THE HOST-PATHOGEN RELATIONSHIP IN RICKETTSIA: EPIDEMIOLOGICAL ANALYSIS OF RMSF IN OHIO AND A COMPARATIVE MOLECULAR ANALYSIS OF FOUR VIR GENES

DISSERTATION

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By

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ABSTRACT

Members of the vector-borne bacterial genus *Rickettsia* represent an emerging infectious disease threat and have continually been implicated in epidemics worldwide. It is of vital importance to understand the geographical distribution of disease and rickettsial-infected arthropods vectors. In addition, understanding the dynamics of the relationship between rickettsiae and their arthropod hosts will help aid in identifying important factors for virulence.

*Dermacentor variabilis* dog ticks are the main vector in the eastern United States for *Rickettsia rickettsii*, the etiological agent of Rocky Mountain spotted fever. The frequency of rickettsial-infected ticks and their geographical location in Ohio over the last twenty years was analyzed. The frequency of rickettsial species was found to remain relatively constant (about 20%), but the incidence of *R. rickettsii* has increased from 6 to 16%. Also, the geographic distribution of rickettsial-positive ticks has expanded, corresponding to a rise of RMSF in these new areas.

Type IV secretion system genes, like the *vir* group, are important for pathogenicity in many pathogens, but have not been analyzed in *Rickettsia*. Four *vir* genes, *virB8, virB11, virB4*, and *virD4* were analyzed in *Rickettsia amblyommii* infected *Amblyomma americanum* Lone Star ticks from across the Northeast United States. Results showed that important protein motifs are mutated or absent. This evidence
suggests the *vir* genes may not be functional in *Rickettsia* or they have evolved a novel role. In addition, analysis of *virD4* showed that isolates were most similar to *Rickettsia conorii* and *R. rickettsii*, two highly pathogenic species.

To determine if these genes are important for virulence, a novel qPCR approach was designed to compare the RNA expression in *R. amblyommii*-infected *A. americanum* tick and Vero cells. Here, expression of *virB8* and *virD4* was increased in Vero cells, while *virB4* and *virB11* had decreased expression. Since rickettsiae cause disease in humans, increases in expression levels in the mammalian Vero cells may be important for pathogenicity.

Understanding all levels of disease, from basic biology and epidemiology, analysis of potential virulence genes, and functional analysis via RNA expression, contributes information that leads us closer to determining the process of rickettsial pathogenicity.
Dedicated to my parents, Becky and John, and my sisters, Susie and Stephanie, without them none of this would have been possible
Dedicated to Stephen Nye, who in death gave purpose to my work
ACKNOWLEDGMENTS

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Balamuthia mandrillaris: Identification of Clinical and Environmental Isolates Using


**FIELDS OF STUDY**

Major Field: Molecular Genetics
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Introduction: Aims</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>2.</td>
<td>Trends in <em>Dermacentor variabilis</em> dog ticks infected with the agent of Rocky Mountain spotted fever, <em>Rickettsia rickettsii</em>, and cases of clinical disease in Ohio over a twenty year time period</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Materials and methods</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Summary</td>
<td>56</td>
</tr>
<tr>
<td>3.</td>
<td>Variability in the mitochondrial control region and 12S rDNA gene among <em>Dermacentor variabilis</em> ticks</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Materials and methods</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>73</td>
</tr>
</tbody>
</table>

Abstract ........................................................................................................ ii
Dedication ........................................................................................................ iv
Acknowledgements ............................................................................................... v
Vita ................................................................................................................... vi
List of Tables ...................................................................................................... x
List of Figures .................................................................................................... xii
List of Abbreviations .......................................................................................... xv

Chapters:
4. Molecular and Phylogeographical analysis of the *vir* genes, *virB8*, *virB11*, *virB4*, and *virD4*, from *Rickettsia amblyommii* isolates from the northeast United States ............................................................... 81
   Materials and methods .................................................... 86
   Results ................................................................................... 90
   Discussion ............................................................................... 100

5. Differential expression of *vir* genes between mammalian and arthropod cells infected with *Rickettsia amblyommii* ................................................................. 104
   Materials and Methods ....................................................... 107
   Results ................................................................................... 117
   Discussion ............................................................................... 140

6. Summary and Conclusions .................................................... 147

Bibliography ............................................................................. 151

Appendix A: Nucleotide Sequences ........................................... 166
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Comparison of number of <em>Dermacentor variabilis</em> ticks positive for <em>Rickettsia</em> sp. by the 17kDa surface antigen gene and DFA methods…</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>Number of rickettsial-infected <em>Dermacentor variabilis</em> submitted from Ohio counties in the years 1983-6 and 2003-4</td>
<td>48</td>
</tr>
<tr>
<td>3</td>
<td>RMSF cases per county in Ohio for the years 1981-86 and 2003-4</td>
<td>53</td>
</tr>
<tr>
<td>4</td>
<td>Number of <em>Dermacentor variabilis</em> ticks analyzed for the 3’ 12S rDNA/D-loop gene region classified by geographical location</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>Number of <em>Dermacentor variabilis</em> ticks analyzed for the 5’12S rDNA gene region classified by geographical region</td>
<td>71</td>
</tr>
<tr>
<td>6</td>
<td>Molecular analysis of the 17kDa surface antigen gene in <em>Dermacentor variabilis</em> ticks analyzed for the D-loop region and 12 rDNA</td>
<td>78</td>
</tr>
<tr>
<td>7</td>
<td><em>vir</em> genes PCR and sequencing primers and annealing temperatures</td>
<td>89</td>
</tr>
<tr>
<td>8</td>
<td>Geographical locations of <em>Rickettsia amblyommii</em>-infected <em>Amblyomma americanum</em> ticks analyzed for <em>vir</em> genes</td>
<td>91</td>
</tr>
<tr>
<td>9</td>
<td>Trypsin diluent ingredients per 2.5 L solution</td>
<td>109</td>
</tr>
<tr>
<td>10</td>
<td>RNA concentrations and amounts used in RT-PCR of <em>R. amblyommii</em>-infected and uninfected Vero, 34°C, and 24°C <em>Amblyomma americanum</em> cell cultures</td>
<td>111</td>
</tr>
<tr>
<td>11</td>
<td>Sequences of primers used in qPCR for <em>virB8, virB11, virB4, virD4,</em> and <em>gltA</em></td>
<td>112</td>
</tr>
<tr>
<td>12</td>
<td>Sequences and annealing temperatures of PCR primers used to amplify <em>vir</em> genes</td>
<td>114</td>
</tr>
</tbody>
</table>
13  virD4 qPCR amplification and melt curve data........................................ 121
14  virB4 qPCR amplification and melt curve data........................................ 126
15  virB8 qPCR amplification and melt curve data........................................ 130
16  virB11 qPCR amplification and melt curve data...................................... 134
17  gltA qPCR amplification and melt curve data.......................................... 138
18  RNA fold change calculations for virD4, virB4, virB8, and virB11 using the $2^{-\Delta\Delta Ct}$ method................................................................. 139
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Geographical distribution of <em>Dermacentor variabilis</em> ticks determined positive for the 17kDa surface antigen gene in Ohio from the years 1983-6, 2003 and 2004</td>
</tr>
<tr>
<td>2</td>
<td>Multiple peaks present in the nucleotide sequence electropherogram of a partial region of the 17kDa surface antigen gene from tick #03-797</td>
</tr>
<tr>
<td>3</td>
<td>Secondary amplification of the 17kDa surface antigen gene multiplex PCR</td>
</tr>
<tr>
<td>4</td>
<td>Number and geographical distribution of RMSF cases in Ohio in 1983-6 compared to 2003-4</td>
</tr>
<tr>
<td>5</td>
<td>UPGMA phylogeny of the mitochondrial 3’ 12S rDNA/D-loop region from <em>Dermacentor variabilis</em> based on number of nucleotide differences</td>
</tr>
<tr>
<td>6</td>
<td>UPGMA phylogeny of the mitochondrial 5’ 12S rDNA region from <em>Dermacentor variabilis</em> based on number of nucleotide differences</td>
</tr>
<tr>
<td>7</td>
<td>UPGMA phylogeny of the combined mitochondrial 3’ 12S rDNA/D-loop region and 5’ 12S rDNA region from <em>Dermacentor variabilis</em> based on number of nucleotide differences</td>
</tr>
<tr>
<td>8</td>
<td>UPGMA phylogeny of <em>virB8</em> based on number of nucleotide differences</td>
</tr>
<tr>
<td>9</td>
<td>UPGMA phylogeny of <em>virB11</em> based on number of nucleotide differences</td>
</tr>
<tr>
<td>10</td>
<td>UPGMA phylogeny of <em>virB4</em> based on number of nucleotide differences</td>
</tr>
<tr>
<td>11</td>
<td>UPGMA phylogeny of <em>virD4</em> based on number of nucleotide differences</td>
</tr>
<tr>
<td>12</td>
<td>AAE2 <em>Amblyomma americanum</em> cell culture</td>
</tr>
</tbody>
</table>
13 qPCR amplification and melt curves of *virD4* from *Rickettsia conorii* standard dilutions

14 qPCR amplification and melt curves of *virD4* from *Rickettsia amblyommii*-infected cell cultures

15 qPCR amplification and melt curves of *virD4* from uninfected cDNA and RNA from *R. amblyommii*-infected cell cultures

16 qPCR standard curve analysis of *virD4*

17 qPCR amplification and melt curves of *virB4* from *Rickettsia conorii* standard dilutions

18 qPCR amplification and melt curves of *virB4* from *Rickettsia amblyommii*-infected cell cultures

19 qPCR amplification and melt curves of *virB4* from uninfected cDNA and RNA from *R. amblyommii*-infected cell cultures

20 qPCR standard curve analysis of *virB4*

21 qPCR amplification and melt curves of *virB8* from *Rickettsia conorii* standard dilutions

22 qPCR amplification and melt curves of *virB8* from *Rickettsia amblyommii*-infected cell cultures

23 qPCR amplification and melt curves of *virB8* from uninfected cDNA and RNA from *R. amblyommii*-infected cell cultures

24 qPCR standard curve analysis of *virB8*

25 qPCR amplification and melt curves of *virB11* from *Rickettsia conorii* standard dilutions

26 qPCR amplification and melt curves of *virB11* from *Rickettsia amblyommii*-infected cell cultures

27 qPCR amplification and melt curves of *virB11* from uninfected cDNA and RNA from *R. amblyommii*-infected cell cultures

28 qPCR standard curve analysis of *virB11*
29 qPCR amplification and melt curves of gltA from *Rickettsia conorii* standard dilutions. .......................................................... 136

30 qPCR amplification and melt curves of gltA from *Rickettsia amblyommii*-infected cell cultures.......................................................... 136

31 qPCR amplification and melt curves of gltA from uninfected cDNA and RNA from *R. amblyommii*-infected cell cultures................................. 137

32 qPCR standard curve analysis of gltA.................................................. 137

33 Consensus sequence for *Dermacentor variabilis* 12S ribosomal RNA gene and control region.......................................................... 167

34 Consensus sequence for *Rickettsia amblyommii virB8*................................. 168

35 *Rickettsia montanensis virB8* nucleotide sequence.................................. 169

36 *Rickettsia rhipicephali virB8* nucleotide sequence.................................. 170

37 *Rickettsia australis virB8* nucleotide sequence.................................. 171

38 Consensus sequence for *Rickettsia amblyommii virB11*................................. 172

39 *Rickettsia rhipicephali virB11* nucleotide sequence.................................. 174

40 *Rickettsia australis virB11* nucleotide sequence.................................. 176

41 Consensus sequence for *Rickettsia amblyommii virB4*................................. 178

42 *Rickettsia rhipicephali virB4* nucleotide sequence.................................. 180

43 *Rickettsia australis virB4* nucleotide sequence.................................. 182

44 Consensus sequence for *Rickettsia amblyommii virD4*................................. 184

45 *Rickettsia rhipicephali virD4* nucleotide sequence.................................. 186

46 *Rickettsia australis virD4* nucleotide sequence.................................. 188
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxynucleic acid</td>
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<tr>
<td>dNTPs</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>g</td>
<td>gram(s)</td>
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<td>h</td>
<td>hour(s)</td>
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<td>M</td>
<td>moles per liter</td>
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<td>n</td>
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<td>Abbreviation</td>
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<tr>
<td>N</td>
<td>gram equivalents per liter</td>
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<td>NADH</td>
<td>nicotinamide adenine dinucleotide hydrogenase</td>
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<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
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<td>room temperature</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<td>sec</td>
<td>second(s)</td>
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<tr>
<td>sp</td>
<td>species</td>
</tr>
<tr>
<td>v/v</td>
<td>by volume</td>
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<tr>
<td>x g</td>
<td>force of gravity</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Members of the bacterial genus *Rickettsia* have been implicated in many disease outbreaks throughout history, being responsible for millions of deaths in times of war and famine, as far back as the Athens’ plague and more recently during World Wars I and II (see the classic historical work “Rats, Lice and History” by Hans Zinsser, which details the impact of typhus on world events)[1]. These microbes continue to cause epidemics worldwide and represent an emerging infectious disease threat. With the advent of molecular tools, new species implicated in human disease are increasingly being discovered. Rickettsial species are a serious threat to humans because of their interrelationship with common arthropod species, such as ticks, mites, fleas and lice. Humans may come in contact with these hosts on a fairly regular basis either directly or indirectly through pets, wildlife species, at home or in recreational areas. Bacteria are acquired through the bite of the arthropod, in the case of ticks and mites, or through the scratching of feces into the skin, as is the case with fleas and lice. Public awareness of rickettsiae should be emphasized due to their ability to adapt to new host species, the expansion of the geographic range of vector species, the increased potential for human contact due to urban development, and climatic and ecological changes. Rickettsia are an excellent model for research in studying virulence factors.
Rickettsiae are rod-shaped Gram-negative, obligate intracellular bacteria. They are small in size, around 1 µm by 0.5 µm. The reproductive doubling time of *Rickettsia* is slightly longer than other bacteria at 9-12 hours. Two main species groups of *Rickettsia* have been identified based on both the type of disease they cause in humans and on antigenic properties: the spotted fever group (SFG) and the typhus group (TG). Rickettsiae of the TG are found almost exclusively in the cytoplasm of host cells, while SFG can be found in both the cytoplasm and the nucleus. Furthermore, the optimal growth temperature is 32°C for the SFG, while 35°C for the TG rickettsiae. Rickettsiae contain inner and outer cell membranes, which are separated by peptidoglycan. The outer membrane contains lipopolysaccharide; however, this layer does not seem to exert endotoxic effects, a phenomenon seen in the pathogens *Coxiella burnetii* and *Chlyamydia trachomatic* [2]. Another unique characteristic of rickettsial organisms is the presence of a slime layer [2, 3]. This is an electronlucent zone that seems to separate the rickettsiae from the cytoplasm of host cells. There is evidence that it contains microfilament structures that might interact with the cytoskeleton [4]. The bacteria seem to target mainly human endothelial cells, although they successfully infect human fibroblasts, arthropod, avian, and rodent cells [3]. Rickettsiae use phagocytosis in order to enter the host cell and from there the phagosome membrane is believed to be destroyed by phospholipase activity [3]. The method of cell infection also differs between the two main rickettsial groups. The SFG seems to spread from cell to cell, apparently by actin motility, whereas the TG multiply within the host cell until lysis occurs and then spreads to other cells [2, 3]. The level of cellular damage to the host continues to increase as the rickettsiae multiply leading to irreversible destruction of many tissues and organs.
Infection by rickettsiae can proceed to a variety of sequellae in humans, anywhere from a mild febrile response to a serious life threatening illness. The most characteristic, yet not defining, symptoms are a rash, high fever and headache. These nonspecific symptoms make the early diagnosis of rickettsial diseases very difficult for proper treatment and survival. Globally the SFG rickettsiae are mainly carried by ticks, mites and fleas. Members of this group include *Rickettsia rickettsii*, the etiological agent of Rocky Mountain spotted fever (RMSF) and Brazilian spotted fever, *Rickettsia conorii*, the agent of Mediterranean spotted fever, *Rickettsia felis*, the agent of flea-borne spotted fever, *Rickettsia akari*, the agent of mite-borne rickettsial-pox, *Rickettsia sibirica*, the agent of North Asian tick typhus, *Rickettsia africae*, the agent of African tick-bite fever, *Rickettsia australis*, the agent of Queensland tick typhus, *Rickettsia japonica*, the agent of Oriental spotted fever, *R. honei*, the agent of Flinders Island spotted fever, and *Rickettsia bellii*, a non-pathogenic species, among others [5]. Their most common hosts are Ixodid ticks such as *Dermacentor variabilis*, the American dog tick, *Dermacentor andersoni*, the Rocky Mountain wood tick, *Rhipicephalus sanguineus*, the brown dog tick, and *Amblyomma americanum*, the Lone Star tick. *R. felis*, unlike other members of the SFG, is spread by cat and dog fleas, *Ctenocephalides felis* and *Ctenocephalides canis*, respectively. Opossums, *Didelphis virginiana*, serve as main hosts of fleas infected with *R. felis* in nature, while species such as rabbits, rodents and dogs serve as the main hosts of ticks.

RMSF is fairly common in the United States as well as Canada, Central America and South America. It has been responsible for outbreaks in every U.S. state, accounting for 1,713 cases in 2004, including 11 from Ohio [6]. Most cases in the United States
occur in the Southeastern states with the highest incidence in North Carolina and Oklahoma, but an increase in disease incidence has occurred in urban areas, like New York City [5]. RMSF is caused by *R. rickettsii* and is spread by the bite of an infected tick. *D. andersoni* is the main vector in the western United States, while *D. variabilis* accounts for the majority of the cases in the east. Humans may come into contact with these tick species either through recreational activities in ecotone or grassy areas or by contact with dogs. The role of dogs in spreading the disease is unknown, but it may be acquired by the inadvertent crushing of ticks as one removes them from a pet [7]. Nonetheless, the infection rate of ticks is fairly low, from 1-3%, despite the high potential for acquiring disease [5, 7]. *R. rickettsii* is only found in the Western Hemisphere, but many other spotted fever rickettsial species are found throughout Europe and Asia. RMSF is best characterized by the presence of a rash, usually initiating on the limbs, palms and soles, and then spreading to the trunk and remainder of the body [5, 7]. The rash usually appears after a 5-10 day incubation period and is seen in approximately 90% of patients [5, 8]. Other symptoms include malaise, chills, headache, fever and myalgia. If untreated, the disease can progress to nausea, vomiting, diarrhea and joint pain. The most severe form of disease is illustrated by chronic pulmonary edema, renal failure, hemorrhaging in the skin and mucous membranes, hypotension, neurological symptoms like delirium and seizures, and possibly death. Case fatality rates are 5% in the United States, and 20% worldwide [8]. The most frequent reason for severe disease progression is misdiagnosis in the early stages, as RMSF is easily treated with doxycycline. Increased awareness and education for both the public and physicians on the
epidemiology of RMSF will allow for proper diagnosis of disease, as well as ultimately reduce the number of clinical cases due to more proactive prevention measures.

Typhus group rickettsiae are also found worldwide. There are two bacterial members of this group and TG species are spread by insects, fleas and lice, which thrive in unsanitary, crowded human conditions. Epidemic typhus is caused by *R. prowazekii* and is highly pathogenic. Murine typhus is caused by *R. typhi*, and has been implicated in an endemic outbreak in Hawaii as recently as 2002 [9]. *R. typhi* is carried by the fleas of rodents, such as *Rattus rattus*, *Rattus norvegicus*, and *Mus musculus* [10].

Epidemic typhus, caused by *R. prowazekii*, is the most devastating of the rickettsial diseases, with a case fatality ratio of up to 30% [5, 10]. Epidemic typhus has been responsible for millions of deaths throughout history, including apparent occurrences during the Crusades, World War I and II, the Grenada conquest and potentially the Athens plague [1, 11, 12]. Outbreaks still occur today in Africa, Russia, and areas of South America [13, 14]. Although responsible for millions of deaths during World War II, there have only been 30 cases in the United States since 1976, one being from Ohio [11, 12, 15]. In 1928, Charles Nicolle was awarded the Nobel Peace Prize for discovering that this disease is spread by the human body louse, *Pediculus humanus corporis* [11]. Humans are the main reservoir; however, in the zoonotic cycle in the United States, the flying squirrel, *Glaucomys volans*, serves as the reservoir for *R. prowazekii* [15]. The rickettsiae are spread by the rodent flea, *Orchopeas howardii*, and the louse, *Neohaematopinus sciuropteri*, and causes sylvatic typhus [5, 15, 16]. Lice infestations occur mainly in poor hygienic and cold weather conditions commonly found during times of war. Lice feed up to five times a day and lay eight eggs a day in the folds
of human clothing; thus, humans can quickly become infected with hundreds of lice. The
only way to effectively eliminate an infestation is to remove and launder clothing, and as
a result many laundry workers become inadvertently infected [14]. *R. prowazekii* is
actually spread by the accidental scratching of the louse feces into the skin. Laboratory
studies have shown that rickettsiae are present in the louse feces as early as five days
after infection [17]. The rickettsiae kill their louse host between 6-13 days after infection
and thus transovarial transmission in the insect does not occur. Lice turn bright red from
the leakage of the blood meal due to lysis of the midgut epithelial cells [17]. The
incubation period of epidemic typhus in humans is approximately 10-14 days, afterwards
100% of patients acquire a severe headache and rash. Additional signs include diarrhea,
pulmonary infection, gangrene of extremities, and up to 80% of patients develop central
nervous system symptoms including delirium, coma and seizures [12, 14]. The word
typhos in Greek means smoke or haze, illustrating the confusing neurological state that
patients experience. The disease may also be recrudescent, with the bacteria being
maintained without symptoms for many years. Patients may relapse later in life,
suffering from a type of typhus called Brill-Zinsser disease, which may be brought on by
stressful situations [5]. Treatment for epidemic typhus is the antibiotic doxycycline and
sometimes chloramphenicol; a single dose of either antibiotic is very effective during
outbreaks. Yet, the only way to successfully prevent disease is to improve
socioeconomic conditions and prevent war.

Murine typhus, caused by *R. typhi*, is milder than epidemic typhus and usually
thrives in warm climates. Outbreaks occur worldwide, most recently in Australia, China,
Kuwait, and Thailand. Symptoms are much like other rickettsioses, with a rash being
observed in about half of patients [5]. The main vector of *R. typhi* is the rat flea, *Xenopsylla cheopis*, which is found on several rat species including *R. rattus*, and *R. norvegicus*. *R. typhi* is maintained transstadially and transovarially in the flea without any negative health implications to the flea. The incubation period is 6-14 days; in this time, the rickettsiae spread to the midgut and are eventually transmitted through the feces. *R. typhi* may also be passed by flea bites [18, 19]. In the United States, murine typhus has not been a notifiable disease since 1994 because overall incidence has been reduced due to rat and flea control by use of rat poison and DDT, respectively [20]. Nonetheless, it is still endemic in Texas and Hawaii, with 49 cases reported as recently as 2002 in Hawaii [9, 21].

New species of *Rickettsia* have recently been discovered and species previously considered nonpathogenic have been implicated in disease. *R. felis* has recently been implicated in a handful of human clinical cases [22-24]. Symptoms are fairly vague, like most rickettsioses, and most patients also had contact with either domestic or feral cats. *Rickettsia parkeri*, found in the Gulf Coast tick *Amblyomma maculatum* and also *A. americanum* ticks, has also been recently implicated in human cases of disease. In 2002, a man from Virginia presented with an eschar-associated rash similar to a mild form of RMSF or rickettsialpox; however, *R. parkeri* was identified in a PCR of the tissue biopsy [25]. This resulted in the questioning of previously diagnosed RMSF cases that were not definitive. This study found that *R. parkeri* was found to be the cause of many of these diseases, some as early as 1926 [26, 27]. This helps explain a previous analysis of 16S rDNA data that showed *R. parkeri* is similar to pathogenic rickettsiae [28]. Furthermore, many RMSF diagnoses are caused by yet unidentified rickettsiae. Tick species like
*Rhipicephalus sanguineus* and *A. americanum* have recently been found to carry *R. rickettsii* and cause disease in humans, and antibodies to *R. rickettsii* have been found in people without any prior illness [29-31]. Thus, it is believed that any rickettsial species has the potential to be pathogenic and since it is not known exactly what makes rickettsiae pathogenic, it is vital to focus research on all rickettsial species and not just the historically pathogenic ones.

Rickettsiae are members of the alpha-proteobacteria group of bacteria. These proteobacteria are members of the order Rickettsiales, which includes the family Rickettsiaceae, defined by intracytoplasmic growth, and Anaplasmataceae, defined by growth within an intracellular vacuole. The family Anaplasmataceae includes the genera of *Neorickettsia*, *Wolbachia*, *Anaplasma*, *Cowdria* and *Ehrlichia*. The family Rickettsiaceae includes the genera *Rickettsia* and *Orientia*, the latter of which was only recently removed from the genus *Rickettsia* [32, 33]. They are hypothesized to be the closely related to the progenitor of the eukaryotic mitochondria based on genetic data for ribosomal proteins and NADH dehydrogenase [34]. The genus *Rickettsia* is further broken down into the SFG and TG, which is supported by numerous genetic studies, mainly 16S rRNA data [28, 35]. However, *R. bellii* and *Rickettsia canadensis* do not fit into either of these groups. Depending on the genetic criteria, *R. canadensis* groups with *R. bellii* or with *R. felis*, but also shares similar characteristics to the SFG and the TG [35, 36]. The species are also classified based on the 17kDa surface antigen gene. This gene is specific to the genus *Rickettsia* and has sufficient divergence to provide species-specific nucleotide sequences. Other genes routinely used in phylogenetic analysis include citrate synthase and the outer membrane protein (OMP) A [37].
Genome sequencing is now complete for nine rickettsial species, including the pathogenic *R. rickettsii* and nonpathogenic *R. bellii*. This allows for a unique opportunity to identify and analyze potential virulence genes, as well as to understand how rickettsiae differ from other pathogens. Rickettsiae are characterized by reduced genomes, approximately 1.1-1.3 Mb in size, due to their intracellular lifestyle. The genome is also very A/T rich, ranging from 28.92% G/C for *R. typhi* to 32.47% G/C in *R. sibirica* and *R. rickettsii* [38, 39]. Rickettsiae possess many pseudogenes, split genes, and junk DNA; only about 76-81% of the genome is coding material [34, 40]. However, this varies between the SFG and TG. For example, some genes are retained only in one group or, in extreme cases, only in a single species [39]. *R. conorii* has 552 genes not found in *R. prowazekii*, while *R. prowazekii* has only 30 genes with no counterpart in *R. conorii* [41]. *R. conorii* also has a larger quantity of repeat elements, consisting of over 3% of its genome [41]. Some of the genes notably lost in rickettsial species include those for sugar metabolism, lipid biosynthesis, nucleotide and amino acid synthesis and metabolism, and TCA cycle enzymes [34, 40]. There are also changes in the chromosomes. An approximately 32 kb section of the chromosome is inverted within the TG rickettsiae and not in the SFG, most likely occurring after the divergence of these groups [38]. *R. typhi* possesses a unique 124-kb inversion that shifts the putative origin of replication in respect to the other rickettsiae and is located in a region where many of the SFG rickettsiae also have rearrangements [38]. *R. prowazekii* contains a 12 kb region that is not found in *R. typhi, R. conorii, R. sibirica* or *R. rickettsii*; hence this region may have been acquired due to horizontal gene transfer.
Another interesting rickettsial genome feature includes the presence of two conjugative plasmids, pRF and pRF\(\delta\) in \(R.\) \(felis\) [42]. Plasmid pRF contains 68 ORF’s, 24 more than is found in pRF\(\delta\), an abbreviated version, and all open reading frames are \(R.\) \(felis\)-specific [42]. These plasmids contain homologues to several \(Tra\) genes, found in \(Agrobacterium\) \(tumefaciens\) and the \(Escherichia\) \(coli\) F plasmid, which are involved in initiating and stabilizing transfer of DNA.

Although both \(R.\) \(felis\) and \(R.\) \(prowazekii\) share similar habitats within a flea, their genomes are surprisingly different. The \(R.\) \(felis\) genome consists of approximately 4.3% repeat sequences, the highest among the rickettsiae [42]. The genome also contains orthologs most closely related to \(\gamma\)-proteobacteria and cyanobacteria; thus these genes may have been acquired through a lateral gene transfer. Furthermore, many genes involved in pathogenicity like toxin and antitoxin genes, rarely found in obligate intracellular pathogens, are present in \(R.\) \(felis\). Finally, the presence of pili genes led to the observation of surface pili in \(R.\) \(felis\) by electron microscopy [42]. It contains two types of pili, one involved in conjugation and the second potentially involved in virulence.

The main antigenic group of genes within \(Rickettsia\) is the surface cell antigen (sea) group, also sometimes referred to as the outer membrane proteins. These proteins are found in alpha, beta, epsilon and gamma proteobacteria as well as \(Chlamydia\) \(sp\). These proteins have three main domains: an amino (N)-terminal signal peptide, a carboxy (C)-terminal autotransporter domain, and a passenger domain. The N-terminal domain allows for the protein’s passage through the inner membrane. The C-terminal domain exports the central passenger domain to the outside of the bacterial cell. Finally, the
passenger domain is variable among bacteria and may code for virulence factors such as proteolysis, adhesions, motility or cytotoxins [43]. There are over sixteen members of this protein group that are found either split, truncated or completely absent in most rickettsial species; only OMPB and Sca4 are found in all rickettsiae [44-46]. The two main immunodominant members are OMPA and OMPB. OMPA is a 190-kDa protein that is found exclusively in the SFG. It is uniquely characterized by a large region of tandem repeats in the passenger domain, approximately 1000 amino acids in length, which may be important for pathogenicity [46, 47]. OMPB is an approximately 168-kDa protein that may be cleaved into a mature 135-kDa protein and a 32-kDa fragment. This protein may be involved in pathogenicity for it reacts with monoclonal antibodies and can provide immunity in mice [2]. Analysis of the nine completed Rickettsia genomes suggests the presence of many potential virulence genes. These include genes involved in actin-based motility, antibiotic resistance, type IV secretion system, and other transporters [38, 40]. Since little is known about virulence factors in the genus these genes deserve more attention.

Rickettsiae are found in a variety of arthropod species across the United States. This includes insects as well as acarine (tick and mite) species. In fact, traditionally the SFG and TG rickettsial species were separated and characterized by the type of arthropod they infected; SFG were considered associated with acarines, while TG were associated with insects, like fleas and lice. However, a newly discovered species, R. felis, is molecularly similar to most SFG rickettsiae transovarially transmitted although they infect fleas. Insects appear to be a relatively new vector species for Rickettsia and the host range for species is constantly evolving. This plays a vital role in the spread of the
disease because as rickettsiae exploit more and more hosts the risk of infecting an
arthropod that bites humans increases. *R. rickettsii* is one example of a species that has
adapted a wider host range based on differences in host geographical location. RMSF is
spread by *D. andersoni*, in the western Rocky Mountain area of the United States, while
*D. variabilis* is the main vector in the eastern United States. In fact, *Dermacentor* ticks
represent 34% of the ticks that are recovered from humans, and are higher at 94% in
disease endemic areas like North Carolina [48, 49]. *D. variabilis*’ geographical range is
along the Atlantic, westward to Montana, and southward through Nebraska, Kansas, and
Oklahoma [50, 51]. Other species such as *A. cajennense* are vectors for disease in the
southern United States, and *A. americanum* has been found to harbor *R. rickettsii* and
transovarially transmit both *R. rickettsii* and *R. parkeri* in laboratory studies, and thus
might be implicated as a vector in human clinical cases [31, 52-57]. *A. americanum* is
found along the Gulf Coast, the south Atlantic, and north as far as Maine. The northern
expansion is very recent and may lead to an increase in human contact and thus the
potential for disease [53, 58]. Of these ticks, *D. variabilis* and *A. americanum* are
frequently found in Ohio and are common hosts for *R. rickettsii, R. bellii, R. montanensis*
and *Rickettsia amblyommii*. The Lone Star tick has recently become established in the
southern counties of Ohio, according to the Ohio Department of Health. The most
frequent, if not the only, rickettsial disease of humans found in Ohio is RMSF.

As mentioned, the vectors for rickettsiae are members of the hard tick family
Ixodidae. All members feed by attaching to vertebrates and taking blood meals through
bites. This family is divided into five subfamilies including the Rhipicephalinae, which
contains the genus *Dermacentor* consisting of thirty species, and Amblyomminae,
containing the genus *Amblyomma* and subsequent 120 species. Hard ticks are characterized by having a hard shell-like scutum. They also are distinguished from members of the Class Insecta because they contain four pairs of legs, lack antenna and wings, and have two pairs of mouth appendages. Internal anatomy mainly consists of the presence of a large sac-like organ called the midgut with many branchings called diverticuli that fill with blood as the organism feeds. Large digestive cells act to digest the blood meal, thus making ticks very different from other blood-sucking arthropods. Typically blood is digested in the gut lumen but ticks digest intracellularly. This greatly facilitates infection of ticks by a wide range of pathogens including rickettsiae [51]. The tracheal system branches out over the midgut and other internal organs and opens to the exterior at a pair of spiracles that are guarded by valves. All the internal organs are contained in a circulating fluid called hemolymph; however, there is no hemoglobin present so this fluid is not involved in respiration [59]. The hemolymph contains a large number of amino acids, salts, soluble proteins, phagocytes, and many hemocytes important for immune defense. Any organ of the tick can potentially become infected with rickettsial pathogens, but mainly it is the midgut, hemolymph, salivary glands, and the ovaries in females that are routinely infected.

Both *Dermacentor* and *Amblyomma* ticks have a three-host life cycle, in which the larva, nymph and adult developmental stages all occupy a different host [51, 60]. *Dermacentor* species feed on mammals; immature ticks feed on small mammals like the white footed mouse, *Peromyscus leucopus*, and the meadow vole, *Microtus pennsylvanicus* whereas adult ticks feed on a variety of larger mammals like dogs, raccoons, opossums, groundhogs, horses, and cattle [51, 60, 61]. On the other hand, adult
Amblyomma species infect white tailed deer, Odocoileus virginianus, coyotes and raccoons, while immature ticks feed on birds and mammals of all sizes [8, 52, 62, 63]. The white tailed deer is the main host for Amblyomma, with 80-100% of animals in certain areas being infested with in excess of 300 per deer [52]. At each developmental stage, the ticks must take a blood meal, detach, and then molt to the next developmental stage. Adult ticks can live up to one year without feeding [51]. The larval tick has six legs, which develops into the eight-legged nymph only after its first blood meal. Molting occurs in sheltered habitats such as soil, leaves, or host nests; in fact, over 90% of the tick’s life is spent off host during molting periods [59]. In species requiring three hosts, the completion of the life cycle can take several months, if larvae and adults feed in the same year, to about two years [59, 60]. The delay is due to host-seeking diapause, a phenomenon that allows an organism to survive adverse environments like the cold temperatures of winter and provides increased feeding opportunities by synchronizing tick activity with the seasonal behavior of many vertebrate hosts. During this time, ticks become inactive, reduce metabolic rates, and cease to feed. Decreased photoperiod and ambient temperature are factors that may trigger diapause, while the reverse situations prompt the tick to resume host seeking. This behavior is also very important for achieving optimal reproductive success. Most species take a blood meal prior to mating and since males usually remain on the host, mating also occurs there. Adult females feed only once, after which they mate, detach from the host, ovipositing over several days to weeks, and then die. D. variabilis on average lays about 3,000 – 6,000 eggs, while Amblyomma species lay from 15,000 – 23,000, depending on the species [60, 64].
The feeding event of ticks is a very specific occurrence. Host-seeking behavior requires favorable conditions such as optimal temperature, photoperiod, solar energy, and in some tropical regions, the transition from the dry to rainy seasons. At these optimal conditions, ticks then must successfully recognize an appropriate host. This can be cued by odors like CO$_2$, ammonia, or butyric and lactic acid found in sweat, as well as radiant heat, visual signals, or vibrations [51, 65]. *D. variabilis* is an ambush type of host-seeker; it waits on vegetation for a host and then quests by extending its forelegs anterolaterally so it is able to cling to the host. *A. americanum* acts similarly, but is also more aggressive in seeking a host directly. Once a tick has acquired a host it searches for a suitable feeding site, which may take several hours. It secretes cement, a proteinaceous material, over several days to help keep itself attached to the feeding site [59, 60]. Ticks release substances like anticoagulants, antihistamine, substances that inhibit platelet aggregation, and other enzymes that suppress the immune response of the host, thereby evading the host’s defenses [59, 66, 67]. Increased blood flow to the feeding site also occurs due to secretion of vasodilators [66]. Moreover, some ticks, including *D. variabilis* and *A. americanum*, secrete calreticulin, a calcium-binding protein that may play a role in inhibiting host platelets and increasing vascular permeability [68]. Many additional genes expressed by the tick’s salivary glands during a feeding event are of unknown function, and may play a role in transmitting pathogens [67, 69]. Only after a specific series of events occur can a tick actually begin its blood meal. The attachment time can vary from 3-8 days for nymphs to 7-12 days for adult females [60]. The long period allows for synthesis of new cuticle, the site of muscle attachment on the tick that also helps prevent desiccation and is required for the tick to ingest a large quantity of
blood. A larva can ingest 10-20 times its body weight, while adult females can ingest from 60-200 times [51, 59, 60]. This, however, is concentrated blood since the tick’s salivary glands eliminate excess water and salts during feeding; therefore, the tick actually ingests more volume than that over the entire feeding event. The completion of feeding, shown by dropping off from the host, is synchronized with host behavior; for example, *D. variabilis* larvae and nymphs drop off once night begins [51].

*D. variabilis* and *A. americanum*, like most Ixodidae species, are considered non-nidicolous because they do not remain in the nests and homes of their hosts. The natural habitat of *D. variabilis* is mainly moist secondary growth of deciduous eastern forests, yet they also occupy grassy meadows, alongside roads, the brushy areas surrounding homes, and edges of fields and forests [8, 51]. *A. americanum* is mainly found in woodland areas, specifically disturbed hardwood forests with young trees and/or dense brush [8]. The geographical distribution of these two species depends on several factors, including the presence of enough moisture to prevent desiccation and temperatures above zero degrees Celsius. However, some ticks are able to survive short periods of desiccation [51]. Furthermore, there may be differences in habitat ranges for juvenile versus adult ticks. Juvenile voles are mainly found in woodlands, while adults frequent old-field and ecotone habitats. It is mainly the juvenile voles that are infected with *D. variabilis*, presenting possible differences in location of juvenile versus adult ticks [70]. These differences in spatial dispersion may be due to many factors, such as host drop-off, foraging for resources, general geography, and indirect migration via birds and other wildlife [70]. Host migration events are responsible for expanding the habitat of ticks by potentially introducing them into new geographic regions. The natural range of *D.*
variabilis is approximately 30 meters, while A. americanum can travel only 21 meters [65, 71, 72]. However, this can be greatly expanded due to general travel of their vertebrate hosts. For example, white-footed mice infected with juvenile ticks have been found to travel up to 990 feet, the range of white-tailed deer is 30-75 hectares, and coyotes are also responsible for long-range dispersal of ticks [63, 70, 73]. Nonetheless, tick habitats can also be affected by various changes, all of which potentially explain increases in rickettsial disease incidence and the expansion of host ranges. Shifting and expansion of human populations can greatly affect a tick’s habitat. Development destroys natural habitats and results in increased contact of humans and domestic pets with wildlife, including ticks. The removal of large predators also greatly affects ecological dynamics to an undetermined extent. Yet, one of the greatest human impacts has been the farming industry, which involves the transport of livestock across the country, spreading and introducing tick vectors into new areas [70]. Also, climate changes and dynamics between organism and host species can affect a tick population. It has been shown that the prevalence of ticks infected with the Lyme disease agent, Borrelia burgdorferi, depends on the amount of food source for their mouse hosts [74, 75]. Consequently, these environmental factors can lead to an increase in diseases, like RMSF [76]. Tick activity begins in the spring when larvae begin to feed, while the rise in adult feeding tends to be slightly later in late spring to early summer; females seem to lay the most eggs in July and August [51]. The seasonal peak of RMSF occurs in May or June in the northeast United States, and slightly later in the southeast, in about July. This coincides with the peak abundance of adult D. variabilis ticks, therefore increased public education and vector control should be implemented during these times.
Although public education and increased awareness is mandatory to control the incidence of tick-borne diseases, numerous other methods have been deemed successful. There are several factors that control the dynamics of the zoonotic life cycle and transmission of ticks and tick-borne pathogens. Active surveillance is necessary for identifying geographic, local, and seasonal activities and any shifts occurring in them over time. Limiting tick habitats through controlled burns and cutting vegetation around parks and homes can prevent contact with humans and decrease the incidence of disease. Another common method of population control is the use of chemical pesticides and repellants for both plants and personal human usage, like DEET. However, these methods have negative environmental and health effects. Thus, other physical blocks like wearing long-sleeves shirts, long pants tucked into socks, and protective headwear are safe and more effective at preventing tick bites. The use of topical acaricide on pets and wildlife are also very effective at limiting exposure to ticks. Monthly use of fipronil on dogs and cats decreases tick infestation by tremendous amounts and has now been extrapolated out to treating wildlife, like deer and mice. A 4-poster application device utilized by the USDA passively applies a 2% acaricide to the head, neck and ears of deer as they feed. It has been shown to reduce the number of adult ticks on treated deer by 94% compared to untreated areas [77]. Bait boxes containing acaricide-treated cotton or food also has been used to control tick infestation in mice [62]. Furthermore, the use of fencing to exclude hosts, like deer, from areas proximal to humans is also a fairly efficient means of controlling *A. americanum* tick populations, although large enclosed areas are required and it is most effective only against adult ticks due to small mammals being able to pass through the fencing [78, 79]. The advent of newer means to control
tick populations should be implemented alongside traditional ones to achieve the most efficient vector management program, with control rates up to 99% [80].

The interaction between rickettsiae and their arthropod hosts is of great interest, both genetically and evolutionarily. Many rickettsiae have evolved a stable means of transmission within their tick hosts. Female ticks can lay thousands of eggs and transovarially transmit the rickettsiae infection to each offspring. Up to 100% of infected females can transmit the rickettsiae onto their offspring, called the transovarial rate. In addition, the filial rate, the number of offspring infected, also reaches up to 100% [2, 81, 82, 83]. Thus, ticks are very effective vectors for rickettsial maintenance. The rickettsiae are then maintained transstadially throughout development of the individual tick, and the cycle is repeated. Furthermore, each life stage of the tick has the capability of acquiring a new rickettsial infection by feeding on an infected vertebrate host. However, this acquisition only occurs while this host is considered “rickettsemic”. This interval is very short, usually around 3-4 days, and is characterized by a high bacterial titer [84]. Upon initial infection, rickettsiae infect the epithelial cells of the tick midgut and then go on to infect the salivary glands and ovaries, among other tissues. Bacteria are detected in the hemolymph 3-5 days post infection, while after 7-10 days they are detected in all tissues [84, 85]. After this time period, the ticks are capable of passing on the infection to another host animal, including humans. However, humans are accidental hosts in the life cycles of these species. They are not good hosts because individuals are usually infected with only one or a few ticks at a time and the risk of transmitting a rickettsial infection to an additional individual tick is very low. Vertical transmission is a highly effective means of transmission and is utilized by many low or nonpathogenic organisms.
Therefore, these species are usually found very abundantly in nature, e.g. *R. bellii* represents over 80% of all rickettsial species found infecting ticks in Ohio [85-88].

However, more pathogenic species, such as *R. rickettsii*, tend to exert negative effects on its tick hosts over time, called host lethality. This is illustrated by higher mortality, less successful feedings, smaller egg hatches, and on average only a 39% transmission rate when infected with pathogenic species compared to uninfected ticks [82, 89]. This may explain why *R. rickettsii* is found in such low numbers in nature, less than 1% [2, 84, 90].

Conversely, members of the TG do not utilize vertical transmission. The relationship between TG rickettsiae and their hosts is thought to be more evolutionarily recent than that of the SFG. The invertebrate insect hosts of TG are not well adapted to the presence of the bacteria and die within 1-2 weeks after infection due to damage of the midgut epithelium, allowing for leakage of blood meal into surrounding hemolymph [17]. Thus, lines of ovarian infection are rarely started or maintained [17, 91]. Moreover, transstadial transmission is not very effective in insects, hypothesized to be related to the external changes insects undergo during development [92]. As a result, TG rickettsiae use horizontal transmission, which requires a secondary, or intermediate, host in order to be transmitted from generation to generation. This is usually a vertebrate host, such as a human. The lifestyle of insect hosts, such as fleas and lice, make this form of transmission very feasible; they are normally found in colonies and capable of multiple feedings. There are uninfected as well as rickettsial-infected insects feeding on the same vertebrate host, which consequently becomes infected. Hence, uninfected primary invertebrate hosts have a high probability of becoming infected by feeding on the
vertebrate host. Therefore TG rickettsiae greatly rely on natural reservoirs to be successfully maintained in nature.

*R. rickettsii* may also use horizontal transmission as a secondary means of maintenance. Here, the rickettsiae are passed from a primary invertebrate host (i.e. tick), to a secondary vertebrate host, and then back to an uninfected primary tick. Examples of secondary hosts include many wildlife organisms such as rabbits, birds, rodents and other small mammals. These animals can be parasitized with several ticks at a time. Therefore, an uninfected tick may become infected through feeding and subsequently become capable of initiating a new vertical line of transmission. Ticks in the larval stage play an important role in this situation because they can feed multiple times and are efficient reservoirs for the rickettsiae [2, 85].

Another interesting characteristic of the relationship between rickettsiae and their arthropod hosts is that it is widely accepted that an initial rickettsial infection prevents the acquisition and transmission of a secondary rickettsial infection. This phenomenon is referred to as interference, a cause of which may be due to rickettsiae inducing cellular changes in the ovaries that prevent a secondary infection, has been supported by numerous laboratory studies [93, 94]. It is also supported by the fact that very few, if any, arthropods from nature have been found to be infected with multiple rickettsial species. This idea was first illustrated by ticks from the Bitterroot Valley in Montana. Ticks from the west side of the valley were routinely infected with *R. rickettsii* corresponding to a high occurrence of RMSF. However, both incidence of disease and presence of *R. rickettsii* were low on the east side of the valley. Ticks were later found to harbor a nonpathogenic agent, *R. peacockii*. It was concluded that the initial presence of
R. peacockii in these ticks prevented them from acquiring and transmitting R. rickettsii [95]. However, a more plausible explanation is that there is population isolation due to the geographical barrier of the river within the valley. Another example of this apparent phenomenon is that Rickettsia rhipicephali occurs in high frequency among D. occidentalis ticks from north and central coastal California, prohibiting a high incidence of R. rickettsii [29]. There are also studies that show conflicting evidence for the existence of interference. One laboratory study showed R. rickettsii could infect tissues of R. peacockii infected ticks, where only the ovarian tissues remained infected exclusively with the east-side agent (R. peacockii) [95]. Another study determined that in the laboratory R. felis-infected fleas could also acquire a R. typhi infection, but at a reduced rate relative to uninfected fleas [96]. Finally, it is not clear if interference extends to other microbes beyond the genus Rickettsia since superinfection is very common in many Ehrlichia, insect and viral species; however it has recently been shown that interference occurs within Anaplasma species [86, 97-103]. Also a D. reticulatus tick infected with R. sibirica was found to additionally harbor a viral entity [104]. Furthermore, individual ticks have been found in various geographical locations harboring several rickettsial species [86, 105, 106]. Previous studies that analyzed the idea of interference focused mainly on the ovarian tissue and transmission and not analysis of all tissues. Interference, as understood to occur in rickettsiae, is usually believed to be specific to the prevention of transovarial transmission of two bacterial species. It does not exclude the possibility that a host could acquire a multiple infection. However, interference may prevent two or more species from being passed to the offspring of the infected host. That does not negate the fact the both species can exist in
the same host. Thus, more sensitive assays, like nested and quantitative PCR, may identify rickettsial mixed infections.

The relationship between rickettsiae and arthropod host serves as an excellent model system in which to study co-evolution patterns. The longer rickettsiae are maintained within their host, the stronger the co-evolutionary relationship. Since the rickettsiae are transmitted vertically from mother to offspring within the arthropod for many generations, their genes are under the same selective pressures as the arthropod’s and hence can evolve in tandem. This relationship is best illustrated by comparison with arthropod mitochondrial genes which also have a direct maternal to offspring relationship. However, rickettsiae that are transmitted horizontally never establish a genetic relationship with their host and thus the genes evolve independently. Due to this, one can distinguish clones of rickettsiae based on phylogenetic evolution. One can also better understand the transmission patterns different species of rickettsiae utilized by analyzing and comparing genes of rickettsiae isolates with their respective arthropod hosts. This information is useful as it helps shed light on the host-pathogen relationship in addition to the potential virulence of the species. It is believed that species utilizing vertical transmission are less virulent than species maintained horizontally [107, 108]. This is partly because a transovarially maintained species must not negatively affect their host in order to establish a long term relationship to be successfully transmitted for many generations. Since horizontally transmitted rickettsiae do not need to evolve a long term relationship with their host, they tend to be more virulent [108]. Therefore, by analyzing this type of phylogenetic relationship, one can better understand the potential virulence of rickettsiae, especially since new species are being discovered and implicated in clinical
cases. Since some arthropod species are found co-infected, either with one rickettsiae and a secondary bacterial or virus or possibly two rickettsial species, understanding the virulence of these species may aid in disease diagnosis and treatment, and in the future, vaccine development and vector control or eradication [86, 104-106].

Since less is known about the non-pathogenic, or more possibly low or “yet to be determined” pathogenicity, rickettsiae, they are important to study. Due to the recent genome sequencing of several *Rickettsia* species, it is easy to identify similarities and differences between species as well as between the SFG and TG. Furthermore, potential virulence genes may be identified for analysis based on their role in other alpha-proteobacteria or other bacterial groups. For example, the *vir* class of genes is vital for pathogenicity and tumorogenesis in *Agrobacterium*, and is also found in *Rickettsia* [109-113]. One way to identify potential virulence factors is to analyze and compare genes between known pathogenic rickettsiae and the lower virulence ones. Any differences found, especially in amino acid sequences, may be key in identifying the roles of these genes in virulence. Also, if any gene is mutated or absent it may illustrate that the gene is no longer important in rickettsial pathogenicity. This information may also shed more light on the evolution of the genus and help predict which, if any, species are more likely to emerge as pathogens and have a public health risk in the future.

**AIMS**

The goal of the project was to focus on the entire process of disease, starting with the natural ecology of the disease agent, identifying and analyzing potential virulence genes, to comparing the expression of these genes in rickettsial infected tick and
mammalian cells in order to relate the molecular gene structure to its function in various hosts. Therefore, the aims of the research described here are to (1) determine the epidemiology of rickettsial species present in Ohio and surrounding states in the northeast United States. In addition, it serves to (2) gain information on potential virulence genes in Rickettsia and (3) investigate the evolution and expression among different hosts in nature.

The prevalence and geographical distribution of rickettsial-infected D. variabilis ticks in Ohio was analyzed and shifts that have occurred over the past twenty years were identified. The primary aim of this analysis was to better understand the epidemiology of RMSF which can identify potential changes in host range and species transmission rates. This information also directly correlates to changes in RMSF disease incidence by identifying new focal areas for public education and awareness. The specific superinfection of a single tick was presented at the international meeting of the American Society of Rickettsiology, Logrono, Spain, June 2005. This information was also published in the Annals of the New York Academy of Sciences, 2006.

Further, a population genetic study and phylogenetic analysis was performed on the D. variabilis mitochondrial 12S rRNA gene and the control region. This was performed to identify any subpopulations that could be used to analyze the host-pathogen coevolutionary relationship. Mitochondrial genes are considered to have a higher than average mutation rate and thus increased levels of variability between individuals may be found.

Next, the phylogeographical variation of four vir genes was highlighted in R. amblyommi-infected A. americanum ticks from nature. These genes have not been
directly analyzed in Rickettsia and this research provides the first sequence information for R. amblyommii specifically. The role of the vir genes has not been elucidated and sequencing analysis can demonstrate any nucleotide and amino acid changes in variable regions and in previously determined domains important for virulence compared to other rickettsial and pathogenic species. Part of this information was presented at the annual meeting of the American Society of Rickettsiology, Asilomar, California, 2006.

The expression of these vir genes was also analyzed in rickettsial infected tick and mammalian cell cultures. Differential RNA levels can identify genes important for successful exploitation of ticks and mammalian hosts, determining any shifts that occurred to allow the rickettsiae to successfully infect mammals. It also identifies potential virulence factors, for disease usually occurs in mammalian hosts, like humans.

The results for each stated aim contribute invaluable information to studying rickettsial diseases. The natural epidemiology of rickettsial diseases and rickettsial-infected vectors aids in increasing public health awareness and developing vector control measures. Analysis of the vir genes in R. amblyommii provides novel information on evolutionary relationships among Rickettsia and potential pathogenic species. Finally, understanding the expression of these vir genes allows for the determination of function and role in virulence. These combined analyses satisfy an overall goal of examining the relationship between rickettsiae and arthropod hosts.

REFERENCES


CHAPTER 2

TRENDS IN DERMACEPTOR VARIABILIS DOG TICKS INFECTED WITH THE AGENT OF ROCKY MOUNTAIN SPOTTED FEVER, RICKETTSIA RICKETTISII, AND CASES OF CLINICAL DISEASE IN OHIO OVER A TWENTY YEAR TIME PERIOD

INTRODUCTION

RMSF is a highly pathogenic and often fatal tick-borne disease that is commonly throughout the United States. It is a reportable disease for the Centers for Disease Control and Prevention (CDC), and in 2004 there were 11 cases from Ohio, or 0.64% of all national cases [1]; in addition, one death was reported of a 5 year old girl from Chillicothe, Ohio (Ross county) in 2002 [2]. Therefore, it is of vital importance to know and understand the geographical distribution of this disease. This information leads to more active surveillance allowing for a better overall awareness of the disease, especially in healthcare providers. Furthermore, by knowing the geographical location of D. variabilis dog ticks infected with the etiological agent R. rickettsii, one can infer possible areas in which patients may have acquired the infection. This information can be analyzed over time to determine the ecology and biology of the tick vector. Vector control measures can then be put into place if specific areas have an abundance of infected ticks, or if many of the human disease cases arise from a particular area.
In the mid-1960s, the case rate for RMSF in Ohio increased dramatically over that reported previously. The year 1964 marked the development of a two step screening procedure called a Gimenez stain which tested tick hemolymph for the presence of members of the SFG genus *Rickettsia* [3]. To better understand the patterns of RMSF in Ohio, to confirm suspected cases of RMSF, and to monitor tick populations for increases in SFG infection rates, the Ohio Department of Health: Zoonotic Disease Program (ODH-ZDP) instituted a tick testing program in 1964. Individual ticks are forwarded to ODH-ZDP from veterinarians, clinicians and other local health departments. Active screening for ticks in localities associated with RMSF is also sometimes performed by the ODH-ZDP. The most prevalent tick species acquired is *D. variabilis*, the main vector for *R. rickettsii*, although *A. americanum* Lone star ticks are sometimes submitted. A Gimenez stain is then performed to detect the presence of any rickettsiae. Ticks found positive by this method are then subjected to a direct fluorescent antibody (DFA) test. Isolates positive for the DFA are considered to contain *R. rickettsii*, the etiological agent of RMSF.

The advent of newer technology like PCR and DNA sequencing allows for a more specific and objective identification of rickettsiae present in tick isolates. These molecular methods may identify ticks infected with a small amount of bacteria possibly missed by the microscopy methods used by the ODH-ZDP. By utilizing such techniques a more accurate picture of the epidemiology of RMSF in Ohio can be acquired. In addition to *R. rickettsii*, two species considered nonpathogenic, *R. bellii* and *R. montanensis*, are commonly found in Ohio [4-6]. Hemolymph positive *D. variabilis* ticks were obtained from the ODH-ZDP from the years 2003 and 2004 to determine the
prevalence of these three rickettsial species within Ohio through molecular analysis.
Ticks were screened for the presence of the 17kDa surface antigen gene to identify the
rickettsial species present. The 17kDa gene was chosen because it is specific to the genus
*Rickettsia* and has species-specific nucleotide sequences. The geographical location of
each tick, provided by the ODH-ZDP, was compared to results obtained in the Fuerst
laboratory from the years 1983-6. This twenty year study determined changes in the
incidence of *R. rickettsii* and RMSF changes. It also identified shifts in geographical
location of infected tick vectors, as well as geographical foci that may be “hotspots” for
disease. Finally, the ODH-ZDP methodology was compared to the molecular techniques
utilized here.

**MATERIALS AND METHODS**

**Samples**

Seventy-one *D. variabilis* ticks collected during ODH-ZDP’s 2003 annual screen
for RMSF and fifty-two ticks from the 2004 annual screen were obtained. All ticks were
hemolymph-positive for *Rickettsia* sp. Additionally seventeen ticks from 2003 and seven
from 2004 were determined *R. rickettsii*-positive by a DFA test. Fifty-six of the seventy-one
ticks acquired from 2003 and thirty-three of the fifty-two ticks from 2004, for a total
of eighty-nine samples, were analyzed in this study.
**DNA extraction**

*D. variabilis* ticks were surface sterilized in 70% ethanol for 10 min., followed by two washes in distilled water (5 min., 1 min.). Ticks were then extracted using one of three methods.

1. Ticks were ground in 180 μl of PBS using a sterile mortar and pestle. DNA was then extracted using the animal cell protocol of the DNeasy® Tissue Kit (QIAGEN, Valencia, CA) and eluted in 100 μL Buffer AE (QIAGEN, Valencia, CA) followed by a secondary elution in 50 μL.

2. Ticks were cut into several pieces using a 1.5 inch 18G admix needle (Becton Dickinson, Franklin Lakes, NJ). DNA was extracted using the IsoQuick® Nucleic Acid Extraction Kit (ORCA Research Inc., Boswell, WA) following the Rapid DNA Extraction protocol and eluted in 50 μL of the provided RNAase free water.

3. Ticks were cut into several pieces using a 1.5 inch 18G admix needle. DNA was extracted following the IsoQuick® Nucleic Acid Extraction Kit following the Rapid DNA Extraction protocol. The initial aqueous phase was removed according to step nine and added to a DNeasy® Mini spin column (QIAGEN, Valencia, CA) from the DNeasy® Tissue kit. DNA was then extracted according using the animal cell protocol, starting with step five. DNA was eluted in 100 μL Buffer AE followed by a secondary elution in 50 μL.
17kD surface antigen gene semi-nested PCR

10 µL-primary amplification reactions contained: 1 µL DNA template, 1 µL 10X buffer (Invitrogen, Carlsbad, CA), 1 µL 50mM MgCl₂ (Invitrogen, Carlsbad, CA), 2 µL 100mM dNTPs (Invitrogen, Carlsbad, CA), 1 µL 100ng/µL BSA, 0.5 µL 20µM forward primer, 0.5 µL 20µM reverse primer, 2.9 µL water, and 0.1 µL Taq DNA polymerase (Invitrogen, Carlsbad, CA). A negative control contained 1 µL of sterile water in place of DNA template. 25 µL-secondary amplification reactions contained: 1 µL of a 1:10 dilution of the primary amplification as template, 2.5 µL 10X buffer, 2 µL 50mM MgCl₂, 4 µL 100mM dNTPs, 2.5 µL 100 ng/µL BSA, 1 µL 20µM forward primer, 1 µL 20µM reverse primer, 10.8 µL water, and 0.2 µL Taq DNA polymerase. A negative control contained 1 µL of a 1:10 dilution of the primary negative control as template. Amplification was done in a Whatman Biometra T-Gradient Thermocycler (Biometra, UK) using conditions of 95°C for 5 min.; 35 cycles of 95°C for 1 min., 53°C (primary)/56°C (secondary) for 1 min., and 60°C for 1.5 min., followed by a final extension at 68°C for 15 min. The primers used in the primary amplification were 17k-5' 5' GCTTTACAAAATTCTAAAAACCATATA [7] and 17kD3' 5'CTTGCCATTGTCCRTCAGGTTG. Primers used in the secondary amplification were 17k-5' and 17kD3'nest 5 'TCACGGCAATATTGACC.

17kD surface antigen gene multiplex PCR

Reactions were performed as described above for the semi-nested PCR with the following adjustments. The primers used in the primary amplification were 17k-5’ and 17kD3’. Secondary amplification contained 2 µL of a 1:10 dilution of the primary
amplification as template, 7.8 µL water, 2 µL of 17k-5’, 1 µL of 17kD3’montA
5’GCCTATTACAACGTGTGAGGTGTAC or 17kD3’montB
5’GCCTATTACAACGTGTGAGGTGTAC and 1 µL of 17kD3’bellii
5’ACTGCTACCACCTGGTGCAGC, specific to *R. montanensis/R. rickettsii* and *R. bellii*, respectively. Conditions were the same as for primary amplification, but using a 68°C annealing temperature. The PCR product using primer 17kD3’montB was the only one used in subsequent studies.

**Citrate synthase PCR**

10 µL-primary amplification reactions contained: 1 µL DNA template, 1 µL 10x TITANIUM™ Taq PCR buffer (Clontech, Palo Alto, CA), 1.5 µL 100mM dNTPs, 0.3 µL 20µM forward primer, 0.3 µL 20µM reverse primer, 1 µL 100ng/µL BSA, 4.7 µL water and 0.2 µL TITANIUM™ Taq DNA polymerase (Clontech, Palo Alto, CA). The primers used were CS1dvar 5’ATGACCAATGAAAATAATAAT and CS1273r 5’CATAACCAGTGTAAGCTG [8]. A negative control contained 1 µL of sterile water in place of DNA template. Amplification was done in a Whatman Biometra T-Gradient Thermocycler using conditions of 95°C for 5 min.; 35 cycles of 95°C for 1 min., 54°C for 1 min., and 68°C for 1.5 min., followed by a final extension at 68°C for 15 min. Secondary amplification contained 2 µL of a 1:10 dilution of the primary amplification as template, 2.5 µL 10x TITANIUM™ Taq PCR buffer, 3.5 µL 100mM dNTPs, 0.8 µL 20µM forward primer, 0.8 µL 20µM reverse primer, 2.5 µL 100 ng/µL BSA, 12.7 µL water and 0.2 µL TITANIUM™ Taq DNA polymerase. Primers used were CS409d 5’CCTATGGCTATTATGCTTC and either CS956rSFG
5’GAATATACTCAGAACTACCG or CS856rBellii
5’AAAGCGATGCAATACCAGTACTGAC. Thermocycling conditions were the same as for the primary amplification but with a 56°C annealing temperature.

Agarose electrophoresis

PCR products were visualized in a 0.55% Synergel™ (Diversified Biotech, Boston, MA) and 0.7% agarose mixed gel.

Amplimer purification

PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN, Valencia, CA) protocol and eluted in 30 µL of elution buffer. PCR products from the 17kDa multiplex reaction were extracted from the synergel/agarose gel using a sterile razor blade. Band isolates were placed on an approximately 0.5 inch square piece of nylon and spun at 14,000g for 1 min. to elute the DNA.

DNA sequencing

Purified PCR products were directly sequenced with 2µM primer 17k-5’, 17kD5’seq 5’GGTTCTCAATTYGG or Cs409d, as appropriate, using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) following manufacturer’s instructions. The nucleotide sequence was visualized using Sequencing Analysis Software v.3.7 with the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).
Vector cloning

1 µL of a 1:10 dilution of the purified 17kD surface antigen gene PCR product was cloned into the pCR® 2.1-TOPO® vector using the TOPO® TA Cloning® Kit (Invitrogen, Carlsbad, CA). All tentatively positive colonies were purified with the QIAprep® Spin Miniprep Kit (QIAGEN, Valencia, CA) and subjected to an EcoRI restriction digest using 5 µL DNA, 1 µL React® 3 Buffer (Invitrogen, Carlsbad, CA), 1 µL EcoRI (Invitrogen, Carlsbad, CA), and 3 µL water. Samples were incubated at 37°C for one hour, and positive samples were confirmed by the presence of an insert of the correct size using agarose electrophoresis. Positive clones were then sequenced using the M13 forward primer (Invitrogen, Carlsbad, CA) following sequencing methods described above.

RESULTS

Of the seventy-one *D. variabilis* ticks acquired in 2003, fifty-six were screened using the 17kDa gene. Twenty-five were found positive (44.6%) for the presence of *Rickettsia*; nine ticks were found to contain *R. rickettsii* (16.1%), thirteen *R. bellii* (23.2%), one *R. montanensis* (1.8%), and one tick (#03-797) was superinfected with *R. rickettsii*, *R. montanensis* and *R. bellii* (1.8%) (Table 1). In contrast, the initial DFA screen performed by the ODH-ZDP determined that seventeen ticks of the seventy-one total ticks obtained (23.9%) were *R. rickettsii*-positive, compared to the 16.1% found positive here (Table 1). The rickettsial positive ticks from 2003 originated in the following counties of Ohio: Clark, Delaware, Fayette, Franklin, Hocking, Knox, Lorain, Lucas, Marion, Morrow, Richland, Ross, Vinton, and Wyandot (Figure 1). Of the fifty-
two *D. variabilis* ticks acquired in 2004, thirty-three ticks were screened using the 17kDa gene; fifteen were found positive (45.5%) for the presence of *Rickettsia*. Six were found to contain *R. bellii* (11.5%); however the remaining nine samples did not yield sequences that could be analyzed (Table 1). Here, the initial screen performed by the ODH-ZDP determined seven of the fifty-two total ticks obtained (13.5%) were *R. rickettsii*-positive by the DFA test (Table 1). The positive ticks from 2004 originated in the following counties of Ohio: Crawford, Darke, Delaware, Fayette, Geauga, Knox, Lucas, Morrow, Pike, Ross, Sandusky, and one from an unknown source (Figure 1). The number of rickettsial-positive ticks found in each county from 2003-4 is shown in Table 2.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>1983-6</th>
<th>2003</th>
<th>2004</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rickettsia</em> positive by 17kDa surface antigen gene</td>
<td>N/A</td>
<td>44.6% (25/56)</td>
<td>45.5% (15/33)</td>
</tr>
<tr>
<td><em>R. rickettsii</em></td>
<td>6%</td>
<td>16.1%</td>
<td>N/A</td>
</tr>
<tr>
<td><em>R. bellii</em></td>
<td>82.1%</td>
<td>23.2%</td>
<td>11.5%</td>
</tr>
<tr>
<td><em>R. montanensis</em></td>
<td>11.9%</td>
<td>1.8%</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Rickettsia</em> positive by DFA</td>
<td>22% (10/45)</td>
<td>23.9% (17/71)</td>
<td>13.5% (7/56)</td>
</tr>
</tbody>
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Table 1. Comparison of number of *Dermacentor variabilis* ticks positive for *Rickettsia* sp. by the 17kDa surface antigen gene and DFA methods.

The RMSF screen performed by the ODH-ZDP in the years 1983-6 identified eighty-four hemolymph positive ticks, of which only forty-five were tested using the DFA assay. Ten of these forty-five ticks (22.2%) were DFA positive for *R. rickettsii* (Table 1). All eighty-four ticks were analyzed with the 17kDa gene in the Fuerst laboratory. Of these, sixty-nine were identified as *R. bellii* (82.1%), ten were *R. montanensis* (11.9%), and only 5 (6%) were *R. rickettsii* (Table 1) [9]. The positive ticks originated in the
following counties of Ohio: Butler, Clark, Clermont, Coshocton, Delaware, Defiance, Fairfield, Franklin, Highland, Jefferson, Knox, Licking, Lucas, Madison, Marion, Meigs, Montgomery, Morrow, Muskingum, Perry, Preble, Warren, Williams and Wyandot (Figure 1).

As described previously, tick #03-797 submitted from Hocking County in 2003 was found positive for multiple rickettsial species. Initial analysis of the 17kDa nucleotide sequence suggested the presence of multiple amplimers. This was observed by the presence of dual electropherogram peaks at numerous base positions throughout the nucleotide sequence (Figure 2). Further analysis revealed that the superimposed sequences corresponded to *R. bellii* as well as *R. montanensis*. Although not conclusive, results were consistent with the additional presence of *R. rickettsii*. Clones were found to contain *R. bellii*, *R. montanensis*, or *R. rickettsii*-specific sequence inserts, at proportions of 3/6, 2/6, and 1/6, respectively. Subsequent sequence analysis of the 17kDa gene product showed multiple electropherogram peaks, consistent with previous results. Individual clones from the vector cloning of this PCR product were found to contain sequence inserts specific to *R. bellii* (4/12), *R. montanensis* (7/12), or *R. rickettsii* (1/12). Species-specific PCR primers were then designed to analyze this sample in a multiplex PCR. One primer was specific for *R. bellii*, while the second was specific to *R. rickettsii* and *R. montanensis*. Three separate primers could not be designed due to the close sequence identity between *R. montanensis* and *R. rickettsii* and the location of these sequence differences between these species. These primers produced PCR products of 362 bp for primer 17kD3’bellii, 430 bp for primer 17kD3’montA, and 475 bp for primer
Figure 1. Geographical distribution of *Dermacentor variabilis* ticks determined positive for the 17kDa surface antigen gene in Ohio from the years 1983-6, 2003, and 2004. Counties positive for *Rickettsia* are illustrated in blue. Counties positive for *Rickettsia rickettsii* are illustrated in red.
<table>
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<tr>
<th>Ohio County</th>
<th>1983-6</th>
<th>2003-4</th>
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<tr>
<td>Butler</td>
<td>11</td>
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</tr>
<tr>
<td>Clark</td>
<td>9</td>
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</tr>
<tr>
<td>Clermont</td>
<td>5</td>
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</tr>
<tr>
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</tr>
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</tr>
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</table>

Table 2. Number of rickettsial-infected *Dermacentor variabilis* submitted from Ohio counties in the years 1983-6 and 2003-4.
17kD3’montB when used with primer 17k-5. Multiplex PCR resulted in the amplification of two products with the predicted sizes for each primer set (Figure 3). The individual bands were isolated and sequenced. The *R. bellii* specific amplimer resulted in a nucleotide sequence consistent with *R. bellii*. The *R. montanensis/R. rickettsii* specific product from primer 17kD3’montB resulted in a mixed nucleotide sequence. When cloned, one clone contained an *R. montanensis*-specific insert while the second one was specific to *R. rickettsii*. Finally, the citrate synthase gene was analyzed to further support the presence of multiple rickettsial species. Once again species-specific primers were designed for analysis of the sample. Separate PCR reactions for each primer set were performed and resulted in products differing in size from each other, as expected. The nucleotide sequences for these PCR products were consistent with *R. montanensis* and *R. bellii*. The *R. montanensis* product was then cloned to determine if *R. rickettsii* was present in small amounts, but no *R. rickettsii* specific clones were found (0/9).

**DISCUSSION**

The frequency of *Rickettsia* sp. in Ohio was found to remain relatively constant from the mid-1980’s to 2004, according to both the 17kDa gene analysis and DFA testing. However, the two analyses are slightly discordant in their findings, with the 17kDa analysis results showing a higher rickettsial infection rate (Table 1). According to the ODH-ZDP the DFA analysis identifies *R. rickettsii* specifically, while here it is presented as SFG specific; this conflict is addressed in more detail below. These data are also in concordance with the overall number of RMSF cases in Ohio, which has also been relatively constant, if not slightly decreasing (Figure 4). However, the specific
Figure 2. Multiple peaks present in the nucleotide sequence electropherogram of a partial region of the 17kDa surface antigen gene from tick #03-797

Figure 3. Secondary amplification of the 17kDa surface antigen gene multiplex PCR. Lane 1: 1 kb DNA Ladder (Invitrogen, Carlsbad, CA), Lane 2: Tick #03-797 with primers 17kD5', 17kD3'bellii and 17kD3'montA, Lane 3: empty, Lane 4: Tick #03-797 with primers 17kD5', 17kD3'bellii and 17kD3'montB, Lane 5: empty, Lane 6: Negative control using primers 17kD5', 17kD3'bellii and 17kD3'montB. The *R. bellii* specific band is 356 bp. The *R. montanensis/R. rickettsii* band is at 430 and 475 bp for primers 17kD3'montA and 17kD3'montB, respectively.
frequency of *R. rickettsii* was found to have increased over the past twenty years, 6% in 1985 and 16.1% in 2003. This data is admittedly small so caution must be used in drawing too broad a conclusion. Furthermore, the data suggest that the geographical distribution of rickettsial positive ticks has become more diverse in Ohio over the past twenty years. Few rickettsial positive ticks were found in south-central Ohio from 1983-6, whereas a large number were identified in 2003-4 (Figure 1). This also corresponds to a rise in RMSF cases from this region (Figure 4, Table 3). For example, Ross County had only two RMSF cases from 1980-89, but ten from 2000-2006, including a death in 2002 [10, 11]. More proactive screening may have been done in this area afterwards accounting for the spike in positive ticks in 2003-4. Further, the number of rickettsial-positive ticks has increased in the north-central/northeastern regions (Figure 1). This is also consistent with the apparent increase in RMSF cases in the northeastern areas of Ohio (Figure 4). Cuyahoga county in the north-east did not have any cases in 1980-89 but nine were reported from 2000-2006; Knox county in the central area of the state went from two cases in 1980-89 to five in 2000-2006 [10, 11]. Yet, the location of *R. rickettsii*-infected ticks does not correlate to the location of RMSF cases (Figures 1 and 4), as shown here and in past studies [6]. For example, from 1983-6 both Clermont and Lucas counties had a high number of RMSF cases with a low prevalence of *R. rickettsii* (Tables 2 and 3). Nevertheless, from 1983-6 to 2003-4, there appears to be only a moderate concordance between the amount of RMSF cases (average 24.5/year to an average 10/year) and the frequency of DFA-positive ticks (22.2% to an average 18.7%) identified through the tick screening program. In conclusion, although the geographic
Figure 4. Number and geographical distribution of RMSF cases in Ohio in 1983-6 compared to 2003-4

- 1983-6 RMSF Cases:
  - Total N = 98
  - 1983: N = 25
  - 1984: N = 22
  - 1985: N = 22
  - 1986: N = 29

- 2003-4 RMSF Cases:
  - Total N = 20
  - 2003: N = 9
  - 2004: N = 11
<table>
<thead>
<tr>
<th>Ohio County</th>
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Table 3. RMSF cases per county in Ohio for the years 1983-6 and 2003-4
ecology of rickettsial-infected *D. variabilis* ticks has shifted over this twenty year time period, the number of RMSF cases has declined, alluding that there may be a decrease in their contact with humans.

The observation of superinfection in tick #03-797 with *R. bellii*, *R. montanensis*, and *R. rickettsii* is the first report of such an occurrence with multiple rickettsiae from arthropods in nature, though superinfection is common with many other bacterial species in nature [12-16]. Additionally, insects are commonly infected with several symbionts and different viral and/or bacterial species can infect the same host, including a *D. reticulatus* tick that was laboratory infected with *R. sibirica* and found to harbor an additional viral entity [17-19]. Each symbiont has co-evolved a different mechanism for maintenance within the host [20]. It is hypothesized that the less virulent organisms evolved a stable transovarial transmission [21], such as would occur for *R. bellii* in the case seen in this report. *R. bellii*, an organism considered to be nonpathogenic for mammals, is commonly found in ticks in Ohio, at frequencies up to 80% [4-6]. Subsequently acquired organisms may be acquired horizontally, as we hypothesize for *R. montanensis* and *R. rickettsii*. Pathogenic species such as *R. rickettsii* tend to have negative effects (including host lethality) on their hosts, effects that are thought to arise from the transovarial transmission of the pathogenic organism [22-26]. As a result, horizontal transmission may play a greater role in the maintenance of these species in arthropods, and may explain the low frequency of infected ticks in nature, less than 1% [22, 25, 26]. The presence of *R. rickettsii* in this tick may represent the intermediate step in its transmission cycle. There exists numerous consequences for a superinfected host due to interspecific competition among rickettsiae, including fitness tradeoffs and
evolution of virulence [27]. Since survival of some rickettsiae depends on occasional horizontal transmission events, multiply infected arthropods may serve a vital role in nature for the maintenance of more virulent species.

There are discrepancies in the ticks identified as *R. rickettsii* between the 17kDa molecular analysis and the microbiological assays of Gimenez and DFA performed by the ODH-ZDP. The DFA assay is based on antibodies raised against *R. rickettsii*; however, the test is apparently not *R. rickettsii* specific. Many antibodies for SFG species are cross-reactive [28-31]. This explains why individual DFA-positive ticks were found to harbor *R. bellii* or *R. montanensis* by the 17kDa gene analysis. Furthermore, PCR is a more sensitive assay and can identify a lower quantity of organisms that a DFA may have missed. Therefore, DFA analysis should be considered to detect SFG positive organisms, and be used in conjunction with more specific assays like PCR and DNA sequencing to correctly identify the species present. Additionally, the hemolymph test in itself is a very subjective assay. The ability to detect the presence of specific rickettsiae within a cell and not other similarly small rod-shaped bacteria may lead to false positive results. This helps explain why not all hemolymph-positive ticks yielded positive amplicons for the 17kDa gene. Another explanation for the low proportion of positive PCR results from many hemolymph-positive ticks may also be due to the quality of the DNA. Whole ticks were obtained from the ODH-ZDP without the addition of ethanol or other buffered solution. It is not known how long the ticks were kept at the ODH facilities. All of the positive ticks from each year were obtained from the ODH-ZDP as a group and not individually at the determination of each positive result; thus some ticks may have been acquired and tested months prior. Moreover, some ticks may have had
low concentrations of rickettsiae or be in different infection stages. This may also explain why PCR was not successful at amplifying the rickettsiae, even with performing a very sensitive assay like semi-nested PCR. All of these reasons should be taken into account for future RMSF analyses performed jointly by the Fuerst laboratory and the ODH-ZDP. The addition of molecular analysis to the current methods of the ODH-ZDP would increase reliability, especially in detecting the prevalence of *R. rickettsii*.

RMSF remains a disease of interest in Ohio and the country even with the improvement of awareness and diagnosis of clinical cases. Since 1983, there have been approximately 200 RMSF-associated deaths in the United States alone, even after proper diagnosis and treatment in some cases [32, 33]. Therefore, it is imperative to survey natural tick populations for the presence of *R. rickettsii* as well as other SFG-rickettsiae.

**SUMMARY**

In conjunction with the ODH-ZDP, *D. variabilis* ticks collected in 2003 and 2004 were molecularly analyzed to determine the rickettsial species present. The geographical location origin of each tick was noted to identify epidemiological shifts in *R. rickettsii* that has occurred since a similar study was performed from 1983-6. There were no infected ticks found in the southwest area of the state in recent years, but their presence was found in the north-central/north-east and south-central counties for the first time (Figure 1). Overall, however, the number of affected counties has decreased, comparable to a decrease in RMSF cases (RMSF Disease Fact Sheet available @ [http://www.odh.ohio.gov/odhPrograms/idc/zoodis/vbdp/vbfdfacts.aspx](http://www.odh.ohio.gov/odhPrograms/idc/zoodis/vbdp/vbfdfacts.aspx)). The reason for the shift in location of infected *D. variabilis* populations could be due to human
interference, environmental or climate alterations, or even the availability of primary vertebrate hosts. This information can lead to more awareness of the disease, especially if cases are documented in more urban areas, such as Columbus, Cleveland, or Cincinnati. The utilization of molecular analysis is of vital importance to properly identify the presence of \textit{R. rickettsii} and to determine its relationship to the locations of RMSF cases. The results of this study are informative for implementing vector control measures, public education initiatives, and in developing an effectual and conclusive test for identifying this disease agent.

**ACKNOWLEDGMENTS**

I would like to thank the Ohio Department of Health- Zoonotic Disease Program for kindly providing the \textit{Dermacentor variabilis} ticks used in this study.

**REFERENCES**


CHAPTER 3

VARIABILITY IN THE MITOCHONDRIAL CONTROL REGION AND 12S RDNA GENE AMONG *DERMACENTOR VARIABILIS* TIKCS

INTRODUCTION

Ticks are a major vector worldwide in the transmission of infectious diseases to humans and livestock, being surpassed only by mosquitoes [1]. In addition to transmitting disease, ticks also decrease the value of livestock by causing physical damage to the animals’ hides. There are over fifty species of ticks that are known to bite humans, including *D. variabilis*, the American dog tick. This species is one of the main vectors for RMSF which affects only countries in the western hemisphere. In the United States, this tick species is found along the Atlantic coast westward to the Rocky Mountains and along parts of the Pacific coast [1, 2]. Large tick populations are found primarily in the southeastern states along the Atlantic coast, such as Maryland and North Carolina. Infection rates of *R. rickettsii*, the etiological agent of RMSF, in *D. variabilis* ticks range from as low as 2% in Connecticut to 10% in Alabama [3-5]. This also coincides with the yearly number of RMSF cases per state. In 2005 there were 1,936 clinical cases, of which 625 occurred in North Carolina (32.3%) while only 21 cases were from Ohio (1.1%) [6]. Important vertebrate hosts for immature *D. variabilis* are the white footed mouse, *Peromyscus leucopus*, and the meadow vole, *Microtus*.


*ppenysylvanicus [4, 7, 8]. However, adults feed on dogs and many other mammals, like “cattle, opossum, coyote, hog, horse, raccoon, wild cat, squirrel, sheep, skunk, deer, fox, domestic cat, mule, rabbit…” [9].

Although much is known about the morphology, behavior and ecology of *D. variabilis* ticks, little molecular analysis has been done, as is also the case with most tick species. In fact, a long-standing hypothesis on arthropod evolution is based on morphological characteristics. Here, it is thought the tick’s mouthparts evolved as they became specialized with host adaptation [10]. Yet, with the advent of newer molecular technologies, one can compare morphological data to molecular ones. Phylogenetic analyses of ticks, including *Dermacentor*, have mainly focused on the mitochondrial genome. Analysis of the entire mitochondrion has found that the placement of NADH dehydrogenase subunit 4 (ND4) and the 12S-16S rRNA genes in the Metastriata groups differ from other arthropods as well as from the ancestral gene order [11]. Several analyses have been done using the 12S, 16S, and 18S ribosomal DNA genes [12-14]. These results differ from each other in several criteria, including the monophyly of *Amblyomminae*, and the relationship of *Dermacentor* to *Haemaphysalinae* and *Rhipicephalinae*. This is mainly due to the fact that these gene regions do not possess enough variable sites for analysis. Furthermore, outside of these three regions, there is not much additional sequence information available for *D. variabilis*.

Therefore, in order to better understand the genetics and evolution of ticks, a population genetic study of rickettsial-infected *D. variabilis* ticks from nature was performed. Examining the mitochondrial sequence in more detail will greatly aid in determining the phylogenetic relationships among ticks, as well as providing novel
information to the database that can be utilized on a larger scale. These organisms can also provide vital information on how tick genes may be affected by a natural rickettsial infection. The potential co-evolution of the tick vector with its rickettsial pathogen can be analyzed by identifying the presence of sub-populations of ticks that have harbored rickettsiae for many generations. Knowing how rickettsial species are transmitted may shed light on their virulence or potential for virulence [15, 16].

*D. variabilis* ticks were acquired from the ODH-ZDP as part of their annual screen for RMSF. The mitochondrial genes for the control region (D-loop) and the 12S rDNA were chosen for analysis based on the potential high variability that is required for phylogenetic analysis. Also, since the ticks originated from various geographical areas, any phylogeographical differences could be determined among the studied genes. Overall, the D-loop region was analyzed in seventy-one ticks, while the 12S rDNA was analyzed in forty-four ticks. Little variation was found among individuals for each gene analyzed individually or in the combined data set; additionally no direct correlation was found between sequence type and geographical location.

**MATERIALS AND METHODS**

**Samples**

Seventy-one *D. variabilis* ticks collected during ODH-ZDP’s 2003 annual screen for RMSF and fifty-two ticks from the 2004 annual screen were obtained. All ticks were hemolymph-positive for *Rickettsia* sp. Seventeen ticks from 2003 and seven from 2004 were additionally determined *R. rickettsii*-positive by a DFA test. From 2003, thirty-five ticks were analyzed for the D-loop, while twelve were analyzed for the 12S rDNA, with
eight of these being duplicates between the two genes. Furthermore, from the 2004 ticks, thirty-six were analyzed for the D-loop and thirty-two for the 12S rDNA, with twenty-five being analyzed for both genes.

**DNA extraction**

*D. variabilis* ticks were surface sterilized in 70% ethanol for 10 min., followed by two washes in distilled water (5 min., 1 min.). Ticks were then extracted using one of three methods.

1. Ticks were ground in 180 µL of PBS using a sterile mortar and pestle. DNA was then extracted using the animal cell protocol of the DNeasy® Tissue Kit and eluted in 100 µl Buffer AE followed by a secondary elution in 50 µL.

2. Ticks were cut into several pieces using a 1.5 inch 18G admix needle. DNA was extracted using the IsoQuick® Nucleic Acid Extraction Kit following the Rapid DNA Extraction protocol and eluted in 50 µL of the provided RNAase free water.

3. Ticks were cut into several pieces using a 1.5 inch 18G admix needle. DNA was extracted following the IsoQuick® Nucleic Acid Extraction Kit using the Rapid DNA Extraction protocol. The initial aqueous phase was removed according to step nine and added to a DNeasy® Mini spin column from the DNeasy® Tissue kit. DNA was then extracted according using the animal cell protocol, starting with step five. DNA was eluted in 100 µL Buffer AE followed by a secondary elution in 50 µL.
PCR

All reactions were performed under the following standard conditions. 25 µL reactions contained 2 µL DNA template, 2.5 µL 10x TITANIUM™ Taq PCR buffer (Clontech, Palo Alto, CA), 3.5 µL 100mM dNTPs, 0.8 µL 20µM forward primer, 0.8 µL 20µM reverse primer, 2.5 µL 100ng/µL BSA, 12.7 µL water and 0.2 µL TITANIUM™ Taq DNA polymerase (Clontech, Palo Alto, CA). A negative control contained 2 µL of sterile water in place of DNA template. Exceptions to this protocol are noted as appropriate. All amplifications were done in a Whatman Biometra T-Gradient Thermocycler.

D-loop PCR

Primers used were mt(+) 5’ AATAGGGTATCTAATCCTAGTTTAA and mt(-) 5’ TACATWATTATCCTATCAAGATA. Thermocycling conditions used were 95°C for 5 min.; 35 cycles of 95°C for 1 min., 53°C for 1 min., and 72°C for 1 min., followed by a final extension at 72°C for 15 min.

12S rDNA PCR

Primers used were 12S(+) 5’ AGAGYGACGCGGCGATATGT and 12S(-) 5’ TATCTTGATAGGATAAATWATGTA. Thermocycling conditions used were 95°C for 5 min.; 35 cycles of 95°C for 1 min., 58°C for 1 min., and 72°C for 1 min., followed by a final extension at 72°C for 15 min.
Agarose electrophoresis

PCR products were visualized in a 0.55% Synergel™ and 0.7% agarose mixed gel.

Amplimer purification

PCR products were purified using the QIAquick® PCR Purification Kit protocol and eluted in 30 µL of elution buffer.

DNA Sequencing

Purified PCR products were directly sequenced using primers mt(+), mt(-), 12S(+) or 12S(-), as appropriate, using the BigDye® Terminator v1.1 Cycle Sequencing Kit following manufacturer’s instructions. The nucleotide sequence was visualized using Sequencing Analysis Software v.3.7 with the ABI 310 Genetic Analyzer.

Phylogenetic analysis

Sequences were manually aligned initially with XESEE [17] and then updated using Bioedit 7.0.8 [18]. UPGMA phylogenetic trees based on number of nucleotide differences were produced using Mega 3.1 [19].

RESULTS

The D-loop control region and the partial adjacent 3’ 12S rRNA gene were analyzed in seventy-one ticks; thirty-five of which were acquired from 2003 and thirty-six from 2004. All but two ticks were from Ohio across twenty-six counties; one tick
was from Pennsylvania and one from Virginia (Table 4). A total of 246 bp in the 3’ 12S rRNA gene region and 204 bp, with up to 300 bp for some samples, of the D-loop were sequenced. Twenty-six variable sites or 10.4% were found in the 12S rRNA gene, and only 7 variable sites or 3.4% were found in the D-loop region, having an overall variation of 7.3% for this region. The two gene regions were combined into one region and the full consensus sequence was submitted to GenBank (Accession #AY520138) (Appendix A; Figure 33). UPGMA phylogenetic analysis was performed on the combined gene region and is shown in Figure 5.

For the 3’ 12S rDNA/D-loop gene region, there was considerable phylogenetic variation across all samples, although there were also numerous groupings of identical sequences. There was one large grouping of sixteen identical sequences, one with six identical samples and three clades containing five samples each; however, there was no overall relationship among tick samples in regards to geographical location (Figure 5). Present in the grouping of sixteen individuals were four of the fifteen ticks obtained from Ross County (03-831, 03-736, 03-693, 03-875), two of the six ticks from Lucas County (03-1030 and 04-614), and the single tick from Pennsylvania. In addition, two of the four ticks obtained from Delaware County (04-146 and 03-713) were grouped together were two of the fifteen ticks obtained from Ross County (03-115 and 03-170) and two of the four ticks obtained from Fayette County (03-322 and 04-352). This lack of specific geographical sequence type was seen for numerous additional ticks. However, the single tick from Virginia (04-589) and the single ticks collected from Fulton (03-822) and Washington (03-846) counties had unique sequences.
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Table 4: Number of *Dermacentor variabilis* ticks analyzed for the 3’ 12SrDNA/D-loop gene region classified by geographical location.
Figure 5. UPGMA phylogeny of the mitochondrial 3’ 12S rDNA/D-loop region from *Dermacentor variabilis* based on number of nucleotide differences. Color of individual tick number denotes geographical location.
The 5’ partial 12S rRNA gene was sequenced and analyzed in forty-four ticks, twelve were acquired from 2003 and thirty-two from 2004. All of the ticks except one were from Ohio spanning nineteen counties, with one tick from Pennsylvania (Table 5). A total of 330 bp were sequenced and fifteen variable sites were found or 4.5%. The total amount of variation in the 12S rRNA gene was 7.1% when the 5’ and 3’ regions were combined. The consensus sequence was submitted to GenBank (Accession #EF690463) (Appendix A; Figure 29). UPGMA phylogenetic analysis was performed on this gene region and is shown in Figure 6.

Due to there being little variation found in the D-loop region, but more variation found in the adjacent 12S rDNA region, the remaining 5’ region of the 12S rDNA was analyzed. Here, less variation was found overall compared to the 3’ 12S rDNA region, 4.5%, compared to 10.4%. The two 12S rDNA regions combined yielded 7.1% variability, over twice as much as the D-loop region with 3.4%. There were five main sequence types, a group of nineteen individuals, a group of six, two groups of four and one containing three samples; yet, there was no correlation with geographical location (Figure 6). However, present in the nineteen identical sequences were both ticks from Knox County (03-372 and 04-168), both ticks from Marion County (03-093 and 03-403), two of the four ticks obtained from Fayette County (03-061 and 04-352), two of the three ticks obtained from Lucas County (04-189 and 04-364) and two of the eight ticks obtained from Ross County (03-433 and 03-651). Also, three of the eight ticks obtained from Ross County were also identical in sequence to each other (04-103, 04-416 and 04-581). For this gene region, unique sequences were found for the single tick from Pennsylvania (04-325) and the single tick collected from Wyandot County (03-370) as
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Table 5. Number of *Dermacentor variabilis* ticks analyzed for the 5’12S rDNA gene region classified by geographical region.
Figure 6. UPGMA phylogeny of the mitochondrial 5' 12S rDNA region from *Dermacentor variabilis* based on number of nucleotide differences. Color of individual tick number denotes geographical location.
well as the only ticks analyzed for this gene from Franklin (04-323) and Pike (04-122) counties.

Sequence data was combined for the thirty-three samples in which both gene regions had been analyzed to determine if further separation of individual sequences could be identified. UPGMA phylogenetic analysis was performed and is shown in Figure 7. More unique sequences were identified using the combined data, as there was only one main grouping of nine identical sequences with the remaining mostly being unique sequence types (Figure 7). Here, there was slight phylogeographical variation observed due to the increased separation of sequence types. The single ticks analyzed for all three gene regions from Ashland (04-608), Delaware (04-146), Mahoning (04-584), Marion (03-093), and Morrow (04-381) counties all had unique sequences, as did the ticks from Pennsylvania (04-325) and Franklin County (04-323), which were also identified as having unique sequence types in the 5’ 12S rDNA region analysis. Nonetheless, the group of nine identical sequences contained both ticks from Knox County (03-372 and 04-168) and two of the five ticks from Ross County (04-103 and 04-416) as well as ticks from four other counties.

DISCUSSION

The analysis of the mitochondrial 12S rDNA and the control region (D-loop) in D. variabilis greatly adds novel information to the public database. In fact, the 3’ region of the 12S rDNA and the control region had not been previously analyzed in this tick species, and the sequences presented here are the first of these gene regions available in GenBank.
Figure 7. UPGMA phylogeny of the combined mitochondrial 3’ 12S rDNA/D-loop region and 5’ 12S rDNA region from *Dermacentor variabilis* based on number of nucleotide differences. Color of individual tick number denotes geographical location.
No strong phylogeographical correlation was found for either of the tick mitochondrial genes analyzed here. Although some ticks obtained from the same county grouped together, the remaining ticks from the same location were not necessarily identical to them or to each other. Also, even if two or more ticks from the same county were identical they also may have been identical to ticks from other Ohio counties. Furthermore, molecular analysis for both gene regions was not performed on all ticks obtained from a single geographical location, which compounds the results of each separate phylogenetic analysis. Yet, in the combined data for the 3’ 12S rDNA/D-loop and 5’ 12S rDNA gene regions, some separation due to geographical location was observed. Unique sequences were found for geographical locations where only one tick was obtained overall, as seen for Ashland County (04-608) and the tick from Pennsylvania (04-325). However, some of the ticks with unique sequence types were not the only individuals obtained from the corresponding county but were the only individuals analyzed for both gene regions. Therefore, from this data alone, it is not conclusive that some counties are phylogeographically distinct. Also, some solely analyzed individuals grouped with either one additional tick or were present in the large group of nine individuals.

Finally, each phylogenetic analysis was critiqued for differences across larger geographical regions of the entire state of Ohio, compared to individual counties. For both the single analysis of the 3’ 12S rDNA/D-loop region and the 5’ 12S rDNA region, there was no overall regional phylogeographical variation, nor within any subgrouping. Conversely, when the two data sets were combined, some regional geographical variation could be discerned. For example, as the sequence types diverged from the main grouping
of nine individuals, there was a slight correlation to geography (Figure 7). The first few branches contained individuals from the central part of Ohio; Franklin, Delaware and Morrow counties; the next subset included individuals from the south-central to south-west part of the state; Ross, Hocking, Fayette, Clark and Darke counties; and the last subsets contained individuals from the far north region of Ohio; Sandusky and Geauga counties. There were some “outliers” to this analysis though; for individuals from Ross County were present in several of the subsets, while individuals form Lucas County in the extreme north-west and Mahoning County in the north-east were present in the earlier branch groups.

In addition to the lack of phylogeographical variation, there was also no separation due to general rickettsial infection or even for specific rickettsial species. Although all ticks obtained from the ODH-ZDP were considered rickettsial positive, infections were only confirmed through PCR for some individuals, while others yielded negative PCR and others were not molecularly analyzed (Table 6). For both separate gene region analyses as well as the combined data, rickettsial-positive ticks grouped with ticks considered to be rickettsial negative through molecular analyses (see Chapter 1). Furthermore, ticks infected with \textit{R. bellii}, \textit{R. rickettsii}, or \textit{R. montanensis} also contained no unique phylogenetic variation. The only possible exception was a tick from Hocking County (03-797), which had a mixed infection containing three rickettsial species, \textit{R. bellii}, \textit{R. montanensis}, and \textit{R. rickettsii}. This individual had a unique sequence type for the 5’ 12S rDNA but not for the 3’ 12S rDNA/D-loop gene region (Figures 5 and 6). Thus, the 12S rDNA and the D-loop gene region are either not affected by the presence of rickettsiae, or there is not enough variation present to identify distinct tick lineages.
Additional genes will need to be analyzed to determine unique sequence types and the amount of co-evolution present between tick and their rickettsial pathogens.

Overall, the two mitochondrial genes analyzed here, the 12S rDNA and the D-loop control region, do not provide enough variation to separate all tick samples from one another. However, this is the first time the 3’ 12S rDNA/D-loop gene region sequence has been analyzed in D. variabilis. This adds new information to the database allowing for more accurate phylogenetic relationships among various tick species to be determined. For both gene regions analyzed individually, there was no correlation with geographical location, although slight phylogeographical differences were discerned in the combined data set. This could be because both of these genes are under strong evolutionary constraints and thus will not vary significantly across individuals. This is expected for the D-loop gene region, also known as the control region, because it contains the signals needed to initiate DNA synthesis. The DNA forms a loop structure as the newly synthesized strand displaces the original strand. Evolutionary, these signals are important and most likely do not exhibit changes over time. Although this study provides novel gene sequence information, further gene regions should be analyzed to allow for a greater understanding of tick population structures, as well as overall phylogenetic relationships with other acarine species.

ACKNOWLEDGMENTS

I would like to thank the Ohio Department of Health: Zoonotic Disease Program for kindly providing the Dermacentor variabilis ticks used in this study.
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Continued

Table 6. Molecular analysis of the 17kDa surface antigen gene in *Dermacentor variabilis* ticks analyzed for the D-loop region and 12 rDNA
Table 6 continued

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REFERENCES


CHAPTER 4

MOLECULAR AND PHYLOGEOGRAPHICAL ANALYSIS OF THE VIR GENES, VIRB8, VIRB11, VIRB4, AND VIRD4 FROM RICKETTSIA AMBLYOMMI ISOLATES FROM THE NORTHEASTERN UNITED STATES

INTRODUCTION

Many bacterial pathogens utilize type IV secretion systems (T4SS) to export protein or DNA substrates across bacterial and eukaryotic membranes into the cytosol of host cells. These systems are important for virulence in many pathogens such as Agrobacterium tumefaciens, Wolbachia pipientis, Brucella spp., Bartonella henselae, Helicobacter pylori and Legionella pneumophila [1-3]. The best characterized system is that of Agrobacterium, where genes of the vir operons (virA-G) are essential for tumorigenesis [3]. The recent completion of genome sequencing for nine rickettsial species provides a useful tool for analyzing and comparing genes among rickettsiae and their relationship with other pathogens. The presence of many T4SS genes, including the vir gene group, has been identified within rickettsial genomes. The virulence determinants in rickettsiae and how rickettsial species differ in their pathogenicity are mostly unknown, but are crucial for better understanding the bacteria and the diseases they cause.
T4SS are bacterial organelles, ancestrally related to bacterial conjugation methods, that translocate DNA or protein substrates across the cell envelope often for pathogenesis [1]. This system was originally discovered and has since been extensively characterized in *A. tumefaciens*, the bacterium that causes crown gall disease in numerous dicotyledonous plants. The genes for tumorogenesis are encoded on the 200 kb extrachromosomal Ti (tumor inducing) plasmid [4]. There are five regions of this plasmid including the T region, *vir* region, *rep* region, the *tra* and *trb* loci, and the genes for catabolism of opines, bacterial nutrients [3]. The T region is processed into a translocation competent substrate and transferred into the plant host nuclei via the direction of the *vir* genes [3, 5]. There are eight *vir* operons, *virA*, -B, -C, -D, -E, -F, -G, and -H, spanning 40 kb, although only six are essential for pathogenesis [3-5]. Homologues to these *vir* genes are present in other bacterial pathogens, including the *ptl* genes in *Bordetella pertussis*, the *dot* and *icm* genes in *L. pneumophila* and *Coxiella burnettii*, the *cag* genes in *H. pylori*, and the *tra*, *trb*, and *trw* genes in many self-transmissible plasmids [1, 2, 6]. The location of these genes and the specific DNA/protein substrates differ in each organism, yet the processes are analogous.

The *vir* genes form complexes to successfully transfer the T region; this is accomplished through the steps of substrate processing, recruitment to the transfer apparatus and translocation through a transenvelope, known as the mating pore [2]. This is mainly performed by the *virB* and *virD* operon genes, although *virA* and *virG* induce expression of the *vir* genes, and *virE1* and *virE2* help protect the T-strand substrate [1, 7]. A set of *vir* genes referred to as DNA transfer and processing proteins form a unit called a relaxosome, and bind to the origin of transfer sequence for the processing of the DNA
substrates. Here, the relaxase, either \textit{virD1} or \textit{virD2}, nicks the T-strand, remains bound to the 5’ end of the DNA, and recruits a coupling protein, like \textit{virD4} [1-3]. This protein is characterized by an amino-terminal proximal region that contains two transmembrane helices and a small periplasmic domain, as well as a cytoplasmic carboxy-terminal domain [1]. Some bacterial systems, however, function independent of a coupling protein, including \textit{B. pertussis}, \textit{Brucella} sp., and \textit{H. pylori} [1, 2]. The coupling protein is an inner membrane protein that localizes to the cellular poles, has potential ATPase activity, and is required for virulence in \textit{A. tumefaciens} [2, 8-10]. It interacts with the DNA substrate through the effector protein \textit{virE2} and the relaxosome complex in order to recruit the bacterial DNA to the transfer apparatus [1-3]. The carboxy-terminal domain of \textit{virE2} binds tightly to single stranded nucleic acids forming the T-complex, and contains nuclear localization sites which help mediate transport of the T-DNA from the cytoplasm to the nucleoplasm [1-3, 7]. The chaperone protein \textit{virE1} is necessary for proper translocation of \textit{virE2} although it does not interact directly with the DNA substrate [2, 11]. \textit{virD4} directly interacts with \textit{virB10}, a member of the mating pair formation (mpf) proteins, and these two proteins may act to help recruit and direct the DNA to and through the mpf channel [1, 3, 12, 13].

Complete translocation of the DNA or protein substrate occurs through the mating pore or transenvelope structure, which spans both the inner and outer membranes of the host cells. This is a stable core complex that contains the inner membrane proteins \textit{virB4}, \textit{virB8} and \textit{virB10} as well as the outer membrane proteins, \textit{virB7} and \textit{virB9} [1, 2]. The outer membrane proteins \textit{virB7} and \textit{virB9} (similar to \textit{Escherichia coli}’s \textit{TraV} and \textit{TraK} proteins, respectively) form disulfide bonds to produce a heterodimer structure that
stabilizes the other \textit{virB} proteins [1, 5, 8]. In fact, the remaining \textit{virB} proteins accumulate in proportion to the \textit{virB7-virB9} heterodimer [7]. \textit{virB9} may also function in recruiting additional \textit{virB} proteins in order to form the core complex [5]. The inner membrane protein \textit{virB8} (\textit{ptlE} in \textit{B. pertussis}) functions in recruiting \textit{virB1}, a transglycosylase, for lysis of the peptidoglycan, the bacterial outer cell wall layer [1, 6]. It localizes to the cellular poles, is essential for virulence, and has been shown to homodimerize and interact with \textit{virB10} [11, 14, 15]. This function may aid in delivering the DNA substrate to the plant cell since most bacteria attach to the plant cell itself at the cellular poles. The energy for substrate transport and assembly of the core complex may come from the ATPase, \textit{virB4}, or homologue \textit{PtlC}, for mutations result in a loss of conjugation frequency [8]. It contains two domains, which protrude across the cytoplasmic membrane allowing it to interact with other mpf proteins and possibly \textit{virB11} [1, 3, 5].

Finally, the translocation of the DNA or protein substances through the pore may be controlled by \textit{virB11}, a cytoplasmic hydrophilic ATPase, homologue to \textit{PtlH} [6]. The structure of \textit{virB11} is a homomultimer that forms hexameric pores, of which the opening and closing are regulated by ATP [1-3, 5]. Since \textit{virB11} can autoprophosphorylate through its ATP binding domain, these conformational changes may provide the energy to translocate the DNA substrate through the pore, although some studies have shown this may occur in the presence of additional associated proteins [1, 5, 7, 16]. Both ATPases, \textit{virB4} and \textit{virB11}, as well as the coupling protein \textit{virD4}, contain Walker A nucleotide binding domains (GxxGxGKT/S, sometimes also referred to as the Walker Box domain), which are required for recruitment of substrates and ultimately virulence [5, 9, 16-18].
There are two hypothesized models for how the translocation process occurs. In the one-step model, the mpf proteins assemble in one step, and the coupling protein recruits and coordinates with an ATPase to drive the substrate through the channel [1, 2]. Alternatively, the process could occur in two steps; here the coupling protein first moves the substrate across the inner membrane and then the mpf proteins deliver it across the outer membrane; this model has also been referred to as the piston model [1, 2]. This latter model alludes to versatility of T4SS, for it explains how both DNA and protein substrates can be utilized. In addition, some proteins can possess multiple binding domains, allowing for increased adaptability to novel or changing conditions. Future work will uncover how the coupling protein interacts with other mpf proteins and with DNA or protein substrates. Nonetheless, whether the vir genes present in Rickettsia sp. are functional and play the same role as illustrated here is yet to be determined.

Therefore, four vir genes, virB8, virB11, virB4 and virD4, were chosen and analyzed in a population study of R. amblyommii-infected A. americanum ticks from nature. Ticks were obtained from the U.S. Army Center for Health Promotion & Preventive Medicine (CHPPM). Individuals originated from the north-east United States including Kentucky, New Jersey, and Maryland, with the majority being from Virginia. Since these genes have not been previously analyzed directly in Rickettsia sp., they were compared between the nine completed rickettsial genomes, and PCR primers were designed for their amplification. This information represents the first analysis of vir genes from rickettsiae. We present population and phylogeographical variation, as well as analysis of the Walker A motif in virB11, virB4, and virD4. This data identifies the
potential virulence of *R. amblyommii* allowing for a greater understanding of virulence patterns among all Rickettsiae.

**MATERIALS AND METHODS**

**Samples**

Pools and individual patient samples harboring *A. americanum* ticks were obtained from CHPPM. Samples were numbered according to individual patients and thus, not all tubes contained single ticks. Most samples only contained one specimen, however seven contained multiple ticks, from 2 – 4. Ticks arrived stored in IsoQuick® Reagent A (Sample Buffer) and Reagent 1 (Lysis Solution) (ORCA Research Inc., Boswell, WA). *Rickettsia* isolates *R. rhipicephali* CWPP, *R. australis* JC, and a *R. montanensis*-positive *D. variabilis* tick were also analyzed in this study.

**DNA extraction**

*A. americanum* ticks were extracted using one of the following two methods.

1. DNA was extracted using the IsoQuick® Nucleic Acid Extraction Kit starting with step two of the Rapid DNA Extraction protocol and eluted in 50 µL of the provided RNAase free water.

2. DNA was extracted using the IsoQuick® Nucleic Acid Extraction Kit starting with step two of the Rapid DNA Extraction protocol. The initial aqueous phase was removed according to step nine and added to a DNeasy® Mini spin column from the DNeasy® Tissue kit. DNA was then extracted according using
the animal cell protocol, starting with step five. DNA was eluted in 100 \( \mu \)L Buffer AE followed by a secondary elution in 50 \( \mu \)L.

**17kD surface antigen gene semi-nested PCR**

A total of 182 patient samples, either in pools or individually were screened for the presence of rickettsiae using analysis of the 17kD surface antigen gene. Individual samples from positive pools were subsequently screened. 10 \( \mu \)L-primary amplification reaction contained: 1 \( \mu \)L DNA template, 1 \( \mu \)L 10X buffer, 1 \( \mu \)L 50mM MgCl\(_2\), 2 \( \mu \)L 100mM dNTPs, 1 \( \mu \)L 100 ng/\( \mu \)L BSA, 0.5 \( \mu \)L 20\( \mu \)M forward primer, 0.5 \( \mu \)L 20\( \mu \)M reverse primer, 2.9 \( \mu \)L water, and 0.1 \( \mu \)L Taq DNA polymerase. A negative control contained 1 \( \mu \)L of sterile water in place of DNA template. 25 \( \mu \)L-secondary amplification reaction contained: 2 \( \mu \)L of a 1:10 dilution of the primary amplification as template, 2.5 \( \mu \)L 10X buffer, 2 \( \mu \)L 50mM MgCl\(_2\), 4 \( \mu \)L 100mM dNTPs, 2.5 \( \mu \)L 100 ng/\( \mu \)L BSA, 1 \( \mu \)L 20\( \mu \)M forward primer, 1 \( \mu \)L 20\( \mu \)M reverse primer, 9.8 \( \mu \)L water, and 0.2 \( \mu \)L Taq DNA polymerase. A negative control contained 1 \( \mu \)L of a 1:10 dilution of the primary negative control as template. Amplification was done in a Whatman Biometra T-Gradient Thermocycler using conditions of 95°C for 5 min.; 35 cycles of 95°C for 1 min., 53°C (primary)/ 56°C (secondary) for 1 min., and 60°C for 1.5 min., followed by a final extension at 60°C for 15 min. The primers used in the primary amplification were 17k-5' and 17KD3'. Primers used in the secondary amplification were 17k-5' and 17KD3'nest.
vir genes PCR

All reactions were performed under the following standard conditions. 25 µL reactions contained: 2 µL DNA template, 2.5 µL 10x TITANIUM™ Taq PCR buffer, 4 µL 100mM dNTPs, 0.5 µL 20µM forward primer, 0.5 µL 20µM reverse primer, 2.5 µL 100 ng/µL BSA, 12.8 µL water and 0.2 µL TITANIUM™ Taq DNA polymerase. A negative control contained 2 µL of sterile water in place of DNA template. A positive control consisted of 2 µL of a 1:10 dilution of either R. montanensis strain OSU 85-939 or R. conorii strain VR141 Moroc. Amplification was done in a Whatman Biometra T-Gradient Thermocycler using conditions of 95°C for 5 min.; 35 cycles of 95°C for 1 min., X°C (annealing temperatures shown in Table 1) for 1 min., and 68°C for 1.5 min., followed by a final extension at 68°C for 15 min. The primers used for amplification of each gene are as follows: virB8: virB8-5’ and virB8-3’; virB11: virB11-5’ and virB11-3’; virB4: virB4-5’ and virB4-3’; virD4: virD4-5’ and virD4-3’ (Table 7).

Agarose electrophoresis

PCR products were visualized in a 0.55% Synergel™ (Diversified Biotech, Boston, MA) and 0.7% agarose mixed gel.

Amplimer purification

PCR products for virB4 and virD4 were purified using the QIAquick® PCR Purification Kit and eluted in 30 µL of elution buffer. PCR products for virB8 and virB11 were physically extracted from the agarose gel using a sterile razor and purified using the QIAquick® Gel Extraction Kit and eluted in 30 µL of elution buffer.
Table 7. *vir* genes PCR and sequencing primers and annealing temperatures. Sequencing primers are internal to the PCR primers and marked by “seq” in the primer name.

**DNA sequencing**

Purified PCR products were directly sequenced using 2μM of either the PCR primers for each gene respectively, in addition to internal sequencing primers (Table 7), as appropriate and necessary, using the BigDye® Terminator v1.1 or v3.1 Cycle Sequencing Kit following manufacturer’s instructions. The nucleotide sequence was visualized using the Sequencing Analysis Software v3.7 with the ABI 310 Genetic Analyzer or ABI 3100 Genetic Analyzer.
Phylogenetic analysis

Sequences were manually aligned using Bioedit 7.0.8 [19] UPGMA phylogenetic trees based on number of nucleotide differences were produced using Mega 3.1 [20].

Vector cloning

1 µL of a 1:10 dilution of each purified PCR product was used cloned into the pCR® 2.1-TOPO® vector using the TOPO® TA Cloning® Kit. All tentatively positive colonies were purified with the QIAprep® Spin Miniprep Kit and subjected to an EcoR I restriction digest using 5 µL DNA, 1 µL React® 3 Buffer, 1 µL EcoR I, and 3 µL water. Samples were incubated at 37°C for one hour, and positive samples were confirmed by the presence of an insert of the correct size using agarose electrophoresis. Positive clones were then sequenced using the M13 forward primer following sequencing methods described above.

RESULTS

17kD surface antigen gene

Forty-six patient samples (25.3%) were found to contain A. americanum ticks positive for rickettsial species. Sequencing analysis of the 17kD surface antigen gene revealed all ticks contained R. amblyommii. The positive ticks originated from eleven geographical locations: Ft. Hill AP, Virginia, Norfolk NS, Virginia, Washington Arng., Virginia, Langley AFB, Virginia, Ft. Knox, Kentucky, W.H. Ford RTC, Kentucky, Ft. Campbell, Kentucky, Aberdeen Proving Ground, Maryland, McGuire AFB, New Jersey, Ft. Dix, New Jersey, and Lakehurst NAES, New Jersey (Table 8). Two samples,
#011238 and 011417, contained very low amounts of DNA and thus, were not subsequently analyzed for any of the vir genes. Nine samples, 010200, 010257, 010264, 010265, 010266, 010267, 010276, 010300, and 010627, contained only enough DNA for analysis of virB8 and virB11.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Geographical Location</th>
<th># positive for Rickettsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Ft. Hill, AP, Virginia</td>
<td>14</td>
</tr>
<tr>
<td>Sample</td>
<td>Norfolk, NS, Virginia</td>
<td>1</td>
</tr>
<tr>
<td>Sample</td>
<td>Washington Arng, Virginia</td>
<td>1</td>
</tr>
<tr>
<td>Sample</td>
<td>Langley, AFB, Virginia</td>
<td>1</td>
</tr>
<tr>
<td>Sample</td>
<td>Ft. Campbell, Kentucky</td>
<td>13</td>
</tr>
<tr>
<td>Sample</td>
<td>W.H.Ford, RTC, Kentucky</td>
<td>6</td>
</tr>
<tr>
<td>Sample</td>
<td>Ft. Knox, Kentucky</td>
<td>3</td>
</tr>
<tr>
<td>Sample</td>
<td>Aberdeen Proving Ground, Maryland</td>
<td>2</td>
</tr>
<tr>
<td>Sample</td>
<td>McGuire, AFB, New Jersey</td>
<td>2</td>
</tr>
<tr>
<td>Sample</td>
<td>Ft. Dix, New Jersey</td>
<td>2</td>
</tr>
<tr>
<td>Sample</td>
<td>Lakehurst NAES, New Jersey</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 8. Geographical locations of Rickettsia amblyommii-infected Amblyomma americanum ticks analyzed for vir genes.

**virB8:**

The virB8 gene was analyzed in forty R. amblyommii isolates from A. americanum ticks. The ticks originated from all eleven geographical locations (Table 8). A total of 710 bp was sequenced, and thirty-six variable sites were found, for a total variation of 5.1%. This gene region was submitted to GenBank and represents novel information for virB8 from R. amblyommii (Accession #EF026585), R. montanensis (Accession #EU496526), R. rhipicephali (Accession #EU523149), and R. australis (Accession #) (Appendix A; Figures 34-37). UPGMA phylogenetic analysis was
performed based on the number of nucleotide differences (Figure 8). The \textit{R. amblyommii} isolates were found to be most similar to \textit{R. rhipicephali}. \textit{R. montanensis} was found to be divergent from the group containing \textit{R. rickettsii}, \textit{R. conorii}, and \textit{R. sibirica}. The \textit{R. australis} isolate was found to be most related to \textit{R. felis}.

There was considerable phylogenetic variation found in \textit{virB8}. Eight samples, 010276, 010804, 010813, 0101063, 010257, 010810, 010791 and 010807, had unique sequences; however, there was no overall relationship among tick samples in regards to geographical location, either by location or by state (Figure 8). Ticks originating from Ft. Hill AP, Virginia and Ft. Campbell, Kentucky were present in numerous clades, being similar to ticks from all other locations. The single isolate from Langley AFB, Virginia, 010791, did contain a unique sequence. None of the other sole isolates from Norfolk NS, Virginia, Washington Arng, Virginia, McGuire AFB, New Jersey, and Lakehurst NAES, New Jersey had unique sequences. Most of the isolates from W.H. Ford RTC, Kentucky were highly similar to one another with isolate 011053 being the only exception.

\textit{virB11}

The gene \textit{virB11} was analyzed in thirty-nine \textit{R. amblyommii} isolates from \textit{A. americanum} ticks. Ticks originated from all eleven geographical locations (Table 8). A total of 910 bp was sequenced and thirty-one variable sites were found, for a total of 3.4\% variability. This gene region was submitted to GenBank and represents novel information on \textit{virB11} from \textit{R. amblyommii} (Accession #EF026586), \textit{R. rhipicephali} (Accession #EU523150), and \textit{R. australis} (Appendix A; Figures 38-40).
Figure 8. UPGMA phylogeny of \textit{virB8} based on number of nucleotide differences. Color of sample number depicts geographical location.
UPGMA phylogenetic analysis was performed based on the number of nucleotide differences (Figure 9). The *R. amblyommii* isolates were found most similar to *R. rhipicephali*. The *R. australis* isolate was found to be most similar to *R. akari*, both divergent from *R. felis*.

There was considerable phylogenetic variation present in *virB11*. Thirteen samples had unique sequences, 010257, 010749, 010276, 010300, 010302, 010200, 010849, 010812, 010806, 010810, 010813, 010809, and 010804; there was a relationship present among tick isolates in regards to geographical location by location and by state (Figure 9). Five of the six isolates from W.H. Ford RTC, Kentucky were identical in sequence, with isolate 0102581 being the only exception. Six of the ten isolates from Ft. Campbell, Kentucky not only had unique sequence but were the most divergent of all the *R. amblyommii* isolates. Two of the three isolates from locations in New Jersey, 010846 and 011050, were identical in sequence. Finally, there was a group of four isolates from Ft. Hill AP, Virginia that were also divergent from the rest of the isolates next to the Ft. Campbell, Kentucky isolates previously described.

*virB4*

The *virB4* gene was analyzed in nineteen *R. amblyommii* isolates from *A. americanum* ticks. Ticks originated from eight geographical locations, Ft. Hill AP, Virginia, Norfolk NS, Virginia, W.H. Ford RTC, Kentucky, Ft. Campbell, Kentucky, Aberdeen Proving Ground, Maryland, McGuire AFB, New Jersey, Ft. Dix, New Jersey, and Lakehurst NAES, New Jersey. A total of 2300 bp was sequenced and 119 variable sites were found, for a total variation of 5.2%. This gene region was submitted to
Figure 9. UPGMA phylogeny of *virB11* based on number of nucleotide differences. Color of sample number depicts geographical location.
GenBank and represents novel information for virB4 from R. amblyommii (Accession #EU496524), R. rhipicephali, and R. australis (Appendix A; Figures 41-43). UPGMA phylogenetic analysis was performed based on the number of nucleotide differences (Figure 10). The R. amblyommii isolates were found to be most similar to R. rhipicephali. The R. australis isolate was most related to R. felis, both divergent from R. akari.

There was considerable phylogenetic variation present among isolates. Six isolates had unique sequences, 010791, 010804, 010302, 011050, 010749, and 012583; there was no overall relationship among samples in regards to geographical location, although some of the unique samples were the only isolates from their geographical local (Figure 10). The isolates with unique sequences included isolate 010791, the only isolate from Norfolk NS, Virginia and isolate 011050, the only isolate from Lakehurst NAES, New Jersey. The two isolates from Aberdeen Proving Ground, Maryland, 010819 and 012612, were highly similar, although not identical. Finally, isolate 012583 was divergent from the remaining isolates, and was the most similar to the R. rhipicephali isolate.

**virD4**

The virD4 gene was analyzed in twenty-eight R. amblyommii isolates from A. americanum ticks. The ticks originated from nine geographical locations, Ft. Hill AP, Virginia, Langley AFB, Virginia, Ft. Knox, Kentucky, W.H. Ford RTC, Kentucky, Ft. Campbell, Kentucky, Aberdeen Proving Ground, Maryland, McGuire AFB, New Jersey, Ft. Dix, New Jersey and Lakehurst NAES, New Jersey. A total of 1760 bp were
Figure 10. UPGMA phylogeny of \textit{virB4} based on number of nucleotide differences. Color of sample number depicts geographical location.
sequenced and fifty-two variable sites were found, for a total variation of 6.8%. This gene region was submitted to GenBank and represents novel information for virD4 from *R. amblyommii* (Accession #EU496525), *R. rhipicephali*, and *R. australis* (Appendix A; Figures 44-46). UPGMA phylogenetic analysis was performed based on the number of nucleotide differences (Figure 11). The *R. amblyommii* isolates were found to be most similar to *R. rhipicephali*. The *R. australis* isolate was found to be most similar to *R. akari*, both divergent from *R. felis*.

The virD4 gene had the most variability of all the vir genes analyzed resulting in a high amount of phylogenetic variability (Figure 11). Most of the isolates analyzed had unique sequences, sixteen of the twenty-eight. This included isolates 010796, 010812, 011050, 010844, 010819, 012587, 012646, 010780, 010804, 010748, 010810, 010295, 010750, 011052, 010302, and 010813. There was no overall relationship present to isolates in regards to geographical locations (Figure 11). However, the single isolates from McGuire AFB, New Jersey, 010796, Langley AFB, Virginia, 010780, and Lakehurst NAES, New Jersey, all contained unique sequences. There was no other phylogeographical variation present as the samples from Ft. Hill AP, Virginia, W.H. Ford RTC, Kentucky, and Ft. Campbell, Kentucky were similar to one another and the remaining samples.

**Walker A box**

The presence of a Walker A box was found in *virB11* and *virB4*, but no similar motif could be detected in *virD4*. This domain is found in *virB11* from amino acid residues 163-170. The amino acid sequence, GGTSTGKT, is conserved among all
Figure 11. UPGMA phylogeny of virD4 based on number of nucleotide differences. Color of sample number depicts geographical location.
rickettsial species analyzed; however, there is a serine and not a glycine at residue 166. The Walker A domain is found in virB4 at amino acid residues 456-463. The amino acid sequence, GPTGAGKT, is conserved among all rickettsial species analyzed.

**DISCUSSION**

The analysis of the genes virB8, virB11, virB4 and virD4 in R. amblyommii isolates represents the first report of these genes in this species. Also, the analysis of these genes in R. montanensis, R. australis, and R. rhipicephali also contribute novel information to the database. The phylogenetic analysis of these potential virulence genes demonstrates the relationship of R. amblyommii among rickettsial species and lends insight into the role of these genes in rickettsial pathogenesis.

The phylogenetic analyses showed there was little variation based on geographical location. However, the isolates 010302, 010810, 010813, and 010804 had unique sequences for three or more of the vir genes. Three of these isolates all originated from Ft. Campbell, Kentucky; thus, there may be more subpopulations present in this location or potentially less selective pressure on these populations allowing the genes to mutate. R. amblyommii is most related to R. rhipicephali, another species of unknown pathogenicity [21, 22].

In addition, no functional Walker A motif was present in virD4 for any of the rickettsial species. Rickettsiae may not utilize the function of a coupling protein in the process of virulence or virD4 may have evolved a novel role among rickettsiae. The Walker A domain was also found to be mutated in virB11. This mutation to a serine was at one of the residues shown to be vital for pathogenicity in Agrobacterium [16]. Thus,
this gene also may not be functional in rickettsiae due to this mutation being present in all rickettsial species. Another protein may provide the energy for the opening and closing of the transmembrane pore. The presence and conservation of the Walker A domain in \textit{virB4} lends support to the functional importance of this gene for virulence among the rickettsiae. These genes need to be studied in more depth to determine their overall function. This is of importance because the essential virulence determinants are yet to be identified for the Rickettsiae.

The sequence data presented here reports the first direct analysis of \textit{vir} genes in \textit{Rickettsia}, and the first information on these genes in \textit{R. amblyomnii}, \textit{R. rhipicephali}, \textit{R. montanensis}, and \textit{R. australis}. This knowledge allows for a greater understanding of the phylogenetic relationship among \textit{Rickettsia} and helps elucidate their role in virulence. The molecular analysis of these \textit{vir} genes, which are important in pathogenicity within many bacterial species, aids in understanding the evolutionary relationship among pathogens and the evolution of virulence.

\textbf{ACKNOWLEDGMENTS}

I would like to thank Ellen Stromdahl from the United States Army Center for Health Promotion and Preventative Medicine for kindly providing the \textit{Amblyomma americanum} ticks used in this study and Jose Diaz from The Ohio State University for analyzing the sequencing reactions on an ABI 3100 Genetic Analyzer.
REFERENCES


CHAPTER 5

DIFFERENTIAL EXPRESSION OF *VIR* GENES BETWEEN MAMMALIAN AND ARTHROPOD CELLS INFECTED WITH *RICKETTSIA AMBLYOMMII*

INTRODUCTION

Rickettsial diseases represent an emerging threat to humans worldwide yet the factors important for virulence are largely unknown. The agents of these vector-borne diseases are bacteria of the genus *Rickettsia*. They are successfully maintained within invertebrate vectors, like ticks, mites and fleas, and also cycle within vertebrate hosts. These relationships are vital for successful transmission of the rickettsiae from generation to generation. However, disease usually occurs when the bacteria are inadvertently transmitted to humans, an evolutionary dead-end. Genes important for this shift in host species are largely unknown. In addition, it is not known whether the rickettsial infection in humans mimics the infection in naturally infected vertebrates, like deer, dogs, opossums, and rabbits. Arthropod hosts like ticks and mites are able to maintain low or nonpathogenic rickettsiae for many generations; however, more pathogenic and louse-borne rickettsiae exert negative effects on their arthropod host or even cause death [1-5]. Negative effects are also demonstrated in human hosts by the process of disease. If rickettsiae are not adapted to surviving in human hosts, this may cause more damage to host cells than they do for their arthropod hosts. Therefore, disparity in the interaction
between rickettsiae with mammalian versus arthropod hosts may identify genes important for host adaptation. Additionally, genes important for pathogenicity may have higher expression in mammalian cells.

The development of cell cultures for various tick species provides an invaluable resource for studying the relationship between pathogen and host [6-12]. Most available culture types are embryonic in nature and thus are a mixture of several different cell types. These cultures replicate slowly and can be maintained at high cell densities for a long period of time [6]. This allows for the analysis of rickettsial replication and its effect on tick cell viability and survival as it might occur in nature. Also, it represents a novel way to primarily isolate and continually culture rickettsiae from clinical or host samples and when routine cell culturing methods are not successful. Here, an increased amount of rickettsiae can be obtained in order to further analyze pathogenesis using molecular and immunological methods. Genes involved in potential virulence and host-adaptation may be identified through comparative studies between vector and vertebrate hosts.

T4SS genes have been shown to be important for virulence in many bacterial pathogens due to their role in exporting protein or DNA substrates across bacterial and eukaryotic membranes into the cytosol of host cells [13-16]. The \textit{vir} gene groups has been identified in \textit{Rickettsia}, however their role has not been directly analyzed. Understanding the role of these genes in rickettsiae is important for elucidating factors critical for pathogenesis. Therefore, a comparative analysis of the RNA expression of the \textit{vir} genes, \textit{virB8}, \textit{virB11}, \textit{virB4}, and \textit{virD4} was performed using \textit{R. amblyommii}-infected \textit{A. americanum} and Vero cell cultures. \textit{A. americanum} represents the natural tick vector
of *R. amblyommii* and rickettsial genes highly expressed here may be important for host specificity and successful maintenance of the bacteria. Vero cells represent a mammalian host, and rickettsial genes highly expressed in this host may be important in virulence. The RNA expression level of these genes was also compared in the *A. americanum* cell line maintained at a lower temperature. Since ticks are poikilothermic, this lower temperature represents the ambient temperature present when the ticks are not associated with any hosts [17]. It has previously been shown that rickettsiae have a lower survival rate in Vero cells compared to two different arthropod cell lines, in addition, several antigenic genes were not expressed when the tick cells were grown at a lower temperature [17]. Although *R. amblyommii* is considered nonpathogenic, identification of genes expressed in mammalian hosts is important, as other nonpathogenic species have recently been implicated in human clinical cases [18, 19].

A novel qPCR approach was developed to analyze the RNA expression of these four *vir* genes of interest. This is a highly sensitive and very specific assay with good reproducibility that has been utilized for many different systems [20, 21]. It was found that *virD4* and *virB8* had a higher mRNA expression in Vero cells compared to the *A. americanum* cells, while *virB4* and *virB11* had a lower expression level. Also *virB4* and *virD4* had a higher mRNA expression level in the tick cells grown at the lower temperature. *virB4* and *virB11* may play roles in the transport of bacterial substrates important for adaptation to a tick host, while *virB8* may be important in mammalian hosts. Finally *virD4* may function in stress response, since it has a higher expression level in both the Vero and lower temperature tick cells. Both the mammalian cell and lower tick temperature represent environments that the rickettsiae might not be best
suited. As a result, the transmission of bacterial substrates in these hosts might occur by a different method or be severely disrupted compared to the optimal tick-on-host environment. This research represents the first analysis of these vir genes in *Rickettsia* and the differential analysis found between pathogen and host type contributes to elucidating factors important for virulence.

**MATERIALS AND METHODS**

**Cell Cultures**

*A. americanum* tick cell line AAE2 subculture 32 was acquired and maintained at 34°C in 25-cm² tissue culture flasks (Figure 12). Cells were cultured in L15B media as previously described by Munderloh and Kurtti, using 80mM glucose concentration [22]. The media was supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Harlan Biosciences, Indianapolis, IN), 5% tryptose phosphate broth (TPB) (Becton Dickinson, Sparks, MD), and 1% of a 1:10 dilution of bovine lipoprotein cholesterol concentrate (MP Biomedicals, Aurora, OH) equivalent to 10 µg/mL in L15B media. The pH was adjusted to 7.5 by adding 1 N NaOH drop wise to the complete media; 5 mL complete medium was used per flask. The culture medium was replaced once a week leaving some media in the flask as not to remove the nonadherent cells. Individual flasks were subcultured every two weeks.
Vero cells were acquired and maintained at 37°C in 10 mL complete media consisting of low glucose Dulbecco's Modified Eagle Medium (GIBCO-Invitrogen, Carlsbad, CA), supplemented with 6.5% FBS, 1% 100X L-glutamine (GIBCO-Invitrogen, Carlsbad, CA), and 0.25% penicillin-streptomycin (GIBCO-Invitrogen, Carlsbad, CA). Cultures were subcultured every three-four days by completely removing the media, washing cells twice with trypsin diluent (Table 9), and adding 0.5 ml trypsin-EDTA (GIBCO-Invitrogen, Carlsbad, CA) to detach the cells by incubating at 37°C for no longer than two minutes, and adding new media to inactivate the trypsin.
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄ · 7 H₂O</td>
<td>0.245 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.5 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.015 g</td>
</tr>
<tr>
<td>KCl</td>
<td>1 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>20 g</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.05 g</td>
</tr>
</tbody>
</table>

Table 9: Trypsin diluent ingredients per 2.5 L solution

**Rickettsial infection**

*R. amblyommii* infected *Ixodes scapularis* tick cell line, ISE6 was obtained and used to infect the AAE2 and Vero cell cultures. After complete lysis of the ISE6 cells, observed by accumulation of extracellular rickettsiae, the culture was passaged 1:100 to AAE2 cells. Infected cultures were grown in 5 mL complete media consisting of L15B300 media, L15B diluted in one-third volume of water, supplemented with 5% FBS, 5% TPB, 1% diluted lipoprotein, 25mM Hepes buffer (GIBCO-Invitrogen, Carlsbad, CA), and 5% v/v of a 5% sodium bicarbonate solution. The pH was adjusted to 7.2-7.5 by adding 1 N NaOH drop wise to the media. The sixth passage of *R. amblyommii* in the AAE2 cells was used to infect two flasks of AAE2 cells, one flask of AAE2 cells that had been grown at 27°C for thirteen days, and two flasks of Vero cells. The infected flasks, along with uninfected flasks for each cell culture type, were placed at the corresponding temperature and maintained for three days.

**RNA extraction**

RNA was extracted from each infected culture flask using the RNeasy® Plus Mini Kit (QIAGEN, Valencia, CA). Cells from the AAE2 cultures were harvested by
resuspending the cells in the existing media, distributing to 1.5 mL Eppendorf tubes and centrifuging for 5 min. at 22°C at 300 x g (1600 rpm) using a JA-18.1 rotor in a Beckman J2-MC centrifuge. Cells from the Vero cell cultures were collected by removing the existing media, washing twice with trypsin diluent, and adding 0.5 mL trypsin-EDTA to detach the cells. Cultures were incubated at 37°C for approximately two min. and 5 mL new media was added. The culture was then distributed to 1.5 mL Eppendorf tubes and centrifuged for 5 min. at 22°C at 300 x g (1600 rpm) using a JA-18.1 rotor in a Beckman J2-MC centrifuge. Cell pellets were lysed in 350 µL Buffer RLT Plus (QIAGEN, Valencia, CA) and homogenized using a 1.5 inch 21G admix needle (Becton-Dickinson, Sparks, MD). The animal cell protocol was followed and RNA was eluted in 50 µL of the provided RNase-free water, followed by a secondary elution in 30 µL. RNA was immediately stored at -70°C and aliquots were stored at -20°C. RNA concentrations were determined spectrophotometrically using an Eppendorf BioPhotometer (Eppendorf, Westbury, NY) (Table 10).

**DNase treatment**

RNA from the *R. amblyomnii*-infected Vero and *A. americanum* cell cultures were DNase treated using the TURBO DNA-free™ Kit (Ambion, Austin, TX) following the routine DNase treatment. 10 µL of RNA from the Vero cell culture was treated with 1 µL TURBO DNase Buffer (Ambion, Austin, TX) and 1 µL TURBO DNase (Ambion, Austin, TX). 20 µL RNA was used for the 34°C and 27°C *A. americanum* cell cultures using 2 µL TURBO DNase Buffer and 1 µL TURBO DNase. 2 µL DNase Inactivation Reagent (Ambion, Austin, TX) was used for each sample.
RT-PCR

500 ng RNA from each sample was used in a RT-PCR (Table 10). cDNA was generated using the Omniscript® Reverse Transcriptase Kit (QIAGEN, Valencia, CA) using 75 µM random primers (Invitrogen, Carlsbad, CA) and 1 unit/µL RNase inhibitor (QIAGEN, Valencia, CA). Samples were incubated at 37°C for 60 min. and stored at -20°C.

<table>
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<th>RNA Sample</th>
<th>RNA concentration (µg/mL)</th>
<th>Amount used in RT-PCR</th>
<th>Amount used in qPCR</th>
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</thead>
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</tbody>
</table>

Table 10: RNA concentrations and amounts used in RT-PCR of *R. amblyommii*-infected and uninfected Vero, 34°C, and 27°C *Amblyomma americanum* cell cultures

qPCR primers

Primers used in the qPCR reactions were designed using the Plexor™ Primer Design Software (Promega, Madison, WI). The nucleotide sequence for each gene’s PCR product was used as the input and ten primer sets were provided. Resulting primers were then analyzed using NetPrimer [23], and the primer set for each gene that contained the least amount of hairpins and primer dimers was chosen (Table 11). Product sizes for each gene are as follows: *virB8* - 87 bp, *virB11* - 99 bp, *virB4* - 84 bp, *virD4* - 130 bp, and
gltA- 100 bp. Primers were ordered from Integrated DNA Technologies, Coralville, IA and one primer from each set contained the fluorescent label FAM adjacent to an iso-dC residue at the 5’ terminus.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>virB8-forward</td>
<td>5’ ACGAGGCAGTTGTTACCTTTTGAAATCA</td>
</tr>
<tr>
<td>virB8-reverse</td>
<td>5’ 56-FAM/iMe-isodC/ATCCGATTCTTGTTCCCATAACC</td>
</tr>
<tr>
<td>virB11-forward</td>
<td>5’ GGCACTCAGTGAATACCTGCAATAG</td>
</tr>
<tr>
<td>virB11-reverse</td>
<td>5’ 56-FAM/iMe-isodC/GCCAGTAAATGCACTCTGTTAGGAT</td>
</tr>
<tr>
<td>virB4-forward</td>
<td>5’ GCACCTAGATCAAGATTGGTTAAAAGTG</td>
</tr>
<tr>
<td>virB4-reverse</td>
<td>5’ 56-FAM/iMe-isodC/GGCATCTTCAACACTCTGCGTAG</td>
</tr>
<tr>
<td>virD4-forward</td>
<td>5’ AGTAATACAGTTCCGCAGAGATGAGCA</td>
</tr>
<tr>
<td>virD4-reverse</td>
<td>5’ 56-FAM/iMe-isodC/AGGTACAAAAGTCGGCGGTAATAAT</td>
</tr>
<tr>
<td>gltA-forward</td>
<td>5’ CACTAAACAGGTGTCATCATCATCAT</td>
</tr>
<tr>
<td>gltA-reverse</td>
<td>5’ 56-FAM/iMe-isodC/CGCAAGCATAATAGCCATAGGAGAA</td>
</tr>
</tbody>
</table>

Table 11. Sequences of primers used in qPCR for virB8, virB11, virB4, virD4, and gltA

qPCR standards

*R. conorii* strain VR141 Moroc was used to amplify the *vir* genes, *virB8*, *virB11*, *virB4* and *virD4*, and the citrate synthase, *gltA*, gene to use as standard controls in the qPCR reactions.

*vir* genes PCR: All reactions were performed under the following standard conditions. 25 µL reactions contained: 2 µL 1:10 dilution of *R. conorii*, 2.5 µL 10x TITANIUM™ Taq PCR buffer, 4 µL 100mM dNTPs, 0.5 µL 20µM forward primer, 0.5 µL 20µM reverse primer, 2.5 µL 100ng/µL BSA, 12.8 µL water and 0.2 µL TITANIUM™ Taq DNA polymerase. A negative control contained 2 µL of sterile water in place of DNA template. Amplification was
done in a Whatman Biometra T-Gradient Thermocycler using conditions of 95°C for 5 min.; 35 cycles of 95°C for 1 min., X°C (annealing temperatures shown in Table 12) for 1 min., and 68°C for 1.5 min., followed by a final extension at 68°C for 15 min. The primers used for amplification of each gene are as follows: 

\[ \text{virB8}: \text{virB8-5'} \text{ and virB8-3'}; \text{virB11}: \text{virB11-5'} \text{ and virB11-3'}; \text{virB4}: \text{virB4-5'} \text{ and virB4-3'}; \text{virD4}: \text{virD4-5'} \text{ and virD4-3'} \] (Table 12).

\text{gltA} \text{ PCR: } 25-\mu\text{L amplification reactions contained: } 2 \mu\text{L DNA template, 2.5 }\mu\text{L 10x TITANIUM}^{\text{TM}} \text{ Taq PCR buffer, 4 }\mu\text{L 100mM dNTPs, 0.5 }\mu\text{L 20}\mu\text{M forward primer, 0.5 }\mu\text{L 20}\mu\text{M reverse primer, 2.5 }\mu\text{L 100ng/\muL BSA, 12.8 }\mu\text{L water and 0.2 }\mu\text{L TITANIUM}^{\text{TM}} \text{ Taq DNA polymerase. The primers used were } \text{conorii-gltA-5'} \text{ 5'} \text{ ATGACCAATGAAAATAAT and conorii-gltA-3'} \text{ 5'} \text{ TTACCTCTCCCGAATACT. A negative control contained 1 }\mu\text{L of sterile water in place of DNA template. Amplification was done in a Whatman Biometra T-Gradient Thermocycler using conditions of 95°C for 5 min.; 35 cycles of 95°C for 1 min., 54°C for 1 min., and 68°C for 1.5 min., followed by a final extension at 68°C for 15 min.}

\text{Agarose electrophoresis: } \text{PCR products were visualized in a 0.55% Synergel}^{\text{TM}} \text{ and 0.7% agarose mixed gel.}
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>virB8-5’</td>
<td>5’ ATGGAYCMATTAACMAAYGC</td>
<td>55 °C</td>
</tr>
<tr>
<td>virB8-3’</td>
<td>5’ TTATTKCTGATTTTTATTYC</td>
<td>55 °C</td>
</tr>
<tr>
<td>virB11-5’</td>
<td>5’ ATGARTGAAGAATTTGCAGC</td>
<td>58 °C</td>
</tr>
<tr>
<td>virB11-3’</td>
<td>5’ ACTTCYGAAACATATCTTYTYCC</td>
<td>58 °C</td>
</tr>
<tr>
<td>virB4-5’</td>
<td>5’ ATGAARTTATTTAGAAGACTAG</td>
<td>58 °C</td>
</tr>
<tr>
<td>virB4-3’</td>
<td>5’ TATAATGTTTTAACTGC</td>
<td>58 °C</td>
</tr>
<tr>
<td>virD4-5’</td>
<td>5’ ATGGAATGGCATTAAGATAC</td>
<td>56 °C</td>
</tr>
<tr>
<td>virD4-3’</td>
<td>5’ TTACTCATTATTITTSRKARC</td>
<td>56 °C</td>
</tr>
</tbody>
</table>

Table 12. Sequences and annealing temperatures of PCR primers used to amplify vir genes.

Amplimer purification: PCR products for virB4 and virD4 were purified using the QIAquick® PCR Purification Kit and eluted in 30 µL of elution buffer. PCR products for virB8 and virB11 were physically extracted from the agarose gel using a sterile razor, purified using the QIAquick® Gel Extraction Kit, and eluted in 30 µL of elution buffer.

Vector cloning: 1 µL of a 1:10 dilution of each purified PCR product was cloned into the pCR® 2.1-TOPO® vector using the TOPO® TA Cloning® Kit. All tentatively positive colonies were purified with the QIAprep® Spin Miniprep Kit and subjected to an EcoR I restriction digest using 5 µL DNA, 1 µL React® 3 Buffer, 1 µL EcoR I, and 3 µL water. Samples were incubated at 37°C for one hour, and positive samples were confirmed by the presence of an insert of the correct size using agarose electrophoresis.
Serial dilutions: RNA concentration was determined spectrophotometrically using an Eppendorf BioPhotometer (Eppendorf, Westbury, NY). The corresponding number of gene copies per microliter was calculated for each cloned product. Samples were diluted in water as appropriate to contain from $2 \times 10^{-1}$ to $2 \times 10^5$ number of copies per microliter.

**qPCR**

qPCR was performed using the Plexor™ qPCR system (Promega, Madison, WI). 20 µL reactions contained 12.5 µL 2X Plexor™ Master Mix, 1 µL 5 µM forward primer, 1 µL 5 µM reverse primer, and 5.5 µL provided nuclease-free water. 5 µL of template was added to each reaction; this consisted of 2 µL cDNA and 3 µL MOPS/EDTA buffer (Promega, Madison, WI) for each experimental sample and 5 µL DNA for each standard control, for a total of $10^0$ – $10^6$ number of copies per reaction. A no template control (NTC) was performed using MOPS/EDTA buffer in place of template. All cDNA samples, standard controls, and NTC were performed in triplicate in order to account for any inaccuracies in pipetting. RNA was also used as a control for each *R. amblyommii*-infected sample using 500 ng RNA or 2 µL per sample (Table 10). A positive control consisted of 12.5 µL 2X Plexor™ Master Mix, 7.5 µL provided nuclease-free water, and 5 µL 5X Plexor™ qPCR control. Samples were analyzed on a iCycler iQ Real-time PCR Detection System (Bio-Rad, Hercules, CA) using thermocycling conditions of 95°C for 3 min., followed by 40 cycles of 95°C for 30 sec. and 60°C for 30 sec. A melt curve was performed consisting of 70 cycles of 60°C for 16 sec., increasing the set point temperature by 0.5°C with every cycle. Standard controls were run in a separate qPCR
reaction to optimize conditions. This data was then combined with the qPCR data from all experimental samples in order to calculate concentrations.

**qPCR analysis**

Raw data for the relative fluorescent units (RFU) and melt curve analysis was obtained using the iCycler IQ™ Optical System Software v3.1 (Bio-Rad, Hercules, CA) and was subsequently analyzed using the Plexor® Analysis Software (Promega, Madison, WI). Ct (cycle threshold) values represent the PCR cycle number at which the fluorescence passes a fixed or constant level.

**Relative Expression Calculations**

The average Ct value of each *vir* gene for each *R. amblyommi*-infected cDNA sample was normalized to the corresponding average Ct value of the reference gene, *gltA* considered a housekeeping gene in *Rickettsia* [21]. This was performed by calculating the \( \Delta C_t \) between the average Ct values for each *vir* gene and the average Ct for the reference gene. Normalization removes sampling differences that occur from variations in RNA extraction procedures, variation in inherent RNA integrity and quantity, PCR set-up variability, and differences in thermocycling efficiencies in order to identify real gene-specific variation [20, 24-26]. Variability between different samples also exists in the reference gene, which represents the cumulative error of the qPCR process as a whole. This is due to innate differences in transcriptional activity and imposed experimental error [24, 25]. The relative fold change of each *vir* gene for the *R. amblyommi*-infected Vero and 27°C *A. americanum* samples (variables, V) was calculated relative to
expression in the *R. amblyommii*-infected 34°C *A. americanum* sample (control, C). The
ΔCt (VΔCt - CΔCt) was first calculated between the normalized ΔCt for each *vir* gene in
each variable sample and the normalized ΔCt for the corresponding *vir* gene in the
control sample. Relative expression was then calculated using the ΔΔCt method,
calculated as $2^{-\left(\frac{V\Delta Ct - C\Delta Ct}{C\Delta Ct}\right)}$ [20, 27].

**RESULTS**

*virD4*

The analysis of the standard controls was 98.5% efficient using a 6-point curve
containing $10^1$ - $10^6$ total number of copies, and there was no amplification in the NTC;
the melt temperature of the expected product was 77°C (Figure 13). Experimental
analysis contained a positive control of $10^4$ total number of copies, cDNA from the *R.
amblyommii*-infected and uninfected Vero, 34 °C and 27 °C *A. americanum* samples,
RNA from the *R. amblyommii*-infected Vero, 34 °C and 27 °C *A. americanum* samples, a
NTC, and the Promega positive control. The positive control of $10^4$ total number of
copies amplified at the equivalent Ct value to the standard control curve (Figure 13). The
uninfected cDNA samples amplified but there was no true product, although there were
primer dimmers present at a melt temperature of 68°C and nonspecific amplification was
present at a melt temperature of 75°C (Figure 14). The Promega positive control
amplified at the expected level and had a melt temperature of 78°C; there was no
amplification in the NTC (Figure 13). RNA from the *R. amblyommii*-infected samples
had amplification at almost equivalent levels to the corresponding cDNA from these
samples; this was due to genomic DNA contamination. As a result, these samples were
treated with DNase and then used for all subsequent qPCR analyses, including repeating the \textit{virD4} analysis. The uninfected RNA samples were not DNase-treated because of the absence of cDNA amplification. A secondary qPCR analyses was performed using a positive control of $10^5$ total number of copies, the DNase-treated cDNA of the \textit{R. amblyommii}-infected Vero, 34 \degree C and 27 \degree C \textit{A. americanum} samples, a NTC, and the Promega positive control. The positive control of $10^5$ total number of copies amplified at a slightly lower, but still appropriate, $C_t$ value than in the standard control curve (Figure 13). The \textit{R. amblyommii}-infected cDNA samples amplified but primer dimers were present (Figure 15), and there was no amplification in the corresponding RNA (Figure 14). The Promega positive control amplified at the expected $C_t$ value and melt temperature, and there was no amplification in the NTC (Figure 13).

Experimental data was combined with the external standard controls in order to calculate the concentrations of the unknowns (Figure 16, Table 13). The triplicates of the \textit{R. amblyommii}-infected Vero sample had $C_t$ values of 32.47, 32.57, and 32.54 corresponding to concentrations of $6.09 \times 10^2$, $5.73 \times 10^2$, and $5.81 \times 10^2$ number of copies, respectively. The triplicates of the \textit{R. amblyommii}-infected 34 \degree C \textit{A. americanum} sample had $C_t$ values of 29.99, 30.15, and 30.11 corresponding to concentrations of $2.94 \times 10^3$, $2.65 \times 10^3$, and $2.73 \times 10^3$ number of copies, respectively. The triplicates of the \textit{R. amblyommii}-infected 27 \degree C \textit{A. americanum} sample had $C_t$ values of 31.03, 31.26, and 31.35 corresponding to concentrations of $1.52 \times 10^3$, $1.31 \times 10^3$, and $1.24 \times 10^3$ number of copies, respectively.
Figure 13. qPCR amplification and melt curves of *virD4* from *Rickettsia conorii* standard dilutions. Dilution series and positive controls are shown in blue. NTC are shown in yellow. The Promega positive control is shown in red.

Figure 14. qPCR amplification and melt curves of *virD4* from *Rickettsia amblyommii*-infected cell cultures. Vero cells are shown in light blue. 34°C *A. americanum* cells are shown in orange. 27°C *A. americanum* cells are shown in purple.
Figure 15. qPCR amplification and melt curves of virD4 from uninfected cDNA and RNA from R. amblyommi-infected cell cultures. Vero samples are shown in light blue. 34°C A. americanum samples are shown in orange. 27°C A. americanum samples are shown in purple.

Figure 16. qPCR standard curve analysis of virD4. Standard dilutions are shown in blue. Vero samples are shown in light blue. 34°C A. americanum samples are shown in orange. 27°C A. americanum samples are shown in purple. The Promega positive control is shown in red.
<table>
<thead>
<tr>
<th>Sample ID</th>
<th>FAM Type</th>
<th>Ct</th>
<th>Tₘ</th>
<th>Concentration</th>
<th>Tm?</th>
<th>Tm#</th>
</tr>
</thead>
<tbody>
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<td>Standard</td>
<td>20.86</td>
<td>77</td>
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<td>1</td>
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<td><em>R. conorii</em></td>
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<td>1</td>
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<td><em>R. conorii</em>-2</td>
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<td>77</td>
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<td>1</td>
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<td>22.14</td>
<td>77</td>
<td>4.25E+05</td>
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<td>77</td>
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<td>6.09E+02</td>
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<td>N/A</td>
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<td>28.11</td>
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<td>78.5</td>
<td>5.82E+03</td>
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<td>77</td>
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<td>Standard</td>
<td>32.78</td>
<td>77</td>
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<td>1</td>
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<tr>
<td><em>AAE27</em></td>
<td>Unknown</td>
<td>31.03</td>
<td>77</td>
<td>1.52E+03</td>
<td>Yes</td>
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<td>31.35</td>
<td>77</td>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Promega -2</td>
<td>Pos. Control</td>
<td>31.15</td>
<td>78.5</td>
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<td><em>Vero-U</em></td>
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<td><em>Vero-U</em></td>
<td>Unknown</td>
<td>35.10</td>
<td>75.0</td>
<td>1.15E+02</td>
<td>No</td>
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<td><em>Vero-U</em></td>
<td>Unknown</td>
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<td>N/A</td>
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<td>1</td>
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<td>1.00E+01</td>
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Table 13. *virD4* qPCR amplification and melt curve data. Tₘ refers to melt temperature.

121
The analysis of the standard controls was 96.9% efficient using a 6-point curve containing from $10^1$ – $10^6$ total number of copies, and there was no amplification in the NTC; the melt temperature of the expected product was 75.9°C (Figure 17). Experimental analysis included a positive-control of $10^5$ total number of copies, cDNA from the *R. amblyommii*-infected and uninfected Vero, 34°C and 27°C *A. americanum* samples, RNA from the *R. amblyommii*-infected Vero, 34°C and 27°C *A. americanum* samples, a NTC, and the Promega positive control. The positive control of $10^5$ number of copies amplified at the equivalent C$_t$ value to the standard control curve (Figure 17). The *R. amblyommii*-infected cDNA samples amplified and had a melt temperature of 75.9°C (Figure 18). The uninfected cDNA samples amplified, but did not contain true product, yet primer dimers were present at a melt temperature of 70°C and nonspecific amplification was present at a melt temperature of 77°C (Figure 19). One of the triplicates for the uninfected 27°C *A. americanum* sample did contain true product according to the melt curve analysis. Upon further analysis, the melt curves for all three replicates were shown to be highly similar, thus it was concluded there was no true product (Figure 19). There was no amplification in the *R. amblyommii*-infected RNA samples and the NTC (Figures 17 and 19). The Promega positive control amplified at the expected C$_t$ value and melt temperature (Figure 17).

Experimental data was combined with the external standard controls in order to determine concentrations of the unknowns (Figure 20, Table 14). The triplicates of the *R. amblyommii*-infected Vero sample had C$_t$ values of 32.26, 32.28, and 32.50 corresponding to concentrations of $2.73 \times 10^3$, $2.69 \times 10^3$, and $2.29 \times 10^3$ number of
copies, respectively. The triplicates of the *R. amblyommii*-infected 34 °C *A. americanum* sample had C\textsubscript{t} values of 27.41, 27.65, and 27.56 corresponding to concentrations of 8.55 x 10\textsuperscript{4}, 7.21 x 10\textsuperscript{4}, and 7.69 x 10\textsuperscript{4} number of copies, respectively. The triplicates of the *R. amblyommii*-infected 27 °C *A. americanum* samples had C\textsubscript{t} values of 28.24, 28.54, and 28.46 corresponding to concentrations of 4.75 x 10\textsuperscript{4}, 3.82 x 10\textsuperscript{4}, and 4.04 x 10\textsuperscript{4} number of copies, respectively.

**virB8**

The analysis of the standard controls was 98.8% efficient using a 5-point curve containing from 10\textsuperscript{2} – 10\textsuperscript{6} total number of copies, and there was slight contamination in the NTC equivalent to 18.2 – 25.0 number of copies; the melt temperature of the expected product was 77°C (Figure 21). Experimental analysis included a positive control of 10\textsuperscript{4} total number of copies, cDNA from the *R. amblyommii*-infected and uninfected Vero and 34°C *A. americanum* samples, RNA from the *R. amblyommii*-infected Vero and 34°C *A. americanum* samples, a NTC, and the Promega positive control. The positive control of 10\textsuperscript{4} total number of copies amplified at an equivalent C\textsubscript{t} value to the standard curve (Figure 21). The *R. amblyommii*-infected cDNA samples amplified and had a melt temperature of 77°C (Figure 22). The uninfected cDNA samples amplified, this was not true product but primer dimers were present at a melt temperature of 70°C and nonspecific amplification was present at a melt temperature of 75.5°C (Figure 23). There was no amplification in the *R. amblyommii*-infected RNA samples and the NTC (Figures 21 and 23). The Promega positive control amplified at the expected C\textsubscript{t} value and melt temperature (Figure 21).
Sample \( C_t \)

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\( R.c. = R. conorii \)

positive controls

Figure 17. qPCR amplification and melt curves of \textit{virB4} from \textit{Rickettsia conorii} standard dilutions. Standard dilutions and positive controls are shown in green. NTC are shown in yellow. The Promega positive control is shown in red.

Figure 18. qPCR amplification and melt curves of \textit{virB4} from \textit{Rickettsia amblyommii}-infected cell cultures. Vero samples are shown in light blue. 34°C \textit{A. americanum} samples are shown in orange. 27°C \textit{A. americanum} samples are shown in purple.
Figure 19. qPCR amplification and melt curves of *virB4* from uninfected cDNA and RNA from *R. amblyommii*-infected cell cultures. Vero samples are shown in light blue. 34°C *A. americanum* samples are shown in orange. 27°C *A. americanum* samples are shown in purple.

Figure 20. qPCR standard curve analysis of *virB4*. Standard dilutions are shown in green. Vero samples are shown in light blue. 34°C *A. americanum* samples are shown in orange. 27°C *A. americanum* samples are shown in purple. The Promega positive control is shown in red.
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Table 14. virB4 qPCR amplification and melt curve data. Tm refers to melt temperature.
Experimental data was combined with the external standard controls to calculate concentrations of the unknowns (Figure 24, Table 15). The triplicates of the *R. amblyommii*-infected Vero samples had *Ct* values of 33.04, 33.17, and 33.13 corresponding to concentrations of $1.91 \times 10^3$, $1.76 \times 10^3$, and $1.80 \times 10^3$ number of copies, respectively. The triplicates of the *R. amblyommii*-infected *A. americanum* samples had *Ct* values of 30.95, 31.07, and 31.15 corresponding to concentrations of $7.50 \times 10^3$, $6.93 \times 10^3$, and $6.60 \times 10^3$ number of copies, respectively.

**virB11**

The analysis of the standard controls was 95.7% efficient using a 6-point curve containing from $10^1$ – $10^6$ total number of copies, and there was no amplification in the NTC; the melt temperature of the expected product was 79.5°C (Figure 25). Experimental analysis included cDNA from the *R. amblyommii*-infected and uninfected Vero and 34°C *A. americanum* samples, RNA from the *R. amblyommii*-infected Vero and 34°C *A. americanum* samples, a NTC and the Promega positive control. The uninfected cDNA samples were run in duplicate due to low remaining quantity of the qPCR master mix. The *R. amblyommii*-infected cDNA samples amplified and had a melt temperature of 79.5°C (Figure 26). The uninfected cDNA samples amplified, but the melt curve showed there was no true product but primer dimers were present at a melt temperature of 68°C (Figure 27). There was no amplification in the *R. amblyommii*-infected RNA samples or in the NTC (Figures 25 and 27). The Promega positive control amplified at the expected *Ct* value and melt temperature (Figure 25).
Figure 21. qPCR amplification and melt curves of \textit{virB8} from \textit{Rickettsia conorii} standard dilutions. Standard dilutions and positive controls are shown in light green. NTC are shown in yellow. The Promega positive control is shown in red.

Figure 22. qPCR amplification and melt curves of \textit{virB8} from \textit{Rickettsia amblyommii}-infected cell cultures. Vero samples are shown in light blue. 34°C \textit{A. americanum} samples are shown in orange.
Figure 23. qPCR amplification and melt curves of \textit{virB8} from uninfected cDNA and RNA from \textit{R. amblyommii}-infected cell cultures. Vero samples are shown in light blue. 34°C \textit{A. americanum} samples are shown in orange.

Figure 24. qPCR standard curve analysis of \textit{virB8}. Standard dilutions are shown in light green. Vero samples are shown in light blue. 34°C \textit{A. americanum} samples are shown in orange. The Promega positive control is shown in red.
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Table 15. *virB8* qPCR amplification and melt curve data. Tm refers to melt temperature.
Experimental data was combined with the external standard controls in order to calculate concentrations of the unknowns (Figure 28, Table 16). The triplicates of the *R. amblyommii* -infected Vero samples had C\textsubscript{t} values of 39.34, 39.43, and 39.11 corresponding to concentrations of 3.64, 3.43, and 4.24 number of copies, respectively. The triplicates of the *R. amblyommii* -infected 34\degree C *A. americanum* samples had C\textsubscript{t} values of 32.71, 33.10, and 35.37, corresponding to concentrations of 2.67 \times 10^{2}, 2.14 \times 10^{2}, and 1.74 \times 10^{2} number of copies, respectively.

\textit{gltA}

The analysis of the standard controls was 91.3\% efficient using a 5-point curve containing from 10\textsuperscript{1} - 10\textsuperscript{5} total number of copies, and there was no amplification in the NTC; the melt temperature of the expected product was 77\degree C (Figure 29). Experimental analysis included a positive control of 10\textsuperscript{4} total number of copies, cDNA from the *R. amblyommii* -infected and uninfected Vero, 34\degree C and 27\degree C *A. americanum* samples, RNA from the *R. amblyommii* -infected Vero, 34\degree C and 27\degree C *A. americanum* samples, a NTC and the Promega positive control. The positive control of 10\textsuperscript{4} total number of copies amplified at a higher but appropriate C\textsubscript{t} value than the standard curve (Figure 29). The *R. amblyommii* -infected cDNA samples amplified and had a melt temperature of 77\degree C (Figure 30). The uninfected cDNA samples contained true product in addition to primer dimers at a melt temperature of 70\degree C (Figure 31). However, the average C\textsubscript{t} values for the uninfected samples (35.45, 34.19, and 35.31, respectively, for Vero, 34\degree C and 27\degree C *A. americanum*) were 6-10 C\textsubscript{t} values less than the corresponding C\textsubscript{t} values for the infected samples (29.12, 25.31, and 28.47, respectively), low enough to be ignored. There was no
Figure 25. qPCR amplification and melt curves of virB11 from Rickettsia conorii standard dilutions. Standard dilutions and positive controls are shown in teal. NTC are shown in yellow. The Promega positive control is shown in red.

Figure 26. qPCR amplification and melt curves of virB11 form Rickettsia amblyomnii-infected cell cultures. Vero samples are shown in light blue. 34°C A. americanum samples are shown in orange.
Figure 27. qPCR amplification and melt curves of \textit{virB11} from uninfected cDNA and RNA from \textit{R. amblyommii}-infected cell cultures. Vero samples are shown in light blue. 34°C \textit{A. americanum} samples are shown in orange.

Figure 28. qPCR standard curve analysis of \textit{virB11}. Standard dilutions are shown in teal. Vero samples are shown in light blue. 34°C \textit{A. americanum} samples are shown in orange. The Promega positive control is shown in red.
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Figure 16. *virB11* qPCR amplification and melt curve data. Tm refers to melt temperature.
amplification in the *R. amblyommii*-infected RNA samples or in the NTC (Figures 29 and 31). The Promega positive control amplified at the expected Ct value and melt temperature (Figure 29).

Experimental data was combined with the external standard controls in order to calculate concentrations of the unknowns (Figure 32, Table 17). The triplicates of the *R. amblyommii*-infected Vero samples had Ct values of 28.86, 28.85, and 29.65 corresponding to concentrations of $2.90 \times 10^4$, $2.90 \times 10^4$, and $1.64 \times 10^4$ number of copies, respectively. The triplicates of the *R. amblyommii*-infected 34°C *A. americanum* sample had Ct values of 25.06, 25.50, and 25.36 corresponding to concentrations of $4.21 \times 10^5$, $3.12 \times 10^5$, and $3.41 \times 10^5$ number of copies, respectively. The triplicates of the *R. amblyommii*-infected 27°C *A. americanum* samples had Ct values of 28.35, 28.55, and 28.50 corresponding to concentrations of $4.12 \times 10^4$, $3.57 \times 10^4$, and $3.70 \times 10^4$ number of copies, respectively.

**Relative expression**

The average Ct values for each *vir* gene were compared to the average Ct value for *gltA* in each *R. amblyommii*-infected sample. The normalized ΔCt values are shown in Table 16. For the *R. amblyommii*-infected Vero sample, *virD4* had a 2.58-fold higher expression level and *virB8* had a 3.38-fold higher expression level compared to the *R. amblyommii*-infected 34°C *A. americanum* sample, while *virB4* had a 2-fold lower expression level and *virB11* had a 5.26-fold lower expression level (Table 18). For the *R. amblyommii*-infected 27°C *A. americanum*, *virD4* had a 4.09-fold higher expression level
Sample | $C_t$
---|---
$10^4$ | 32.80
$10^4$ | 32.62
$10^4$ | 32.63
*R.c.-1* | 31.18
*R.c.-1* | 31.36
*R.c.-1* | 31.63

*R.c.* = *R. conorii*
positive control

---

Figure 29. qPCR amplification and melt curves of *gltA* from *Rickettsia conorii* standard dilutions. Standard dilutions and positive controls are shown in dark purple. NTC are shown in yellow. The Promega positive control is shown in red.

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Figure 30. qPCR amplification and melt curves of *gltA* from *Rickettsia amblyommii*-infected cell cultures. Vero samples are shown in light blue. 34°C *A. americanum* samples are shown in orange. 27°C *A. americanum* samples are shown in purple.
Figure 31. qPCR amplification and melt curves of gltA from uninfected cDNA and RNA from *R. amblyommii*-infected cell cultures. Vero samples are shown in light blue. 34°C *A. americanum* samples are shown in orange. 27°C *A. americanum* samples are shown in purple.

Figure 32. qPCR standard curve analysis of gltA. Standard dilutions are shown in dark purple. Vero samples are shown in light blue. 34°C *A. americanum* samples are shown in orange. 27°C *A. americanum* samples are shown in purple. The Promega positive control is shown in red.
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Table 17. *gltA* qPCR amplification and melt curve data. $Tm$ refers to melt temperature.
Table 18. RNA fold change calculations for *virD4*, *virB4*, *virB8*, and *virB11* using the \(2^{\Delta \Delta Ct}\) method. SD refers to standard deviation. For \(2^{-(\Delta \Delta Ct)}\) values less than 1, relative fold change is equivalent to \(1/x\).

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and *virB4* had a 4.88-fold higher expression level compared to the *R. amblyommi*-infected 34°C *A. americanum* sample (Table 18).

**DISCUSSION**

The expression of four rickettsial *vir* genes was investigated within differential host species. The mRNA expression levels of *virB8* and *virD4* in *R. amblyommi*-infected Vero cell cultures, and of *virB4* and *virD4* in 27°C *A. americanum* cell cultures were found to be higher compared to expression in the 34°C *A. americanum* cell cultures. The genes *virB4* and *virB11*, however, were found to have lower expression in the Vero cell culture. The 34°C *A. americanum* culture was chosen as the control sample since this represents the natural host species of *R. amblyommi* at the optimal temperature for the cell culture. These findings suggest that the genes *virB4* and *virB11* may be important for host specificity due to differential expression between the two host species. Additionally, the *virB8* and *virD4* genes may play a role in stress due to increased expression levels in the 27°C *A. americanum* and Vero cell cultures. These represent unlikely or inadvertent environments for rickettsiae in nature. However, one should remember that qPCR does not measure an increase or decrease in mRNA levels over the rickettsial infection period but represents an overall level of steady-state mRNA. This may better explain the higher expression in both the Vero cells and reduced temperature tick cells for both genes may be highly important for rickettsial survival within the host.

The four *vir* genes analyzed represent T4SS genes important for virulence in many pathogenic bacteria, mainly studied in *Agrobacterium* [13, 28-30]. Two of these genes, *virD4* and *virB8*, were found to have higher expression in the Vero cell cultures.
compared to the 34°C *A. americanum* cell cultures. The gene *virD4* is a coupling protein with ATPase activity that interacts with the DNA substrate in order to recruit it to the transfer apparatus [14, 15, 31, 32]. The expression level of *virD4* was 2.58-fold greater in the Vero cells and 4.09-fold greater in the 27°C *A. americanum* cells. The increase in the mRNA level may be due to increased virulence in the mammalian cells, but it does not explain the increase in the lower tick temperature. Another explanation may be an increased necessity for ATP if the rickettsiae are unable to utilize the host cell’s energy. Also, substrate transfer activity may be increased if the rickettsiae are not fully able to utilize a mammalian host’s energy. The gene *virB8* is a vital member of the inner core of the transmembrane complex. It has been shown to function in recruiting *virB1*, a transglycosylase, for lysis of the peptidoglycan, the bacterial outer cell wall layer [16, 28]. The expression level of *virB8* was 3.38-fold greater in Vero cells compared to the 34°C *A. americanum* cells. Thus, this gene may be important for virulence due to its role in breaking down the bacterial cell wall and may help aid in the transfer of important bacterial substrates to the host cell. Therefore, *virD4* and *virB8* may be crucial for increased pathogenicity in mammalian cells.

Conversely, the remaining two *vir* genes, *virB4* and *virB11*, may play a greater role in host-specificity due to their decreased expression in the Vero cell cultures compared to the 34°C *A. americanum* cell culture. The gene *virB4*, another ATPase, provides the energy for substrate transport and assembly of the core complex in the transfer apparatus [14, 33]. The expression level of *virB4* was 2-fold lower in the Vero cells but 4.88-fold greater in the 27°C *A. americanum* cells. Thus, this gene may be important for adaptation to the tick host; the energy for substrate transport in mammalian
cells may be provided by an alternate gene. The translocation of the DNA or protein substrates through the transmembrane pore may be controlled by *virB11*, a cytoplasmic hydrophilic ATPase. The structure of *virB11* is a homomultimer that forms hexameric pores, of which the opening and closing are regulated by ATP [15, 28, 30, 33]. Since *virB11* can autophosphorylate through its ATP binding domain, these conformational changes may provide the energy to translocate the DNA substrate through the pore. The expression level of *virB11* was 5.26-fold lower in the Vero cell culture. Thus, the physical movement of the bacterial substrates across the transmembrane pore may occur by a completely different process in mammalian hosts than in a tick host. Nonetheless, this result is not conclusive due to the very low overall amplification levels of this gene in both the Vero and 34°C *A. americanum* cell cultures (Figure 26). Amplification could be problematic due the inherent nature of the primer set used or from an actual low amount of mRNA present in both cell types. Therefore, these low values might not accurately represent the true expression differences, especially when further compounded by normalization to *gltA* and compared between host types.

These results represent a novel qPCR approach for analyzing the expression levels of rickettsial *vir* genes between different host types. Two of these genes, *virB4* and *virB11*, were shown to have a lower expression level in Vero cells than in the tick cells and are most likely important for host adaptation. Both of these genes are found peripherally bound compared to other T4SS genes involved in the transmembrane core complex and may represent a dichotomous role for these proteins. *Rickettsia* may have evolutionarily adapted two different proteins that serve the same function but for different host species environments. This would increase their versatility in successfully infecting,
surviving, and being transmitted within new hosts. Moreover, \textit{virD4} and \textit{virB8} were found to have a higher expression level in the Vero cells. These genes may be important in virulence, or more plausible, crucial members in the process for translocation of bacteria substrates; for \textit{virD4} was also found to have a higher expression level in the tick cells at the reduced temperature (Table 18). This higher level of expression may be a rickettsial response to an undesirable environment and may lead to the recognition of the bacteria by the host. Ultimately this can cause clearing of the bacteria by the host preventing an evolutionary relationship. Since little is known about the pathogenesis of rickettsiae these results provide novel and key information to the field. The differential expression levels illustrated here provide support for the role of these genes in host-adaptation and potential virulence. The relationship between pathogen and host is vital for understanding the evolutionary mechanisms of virulence that allows for development of more effective treatments. The highly sensitive molecular analysis of qPCR allows for the rapid and specific analysis of RNA expression between many samples and can potentially be used to analyze and compare a high number of human clinical cases. The addition of this approach to the study of \textit{Rickettsia} is an invaluable resource for increasing our knowledge of this emerging disease threat.

\textbf{ACKNOWLEDGMENTS}

I would like to thank Dr. Ulrike Munderloh at the University of Minnesota for kindly providing \textit{Amblyomma americanum} tick cell cultures and the \textit{Rickettsia amblyommii} isolate and for all her help and advice. I would like to thank Dr. Deborah Parris at The Ohio State University for kindly providing Vero cell cultures. Finally, I
would like to thank Michael Zianni at The Ohio State University for performing the qPCR runs, help with data analysis, and for his invaluable time and advice.

REFERENCES


CHAPTER 6

SUMMARY AND CONCLUSIONS

This analysis of the host-pathogen relationship using *Rickettsia* and their arthropod vectors has provided novel information on the epidemiology of RMSF and the molecular characterization and expression of potential virulence genes. The results of these analyses provide a better understanding of the process of disease pathogenesis by using a multi-part perspective. First, understanding the natural ecology of the disease agent helps identify shifts in frequency, geographical location, and outbreaks over time. Identifying how, when and where a disease occurs is the first step in preventing clinical cases and improving public health. Secondly, identifying and analyzing potential virulence genes provides data on how rickettsiae are similar to other pathogens. Finally, studying the expression of these potential virulence genes in vitro helps elucidate how they function in nature and how rickettsiae adapt to different hosts and potentially cause disease. This analysis helps elucidate the function of these genes and their role in pathogenesis, ultimately leading to more targeted disease treatments. The overall goal of the analyses presented here is to increase our understanding of rickettsial diseases that pose an emerging threat to global public health.

The epidemiology of RMSF in Ohio was analyzed over a past twenty year period (1983 – 2004). It was demonstrated the overall incidence of *R. rickettsii* has increased
from 6% to 16%. Furthermore, the geographical ecology of *R. rickettsii*-infected *D. variabilis* ticks has increased into the northern parts of Ohio, corresponding to a rise of RMSF cases in these areas. The overall number of clinical cases has declined most likely due to increased public awareness in areas of high potential for disease. Also, ticks in general may be forced into new geographical niches from human expansion and development. Thus, public education should be focused in these areas where disease has recently occurred. These results illustrate the continual threat from rickettsial diseases.

The molecular analyses of two *D. variabilis* mitochondrial genes contribute novel information to the field. This information allows for a better understanding of the evolutionary relationship among tick species, which is currently not completely understood. As available information increases, subsequent studies can be done on coevolutionary analyses between tick vectors and rickettsial species. These analyses will allow for a better understanding of the transmission patterns of many new rickettsial species and their potential for virulence.

The factors important for rickettsial virulence are not well known, and the molecular and expression analyses of four *vir* genes help elucidate factors important for this process. The T4SS genes, *virB8, virB11, virB4*, and *virD4* have been shown to be important virulence factors in many other bacterial species. Their presence in all available rickettsial genomes lends support for their role in pathogenesis, especially in light of the fact that rickettsiae have undergone massive reduction in their genomes. In addition, molecular analyses for *R. amblyommii, R. rhipicephali, R. montanensis* and *R. australis* are not complete, and the complete understanding of the virulence potential of many “nonpathogenic” species is not known. Thus, the molecular and phylogenetic
analyses of these genes provide novel information to the database, help identify evolutionary relationships among the *Rickettsia*, and elucidate their function. It is hypothesized that these *vir* genes will also have a higher expression level in mammalian cells, where disease occurs, if they function in virulence.

Results demonstrated that the *R. amblyommi* isolates were most similar to *R. rhipicephali* for all four *vir* genes analyzed, *virB8*, *virB11*, *virB4*, and *virD4*; these two species have not been implicated in any human disease cases. The absence of a Walker A domain in *virD4* may indicate this gene is not functional or may have evolved a novel role in *Rickettsia* that does not require this domain. This could also explain the increase of expression levels for *virD4* in both the Vero cell and reduced temperature *A. americanum* cells compared to the *A. americanum* cells at the optimal temperature. This gene may not important for virulence but may be necessary for overall survival of rickettsiae in their host. Also, rickettsiae may have evolved a different process of infection in mammalian hosts, one that does not rely on the need for a coupling protein.

The Walker A domain was found to be present but mutated in *virB11*. Thus, this gene may not be important for virulence or may have evolved a novel role in rickettsiae compared to other pathogens. The fact that the expression levels of *virB11* were found to be lower in Vero cells than in the *A. americanum* cells support the idea that it may be important in providing energy for substrate transport only in arthropod hosts. The genes *virD4* and *virB11* may not be important for virulence but provide more information into the host-pathogen relationship.

An intact Walker A domain was found in *virB4*, however. The expression levels of this gene were also found to be lower in Vero cells but higher overall in the reduced
temperature *A. americanum* cells, compared to the *A. americanum* cells at the optimal temperature. Thus, this gene may provide the energy needed for the transport of bacterial substrates necessary for survival only in arthropod hosts. There may be another functional ATPase utilized in the infection of mammalian hosts. An additional explanation may be that *virB4*, and possibly *virB11*, have reduced expression in the Vero cells because rickettsiae have not evolved any successful mechanism of maintenance within a vertebrate host. It is only vital that they survive for generations within their arthropod host and thus, may be immediately recognized by the vertebrate host’s immune system causing disease symptoms. Furthermore, the expression of these *vir* genes was analyzed using *R. amblyommii* as a model system. This species may not utilize genes important for virulence and thus, the *vir* genes may show increased levels of expression in mammalian cells in more pathogenic species, like *R. rickettsii*. Due to the presence of differential expression of these *vir* genes between host species, further functional analyses should be performed to identify their role in the process of pathogenesis.

All the results presented here provide novel information to the study of rickettsial diseases. The comparison of genetic, molecular and evolutionary factors between nonpathogenic and pathogenic species will help identify specific factors important for virulence. In addition, the identification of genes important for adaptation to new host species aid in understanding the evolutionary relationship between SFG and TG rickettsiae as well as the evolution of virulence.


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APPENDIX A

Nucleotide Sequences
Figure 33. Consensus sequence for *Dermacentor variabilis* 12S ribosomal RNA gene and control region
Figure 34. Consensus sequence for *Rickettsia amblyommii virB8*
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ACCESSION  1065176  
VERSION  
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SOURCE    Rickettsia montanensis  
ORGANISM    Rickettsia montanensis  
  Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales;  
  Rickettsiaceae; Rickettsia; spotted fever group.  
REFERENCE  1  (bases 1 to 654)  
  AUTHORS  Carmichael,J.R. and Fuerst,P.A.  
  TITLE  Phylogeographical variation of vir genes from Rickettsia amblyommii  
  isolates from the Northeast United States  
  JOURNAL  Unpublished  
REFERENCE  2  (bases 1 to 654)  
  AUTHORS  Carmichael,J.R. and Fuerst,P.A.  
  TITLE  Direct Submission  
  JOURNAL  Submitted (19-FEB-2008) Molecular Genetics, The Ohio State  
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  CDS             <1..>654  
    /codon_start=2  
    /transl_table=11  
    /product="virB8"  
    /translation="XYFIXARKWYNFKYILPLSRTLLLICTIFILLTLICININM  
LLPINKKSYILKDDGEKQATVTNKHSTLANPYISVANIQLQNYVQEEYNDVL  
KEQFTIKNASTISIVYMQFANFNDNPLSPVYQRKYRBNSTKMKINDNXXXV  
TFESLAKNXTGAILXNVMWETRIGFIMDSIXSIISPMPFXFPTXYNLXLLRNKIXN"  
BASE COUNT  250 a  94 c  76 g  219 t  15 others  
ORIGIN  
  1 cgantatttt attgnngcaa gaaaatggta taattttaag tatatcttac cgttaagtca  
  61 tagaactctt ctgcttttaa tatgcacgat ttttatctta ttattaacct taatttgtat  
  121 aaatatcaac atgttgctac caataaacaa aaaagtaagt tatttaataa aagatgatgg  
  181 cgaaaaacaa gctaccgtta ctaatactaa acattcaaca ttagcaaatc cttatatctc  
  241 tgttgcaaat attatgcttc agaattatgt aaaccaacga gaagaataca attacgatgt  
  301 attaaagga caatattctc tatcaaaa tgcctcagc agatggtt atatgaat  
  361 tgtatatttt ataatattg ataacccatt atacccggtc atgcgtcttttttcttatctctct  
  421 tagacgctca attaatatat tatatatatgct ctttaattata tcaaccttcgg tgccttcttc  
  481 ttgctctttt acactttct atatatata aacttactt acaacttcttctttaatacttgcgt  
  541 aagacaga agatggtt atatatcct taaacttttac aactctttc ggcttttcttttcttct  
  601 ttttcccttt actdtttact ccacattaactt gtaaatcataa ataaataatc aataaataaat  

Figure 35. *Rickettsia montanensis* virB8 nucleotide sequence
LOCUS       bankit1067656            601 bp    DNA     linear   BCT 27-FEB-2008
DEFINITION  Rickettsia rhipicephali virB8, partial cds.
ACCESSION  1067656
VERSION
KEYWORDS
SOURCE       Rickettsia rhipicephali
ORGANISM    Rickettsia rhipicephali
             Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales;
             Rickettsiaceae; Rickettsiae; Rickettsia; spotted fever group.
REFERENCE   1  (bases 1 to 601)
             Carmichael,J.R. and Fuerst,P.A.
             Phylogenetic variation of vir genes among Rickettsia
             Unpublished
REFERENCE   2  (bases 1 to 601)
             Carmichael,J.R. and Fuerst,P.A.
             Direct Submission
             Molecular Genetics, The Ohio State University, 318 West 12th Avenue, Columbus, OH 43210, USA
FEATURES             Location/Qualifiers
source          1..601
             /organism="Rickettsia rhipicephali"
             /mol_type="genomic DNA"
             /strain="CWPP"
             /db_xref="taxon:33992"
CDS             <1..>601
             /codon_start=3
             /transl_table=11
             /product="virB8 protein"
             /translation="SGXYFIDARKWYNFKYILPLSRSLLLICTIFTLLTLCINI
             NULLPINKVSYLIKDAEKQAATNTKHSITLANPYISVANMLQNYVNQREEYNYXI
             LKEQFTFIKASTISVYMQFGNMHDINPSVIRYQKLYRNSINLSSINNDN
             VFESLAKNSTGTILENMLWEARIGFMIDXISTSTSPYMP"
BASE COUNT      232 a     90 c     77 g    199 t      3 others
ORIGIN

1 aatcggcgn atatttgatt gatgcaagaa aatgattat ttaaattga tttttacttt
61 taagctattt cacacctttt ccctaatatt gcagaattt ttccttatatt
121 ccctaatatt gcacatatatt tcctaattttt cctaatattt cacatatttt
181 ccctaatatt gcacatatatt tcctaattttt cctaatattt cacatatttt
241 ccctaatatt gcacatatatt tcctaattttt cctaatattt cacatatttt
301 ccctaatatt gcacatatatt tcctaattttt cctaatattt cacatatttt
361 ccctaatatt gcacatatatt tcctaattttt cctaatattt cacatatttt
421 ccctaatatt gcacatatatt tcctaattttt cctaatattt cacatatttt
481 ccctaatatt gcacatatatt tcctaattttt cctaatattt cacatatttt
541 ccctaatatt gcacatatatt tcctaattttt cctaatattt cacatatttt
601 c

Figure 36. Rickettsia rhipicephali virB8 nucleotide sequence
Figure 37. *Rickettsia australis* virB8 nucleotide sequence
LOCUS       EF026586                 967 bp    DNA     linear   BCT 27-FEB-2008
DEFINITION Rickettsia amblyommii VirB11 (virB11) gene, partial cds.
ACCESSION   EF026586
VERSION     EF026586.2 GI:168989261
KEYWORDS
SOURCE Rickettsia amblyommii
ORGANISM Rickettsia amblyommii
Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales; Rickettsiaceae; Rickettsia; spotted fever group.
REFERENCE 1 (bases 1 to 967)
  AUTHORS Carmichael,J.R. and Fuerst,P.A.
  TITLE Phylogeographical variation of vir genes from Rickettsia amblyommii isolates from the Northeast United States
  JOURNAL Unpublished
REFERENCE 2 (bases 1 to 967)
  AUTHORS Carmichael,J.R. and Fuerst,P.A.
  TITLE Direct Submission
  JOURNAL Submitted (25-SEP-2006) Molecular Genetics, The Ohio State University, 318 West 12th Avenue, Columbus, OH 43210, USA
REFERENCE 3 (bases 1 to 967)
  AUTHORS Carmichael,J.R. and Fuerst,P.A.
  TITLE Direct Submission
  JOURNAL Submitted (27-FEB-2008) Molecular Genetics, The Ohio State University, 318 West 12th Avenue, Columbus, OH 43210, USA
REMARK Sequence update by submitter
COMMENT On Feb 27, 2008 this sequence version replaced gi:116691241.
FEATURES             Location/Qualifiers
source          1..967
  /organism="Rickettsia amblyommii"
  /mol_type="genomic DNA"
  /specific_host="Amblyomma americanum (tick)"
  /db_xref="taxon:33989"
  /PCR_primers="fwd_seq: atgartgaagaatttgcagc, rev_seq: acttcygaaacatatcttytycc"
gene            1..>967
  /gene="virB11"
CDS             1..>967
  /gene="virB11"
  /codon_start=1
  /transl_table=11
  /product="VirB11"
  /protein_id="ABK15689.2"
  /db_xref="GI:168989262"
  /translation="MSEEFAALEALETFLLPFKAKMGAFDDTATESLVDETVILNFFGSKKYILTVVDIVVQLKRGSGKCI"

Figure 38. Consensus sequence for Rickettsia amblyommii virB11
Figure 38 continued

ORIGIN
1 atgagtgga aattgcagc cttagagcg ttttactctcttttaaaaattttgct
61 gaagacggta taacgaatat tgtgttaaat aagccggag aagtatttgaagtattttgaagacggta ttaacgaaat tatggttaat aagcccggag aagtatgggt tgaaaaaaaa
121 ggtggtatag cttaatctac cgaacatag atttaagaag aaaagcctggattttggct tcctttcgc
241 atcttgccga atgcagccct tttccttctctgtctgttgaaatgagacggta ttaacgaaat tatggttaat aagcccggag aagtatgggt tgaaaaaaaa
301 atcatttatt ctaaaaagat gctagcga agtttatttta attttggatgttttttgaagacggta ttaacgaaat tatggttaat aagcccggag aagtatgggt tgaaaaaaaa
361 atggtggtag cttaatctac cgaacatag atttaagaag aaaagcctggattttggct tcctttcgc
421 atatactaatgtagtggaggtggattttttaattttggatgttttttgaagacggta ttaacgaaat tatggttaat aagcccggag aagtatgggt tgaaaaaaaa
481 aaaaaatatcataatttttctctattttctcaacgtaaattaatctttaggatttttttttgaagacggta ttaacgaaat tatggttaat aagcccggag aagtatgggt tgaaaaaaaa
541 ctaatctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
LOCUS bankit1067665  886 bp  DNA  linear  BCT 27-FEB-2008
DEFINITION Rickettsia rhipicephali virB11, partial cds.
ACCESSION 1067665
VERSION
KEYWORDS
SOURCE Rickettsia rhipicephali
ORGANISM Rickettsia rhipicephali
  Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales;
  Rickettsiaceae; Rickettsieae; Rickettsia; spotted fever group.
REFERENCE 1  (bases 1 to 886)
  AUTHORS Carmichael, J.R. and Fuerst, P.A.
  TITLE Phylogenetic variation of vir genes among Rickettsia
  JOURNAL Unpublished
REFERENCE 2  (bases 1 to 886)
  AUTHORS Carmichael, J.R. and Fuerst, P.A.
  TITLE Direct Submission
  JOURNAL Submitted (27-FEB-2008) Molecular Genetics, The Ohio State
  University, 318 West 12th Avenue, Columbus, OH 43210, USA
FEATURES Location/Qualifiers
  source 1..886
    /organism="Rickettsia rhipicephali"
    /mol_type="genomic DNA"
    /strain="CWPP"
    /db_xref="taxon:33992"
  CDS <1..>886
    /codon_start=2
    /transl_table=11
    /product="virB11 protein"
    /translation="FLLPFTNLFAEDGINEIMVNKPGEVWVEKKGiYSQQIPELDSE
    HLLALGRLVAQSTEQMISEEKPLLTSATLPNGYRIQIVFPACEIQIYAIRKPSGM
    LTLDEYAKMGAFDDTATESLVEDAVILNNFLAEKIKEFIRHASSVSSKKNISGGTS
    TGKTFTNAALTEIAPLRITVEDAREVVLSSHPNRVHLLASKGGQGRANVTTQDLI
    EACLRLPRDRIVGELRGKEAFSLRAINTGHPSISTLHADSPAMAIEQLKLMVMQA
    DLGMPPVEVYKYLTVVDI"
BASE COUNT  286 a  146 c  189 g  265 t

Figure 39. Rickettsia rhipicephali virB11 nucleotide sequence
Figure 39 continued

ORIGIN

1 gttttactt ccttttacaa atttatgtc tgaagaggg attaacgaa aaatacgttaa
61 taaccccgga gaagtagggg ttgaaaaaa aggggatata tattctcagc aataaccgga
121 getttcatc gaacatctac ttgagctcag gcgtttagtc aatcatttt cctgtacagg
181 gattctagaa gaaaaacgc ctcttccgcttc aacattttag gttatatggc aataccgga
241 agtttttctcttctgtgttgtat ggtttgattct ccatgcttaaa aatcatttt cctgtacagg
301 tataggtag acatcatttt aatcatttt cctgtacagg aataaccgga
361 gagttttaa gatgagacgtg gattttctc gatgagacgtg aataaccgga
421 aagatattt aagtttctg tttatctgtaa aatcatttt cctgtacagg aataaccgga
481 aacccctgaa acttccttta ctaatcgtgac acttcctgaa aatcatttt cctgtacagg
541 aattttctc aagtttttag ttttttatttttt cctgtacagg aataaccgga
601 acttcctgta ttttttatttttt cctgtacagg aataaccgga
661 ctgcggttc ggtgtttttctt cccttcgttttt ctgcggttc ggtgtttttctt
721 tagttttg ttttttatttttt cctgtacagg aataaccgga
781 tagttttttt ttttttatttttt cctgtacagg aataaccgga
841 ggtttttttttt cctgtacagg aataaccgga

175
LOCUS     bankit1069068  886 bp  DNA linear  BCT 02-MAR-2008
DEFINITION Rickettsia australis virB11 gene, partial cds.
ACCESSION 1069068
VERSION
KEYWORDS
SOURCE     Rickettsia australis
ORGANISM  Rickettsia australis
           Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales;
           Rickettsiaceae; Rickettsieae; Rickettsia; spotted fever group.
REFERENCE 1 (bases 1 to 886)
           AUTHORS  Carmichael,J.R. and Fuerst,P.A.
           TITLE    Phylogenetic variation of vir genes among Rickettsia
           JOURNAL  Unpublished
REFERENCE 2 (bases 1 to 886)
           AUTHORS  Carmichael,J.R. and Fuerst,P.A.
           TITLE    Direct Submission
           JOURNAL  Submitted (02-MAR-2008) Molecular Genetics, The Ohio State
           University, 318 West 12th Avenue, Columbus, OH 43210, USA
FEATURES             Location/Qualifiers
          source          1..886
            /organism="Rickettsia australis"
            /mol_type="genomic DNA"
            /strain="JC"
            /db_xref="taxon:787"
       CDS            <1..>886
            /codon_start=2
            /transl_table=11
            /product="virB11 protein"
            /translation="FLLPFTNLAEDGINEIMVNKPGEAVKGDYKQIPHELDS
           HLLSLGRLVAQSTEQISNEEKLPLSATLPNYRIQIFPAVEIGQIQYISIRKPSGMN
           LTLDEYAKMGAFFDTATESLIDEDTILNNFLADKKIKEFIRHAAVVSKKNIIISGGT
           TGKTNTNAALTEIPARELITVEDAREVVLSSHLNRHVLLASXGGQGRANVTTQDLI
           EACLRLRPDRIVGELRGKEAFSFLRAINTGPGSISTLHADSPAMAIEQLKLMVMQA
           DLGMPPEEVKKYILTVPDI"
BASE COUNT    286 a  139 c  186 g  273 t  2 others

Figure 40. Rickettsia australis virB11 nucleotide sequence
Figure 40 continued

ORIGIN

1  gttttactt ccttttacaa atttatttgc tgaagacggt attaacgaga ttatggttaa
  61 taagcctgga gaagcatggg tgaaaaaaa aggegatatata tattctaaac aaataccgga
  121 attggatagt gacatctac ttcttatagg ggttaagttt gtctaaatca cegaagcatat
  181 aattttggaa gaaaaacctt tacttttgcc aacctttgccc aacggttacc gtattcaaat
  241 agtttctcctcgctgttg agataggaca aatcattatat tctataagaa aacgctgagg
  301 tatgaactca acttagagat agtagcctaa aatgggagca tttgatgata cttcaacaga
  361 gattttata gatgtaagata cagtaatattt aaataatctt ttagagcaca aaaaagatt
  421 agaatttact agacatgcag tttctcctaa gaaaaaatata ataatagtgt gttggacttc
  481 aacggttaaa actacattta caattgcaag acttaatgaa atacccgcaaa gaaaaagatt
  541 aattttgta gaagatgctc gtgggtgtgt gcattcaacca catgaagtc aggtgcattt
  601 acttgtcct aanggnggcac gggtctgtgc aatcttttaactctaatc atatagggggc
  661 gtggtttctgt taagacgcgg atataattat agtagagtgag cttcaggtta aggaaacgtt
  721 tagtttttttc gttgcttaata atacccagta cccggtttca atataaacac tgcctgcca
  781 taggctgtcgt atggctattt acgcgttaaa cggtaggtgt atcgaagcag atctttgtat
  841 gctggcttggc gaagtaaaga agtatatttt aacggttcatgatac
Figure 41. Consensus sequence for *Rickettsia amblyommii* virB4
Figure 41 continued

ORIGIN

1  atgaagttat ttagaactag agcagctaaa gaattaaggt ctaaacaaga aatcccaact
61  tcacatatta tcacctaaaa atgcatctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Figure 42. *Rickettsia rhipicephali* virB4 nucleotide sequence
Figure 42 continued

ORIGIN
1  acatttttac ccttataaat gtcattggga tagtaatact attttaacaa aagataaattc
61  ttatttacaa gtttaaaa aatagttt gatttttgt gatactaga gtaaagctct
121  aaacattaa aagaatatca nnaatgctt acttaaaaat atggettcnga gaaatatntt
181  tattgttttc cataactta gaagagc ttaa agcagtaata ttttgagata cccaaattac
241  tattgatcct acctttaaag tctctaagtct ttttattaatatttattg tttatagttg
301  taagaaacat gtcggtgcta ggtcnnntttta taaccaatta tattttanta tttttttatata
361  nccttatact gcgcgtattgc ccatanntna nnttttctta aanaacatc caacanaaatc
421  taataaaacc nnttttnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn
481  nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn
541  nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn
601  nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn
661  nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn
721  nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn
781  nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn
841  nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn
901  tcaacctacaa attggctaaa taatttacgg cccattataagt gccactagcg gtgatatcgc
961  ttccgggtag catcatttat cgtttaatttg ttcgcaaat aataataaag attttgaagta
1021  tatattttcct actgcttccct acgtccaga atccagccat ctagagaaaa
1081  agttactatag cagactcggc cctggccagaga atatggatc atatatgtcgc
1141  taacttact ataatcact ctcattagc cctgttttggc taacacacta atatccgct
1201  cgcagtaaaatt agagataaca ctggttggaga atatggcactagc gtaatttggta ctattcagg
1261  tacacgc
Figure 43. *Rickettsia australis virB4* nucleotide sequence
Figure 43 continued

ORIGIN

1 aaccatacg acgggttaaa tcgcaattaca gccaaataga atgtacaat cctggtgataa
61 agctacttc gcaaatgctg aaattaatac ggcccttgat atggccacta gcggtgatat
121 eggttctcg aggacattct actgcttttt atgttctgca aataatatta aagcttttga
181 agatatatta tcaatggcac cagtggact tttatattc ggaatcagca gcggtgatat
241 aaaaatttact aatagatagc tctattttc ctgttcttct gtgacgcttt tacattatc
301 aacgtgggct actaaaacta tctattttat ggtgtatttt agcttcttct tctattttt
361 gtcgagatatt agatagatagc cagcgtcttt gcttttttt tcggttctttt tcttttct
421 aggtcgctcg tttatatatt aatttatttct cgcgttcgct gggcattagtt tacatttttt
481 tcaactttgt gcgggttaaa agtttactgt cttggagact acaaaaaatt
541 caaaacctgct acgtttattt tttgatattt cctgagacgc aacatatttt cgtattattt
601 aaacggacct tacagctaca gtaacgctaa tgttttttatt ctgacttttt cttttttttt
661 tgaagcttt tattttttta gtttttttta tttttttttt tttttttttt tttttttttt
721 taacggtgga agtttttaag cacaagatat tttttttttt cttttttttt cttttttttt
781 ttgacactgtat gccggattta cagcgaagcc ctgacttttt cttggttattt tttttttttt
841 tacaccaat agttggtcag atgttttttt tgtttttttt tttttttttt tttttttttt
901 gatttaatg ttgacactgtct gctgttata tttttttttt tttttttttt tttttttttt
961 tatgactgaa ttaccttttt aacgggttaaa cccgggttata attttttttt tttttttttt
1021
1081
LOCUS       bankit1065170       1762 bp    DNA    linear   BCT 19-FEB-2008
DEFINITION  Rickettsia amblyommii virD4 gene, complete cds.
ACCESSION   1065170
VERSION
KEYWORDS
SOURCE      Rickettsia amblyommii
ORGANISM   Rickettsia amblyommii
            Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales;
            Rickettsiaceae; Rickettsiaeae; Rickettsia; spotted fever group.
REFERENCE   1 (bases 1 to 1762)
            AUTHORS   Carmichael,J.R. and Fuerst,P.A.
            TITLE     Phylogeographical variation of vir genes from Rickettsia amblyommii
            isolates from the Northeast United States
            JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 1762)
            AUTHORS   Carmichael,J.R. and Fuerst,P.A.
            TITLE     Direct Submission
            JOURNAL   Submitted (19-FEB-2008) Molecular Genetics, The Ohio State
            University, 318 West 12th Avenue, Columbus, OH 43210, USA
FEATURES             Location/Qualifiers
            source          1..1762
            /organism="Rickettsia amblyommii"
            /mol_type="genomic DNA"
            /specific_host="Amblyomma americanum"
            /db_xref="taxon:33989"
            CDS             1..>1762
            /codon_start=1
            /transl_table=11
            /product="virD4"
            /translation="MEWHKILKVTXNIFGHAIIHPVFICTIWISGAFVAILFTNEVGALG
DINAINIAYKWAYWIVNVWQLQKLADYNLYLKWKLLASLLGPAAIIVFYIKNFERIK
SLQFKQNEKVYGNASASSSIEAAGLRSKKGMLIGVDAGGYFVADGFQHALLFAP
TGSGKGVGFVIPNLFWGDSVYVHDIKLENHELTSGWERKQGQKVFWEPSNPDGV
THCYNPIDWVSTKPGQMVDVVQKISNLIMPEKDFWNNEARSLFLGVTLYLIAADPTKT
KSEVVRTMTSDDLVYNLAVVLDTFGGVHVAAYMNIAAFLQKAKERSGVISTMNSS
LELWANPLIDSATASSDFNIEQEFKKVKTYYVGLTPDNIQRLQKLMQFYYQQATEFLS
KMPDLKEEPYGVMFLLDEPTLKGKMDFFKAGIAFYRGYRVLFLIIQDTQQLKGTY
E
DAGMNSPLSNATYRITFAANNYETANLISQLVGNKTVQEQRFSKPLFDNLNSTRTQNI
SQVQRALLLPQEOQILPRDEQIVLIESFPPKSKRKIKYKDKFFSRRLLPPTVFPTQV
PFDPFRANNNKASEETETITAP"
BASE COUNT      553 a    270 c    362 g    576 t    1 others

Figure 44. Consensus sequence for Rickettsia amblyommii virD4
Figure 44 continued

ORIGIN

1 atggaatggc ataaagatac taaaggttct anaaatatat ttggtcatgc tataattcat
61 cagcgttgtta tttttgtac cattggata aacgcgtgcat tttttgaca ttttttctaa
121 tcaggttgag cttatagggtg aagattaatag gttaataatat gtattctaatg
181 tgcgagagtc ggatattcct gtttaatgta gatttttagc ttatttttagc
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301 gtttttttcc cggggttgaa ttttttttt ttttttttt ttttttttt
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LOCUS       bankit1069148           1330 bp    DNA     linear   BCT 02-MAR-2008
DEFINITION  *Rickettsia rhipicephali* virD4 gene, partial cds.
ACCESSION  1069148
VERSION
KEYWORDS
SOURCE     *Rickettsia rhipicephali*
ORGANISM  *Rickettsia rhipicephali*
          Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales;
          Rickettsiaceae; Rickettsiaceae; Rickettsia; spotted fever group.
REFERENCE  1 (bases 1 to 1330)
          AUTHORS Carmichael,J.R. and Fuerst,P.A.
          TITLE  Phylogenetic variation of *vir* genes in *Rickettsia*
          JOURNAL Unpublished
REFERENCE  2 (bases 1 to 1330)
          AUTHORS Carmichael,J.R. and Fuerst,P.A.
          TITLE  Direct Submission
          JOURNAL Submitted (02-MAR-2008) Molecular Genetics, The Ohio State
          University, 318 West 12th Avenue, Columbus, OH 43210, USA
FEATURES             Location/Qualifiers
          source          1..1330
                             /organism="*Rickettsia rhipicephali*
                             /mol_type="genomic DNA"
                             /strain="CWPP"
                             /db_xref="taxon:33992"
          CDS             <1..>1330
                             /codon_start=2
                             /transl_table=11
                             /product="virD4 protein"
                             /translation="GGYFVADGFQHALLXAPTGSNGKVGIFVQPNLLFWSDSVVVHDIK
LENHGLTSGWREKQGKQVXWEPSNPEGVTHCYNPIDWVSTKPGMQMVDDQKIS
NLIMPEKDFWNNARSLFLGVTLYLIADPTKTSFGEIVRTMRSDDVYNNLVVLDT
LGGVIIHPAYYMNIAAFQKADKERSGVISTMNSSLWPLWNPLIDSATSSDFNIFQFK
KVKTQYVGRTPDNNQLQKLMQVYXQATEFLXKMPDLKEEPGYXMFLLDFXPT
LGKMDTFFKAIYNGRYRGGFLLIIQDTQQLKGTYQDAGINSFNLSNATYPITFAGNLY
ETANLISQGLOOKTVEQRRSFKPLFFDLNISTRSENVSQVQORALLPQEVQIQLPRDEQI
VLLIESFPPIKSRKIKYYEDKFTSXLLPPTFVPTQVFPNFNPXANNNESSEETETITV"*
BASE COUNT      418 a    223 c    261 g    416 t     12 others

Figure 45. *Rickettsia rhipicephali* virD4 nucleotide sequence
Figure 45 continued

ORIGIN

1 cggtggatat ttgtngcag aecgggttca gcagctttta ttagntgctc ctaccggttc
       61 agtgaaggt ttgaggtt gtagctctaa ettgtnattt tgaggtgatt eggtggtat
121 gcagctcata atcacggttt gacaagtgt tggcgtgaga agcaaggca
       181 gaaagttttt gccttggttct cttcatactc tcaggtgttt ctccacgttctg aatatttttt
241 tgaggtttt gtgaccaaac ctggtcctaa ggtggtgac gtctaaaaaa ttcatctat
301 tataagcccg gaaaagtttt ttggcaataa ttaggcagca gctatattt tagcgtaac
361 tcgtatttt atagccgacg ctactaaaaac taatattttt cttggaatag tggcgtctat
421 gaggggtcag gatgtgattt ataactcata cgcgtgactc gatacgcttt ggtgctgtaat
481 cacatgtgtt gcataatgta atattgtgc attttggcag aaaggcgata aagagcgttc
541 cgctgagata ctctacatgta ctctagctg gcaaatccac tttgatgpac
t126 601 acgtcagactct ctctaggtt gtagcttaaa gtagctcttt ctatattttt ttggtggtat
661 gtgaggtttt ttgctctttt tctaaatctg ggtgaaaggt tgcgtactat
721 gaggagtttt gcgcgtaaa gcagcgtgctc gctgtcctgt ggtgcgttct
781 ncagcccaac gaaatatttt tttattatt ttttattatt ggtggttttt tttttatttt
841 gagtttttttt gcaggaagca agatgtgtgt gcggagctttg gttattattttttt ttttttttt
901 aacgtaaaac gcagagattt ttgaatttttt ttaacttttta atccttttttttttttttt
961 tctctctctt ctctctctct ctctctctct ctctctctct ctctctctct ctctctctct
t126 1021 cacggtgagc gataattttt gtcctttttt gtcctttttt gtcctttttt gtcctttttt
t1321 aactggttcc

187
Figure 46. *Rickettsia australis* virD4 nucleotide sequence