582. doi:https://doi.org/10.1016/j.bbagen.2012.12.016

de la Fuente, J., Almazán, C., Canales, M., de la Lastra, J. M. P., Kocan, K. M., & Willadsen, P. (2007a). A ten-year review of commercial vaccine performance for

control of tick infestations on cattle. *Animal Health Research Reviews, 8*(1), 23-28. doi:https://doi.org/10.1017/S1466252307001193

de la Fuente, J., Blouin, E. F., Manzano-Roman, R., Naranjo, V., Almazán, C., de la Lastra, J. M. P., . . . Kocan, K. M. (2007b). Functional genomic studies of tick cells in response to infection with the cattle pathogen, *Anaplasma marginale*. *Genomics, 90*(6), 712-722. doi:https://doi.org/10.1016/j.ygeno.2007.08.009

de la Fuente, J., Estrada-Pena, A., Venzal, J. M., Kocan, K. M., & Sonenshine, D. E. (2008). Overview: ticks as vectors of pathogens that cause disease in humans and animals. *Frontiers in Bioscience, 13*(13), 6938-6946. doi:https://doi.org/10.2741/3200

de la Fuente, J., Garcia-Garcia, J. C., Blouin, E. F., Saliki, J. T., & Kocan, K. M. (2002). Infection of tick cells and bovine erythrocytes with one genotype of the intracellular ehrlichia *Anaplasma marginale* excludes infection with other genotypes. *Clinical and Diagnostic Laboratory Immunology, 9*(3), 658-668. doi:https://doi.org/10.1128/CDLI.9.3.658-668.2002

Diop, A., Raoult, D., & Fournier, P.-E. (2019). Paradoxical evolution of rickettsial genomes. *Ticks and Tick-Borne Diseases, 10*(2), 462-469. doi:https://doi.org/10.1016/j.ttbdis.2018.11.007

Dolado, M., & JC, P. N. (1994). Meningoencephalitis due to *Rickettsia conorii*. Etiopathological, clinical and diagnostic aspects. *Neurologia, 9*(2), 72-75.

Dong, Z., Yang, Y., Wang, Q., Xie, S., Zhao, S., Tan, W., . . . Wang, Y. (2019). A case with neurological abnormalities caused by *Rickettsia raoultii* in northwestern China. *BMC Infectious Diseases, 19*(1), 1-5. doi:https://doi.org/10.1186/s12879-019-4414-4

Droleskey, R., Holman, P., & Wagner, G. (1981). *Babesia-bovis* infection of a tick cell-line studied by electron-microscopy *In Vitro, 17*(3), 209-209.

Duque, V., Ventura, C., Seixas, D., da Cunha, S., Meliço-Silvestre, A., Barai, A., . . . Martins, J. (2012). Mediterranean spotted fever and encephalitis: a case report and review of the literature. *Journal of Infection and Chemotherapy, 18*(1), 105-108. doi:https://doi.org/10.1007/s10156-011-0295-1

Duscher, G. G., Feiler, A., Leschnik, M., & Joachim, A. (2013). Seasonal and spatial distribution of ixodid tick species feeding on naturally infested dogs from Eastern Austria and the influence of acaricides/repellents on these parameters. *Parasites & Vectors, 6*(1), 1-8. doi:https://doi.org/10.1186/1756-3305-6-76

Duscher, G. G., Hodžić, A., Weiler, M., Vaux, A. G., Rudolf, I., Sixl, W., . . . Hubálek, Z. (2016). First report of *Rickettsia raoultii* in field collected *Dermacentor reticulatus* ticks from Austria. *Ticks and Tick-Borne Diseases, 7*(5), 720-722. doi:https://doi.org/10.1016/j.ttbdis.2016.02.022

Edouard, S., Bhengsri, S., Dowell, S. F., Watt, G., Parola, P., & Raoult, D. (2014). Two human cases of *Rickettsia felis* infection, Thailand. *Emerging Infectious Diseases, 20*(10), 1780. doi:https://doi.org/10.3201/eid2010.140905

Elfving, K., Lukinius, A., & Nilsson, K. (2012). Life cycle, growth characteristics and host cell response of *Rickettsia helvetica* in a Vero cell line. *Experimental and Applied Acarology, 56*(2), 179-187. doi:https://doi.org/10.1007/s10493-011-9508-7

Eremeeva, M., Balayeva, N., Ignatovich, V., & Raoult, D. (1995). Genomic study of *Rickettsia akari* by pulsed-field gel electrophoresis. *Journal of Clinical*

Microbiology, 33(11), 3022-3024. doi:https://doi.org/10.1128/jcm.33.11.3022-3024.1995

Eremeeva, M. E., Bosserman, E. A., Demma, L. J., Zambrano, M. L., Blau, D. M., & Dasch, G. A. (2006). Isolation and identification of *Rickettsia massiliae* from *Rhipicephalus sanguineus* ticks collected in Arizona. *Applied and Environmental Microbiology, 72*(8), 5569-5577. doi:https://doi.org/10.1128/AEM.00122-06

Eremeeva, M. E., & Dasch, G. A. (2015). Challenges posed by tick-borne rickettsiae: eco-epidemiology and public health implications. *Frontiers in Public Health, 3*, 55. doi:https://doi.org/10.3389/fpubh.2015.00055

Eremeeva, M. E., Dasch, G. A., & Silverman, D. J. J. J. o. c. m. (2003). Evaluation of a PCR assay for quantitation of *Rickettsia rickettsii* and closely related spotted fever group rickettsiae. *Journal of Clinical Microbiology, 41*(12), 5466-5472. doi:10.1128/JCM.41.12.5466–5472.2003

Estrada-Peña. (2015). Ticks as vectors: taxonomy, biology and ecology. *Revision Science Technology, 34*(1), 53-65. doi:https://doi.org/10.20506/rst.34.1.2345

Estrada-Peña, A., Bouattour, A., Camicas, J., & Walker, A. (2004). Ticks of domestic animals in the Mediterranean region. *University of Zaragoza, Spain, 131*.

Ezpeleta, D., Munoz-Blanco, J., Tabernero, C., & Gimenez-Roldan, S. (1999). Neurological complications of Mediterranean boutonneuse fever. Presentation of a case of acute encephalomeningomyelitis and review of the literature. *Neurologia, 14*(1), 38-42.

Fenollar, F., Maurin, M., & Raoult, D. (2003). *Wolbachia pipientis* growth kinetics and susceptibilities to 13 antibiotics determined by immunofluorescence staining and

real-time PCR. *Antimicrobial Agents and Chemotherapy, 47*(5), 1665-1671. doi:10.1128/AAC.47.5.1665–1671.2003

Földvári, G., Rigó, K., & Lakos, A. (2013). Transmission of *Rickettsia slovaca* and *Rickettsia raoultii* by male *Dermacentor marginatus* and *Dermacentor reticulatus* ticks to humans. *Diagnostic Microbiology and Infectious Disease, 76*(3), 387-389. doi:https://doi.org/10.1016/j.diagmicrobio.2013.03.005

Fournier, P.-E., Dumler, J. S., Greub, G., Zhang, J., Wu, Y., & Raoult, D. (2003). Gene sequence-based criteria for identification of new *rickettsia* isolates and description of *Rickettsia heilongjiangensis* sp. nov. *Journal of Clinical Microbiology, 41*(12), 5456-5465. doi:https://doi.org/10.1128/JCM.41.12.5456-5465.2003

Fournier, P.-E., El Karkouri, K., Leroy, Q., Robert, C., Giumelli, B., Renesto, P., . . . Raoult, D. (2009a). Analysis of the *Rickettsia africae* genome reveals that virulence acquisition in *Rickettsia* species may be explained by genome reduction. *BMC Genomics, 10*(1), 1-15. doi:https://doi.org/10.1186/1471-2164-10-166.

Fournier, P.-E., Gouriet, F., Brouqui, P., Lucht, F., & Raoult, D. (2005). Lymphangitis-associated rickettsiosis, a new rickettsiosis caused by *Rickettsia sibirica mongolotimonae*: seven new cases and review of the literature. *Clinical Infectious Diseases, 40*(10), 1435-1444. doi:https://doi.org/10.1086/429625

Fournier, P.-E., Jensenius, M., Laferl, H., Vene, S., & Raoult, D. (2002). Kinetics of antibody responses in *Rickettsia africae* and *Rickettsia conorii* infections. *Clinical and Vaccine Immunology, 9*(2), 324-328. doi:https://doi.org/10.1128/CDLI.9.2.324-328.2002.

Fournier, P.-E., & Raoult, D. (2004). Suicide PCR on skin biopsy specimens for diagnosis of rickettsioses. *Journal of Clinical Microbiology, 42*(8), 3428-3434. doi:https://doi.org/10.1128/JCM.42.8.3428-3434.2004

Fournier, P.-E., & Raoult, D. (2009b). Current knowledge on phylogeny and taxonomy of *Rickettsia* spp. *Annals of the New York Academy of Sciences*. doi:https://doi.org/10.1111/j.1749-6632.2009.04528.x

Fujisawa, T., Kadosaka, T., Fujita, H., Ando, S., Takano, A., Ogasawara, Y., . . . Seishima, M. (2012). *Rickettsia africae* infection in a Japanese traveller with many tick bites. *Acta Dermato-Venereologica, 92*(4), 443-444. doi:https://doi.org/10.2340/00015555-1313

Galay, R. L., Umemiya-Shirafuji, R., Mochizuki, M., Fujisaki, K., & Tanaka, T. (2015). Iron metabolism in hard ticks (Acari: Ixodidae): The antidote to their toxic diet. *Parasitology International, 64*(2), 182-189. doi:https://doi.org/10.1016/j.parint.2014.12.005

Giangaspero, A., Marangi, M., Papini, R., Paoletti, B., Wijnveld, M., & Jongejan, F. (2015). *Theileria* sp. OT3 and other tick-borne pathogens in sheep and ticks in Italy: molecular characterization and phylogeny. *Ticks and Tick-Borne Diseases, 6*(1), 75-83. doi:https://doi.org/10.1016/j.ttbdis.2014.09.007

Gillespie, J. J., Beier, M. S., Rahman, M. S., Ammerman, N. C., Shallom, J. M., Purkayastha, A., . . . Azad, A. F. (2007). Plasmids and rickettsial evolution: insight from *Rickettsia felis*. *PLoS One, 2*(3), e266. doi:https://doi.org/10.1371/journal.pone.0000266

Giménez, D. F. (1964). Staining rickettsiae in yolk-sac cultures. *Stain Technology, 39*(3), 135-140. doi:https://doi.org/10.3109/10520296409061219.

Gomes, H., Romeiro, N. C., Braz, G. R., de Oliveira, E. A. G., Rodrigues, C., da Fonseca, R. N., . . . Ohashi, K. (2013). Identification and structural-functional analysis of cyclin-dependent kinases of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*. *PLoS One, 8*(10), e76128. doi:https://doi.org/10.1371/journal.pone.0076128

Guo, L.-P., Mu, L.-M., Xu, J., Jiang, S.-H., Wang, A.-D., Chen, C.-F., . . . Wang, Y.-Z. (2015). *Rickettsia raoultii* in *Haemaphysalis erinacei* from marbled polecats, China–Kazakhstan border. *Parasites & Vectors, 8*(1), 1-3. doi:https://doi.org/10.1186/s13071-015-1065-1

Harrison, N., Burgmann, H., Forstner, C., Ramharter, M., Széll, M., Schötta, A. M., . . . Markowicz, M. (2016). Molecular diagnosis of African tick bite fever using eschar swabs in a traveller returning from Tanzania. *Wiener Klinische Wochenschrift, 128*(15), 602-605. doi:https://doi.org/10.1007/s00508-016-1047-0

Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). Real time quantitative PCR. *Genome Research, 6*(10), 986-994. doi:https://doi.org/10.1101/gr.6.10.986

Helmick, C. G., Bernard, K. W., & D'Angelo, L. J. (1984). Rocky Mountain spotted fever: clinical, laboratory, and epidemiological features of 262 cases. *The Journal of Infectious Diseases, 150*(4), 480-488. doi:https://doi.org/10.1093/infdis/150.4.480

Henry, K. M., Jiang, J., Rozmajzl, P. J., Azad, A. F., Macaluso, K. R., & Richards, A. L. (2007). Development of quantitative real-time PCR assays to detect *Rickettsia typhi* and *Rickettsia felis*, the causative agents of murine typhus and flea-borne spotted fever. *Molecular and Cellular Probes, 21*(1), 17-23. doi:https://doi.org/10.1016/j.mcp.2006.06.002

Hernandez, E. P., Kusakisako, K., Talactac, M. R., Galay, R. L., Yoshii, K., & Tanaka, T. (2018). Induction of intracellular ferritin expression in embryo-derived *Ixodes scapularis* cell line (ISE6). *Scientific Reports, 8*(1), 1-12. doi:https://doi.org/10.1038/s41598-018-34860-3

Herrin, B., Mahapatra, S., Blouin, E. F., & Shaw, E. I. (2011). Growth of *Coxiella burnetii* in the *Ixodes scapularis*–derived IDE8 tick cell line. *Vector-Borne and Zoonotic Diseases*, *11*(7), 917-922. doi:https://doi.org/10.1089/vbz.2010.0126

Hodžić, A., Fuehrer, H. P., & Duscher, G. (2017). First molecular evidence of zoonotic bacteria in ticks in Bosnia and Herzegovina. *Transboundary and Emerging Diseases, 64*(4), 1313-1316. doi:https://doi.org/10.1111/tbed.12473

Hofmeester, T. R., van der Lei, P.-B., Van Leeuwen, A. D., Sprong, H., & van Wieren, S. E. (2016). New foci of *Haemaphysalis punctata* and *Dermacentor reticulatus* in the Netherlands. *Ticks and Tick-Borne Diseases, 7*(2), 367-370. doi:https://doi.org/10.1016/j.ttbdis.2015.12.009

Holman, P., & Onald, N. (1980). A new tick cell line derived from *Boophilus microplus*. *Research in Veterinary Science, 29*(3), 383-387. doi:https://doi.org/10.1016/S0034-5288(18)32647-X

Holman, P. J. (1981). Partial characterization of a unique female diploid cell strain from the tick *Boophilus microplus* (Acari: Ixodidae). *Journal of Medical Entomology, 18*(1), 84-88. doi:https://doi.org/10.1093/jmedent/18.1.84

Honda, E., Tanioka, M., & Honda, T. (2003). Nine cases of Japanese spotted fever, including 3 cases with DIC and MOF treated at ICU. *Rinsho Hihu, 57*, 523-526.

Horney, L. F., & Walker, D. H. (1988). Meningoencephalitis as a major manifestation of Rocky Mountain spotted fever. *Southern Medical Journal, 81*(7), 915-918. doi:https://doi.org/10.1097/00007611-198807000-00028

Huebner, R. J., Jellison, W. L., & Pomerantz, C. (1946). Rickettsialpox: A newly recognized rickettsial disease: IV. Isolation of a *rickettsia* apparently identical with the causative agent of rickettsialpox from *Allodermanyssus sanguineus*, a rodent mite. *Public Health Reports*, 1677-1682. doi:https://doi.org/10.2307/4585913

Husin, N. A., AbuBakar, S., & Khoo, J. J. (2021a). Current tools for the diagnosis and detection of spotted fever group *Rickettsia*. *Acta Tropica*, 105887. doi:https://doi.org/10.1016/j.actatropica.2021.105887

Husin, N. A., Khoo, J. J., Zulkifli, M. M. S., Bell-Sakyi, L., & AbuBakar, S. (2021b). Replication kinetics of *Rickettsia raoultii* in tick cell lines. *Microorganisms, 9*(7), 1370. doi:https://doi.org/10.3390/microorganisms9071370

Igolkina, Y., Krasnova, E., Rar, V., Savelieva, M., Epikhina, T., Tikunov, A., . . . Tikunova, N. (2018). Detection of causative agents of tick-borne rickettsioses in Western Siberia, Russia: identification of *Rickettsia raoultii* and *Rickettsia sibirica* DNA in clinical samples. *Clinical Microbiology and Infection, 24*(2), 199. e199-199. e112. doi:https://doi.org/10.1016/j.cmi.2017.06.003

Irwin, P. J., & Jefferies, R. (2004). Arthropod-transmitted diseases of companion animals in Southeast Asia. *Trends in Parasitology, 20*(1), 27-34. doi:https://doi.org/10.1016/j.pt.2003.11.004

Jensenius, M., Fournier, P.-E., Fladby, T., Block Hellum, K., Hagen, T., Priø, T., . . . Myrvang, B. (2006). Sub-acute neuropathy in patients with African tick bite fever.

Scandinavian Journal of Infectious Diseases, 38(2), 114-118. doi:https://doi.org/10.1080/00365540500321579

Jia, N., Zheng, Y.-C., Ma, L., Huo, Q.-B., Ni, X.-B., Jiang, B.-G., . . . Cao, W.-C. (2014). Human infections with *Rickettsia raoultii*, China. *Emerging Infectious Diseases, 20*(5), 866. doi:https://doi.org/10.3201/eid2005.130995

Jiang, J., Chan, T.-C., Temenak, J. J., Dasch, G. A., Ching, W.-M., & Richards, A. L. (2004). Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. *The American Journal of Tropical Medicine and Hygiene, 70*(4), 351-356. doi:https://doi.org/10.4269/ajtmh.2004.70.351

Jiang, J., Sangkasuwan, V., Lerdthusnee, K., Sukwit, S., Chuenchitra, T., Rozmajzl, P. J., . . . Richards, A. L. (2005). Human infection with *Rickettsia honei*, Thailand. *Emerging Infectious Diseases, 11*(9), 1473. doi:https://doi.org/10.3201/eid1109.050011

Jiang, J., Temenak, J. J., & Richards, A. L. (2003). Real-time PCR duplex assay for *Rickettsia prowazekii* and *Borrelia recurrentis*. *Annals of the New York Academy of Sciences, 990*(1), 302-310. doi:https://doi.org/10.1111/j.1749-6632.2003.tb07380.x

Jiang, J., You, B. J., Liu, E., Apte, A., Yarina, T. R., Myers, T. E., . . . Tsertsvadze, N. (2012). Development of three quantitative real-time PCR assays for the detection of *Rickettsia raoultii*, *Rickettsia slovaca*, and *Rickettsia aeschlimannii* and their validation with ticks from the country of Georgia and the Republic of Azerbaijan. *Ticks and Tick-borne Diseases, 3*(5-6), 327-331. doi:doi:10.1016/j.ttbdis.2012.10.004

Jongejan, F., Ringenier, M., Putting, M., Berger, L., Burgers, S., Kortekaas, R., . . . Madder, M. (2015). Novel foci of *Dermacentor reticulatus* ticks infected with *Babesia canis* and *Babesia caballi* in the Netherlands and in Belgium. *Parasites & Vectors, 8*(1), 1-10. doi:https://doi.org/10.1186/s13071-015-0841-2

Jongejan, F., & Uilenberg, G. (2004a). The global importance of ticks. *Parasitology, 129*(S1), S3-S14.

Jongejan, F., & Uilenberg, G. (2004b). The global importance of ticks. *Parasitology, 129*, S3. doi:https://doi.org/10.1017/S0031182004005967

Joshi, S. G., & Kovács, A. D. (2007). *Rickettsia rickettsii* infection causes apoptotic death of cultured cerebellar granule neurons. *Journal of Medical Microbiology, 56*(1), 138-141. doi:https://doi.org/10.1099/jmm.0.46826-0

Kass, E. M., Szaniawski, W. K., Levy, H., Leach, J., Srinivasan, K., & Rives, C. (1994). Rickettsialpox in a New York City hospital, 1980 to 1989. *New England Journal of Medicine, 331*(24), 1612-1617. doi:https://doi.org/10.1056/NEJM199412153312403

Kenny, M. J., Birtles, R. J., Day, M. J., & Shaw, S. E. (2003). *Rickettsia felis* in the United Kingdom. *Emerging Infectious Diseases, 9*(8), 1023. doi:https://doi.org/10.3201/eid0908.030314

Kho, K. L., Koh, F. X., Hasan, L. I. M., Wong, L. P., Kisomi, M. G., Bulgiba, A., . . . Tay, S. T. (2017). Rickettsial seropositivity in the indigenous community and animal farm workers, and vector surveillance in Peninsular Malaysia. *Emerging Microbes & Infections, 6*(1), 1-9. doi:https://doi.org/10.1038/emi.2017.4

Khoo, J. J., Husin, N. A., Lim, F. S., Oslan, S. N. H., Azami, S. N. I. M., To, S. W., . . . Khor, C. S. (2021). Molecular detection of pathogens from ectoparasites recovered from peri-domestic animals, and the first description of a *Candidatus* Midichloria sp. from *Haemaphysalis wellingtoni* from rural communities in Malaysia. *Parasitology International, 80*, 102202. doi:https://doi.org/10.1016/j.parint.2020.102202

Kirk, J. L., Fine, D. P., Sexton, D. J., & Muchmore, H. G. (1990). Rocky Mountain spotted fever. A clinical review based on 48 confirmed cases, 1943-1986. *Medicine, 69*(1), 35-45.

Klitgaard, K., Chriél, M., Isbrand, A., Jensen, T. K., & Bødker, R. (2017). Identification of *Dermacentor reticulatus* ticks carrying *Rickettsia raoultii* on migrating jackal, Denmark. *Emerging Infectious Diseases, 23*(12), 2072. doi:https://doi.org/10.3201/eid2312.170919

Kodama, K., Noguchi, T., & Chikahira, Y. (2000). Japanese spotted fever complicated by acute respiratory failure. *Kansenshogaku zasshi. The Journal of the Japanese Association for Infectious Diseases, 74*(2), 162-165. doi:https://doi.org/10.11150/kansenshogakuzasshi1970.74.162

Kodama, K., Senba, T., Yamauchi, H., Chikahira, Y., & Fujita, H. (2001). Japanese spotted fever associated with multiorgan failure. *Journal of Infection and Chemotherapy, 7*(4), 247-250. doi:https://doi.org/10.1007/s101560170021

Kodama, K., Senba, T., Yamauchi, H., Chikahira, Y., Katayama, T., Furuya, Y., . . . Yamamoto, S. (2002). Fulminant Japanese spotted fever definitively diagnosed by the polymerase chain reaction method. *Journal of Infection and Chemotherapy, 8*(3), 266-268. doi:https://doi.org/10.1007/s10156-002-0185-7

Koh-Tan, H. C., Strachan, E., Cooper, K., Bell-Sakyi, L., & Jonsson, N. N. (2016). Identification of a novel β-adrenergic octopamine receptor-like gene (βAOR-like) and increased ATP-binding cassette B10 (ABCB10) expression in a *Rhipicephalus microplus* cell line derived from acaricide-resistant ticks. *Parasites & Vectors, 9*(1), 1-11. doi:https://doi.org/10.1186/s13071-016-1708-x

Kopecky, J., & Stankova, I. (1998). Interaction of virulent and attenuated tick-borne encephalitis virus strains in ticks and a tick cell line. *Folia Parasitologica, 45*(3), 245-250.

Kulkarni, A. (2011). Childhood rickettsiosis. *The Indian Journal of Pediatrics, 78*(1), 81-87. doi:https://doi.org/10.1007/s12098-010-0255-2

Kuriakose, J. A., Miyashiro, S., Luo, T., Zhu, B., & McBride, J. W. (2011). *Ehrlichia chaffeensis* transcriptome in mammalian and arthropod hosts reveals differential gene expression and post transcriptional regulation. *PLoS One, 6*(9), e24136. doi:https://doi.org/10.1371/journal.pone.0024136

Kurtti, T., Felsheim, R., Mattila, J., Baldridge, G., Burkhardt, N., & Munderloh, U. (2006). Stable transformation of a tick (*Ixodes scapularis*) cell line with the Sleeping Beauty transposon system. *In Vitro Cellular & Developmental Biology-Animal, 42*, 32A-32A.

Kurtti, T., Munderloh, U., & Ahlstrand, G. (1988). Tick tissue and cell culture in vector research. *Advances in Diseases Vector Research, 5*, 87-109.

Kurtti, T., Munderloh, U., & Stiller, D. (1983). The interaction of *Babesia caballi* kinetes with tick cells. *Journal of Invertebrate Pathology, 42*(3), 334-343. doi:https://doi.org/10.1016/0022-2011(83)90172-6

Kurtti, T. J., Felsheim, R. F., Burkhardt, N. Y., Oliver, J. D., Heu, C. C., & Munderloh, U. G. (2015). *Rickettsia buchneri* sp. nov., a rickettsial endosymbiont of the blacklegged tick *Ixodes scapularis*. *International Journal of Systematic and Evolutionary Microbiology, 65*(Pt 3), 965. doi:https://doi.org/10.1099/ijs.0.000047

Kurtti, T. J., Munderloh, U. G., Ahlstrand, G. G., & Johnson, R. C. (1988). *Borrelia burgdorferi* in tick cell culture: growth and cellular adherence. *Journal of Medical Entomology, 25*(4), 256-261. doi:https://doi.org/10.1093/jmedent/25.4.256

Kurtti, T. J., Munderloh, U. G., & Samish, M. (1982). Effect of medium supplements on tick cells in culture. *The Journal of Parasitology*, 930-935. doi:https://doi.org/10.2307/3281008

Kurtti, T. J., Mundertoh, U., Andreadis, T. G., & Magnarelli, L. A. (1996). Tick cell culture isolation of an intracellular prokaryote from the tick *Ixodes scapularis*. *Journal of Invertebrate Pathology*. doi:https://doi.org/10.1006/jipa.1996.0050

Kurtti, T. J., Simser, J. A., Baldridge, G. D., Palmer, A. T., & Munderloh, U. G. (2005). Factors influencing *in vitro* infectivity and growth of *Rickettsia peacockii* (Rickettsiales: Rickettsiaceae), an endosymbiont of the Rocky Mountain wood tick, *Dermacentor andersoni* (Acari, Ixodidae). *Journal of Invertebrate Pathology, 90*(3), 177-186. doi:https://doi.org/10.1016/j.jip.2005.09.001

Labruna, M. B., Whitworth, T., Horta, M. C., Bouyer, D. H., McBride, J. W., Pinter, A., . . . Walker, D. H. (2004). *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of Sao Paulo, Brazil, where Brazilian spotted fever is endemic. *Journal of Clinical Microbiology, 42*(1), 90-98. doi:https://doi.org/10.1128/JCM.42.1.90-98.2004

Labuda, M., & Nuttall, P. (2004). Tick-borne viruses. *Parasitology, 129*(S1), S221. doi:https://doi.org/10.1017/s0031182004005220

Lakos, A. (1997). Tick-borne lymphadenopathy–a new rickettsial disease? *The Lancet, 350*(9083), 1006. doi:https://doi.org/10.1016/S0140-6736(05)64072-X

Lakos, A. (2002). Tick-borne lymphadenopathy (TIBOLA). *Wiener Klinische Wochenschrift, 114*(13/14), 648-653.

Lakos, A., Kőrösi, Á., & Földvári, G. (2012). Contact with horses is a risk factor for tick-borne lymphadenopathy (TIBOLA): a case control study. *Wiener Klinische Wochenschrift, 124*(17), 611-617. doi:https://doi.org/10.1007/s00508-012-0217-y

Lawrie, C., Uzcátegui, N., Armesto, M., Bell-Sakyi, L., & Gould, E. (2004). Susceptibility of mosquito and tick cell lines to infection with various flaviviruses. *Medical and Veterinary Entomology, 18*(3), 268-274. doi:https://doi.org/10.1111/j.0269-283X.2004.00505.x

Lempereur, L., De Cat, A., Caron, Y., Madder, M., Claerebout, E., Saegerman, C., & Losson, B. (2011). First molecular evidence of potentially zoonotic *Babesia microti* and *Babesia* sp. EU1 in *Ixodes ricinus* ticks in Belgium. *Vector-Borne and Zoonotic Diseases, 11*(2), 125-130. doi:https://doi.org/10.1089/vbz.2009.0189

Lim, F. S., Khoo, J. J., Tan, K. K., Zainal, N., Loong, S. K., Khor, C. S., & AbuBakar, S. (2020). Bacterial communities in *Haemaphysalis*, *Dermacentor* and *Amblyomma* ticks collected from wild boar of an Orang Asli Community in Malaysia. *Ticks and Tick-Borne Diseases, 11*(2), 101352. doi:https://doi.org/10.1016/j.ttbdis.2019.101352

Lindblom, A., Severinson, K., & Nilsson, K. (2010). *Rickettsia felis* infection in Sweden: report of two cases with subacute meningitis and review of the literature. *Scandinavian Journal of Infectious Diseases, 42*(11-12), 906-909. doi:https://doi.org/10.3109/00365548.2010.508466

Litchfield, T., Mackenzie, C., & Varma, M. (1991). The conditions required for the maintenance of *Onchocerca lienalis* microfilariae *in vitro*. *Acta Tropica, 48*(4), 293-304. doi:https://doi.org/10.1016/0001-706X(91)90017-E

Liu, D., Wang, Y.-Z., Zhang, H., Liu, Z.-Q., Wureli, H.-z., Wang, S.-W., . . . Chen, C.-F. (2016). First report of *Rickettsia raoultii* and *R. slovaca* in *Melophagus ovinus*, the sheep ked. *Parasites & Vectors, 9*(1), 1-6. doi:https://doi.org/10.1186/s13071-016-1885-7

Low, V. L., Tay, S. T., Kho, K. L., Koh, F. X., Tan, T. K., Lim, Y. A. L., . . . Sofian-Azirun, M. (2015). Molecular characterisation of the tick *Rhipicephalus microplus* in Malaysia: new insights into the cryptic diversity and distinct genetic assemblages throughout the world. *Parasites & Vectors, 8*(1), 1-10. doi:https://doi.org/10.1186/s13071-015-0956-5

Luce-Fedrow, A., Mullins, K., Kostik, A. P., St John, H. K., Jiang, J., & Richards, A. L. (2015). Strategies for detecting rickettsiae and diagnosing rickettsial diseases. *Future Microbiology, 10*(4), 537-564. doi:https://doi.org/10.2217/fmb.14.141

Lynen, G., Zeman, P., Bakuname, C., Di Giulio, G., Mtui, P., Sanka, P., & Jongejan, F. (2007). Cattle ticks of the genera *Rhipicephalus* and *Amblyomma* of economic importance in Tanzania: distribution assessed with GIS based on an extensive field survey. *Experimental and Applied Acarology, 43*(4), 303-319. doi:https://doi.org/10.1007/s10493-007-9123-9

Machado-Ferreira, E., Balsemao-Pires, E., Dietrich, G., Hojgaard, A., Vizzoni, V. F., Scoles, G., . . . Soares, C. A. (2015). Transgene expression in tick cells using *Agrobacterium tumefaciens*. *Experimental and Applied Acarology, 67*(2), 269-287. doi:https://doi.org/10.1007/s10493-015-9949-5

Mahara, F., Koga, K., Sawada, S., Shigemi, F., Suto, T., Tsuboi, Y., . . . Uchiyama, T. (1985). The first report of the rickettsial infections of spotted fever group in Japan; Three clinical cases. *The Journal of the Japanese Association for Infectious Diseases, 59*(11), 1165-1172. doi:https://doi.org/10.11150/kansenshogakuzasshi1970.59.1165

Mangia, C., Vismarra, A., Kramer, L., Bell-Sakyi, L., Porretta, D., Otranto, D., . . . Grandi, G. (2016). Evaluation of the *in vitro* expression of ATP binding-cassette (ABC) proteins in an *Ixodes ricinus* cell line exposed to ivermectin. *Parasites & Vectors, 9*(1), 1-5. doi:https://doi.org/10.1186/s13071-016-1497-2

Marcelino, I., Veríssimo, C., Sousa, M. F., Carrondo, M. J., & Alves, P. M. (2005). Characterization of *Ehrlichia ruminantium* replication and release kinetics in endothelial cell cultures. *Veterinary Microbiology, 110*(1-2), 87-96. doi:https://doi.org/10.1016/j.vetmic.2005.07.012

Marrie, T. J., & Raoult, D. (1992). Rickettsial infections of the central nervous system. *Seminars in Neurology, 12*(03), 213-224. doi:https://doi.org/10.1055/s-2008-1041178

Martinez, J. J., & Cossart, P. (2004). Early signaling events involved in the entry of *Rickettsia conorii* into mammalian cells. *Journal of Cell Science, 117*(21), 5097-5106. doi:https://doi.org/10.1242/jcs.01382

Massey, E., Thames, T., Coffey, C., & Gallis, H. (1985). Neurologic complications of Rocky Mountain spotted fever. *Southern Medical Journal, 78*(11), 1288-1290, 1303. doi:https://doi.org/10.1097/00007611-198511000-00004

Mediannikov, O., Matsumoto, K., Samoylenko, I., Drancourt, M., Roux, V., Rydkina, E., . . . Fournier, P.-E. (2008). *Rickettsia raoultii* sp. nov., a spotted fever group *rickettsia* associated with *Dermacentor* ticks in Europe and Russia. *International Journal of Systematic and Evolutionary Microbiology, 58*(7), 1635-1639. doi:https://doi.org/10.1099/ijs.0.64952-0

Merhej, V., Angelakis, E., Socolovschi, C., & Raoult, D. (2014). Genotyping, evolution and epidemiological findings of *Rickettsia* species. *Infection, Genetics and Evolution, 25*, 122-137. doi:https://doi.org/10.1016/j.meegid.2014.03.014

Merhej, V., & Raoult, D. (2011). Rickettsial evolution in the light of comparative genomics. *Biological Reviews, 86*(2), 379-405. doi:https://doi.org/10.1111/j.1469-185X.2010.00151.x

Mosqueda, J., Cossío-Bayugar, R., Rodríguez, E., Falcón, A., Ramos, A., Figueroa, J. V., & Alvarez, A. (2008). Primary midgut, salivary gland, and ovary cultures from *Boophilus microplus*. *Annals of the New York Academy of Sciences, 1149*(1), 49-52. doi:https://doi.org/10.1196/annals.1428.050

Mouffok, N., Socolovschi, C., Benabdellah, A., Renvoise, A., Parola, P., & Raoult, D. (2011). Diagnosis of rickettsioses from eschar swab samples, Algeria. *Emerging Infectious Diseases, 17*(10), 1968. doi:https://doi.org/10.3201/eid1710.110332.

Moy, W. L., & Ooi, S. T. (2015). Case report: Abducens nerve palsy and meningitis by *Rickettsia typhi*. *The American Journal of Tropical Medicine and Hygiene, 92*(3), 620. doi:https://doi.org/10.4269/ajtmh.14-0559

Mullis, K. B. (1990). The unusual origin of the polymerase chain reaction. *Scientific American, 262*(4), 56-65. doi:https://doi.org/10.1038/scientificamerican0490-56

Munderloh, U., Kurtti, T., & Maramorosch, K. (1982). *Anopheles stephensi* and *Toxorhynchites amboinensis*: aseptic rearing of mosquito larvae on cultured cells. *The Journal of Parasitology*, 1085-1091. doi:https://doi.org/10.2307/3281096

Munderloh, U. G., Blouin, E. F., Kocan, K. M., Ge, N. L., Edwards, W. L., & Kurtti, T. J. (1996). Establishment of the tick (Acari: Ixodidae)-borne cattle pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in tick cell culture. *Journal of Medical Entomology, 33*(4), 656-664. doi:https://doi.org/10.1093/jmedent/33.4.656

Munderloh, U. G., Hayes, S. F., Cummings, J., & Kurtti, T. J. (1998). Microscopy of spotted fever *rickettsia* movement through tick cells. *Microscopy and Microanalysis, 4*(2), 115-121. doi:https://doi.org/10.1017/S1431927698980096

Munderloh, U. G., Jauron, S. D., Fingerle, V., Leitritz, L., Hayes, S. F., Hautman, J. M., . . . Ahlstrand, G. G. (1999). Invasion and intracellular development of the human granulocytic ehrlichiosis agent in tick cell culture. *Journal of Clinical Microbiology, 37*(8), 2518-2524. doi:https://doi.org/10.1128/JCM.37.8.2518-2524.1999

Munderloh, U. G., & Kurtti, T. J. (1989). Formulation of medium for tick cell culture. *Experimental and Applied Acarology, 7*(3), 219-229. doi:https://doi.org/10.1007/BF01194061

Munderloh, U. G., Liu, Y., Wang, M., Chen, C., & Kurtti, T. J. (1994). Establishment, maintenance and description of cell lines from the tick *Ixodes scapularis*. *The Journal of Parasitology, 80*(4), 533-543. doi:10.2307/3283188

Muñoz-Leal, S., Toledo, L. F., Venzal, J. M., Marcili, A., Martins, T. F., Acosta, I. C., . . . Labruna, M. B. (2017). Description of a new soft tick species (Acari: Argasidae: *Ornithodoros*) associated with stream-breeding frogs (Anura: Cycloramphidae: *Cycloramphus*) in Brazil. *Ticks and Tick-Borne Diseases, 8*(5), 682-692. doi:https://doi.org/10.1016/j.ttbdis.2017.04.015

Murray, G. G., Weinert, L. A., Rhule, E. L., & Welch, J. J. (2016). The phylogeny of *Rickettsia* using different evolutionary signatures: how tree-like is bacterial evolution? *Systematic Biology, 65*(2), 265-279. doi:https://doi.org/10.1093/sysbio/syv084

Najm, N. A. (2012). *In vitro culture studies of tick cell lines.* lmu,

Nakata, R., Motomura, M., Tokuda, M., Nakajima, H., Masuda, T., Fukuda, T., . . . Kawakami, A. (2012). A case of Japanese spotted fever complicated with central nervous system involvement and multiple organ failure. *Internal Medicine, 51*(7), 783-786. doi:https://doi.org/10.2169/internalmedicine.51.6214

Narra, H. P., Sahni, A., Walker, D. H., & Sahni, S. K. (2020). Recent research milestones in the pathogenesis of human rickettsioses and opportunities ahead. *Future Microbiology, 15*(9), 753-765. doi:https://doi.org/10.2217/fmb-2019-0266

Nelson, C. M., Herron, M. J., Felsheim, R. F., Schloeder, B. R., Grindle, S. M., Chavez, A. O., . . . Munderloh, U. G. (2008). Whole genome transcription profiling of *Anaplasma phagocytophilum* in human and tick host cells by tiling array analysis. *BMC Genomics, 9*(1), 1-16. doi:https://doi.org/10.1186/1471-2164-9-364

Ngwamidiba, M., Blanc, G., Raoult, D., & Fournier, P.-E. (2006). Sca 1, a previously undescribed paralog from autotransporter protein-encoding genes in *Rickettsia*

species. *BMC Microbiology, 6*(1), 12. doi:https://doi.org/10.1186/1471-2180-6-12

Nijhof, A. M., Taoufik, A., De la Fuente, J., Kocan, K. M., De Vries, E., & Jongejan, F. (2007). Gene silencing of the tick protective antigens, *Bm86*, *Bm91* and *subolesin*, in the one-host tick *Boophilus microplus* by RNA interference. *International Journal for Parasitology, 37*(6), 653-662. doi:https://doi.org/10.1016/j.ijpara.2006.11.005

Nooroong, P., Trinachartvanit, W., Baimai, V., & Ahantarig, A. (2018). Phylogenetic studies of bacteria (*Rickettsia*, *Coxiella*, and *Anaplasma*) in *Amblyomma* and *Dermacentor* ticks in Thailand and their co-infection. *Ticks and Tick-Borne Diseases, 9*(4), 963-971. doi:https://doi.org/10.1016/j.ttbdis.2018.03.027

Nyindo, M., Shatry, A., Awiti, L., Chimtawi, M., & Hendricks, L. (1987). *Leishmania donovani* and *L. major*: cultivation *in vitro* in tick embryonic cell lines. *Experimental Parasitology, 63*(2), 240-242. doi:https://doi.org/10.1016/0014-4894(87)90168-8

Oliver, J. D., Chávez, A. S. O., Felsheim, R. F., Kurtti, T. J., & Munderloh, U. G. (2015). An *Ixodes scapularis* cell line with a predominantly neuron-like phenotype. *Experimental and Applied Acarology, 66*(3), 427-442. doi:https://doi.org/10.1007/s10493-015-9908-1

Oteo, J. A., Portillo, A. J. T., & diseases, t.-b. (2012). Tick-borne rickettsioses in Europe. *Ticks and Tick-Borne Diseases, 3*(5-6), 271-278. doi:https://doi.org/10.1016/j.ttbdis.2012.10.035

Pai, H., Sohn, S., Seong, Y., Kee, S., Chang, W. H., & Choe, K. W. (1997). Central nervous system involvement in patients with scrub typhus. *Clinical Infectious Diseases, 24*(3), 436-440. doi:https://doi.org/10.1093/clinids/24.3.436

Palmer, S., Wiegand, A. P., Maldarelli, F., Bazmi, H., Mican, J. M., Polis, M., . . . Metcalf, J. A. (2003). New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. *Journal of Clinical Microbiology, 41*(10), 4531-4536. doi:https://doi.org/10.1128/JCM.41.10.4531-4536.2003

Palomar, A. M., Premchand-Branker, S., Alberdi, P., Belova, O. A., Moniuszko-Malinowska, A., Kahl, O., & Bell-Sakyi, L. (2019). Isolation of known and potentially pathogenic tick-borne microorganisms from European ixodid ticks using tick cell lines. *Ticks and Tick-borne Diseases, 10*(3), 628-638. doi:10.1016/j.ttbdis.2019.02.008

Paris, D., Aukkanit, N., Jenjaroen, K., Blacksell, S., & Day, N. (2009). A highly sensitive quantitative real-time PCR assay based on the groEL gene of contemporary Thai strains of *Orientia tsutsugamushi*. *Clinical Microbiology and Infection, 15*(5), 488-495. doi:https://doi.org/10.1111/j.1469-0691.2008.02671.x

Paris, D. H., & Dumler, J. S. (2016). State of the art of diagnosis of rickettsial diseases: the use of blood specimens for diagnosis of scrub typhus, spotted fever group rickettsiosis, and murine typhus. *Current Opinion in Infectious Diseases, 29*(5), 433. doi:https://doi.org/10.1097/QCO.0000000000000298.

Parola, P., Paddock, C. D., & Raoult, D. (2005). Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clinical Microbiology Reviews, 18*(4), 719-756. doi:https://doi.org/10.1128/CMR.18.4.719-756.2005

Parola, P., Paddock, C. D., Socolovschi, C., Labruna, M. B., Mediannikov, O., Kernif, T., . . . Fournier, P.-E. (2013). Update on tick-borne rickettsioses around the world: a geographic approach. *Clinical Microbiology Reviews, 26*(4), 657-702. doi:https://doi.org/10.1128/CMR.00032-13

Parola, P., Rovery, C., Rolain, J. M., Brouqui, P., Davoust, B., & Raoult, D. (2009). *Rickettsia slovaca* and *R. raoultii* in tick-borne rickettsioses. *Emerging Infectious Diseases, 15*(7), 1105. doi:https://doi.org/10.3201/eid1507.081449

Parola, P., Vogelaers, D., Roure, C., Janbon, F., & Raoult, D. (1998). Murine typhus in travelers returning from Indonesia. *Emerging Infectious Diseases, 4*(4), 677. doi:https://doi.org/10.3201/eid0404.980423

Parthasarathy, A. (2013). *Textbook of pediatric infectious diseases*: JP Medical Ltd.

Petchampai, N., Sunyakumthorn, P., Banajee, K. H., Verhoeve, V. I., Kearney, M. T., & Macaluso, K. R. (2015). Identification of host proteins involved in rickettsial invasion of tick cells. *Infection and Immunity, 83*(3), 1048-1055. doi:https://doi.org/10.1128/IAI.02888-14

Pether, J., Jones, W., Lloyd, G., Rutter, D., & Barry, M. (1994). Fatal murine typhus from Spain. *The Lancet, 344*(8926), 897-898. doi:https://doi.org/10.1016/s0140-6736(94)92875-4

Philip, R., Casper, E., Ormsbee, R., Peacock, M., & Burgdorfer, W. (1976). Microimmunofluorescence test for the serological study of Rocky Mountain spotted fever and typhus. *Journal of Clinical Microbiology, 3*(1), 51-61. doi:https://doi.org/10.1128/jcm.3.1.51-61.1976

Pohl, P. C., Carvalho, D. D., Daffre, S., da Silva Vaz Jr, I., & Masuda, A. (2014). *In vitro* establishment of ivermectin-resistant *Rhipicephalus microplus* cell line and the contribution of ABC transporters on the resistance mechanism. *Veterinary Parasitology, 204*(3-4), 316-322. doi:https://doi.org/10.1016/j.vetpar.2014.05.042

Policastro, P. F., Munderloh, U. G., Fischer, E. R., & Hackstadt, T. (1997). *Rickettsia rickettsii* growth and temperature-inducible protein expression in embryonic tick cell lines. *Journal of Medical Microbiology, 46*(10), 839-845. doi:https://doi.org/10.1099/00222615-46-10-839

Pornwiroon, W., Pourciau, S. S., Foil, L. D., & Macaluso, K. R. (2006). *Rickettsia felis* from cat fleas: isolation and culture in a tick-derived cell line. *Applied and Environmental Microbiology, 72*(8), 5589-5595. doi:https://doi.org/10.1128/AEM.00532-06

Portillo, A., De Sousa, R., Santibáñez, S., Duarte, A., Edouard, S., Fonseca, I. P., . . . Santos, M. (2017). Guidelines for the detection of *Rickettsia* spp. *Vector-Borne and Zoonotic Diseases, 17*(1), 23-32. doi:https://doi.org/10.1089/vbz.2016.1966.

Prescott, H. (2002). Laboratory exercises in microbiology Fifth Edition. In: McGraw-Hill.

Pudney, M., Varma, M., & Leake, C. (1973). Culture of embryonic cells from the tick *Boophilus microplus* (Ixodidae). *Journal of Medical Entomology, 10*(5), 493-496. doi:https://doi.org/10.1093/jmedent/10.5.493

Radulovic, S., Feng, H.-m., Morovic, M., Djelalija, B., Popov, V., Crocquet-Valdes, P., & Walker, D. H. (1996). Isolation of *Rickettsia akari* from a patient in a region

where Mediterranean spotted fever is endemic. *Clinical Infectious Diseases, 22*(2), 216-220. doi:https://doi.org/10.1093/clinids/22.2.216

Radulovic, S., Price, P., Beier, M., Gaywee, J., Macaluso, J., & Azad, A. (2002). *Rickettsia*-macrophage interactions: host cell responses to *Rickettsia akari* and *Rickettsia typhi*. *Infection and Immunity, 70*(5), 2576-2582. doi:https://doi.org/10.1128/IAI.70.5.2576-2582.2002

Randolph, S., Miklisova, D., Lysy, J., Rogers, D., & Labuda, M. (1999). Incidence from coincidence: patterns of tick infestations on rodents facilitate transmission of tick-borne encephalitis virus. *Parasitology, 118*(2), 177-186. doi:https://doi.org/10.1017/s0031182098003643

Raoult, D., Berbis, P., Roux, V., Xu, W., & Maurin, M. (1997a). A new tick-transmitted disease due to *Rickettsia slovaca*. *The Lancet, 350*(9071), 112-113. doi:https://doi.org/10.1016/S0140-6736(05)61814-4

Raoult, D., Lakos, A., Fenollar, F., Beytout, J., Brouqui, P., & Fournier, P.-E. (2002). Spotless rickettsiosis caused by *Rickettsia slovaca* and associated with *Dermacentor* ticks. *Clinical Infectious Diseases, 34*(10), 1331-1336. doi:https://doi.org/10.1086/340100

Raoult, D., & Roux, V. (1997b). Rickettsioses as paradigms of new or emerging infectious diseases. *Clinical Microbiology Reviews, 10*(4), 694-719. doi:https://doi.org/10.1128/CMR.10.4.694

Raoult, D., Weiller, P. J., Chagnon, A., Chaudet, H., Gallais, H., & Casanova, P. (1986). Mediterranean spotted fever: clinical, laboratory and epidemiological features of 199 cases. *The American Journal of Tropical Medicine and Hygiene, 35*(4), 845-850. doi:https://doi.org/10.4269/ajtmh.1986.35.845

Reck, J., Klafke, G. M., Webster, A., Dall'Agnol, B., Scheffer, R., Souza, U. A., . . . de Souza Martins, J. R. (2014). First report of fluazuron resistance in *Rhipicephalus microplus*: a field tick population resistant to six classes of acaricides. *Veterinary Parasitology, 201*(1-2), 128-136. doi:https://doi.org/10.1016/j.vetpar.2014.01.012

Renvoisé, A., Joliot, A.-Y., & Raoult, D. (2009). *Rickettsia felis* infection in man, France. *Emerging Infectious Diseases, 15*(7), 1126. doi:https://doi.org/10.3201/eid1507.090029

Renvoisé, A., van't Wout, J. W., van der Schroeff, J.-G., Beersma, M. F., & Raoult, D. (2012). A case of rickettsialpox in Northern Europe. *International Journal of Infectious Diseases*

16(3), e221-e222. doi:https://doi.org/10.1016/j.ijid.2011.11.009

Reye, A. L., Stegniy, V., Mishaeva, N. P., Velhin, S., Hübschen, J. M., Ignatyev, G., & Muller, C. P. (2013). Prevalence of tick-borne pathogens in *Ixodes ricinus* and *Dermacentor reticulatus* ticks from different geographical locations in Belarus. *PLoS One, 8*(1), e54476. doi:https://doi.org/10.1371/journal.pone.0054476

Ribeiro, M. F., Bastos, C. V., Vasconcelos, M. M. C., & Passos, L. M. (2009). *Babesia bigemina*: *in vitro* multiplication of sporokinetes in *Ixodes scapularis* (IDE8) cells. *Experimental Parasitology, 122*(3), 192-195. doi:https://doi.org/10.1016/j.exppara.2009.03.011

Richards, A. L. (2012). Worldwide detection and identification of new and old rickettsiae and rickettsial diseases. *FEMS Immunology & Medical Microbiology, 64*(1), 107-110. doi:https://doi.org/10.1111/j.1574-695X.2011.00875.x

Robinson, M. T., Satjanadumrong, J., Hughes, T., Stenos, J., & Blacksell, S. D. (2019). Diagnosis of spotted fever group Rickettsia infections: The Asian perspective. *Epidemiology Infection, 147*. doi:https://doi.org/10.1017/S0950268819001390

Rolain, J.-M., Jensenius, M., & Raoult, D. (2004). Rickettsial infections-a threat to travellers? *Current Opinion in Infectious Diseases, 17*(5), 433-437. doi:https://doi.org/10.1097/00001432-200410000-00008

Rolain, J.-M., Stuhl, L., Maurin, M., & Raoult, D. (2002). Evaluation of antibiotic susceptibilities of three rickettsial species including *Rickettsia felis* by a quantitative PCR DNA assay. *Antimicrobial Agents and Chemotherapy, 46*(9), 2747-2751. doi:https://doi.org/10.1128/AAC.46.9.2747-2751.2002

Rolain, J., Maurin, M., Vestris, G., & Raoult, D. (1998). *In vitro* susceptibilities of 27 rickettsiae to 13 antimicrobials. *Antimicrobial Agents and Chemotherapy, 42*(7), 1537-1541. doi:https://doi.org/10.1128/AAC.42.7.1537

Roux, V., Rydkina, E., Eremeeva, M., & Raoult, D. (1997). Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the rickettsiae. *International Journal of Systematic Bacteriology, 47*(2), 252-261. doi:https://doi.org/10.1099/00207713-47-2-252

Rydkina, E., Roux, V., Fetisova, N., Rudakov, N., Gafarova, M., Tarasevich, I., & Raoult, D. (1999). New Rickettsiae in ticks collected in territories of the former Soviet Union. *Emerging Infectious Diseases, 5*(6), 811. doi:https://doi.org/10.3201/eid0506.990612

Saliki, J. T., Blouin, E. F., Rodgers, S. J., & Kocan, K. M. (1998). Use of tick cell culture-derived *Anaplasma marginale* antigen in a competitive ELISA for serodiagnosis

of anaplasmosis. *Annals of the New York Academy of Sciences, 849*(1), 273-281. doi:https://doi.org/10.1111/j.1749-6632.1998.tb11059.x

Samish, M., Shkap, V., Bin, H., & Pipano, E. M. (1988). Cultivation of *Besnoitia besnoiti* in four tick cell lines. *International Journal for Parasitology, 18*(3), 291-296. doi:https://doi.org/10.1016/0020-7519(88)90136-1

Santibáñez, S., Portillo, A., Palomar, A. M., Bell-Sakyi, L., Romero, L., & Oteo, J. A. (2015). Isolation and maintenance of *Rickettsia raoultii* in a *Rhipicephalus sanguineus* tick cell line. *Microbes and Infection, 17*(11-12), 866-869. doi:https://doi.org/10.1016/j.micinf.2015.09.018

Sayler, K. A., Wamsley, H. L., Pate, M., Barbet, A. F., & Alleman, A. R. (2014). Cultivation of *Rickettsia amblyommii* in tick cells, prevalence in Florida lone star ticks (*Amblyomma americanum*). *Parasites & Vectors, 7*(1), 1-7. doi:https://doi.org/10.1186/1756-3305-7-270

Sekeyová, Z., Danchenko, M., Filipčík, P., & Fournier, P. E. (2019). Rickettsial infections of the central nervous system. *PLoS Neglected Tropical Diseases, 13*(8), e0007469. doi:https://doi.org/10.1371/journal.pntd.0007469

Selmi, M., Bertolotti, L., Tomassone, L., & Mannelli, A. (2008). *Rickettsia slovaca* in *Dermacentor marginatus* and tick-borne lymphadenopathy, Tuscany, Italy. *Emerging Infectious Diseases, 14*(5), 817. doi:https://doi.org/10.3201/eid1405.070976

Selmi, M., Martello, E., Bertolotti, L., Bisanzio, D., & Tomassone, L. (2009). *Rickettsia slovaca* and *Rickettsia raoultii* in *Dermacentor marginatus* ticks collected on wild boars in Tuscany, Italy. *Journal of Medical Entomology, 46*(6), 1490-1493. doi:https://doi.org/10.1603/033.046.0636

Seo, M.-G., Kwon, O.-D., & Kwak, D. (2020). Early Release-High Prevalence of *Rickettsia raoultii* and Associated Pathogens in Canine Ticks, South Korea. *Emerging Infectious Diseases*. doi:https://doi.org/10.3201/eid2610.191649

Sexton, D. J., & Kirkland, K. B. (1998). Rickettsial infections and the central nervous system. *Clinical Infectious Diseases, 26*(1), 247-247. doi:https://doi.org/10.1086/517043

Silaghi, C. (2008). *Prevalence and genetic analysis of Anaplasma phagocytophilum and spotted fever group rickettsiae in the tick Ixodes ricinus in urban and periurban sites in Southern Germany*. lmu,

Silpapojakul, K., Ukkachoke, C., Krisanapan, S., & Silpapojakul, K. (1991). Rickettsial meningitis and encephalitis. *Archives of Internal Medicine, 151*(9), 1753-1757. doi:https://doi.org/10.1001/archinte.1991.00400090051010

Simser, J. A., Palmer, A. T., Munderloh, U. G., & Kurtti, T. J. (2001). Isolation of a spotted fever group *rickettsia*, *Rickettsia peacockii*, in a Rocky Mountain wood tick, *Dermacentor andersoni*, cell line. *Applied and Environmental Microbiology*, 67(2), 546-552. doi:https://doi.org/10.1128/AEM.67.2.546-552.2001

Speck, S., Derschum, H., Damdindorj, T., Dashdavaa, O., Jiang, J., Kaysser, P., . . . Munkhbat, E. (2012). *Rickettsia raoultii*, the predominant *Rickettsia* found in Mongolian *Dermacentor nuttalli*. *Ticks and Tick-Borne Diseases, 3*(4), 227-231. doi:https://doi.org/10.1016/j.ttbdis.2012.04.001

Špitalská, E., Štefanidesová, K., Kocianová, E., & Boldiš, V. (2012). *Rickettsia slovaca* and *Rickettsia raoultii* in *Dermacentor marginatus* and *Dermacentor reticulatus* ticks from Slovak Republic. *Experimental and Applied Acarology, 57*(2), 189-197. doi:10.1007/s10493-012-9539-8

Stafford, I. A., Centeno, F. H., Al Mohajer, M., Parkerson, G., Woc-Colburn, L., Burgos-Lee, A. J., . . . Muldrew, K. (2020). Successful detection of unrecognized *Rickettsia typhi* in pregnancy using cell-free next-generation sequencing. *Case Reports in Obstetrics and Gynecology, 2020*. doi:https://doi.org/10.1155/2020/6767351

Steinfeld, H., Silverstein, J., & Weisburger, W. (1988). Deafness associated with Rocky Mountain spotted fever. *Maryland Medical Journal, 37*(4), 287-288.

Stenos, J., Graves, S. R., & Unsworth, N. B. (2005). A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group Rickettsiae. *The American Journal of Tropical Medicine and Hygiene, 73*(6), 1083-1085. doi:https://doi.org/10.4269/ajtmh.2005.73.1083

Stewart, P. E., & Bloom, M. E. (2020). Sharing the ride: *Ixodes scapularis* symbionts and their interactions. *Frontiers in Cellular and Infection Microbiology, 10*, 142. doi:https://doi.org/10.3389/fcimb.2020.00142

Switaj, K., Chmielewski, T., Borkowski, P., Tylewska-Wierzbanowska, S., & Olszynska-Krowicka, M. (2012). Spotted fever rickettsiosis caused by *Rickettsia raoultii*--case report. *Przeglad Epidemiologiczny, 66*(2), 347-350.

Szekeres, S., van Leeuwen, A. D., Rigó, K., Jablonszky, M., Majoros, G., Sprong, H., & Földvári, G. (2016). Prevalence and diversity of human pathogenic rickettsiae in urban versus rural habitats, Hungary. *Experimental and Applied Acarology, 68*(2), 223-226. doi:https://doi.org/10.1007/s10493-015-9989-x

Texier, P., Rousselot, J., Quillerou, D., Aufrant, C., Robain, D., & Foucaud, P. (1984). Mediterranean boutonneuse fever. Apropos of a fatal case in a newborn infant. *Archives Francaises de Pediatrie, 41*(1), 51-53.

Thepparit, C., Bourchookarn, A., Petchampai, N., Barker, S. A., & Macaluso, K. R. (2010). Interaction of *Rickettsia felis* with histone H2B facilitates the infection of a tick cell line. *Microbiology, 156*(Pt 9), 2855. doi:https://doi.org/10.1099/mic.0.041400-0

Tian, Z.-C., Liu, G.-Y., Shen, H., Xie, J.-R., Luo, J., & Tian, M.-Y. (2012). First report on the occurrence of *Rickettsia slovaca* and *Rickettsia raoultii* in *Dermacentor silvarum* in China. *Parasites & Vectors, 5*(1), 1-4. doi:https://doi.org/10.1186/1756-3305-5-19

Tichopad, A., Dilger, M., Schwarz, G., & Pfaffl, M. W. (2003). Standardized determination of real-time PCR efficiency from a single reaction set-up. *Nucleic acids research, 31*(20), e122-e122. doi:https://doi.org/10.1093/nar/gng122

Tikare, N. V., Shahapur, P. R., Bidari, L. H., & Mantur, B. G. (2010). Rickettsial meningoencephalitis in a child—a case report. *Journal of Tropical Pediatrics, 56*(3), 198-200. doi:https://doi.org/10.1093/tropej/fmp065

Tjale, M. A., Pretorius, A., Josemans, A., Van Kleef, M., & Liebenberg, J. (2018). Transcriptomic analysis of Ehrlichia ruminantium during the developmental stages in bovine and tick cell culture. *Ticks and Tick-Borne Diseases, 9*(1), 126-134. doi:https://doi.org/10.1016/j.ttbdis.2017.09.014

Tzavella, K., Hatzizisis, I. S., Vakali, A., Mandraveli, K., Zioutas, D., & Alexiou-Daniel, S. (2006). Severe case of Mediterranean spotted fever in Greece with predominantly neurological features. *Journal of Medical Microbiology, 55*(3), 341-343. doi:https://doi.org/10.1099/jmm.0.46337-0

Uchiyama, T., Kawano, H., & Kusuhara, Y. (2006). The major outer membrane protein rOmpB of spotted fever group rickettsiae functions in the rickettsial adherence to

and invasion of Vero cells. *Microbes and Infection, 8*(3), 801-809. doi:https://doi.org/10.1016/j.micinf.2005.10.003

Varma, M. (1989). Progress in the study of human and animal pathogens in primary and established tick cell lines. *Invertebrate Cell System Applications*.

Varma, M., Pudney, M., & Leake, C. (1975). The establishment of three cell lines from the tick *Rhipicephalus appendiculatus* (Agari: Ixodidae) and their infection with some arboviruses. *Journal of Medical Entomology, 11*(6), 698-706. doi:https://doi.org/10.1093/jmedent/11.6.698

Vayssier-Taussat, M., Moutailler, S., Michelet, L., Devillers, E., Bonnet, S., Cheval, J., . . . Eloit, M. (2013). Next generation sequencing uncovers unexpected bacterial pathogens in ticks in western Europe. *PLoS One, 8*(11), e81439. doi:https://doi.org/10.1371/journal.pone.0081439

Walker, D. H. (1996). Rickettsiae. In B. S (Ed.), Medical Microbiology (4th edition ed.): University of Texas Medical Branch at Galveston. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK7624/.

Walker, D. H. (2004). Ricketts creates rickettsiology, the study of vector-borne obligately intracellular bacteria. *The Journal of Infectious Diseases, 189*(5), 938-942. doi:https://doi.org/10.1086/381710

Walker, D. H., Herrero-Herrero, J. I., Ruiz-Beltrán, R., Bullon-Sopelana, A., & Ramos-Hidalgo, A. (1987). The pathology of fatal Mediterranean spotted fever. *American Journal of Clinical Pathology, 87*(5), 669-672. doi:https://doi.org/10.1093/ajcp/87.5.669

Walker, D. H., & Ismail, N. J. N. R. M. (2008). Emerging and re-emerging rickettsioses: endothelial cell infection and early disease events. *Nature Reviews Microbiology, 6*(5), 375-386. doi:https://doi.org/10.1038/nrmicro1866

Walker, D. H., Valbuena, G. A., & Olano, J. P. (2003). Pathogenic mechanisms of diseases caused by *Rickettsia*. *Annals of the New York Academy of Sciences, 990*(1), 1-11. doi:https://doi.org/10.1111/j.1749-6632.2003.tb07331.x

Weinert, L. A., Werren, J. H., Aebi, A., Stone, G. N., & Jiggins, F. M. (2009). Evolution and diversity of *Rickettsia* bacteria. *BMC Biology, 7*(1), 1-15. doi:https://doi.org/10.1186/1741-7007-7-6

Weisheit, S., Villar, M., Tykalová, H., Popara, M., Loecherbach, J., Watson, M., . . . Fazakerley, J. K. (2015). *Ixodes scapularis* and *Ixodes ricinus* tick cell lines respond to infection with tick-borne encephalitis virus: transcriptomic and proteomic analysis. *Parasites & Vectors, 8*(1), 1-26. doi:https://doi.org/10.1186/s13071-015-1210-x

Wen, J., Jiao, D., Wang, J.-h., Yao, D.-h., Liu, Z.-x., Zhao, G., . . . Sun, Y. (2014). *Rickettsia raoultii*, the predominant *Rickettsia* found in *Dermacentor silvarum* ticks in China–Russia border areas. *Experimental and Applied Acarology, 63*(4), 579-585. doi:https://doi.org/10.1007/s10493-014-9792-0

Wijnveld, M., Schötta, A.-M., Pintér, A., Stockinger, H., & Stanek, G. (2016). Novel *Rickettsia raoultii* strain isolated and propagated from Austrian *Dermacentor reticulatus* ticks. *Parasites & Vectors, 9*(1), 1-7. doi:https://doi.org/10.1186/s13071-016-1858-x

Wisseman, C., Waddell, A., & Silverman, D. (1976). *In vitro* studies on *Rickettsia*-host cell interactions: lag phase in intracellular growth cycle as a function of stage of

growth of infecting *Rickettsia prowazeki*, with preliminary observations on inhibition of rickettsial uptake by host cell fragments. *Infection and Immunity, 13*(6), 1749-1760. doi:https://doi.org/10.1128/iai.13.6.1749-1760.1976

Wójcik-Fatla, A., Cisak, E., Zajac, V., Sroka, J., Sawczyn, A., Dutkiewicz, J. J. A. o. A., & Medicine, E. (2013). Study on tick-borne rickettsiae in eastern Poland. I. Prevalence in *Dermacentor reticulatus* (Acari: Amblyommidae). *Annals of Agricultural and Environmental Medicine, 20*(2).

Woldehiwet, Z., Horrocks, B., Scaife, H., Ross, G., Munderloh, U. G., Bown, K., . . . Hart, C. (2002). Cultivation of an ovine strain of *Ehrlichia phagocytophila* in tick cell cultures. *Journal of Comparative Pathology, 127*(2-3), 142-149. doi:https://doi.org/10.1053/jcpa.2002.0574

Wong, M. L., & Medrano, J. F. (2005). Real-time PCR for mRNA quantitation. *Biotechniques, 39*(1), 75-85. doi:https://doi.org/10.2144/05391RV01

Yssouf, A., Almeras, L., Terras, J., Socolovschi, C., Raoult, D., & Parola, P. (2015). Detection of *Rickettsia* spp in ticks by MALDI-TOF MS. *PLoS Neglected Tropical Diseases, 9*(2), e0003473. doi:https://doi.org/10.1371/journal.pntd.0003473

Yssouf, A., Flaudrops, C., Drali, R., Kernif, T., Socolovschi, C., Berenger, J.-M., . . . Parola, P. (2013). Matrix-assisted laser desorption ionization–time of flight mass spectrometry for rapid identification of tick vectors. *Journal of Clinical Microbiology, 51*(2), 522-528. doi:https://doi.org/10.1128/JCM.02665-12.

Yunker, C. (1987). Preparation and maintenance of arthropod cell cultures: Acari, with emphasis on ticks [Press release]

Yunker, C., Cory, J., & Meibos, H. (1981). Coitinuous cell lines from embryonic tissues of ticks (Acari: Ixodidae). *In Vitro, 17*(2), 139-142. doi:https://doi.org/10.1007/BF02618071

Zammarchi, L., Farese, A., Trotta, M., Amantini, A., Raoult, D., & Bartoloni, A. (2014). *Rickettsia africae* infection complicated with painful sacral syndrome in an Italian traveller returning from Zimbabwe. *International Journal of Infectious Diseases, 29*, 194-196. doi:https://doi.org/10.1016/j.ijid.2014.10.017

Znazen, A., Sellami, H., Elleuch, E., Hattab, Z., Sassi, L. B., Khrouf, F., . . . Hammami, A. (2015). Comparison of two quantitative real time PCR assays for *Rickettsia* detection in patients from Tunisia. *PLoS Neglected Tropical Diseases, 9*(2), e0003487. doi:https://doi.org/10.1371/journal.pntd.0003487

Zweygarth, E., Josemans, A., Spickett, A., Steyn, H., Putterill, J., Troskie, P., . . . Fish, L. (2006). *In vitro* cultivation of a South African isolate of an *Anaplasma* sp. in tick cell cultures. *The Onderstepoort Journal of Veterinary Research, 73*(4), 251-255.