Identification of the Prevalent Species of Spotted Fever Group Rickettsiae in Indiana Ticks

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Thesis Abstract

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Abstract

In May 2000, *Dermacentor variabilis* ticks were collected in southern Indiana counties and pooled according to sex in groups of three. These pools were used to prepare DNA templates from which the identity of the prevalent species of spotted fever group (SFG) rickettsiae could be revealed. Twenty-eight samples proved to be positive for SFG rickettsiae. These positive samples were subjected to restriction fragment length polymorphism (RFLP) in order to determine the species of SFG rickettsiae that the ticks harbored. Also, seventy-one ticks preserved in alcohol that had been previously determined to be positive for SFG rickettsiae from 1995-2001 using the Gimenez staining technique were surveyed. The mobility pattern from RFLP analysis of positive samples was compared to those published by Regnery et al. 1991, whose work is the basis for characterization of Rickettsiae.

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Chapter I

Introduction & Literature Review

Rickettsial Characteristics

Rickettsiae are obligate, gram negative, intracellular parasites ranging from

harmless endosymbionts to the etiologic agents that are transmitted by arthropods. They

include some of the most devastating diseases know to mankind (Hackstadt 1996). These

bacteria cause zoonoses; diseases that are associated primarily with animals, but which

can also be accidentally transmitted to humans. They are characterized mainly by their

efficient multiplication, long-term maintenance, transstadial and transovarial transmission

in their arthropod vector, and extensive geographic distribution (Azad 1998). The

Rickettsiae are a diverse group of bacteria; their fastidious requirements limit the

methods by which they are classified.

The bacterial groups contained within the family Rickettsiaceae include the

genera *Rickettsia*, which replicate inside the cytoplasm of infected cells, *Coxiella*, which

replicate within phagolysosomes, *Erhlichia*, which replicate within nonlysosomal cells,

and *Bartonella*, which exist in an epicellular fashion (Hackstadt 1996). The genus

Rickettsia has itself been separated into three distinct groups of bacteria based upon the

diseases they cause and their molecular and antigenic characteristics. These groups are

the spotted fever group, the typhus group, and the scrub-typhus group (La Scola & Raoult

1997). The members of these groups, their geographic distribution, and diseases of which

they are the cause are presented in Table 1.

Table 1
Prominent Rickettsial Species and Their Characteristics

Organism	Disease	Distribution	Vector
Spotted Fever Group			
Rickettsia rickettsii	Rocky Mountain Spotted Fever	North & South America	Ticks
Rickettsia conorii	Mediterranean Spotted Fever	Southern Europe, Mideast, Africa	Ticks
Rickettsia akari	Rickettsialpox	Worldwide	Mites
Rickettsia australis	Queensland Tick Typhus	Australia	Ticks
Rickettsia sibirica	North Asian Tick Typhus	Asia	Ticks
Rickettsia montana	Avirulent	North America	Ticks
Rickettsia parkeri	Avirulent	North America	Ticks
Rickettsia rhipicephali	Avirulent	North America	Ticks
Typhus Group			
Rickettsia prowazekii	Epidemic Typhus	Worldwide	Lice
Rickettsia typhi	Murin Typhus	Worldwide	Fleas
Rickettsia canada	Avirulent	North America	Ticks
Scrub Typhus Group			
Rickettsia tsutsugamushi	Scrub Typhus	Southeast Asia	Chiggers

The study of spotted fever group (SFG) rickettsiae is of great importance in Indiana, because one member of this group causes Rocky Mountain spotted fever (RMSF), a disease fatal to humans. Since 1970, 225 confirmed cases of RMSF were reported in Indiana. The presence of *Dermacentor variabilis*, the tick vector of RMSF, has been recorded in all 92 counties in Indiana (Pinger 2000).

Structure & Composition

Rickettsiae were originally considered organisms that were neither bacteria nor viruses, due to their intracellular nature. However, extensive research has determined that members of the genus *Rickettsia* are small, gram-negative coccobacilli measuring 0.3-0.5 um in width and 0.7-1.0 um in length (Ormsbee 1985). *Rickettsiae* contain no plasmids (Hackstadt 1996).

The rickettsial genome is small, but comparable to those of other free-living bacteria. Although the actual size of the genome depends upon the species of rickettsiae, it ranges between 1.2-1.4 Mb in the spotted fever group (Roux 1993). The rickettsial cell wall is typical of gram-negative bacteria, with an inner and outer membrane separated by a peptidoglycan layer (Anacker 1967). Components of this peptidoglycan layer include D-amino acids, muramic acid, and diaminopimelic acid.

The cell wall of rickettsiae contains many interesting features. For example, the cell wall of *Rickettsia rickettsii* possesses two large, immunodominant surface protein antigens (Anaker 1987). These proteins have unknown functions, and have been named rOmpA and rOmpB (rickettsial outer membrane proteins A and B). rOmpA had previously been known as the 155-kDa, 170-kDa, and 190-kDa antigen, and rOmpB had been known as the 120-kDa, 133-kDa, and 135-kDa antigen (Gilmore 1991). These nomenclatures caused confusion within the scientific community, as the actual size of these proteins varies between species of rickettsiae. Both rOmpA and rOmpB are heterogeneous between species, and have been observed in each species of rickettsiae by monoclonal antibodies (Anacker 1987).

The rOmpA protein appears to be present primarily in the spotted fever group. This protein contains a large tandem repeat region that is among the largest known to occur in prokaryotes (Anderson et al. 1990). It includes 72-75 amino acids and 13 repeat units (Gilmore 1993). Differentiation in size of these repeat regions between species makes these species distinguishable via polymerase chain reaction (PCR) analysis of samples. On the other hand, the rOmpB protein is the most abundant surface protein within rickettsiae, and is interesting in that it has a unique surface location, strong

immunogenicity, and reactivity with monoclonal antibodies that protect mice against lethal rickettsial infection (Williams 1986). rOmpB is believed to include a paracrystalline surface array (S-layer) in rickettsiae (Sleytr et al. 1983). Identification of different species using this protein is difficult, as it is so abundant (Hackstadt et al. 1992).

A controversial structure within rickettsiae is the slime layer. This layer is an electron lucent zone that separates the rickettsiae from the cytoplasm. It was initially dismissed as an artifact from electron microscopy, but has been subsequently re-observed to actually exist and perform an important protective function. The slime layer is mostly composed of polysaccharides, and observations using high-voltage electron microscopy suggest that it promotes interactions within the cytoskeleton (Todd et al. 1983). This component is still controversial, and may actually be found to contain both rickettsial and host characteristics, as rickettsiae have coevolved alongside their eukaryotic hosts (Hackstadt 1996). These observations suggest that the slime layer is important in the disease process (Austin & Winkler 1988; Sonenshine 1993).

Growth & Development

Rickettsiae are maintained principally by transovarial transmission, also known as vertical transmission. In this type of transmission rickettsiae are passed from infected female ticks to their ova, which hatch into infected offspring. Transovarial of transmission can occur at rates of 100% (Hackstadt 1996). Transmission can also occur horizontally, when an infected tick ingests rickettsiae laden fluids while feeding on an infected mammal. Once inside a host cell, rickettsiae multiply by binary fission. Rickettsiae reproduce prominently in the cytoplasm and occasionally appear in the

nucleus (Soneneshine 1993). Because rickettsiae are highly adapted to the intracellular environment, they thrive in potassium-rich surroundings like the eukaryotic cytosol (Walker 1988). They have specific membrane-transport systems for acquiring amino acids, ATP, and other metabolites from the host cell. Rickettsiae reproduce every 8-10 hours and, in the laboratory, can be propagated only in living animals, embryonated eggs, or cell cultures. The specificity of the rickettsial metabolic mechanisms will not allow survival in any other type of medium (La Scola et al. 1997).

Epidemiology & Rocky Mountain Spotted Fever

The determining factor in rickettsial prevalence is the ecology of the arthropod vector (Walker 1998). The major vectors of SFG rickettsiae in the United States are *Dermacentor variabilis* (American dog tick) and *D. andersoni* (Rocky Mountain wood tick). Both species can transmit *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever, a disease that occurs in North America, throughout the range of these tick species. The geographic distribution of members of the genus *Rickettsia*, as well as the major rickettsioses that are caused by these bacteria are presented in Table 1.

Rocky Mountain spotted fever (RMSF) is a severe disease caused by the SFG rickettsia, *Rickettsia rickettsii*. The incidence of RMSF is seasonal, occurring only in the spring and early summer (Hackstadt 1996). Tick vectors, once infected, will remain infected for life. The rickettsiae can be found in many of the tick's tissues, including the salivary glands, hemolymph, digestive tissues, and genital tissues. Ingestion of a blood meal stimulates a massive rickettsial multiplication. It has been suggested that an increase

in the diameter of the controversial slime layer is associated with rickettsial reactivation. Transmission of *Rickettsia rickettsii* is by the saliva of feeding ticks (Hayes et al. 1982).

RMSF was first recognized in 1896 in Idaho's Snake River Valley. Initial signs and symptoms include sudden onset of fever, headache, muscle pain, and rash. It can be fatal if not treated quickly and appropriately. This disease is caused by attachment of *Rickettsia rickettsii* to vascular endothelium cells, which in turn engulf the bacteria. Once inside the cell's phagosome, the rickettsiae escape by lysing the phagosomal membrane using a phospholipase. In the cytosol, the bacteria multiply and spread to other cells via a complex network composed of actin filaments that connect cell membranes. The characteristic rash that accompanies RMSF is composed of hemorrhagic spots, which are the effects of damage to foci of contiguous endothelial cells (Walker 1988).

There are many baffling aspects of the epidemiology of RMSF. The severity of the disease has shifted trends numerous times since 1800. In the late 1800s to the early 1900s, RMSF had a mortality rate in the Bitterroot Valley of western Montana of >80% (Parker 1938), while the mortality rate in Idaho was 2-3%. This phenomenon is quite interesting. It may actually be caused by competition between pathogenic and nonpathogenic Rickettsiae (Burgdorfer et al. 1975). The current nationwide mortality rate is somewhere between 2-10% (Hattwik 1970).

Other Spotted Fever Group Rickettsia

In addition to *R. rickettsii*, the SFG includes the following bacteria: *R. conorii, R. akari, R. australius, R. siberica, R. montana, R. parkeri,* and *R. rhipicephali* (Hackstadt 1996). Of these, only three are nonpathogenic; *R. montana, R. parkeri,* and *R. rhipicephali*. Previous research conducted at the Public Health Entomology Laboratory (Ball State University) on serum obtained from dogs in Indiana identified three main species of rickettsiae, *R. rickettsii, R. rhipicephali,* and *R. Montana* (Stauffer 1988). The presence of *Rickettsia* in *D. variabilis* tick samples collected in southern Indiana was confirmed using PCR (Mesina 2001). It was the purpose of my research to characterize the SFG rickettsiae found in *D. variabilis* in Indiana using molecular techniques.

Chapter II

Methods & Materials

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an *in vitro* reaction that produces enzymatic synthesis of millions of copies of a specific DNA segment from a complex template without the use of cloning (Mullis 1990). The reaction is based on the amplification of a DNA segment using DNA polymerase and oligonucleotide primers that hybridize to opposite strands of the sequence to be amplified. There are three basic steps that constitute a PCR cycle, and the amount of amplified DNA produced is limited theoretically only by the number of times the steps are repeated. In the first step of PCR, the DNA to be amplified is denatured into single strands. It is not necessary to purify or clone the DNA sample. The DNA can come from any number of sources, including genomic DNA, forensic samples, samples stored as part of medical records, single hairs, mummified remains, and fossils. The double stranded DNA is separated by heating until it dissociates into single strands. Following denaturation of DNA, each primer hybridizes to one of the two separated strands such that extension from each 3'-hydroxy end is directed toward each other. These primers are synthetic oligonucleotides that anneal to sequences flanking the segment to be amplified. Each primer has a sequence complimentary to only one of the strands of DNA.

In the third step, a heat-resistant DNA polymerase (Taq polymerase) is added to the reaction mixture. The annealed primers are then extended in the 5'-3' direction, using

the single-stranded DNA bound to the primer as a template. The product is a double-stranded DNA molecule with the primers incorporated into the final product.

These three steps constitute a single PCR cycle. Each cycle is generally performed at a different temperature. Repeated cycles of PCR result in the exponential accumulation of a discrete fragment. The process is automated and uses a machine called a thermocycler, which can be programmed to carry out a predetermined number of cycles, producing large amounts of amplified DNA segments in a few hours (Mullis 1990).

Gel Electrophoresis

Electrophoresis is a technique used to separate macromolecules, i.e. proteins and nucleic acids by size, charge, and/or conformation. Electrophoresis may take many forms, but usually involves a medium (agarose gel, polyacrylamide gel, paper, nitrocellulose membrane), a buffer, and an electric current. The medium may be marked and the results visualized following electrophoresis. The basic principle is as follows: a sample is loaded onto the medium, the medium is covered with a buffer solution (in the case of submarine electrophoresis), and an electric current is run through the buffer. This current moves the sample through the medium (Barker 1998).

In the case of nucleic acids, agarose gel or polyacrylamide gel are the mediums of choice. Nucleic acids, which contain a net negative charge, are pulled from the negative (cathode) to the positive (anode) in such a manner that they create specific banding patterns. Larger molecules migrate more slowly than smaller molecules, allowing for noticeable separation.

Agarose gel electrophoresis involves agarose, a polysaccharide extracted from seaweed. This type of medium is typically used in 0.2-5% concentrations, and creates a porous gel through which molecules migrate. Higher concentrations of agarose facilitate the separation of smaller molecules. The concentration must therefore be carefully decided upon before running the gel, as resolution of the bands will differ in diverse agarose concentrations (Barker 1998).

Polyacrylamide gel electrophoresis is used to separate most proteins and small oligonucleotides which require a small gel pore size for retardation. Polyacrylamide gel is made of cross-linked polymers of acrylamide. The concentration of acrylamide used ultimately determines the length of these polymers, much in the same manner as agarose. The resolving power of these gels is rather high, although they have a small range of separation. This type of electrophoresis can resolve differences in one base pair of molecular weight between samples (Vann 2002).

Ethidium bromide (EtBr) is a base analog that can reversibly intercalate between the bases of DNA molecules. The DNA-EtBr complex emits an orange fluorescence upon excitation by UV light. DNA fragments resolved by electrophoresis can be visualized by incubating the gel in a solution containing ethidium bromide and exposing it to UV light (Sharp et al. 1973). The DNA-EtBr complex fluoresces optimally at 254 nm. However, DNA molecules can be cut and dimerized at this wavelength (Brunk & Simpson 1977). Thus, polyacrylamide and agarose gels are generally visualized at 300 nm.

Restriction Fragment Length Polymorphism

In order to differentiate between the species of rickettsiae present in Indiana,

restriction fragment length polymorphism (RFLP) was utilized. This technique involves

the use of PCR to amplify a specific region of DNA, and restriction enzymes

(endonucleases) that then cut the amplified DNA into species-specific fragments that may

be visualized in polyacrylamide gel electrophoresis (Barker 1998).

Restriction endonucleases are a group of enzymes that recognize short,

palindromic, specific sequences in DNA or RNA known as restriction sites. Restriction

enzymes act as "molecular scissors" that cut the double-stranded DNA at specific sites or

within sites adjacent to the recognition sequence. DNA may be cleaved at every instance

of that recognition sequence (Meyers 1995). An example of this activity is presented in

Figure 1.
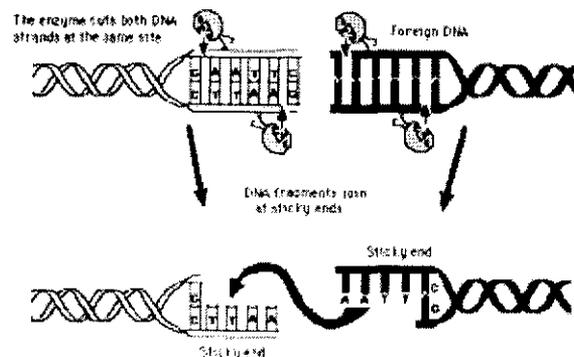
Fig 1. Example of Restriction Enzyme Activity using the Restriction Endonuclease, EcoRI.
(http://www.accessexcellence.org/AB/GG/restriction.html)

Different species of organisms may contain varying numbers of restriction sites for any one restriction endonuclease. This characteristic makes differentiation of species in unknown samples easy, in that differing numbers of fragments with differing sizes will migrate through polyacrylamide gel to create species-specific patterns (Regnery et al. 1991). These techniques were vital in the identification of the species of SFG rickettsiae present in infected ticks from southern Indiana.

Templates

DNA templates for RFLP were previously prepared by Katrina Mesina (Mesina 2001). These templates were subjected to secondary PCR to confirm positive results.

Oligonucleotide Primer Selection

General rickettsial oligonucleotide primers for PCR were derived from two areas of the citrate synthase gene which have significant homologies for both *R. prowazekii* and *Escherichia coli* analogous sequences; the exact nucleotide sequence of both primers were derived from the *R. prowazekii* gene (Ner et al. 1983; Wood et al. 1987). It was initially discovered that sequences conserved between diverse bacterial genera would most likely also be conserved within either of the genera from which the sequences were initially derived (Regnery et al. 1991).

Oligonucleotide primers for PCR amplification of regions of the 190-kDa SFG gene were derived from the *R. rickettsii* (R strain) sequence without prior specific information regarding genus and species conservation (Anderson et al. 1990).

Table 2

Oligonucleotide Primers

Primer	Species	Gene	Nucleotide Sequence	Product Size (bp)
RpCS.877p	R. prowazekii	Citrate synthase	GGGGCCTGCTCACGGCGG	381
RpCS.1258n	R. prowazekii	Citrate synthase	ATTGCAAAAGTACAGTGAACA	381
Rr190.70p	R. rickettsii	190-kDa antigen	ATGGCGAATATTTCTCCAAAA	532
Rr190.602n	R. rickettsii	190-kDa antigen	AGTGCAGCATTCGCTCCCCCT	532

Primer pairs were referred to by the initals of the genus and species from which

they were derived, combined with a simple alphanumeric identifier of the gene amplified

and followed by the position of the first nucleotide of the "positive" strand of the primer

(using the conventional 5'-3' presentation of the sequence data) and then the position of

the last nucleotide of the "negative" strand of the corresponding primer from the

complementary strand of DNA (Regnery et al. 1991). For example, *Rp*CS.877p and

*Rp*CS.1258n refer to the *R. prowazekii* citrate synthase primer pair for a region between

nucleotides 877 and 1258. Similarly, *Rr*190.70p and *Rr*190.602n refer to primers derived

from the *R. rickettsii* (R strain) 190-kDa antigen gene sequence and which prime an

amplified product between nucleotides 70 and 602 of the homologous DNA sequence

(Regnery et al. 1991).

The primers used in this study are included in Table 2. Nucleotide primers were synthesized at Invitrogen Life Technologies, 1600 Faraday Ave Carlsbad, CA (USA) 92008.

Polymerase Chain Reaction Amplifications

PCR amplifications were performed in a Perkin Elmer 2400 thermal cycler according to the method previously described (Mesina 2001) PCR products were used as substrates for restriction enzyme assay. These products were sometimes cleaned further using Promega's Wizard PCR Preps DNA Purification System. Purifications were performed without a vacuum manifold in accordance with the manufacturer's instructions.

Restriction Fragment Length Polymorphism

In order to determine that the 190-kDa gene had been amplified, as well as to determine the species of *Rickettsia* present in the positive samples provided by PCR, restriction fragment length polymorphism was performed. PCR products were digested with the restriction enzyme *Pst*I, whose action cuts at differing numbers of sites in each species of *Rickettsia*. The sizes of these fragments may be observed in Figure 2.
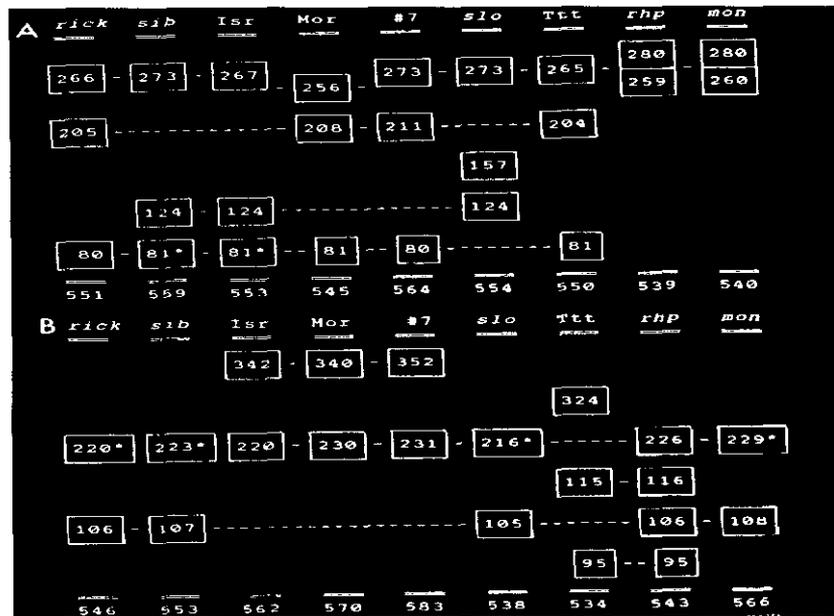
Figure 2: Banding Patterns of Rickettsial Species When Cut With *Pst*I are illustrated in part A. Banding patterns associated with *Rsa*I are illustrated in part B of this diagram (Regnery et al. 1991).

It is possible that three species of *Rickettsia* occur naturally in Indiana, based upon a prior study (Stauffer 1988). The electrophoretic patterns generated after restriction enzyme analysis for these three species are as follows: the pathogenic *R. rickettsii* shows a pattern of three bands, each at 266 bp, 205 bp, and 80 bp, while the nonpathogenic *R. rhipicephali* and *R. montana* show bands of 280 bp, 259 bp, and 280 bp, and 260 bp, respectively (Regnery et al. 1991). Only preliminary results of one sample digested with *Rsa*I were analyzed. Further analysis with the enzyme *Rsa*I were not performed due to time constraints, although this digestion would have further clarified the exact species of *Rickettsia* present in the tick pools.

Restriction digestions were performed in 1.5ml microcentrifuge tubes, using a 15ul reaction volume. The reaction mixture included 13 ul of the PCR product, 1.5ul of

REAct2, and 0.5ul of the restriction enzyme *Pst*I. Master mixes including the enzyme and

REAct2 were created to ensure equal distribution of reagents. The digestion mixture was

incubated at 37°C for 1 hour. Each digestion contained one positive control (*R. slovaca*)

and one negative control (water blank). Products were segregated in a 6% polyacrylamide

gel electrophoresis, and the image was captured on the Gel Print 2000 Imaging system

(Biophotomic, Ann Arbor, MI).

Template Preparation and DNA Survey of Gimenez Positive Ticks Preserved in Alcohol

The second half of this study was devoted to determining the identity of the

species of *Rickettsia* present within ticks sent to the Public Health Entomology

Laboratory that had previously tested positive for SFG rickettsiae using the Gimenez

stain microscopic technique (Gimenez 1964). These samples were further tested with an

Immunofluorescent Assay that identified those ticks which carried the pathogenic *R.*

rickettsii bacteria. All Gimenez positive ticks from 1995-2001 were subjected to testing

via the same methods as previously described. The only significant change was the

method of template preparation, which was based upon the method of template

preparation described in Persing et al. 1990. Ticks to be studied were removed from the

70 % ethanol preservation solution in which they were placed following Gimenez stain

analysis, and air-dried on Whatman filter paper for 5 min. The individual ticks were then

frozen with liquid nitrogen inside sterile mortar and pestles containing a small amount of

sterile sand (1 mortar and pestle per tick). The ticks were then ground into a fine powder,

which was transferred to a new 1.5ml microcentrifuge tube. Following placement into the

tube, the PCR templates were prepared in the same fashion as described by Burket

(Burket et al 1998). Agarose gels were analyzed in the same fashion, and positive PCR

products were subjected to the same restriction digestion protocol as previously

described. This study provided data regarding the prevalent species of SFG Rickettsiae in

Indiana. A list of these samples is presented in Table 3.

Table 3: Gimenez Positive Tick Samples Tested for SFG Rickettsiae

Sample Number '95-'96	Indiana County	Sample Number '99	Indiana County	Sample Number '00-'01	Indiana County
95 0042	Lawrence	99 0027	Bartholomew	00 0262	Johnson
95 0051	Vanderburg	99 0043	Franklin	00 0393	Bartholomew
95 0073	Gibson	99 0081	Decatur	00 0397	Bartholomew
95 0074	Lawrence	99 0106	Bartholomew	00 0718	Orange
95 0075	Lawrence	99 0110	Franklin	01 0008	Monroe
95 0187	Lawrence	99 0112	Franklin	01 0022	Morgan
95 0193	Jennings	99 0113	Franklin	01 0073	Franklin
95 0228	Brown	99 0155	Lawrence	01 0198	Jasper
95 0232	Lawrence	99 0163	Fayette	01 0231	Franklin
95 0247	Lawrence	99 0174	Porter	01 0383	Lake
95 0262	Morgan	99 0188	Franklin		
95 0272	Franklin	99 0209	Lawrence		
95 0311	Brown	99 0213	out of state		
95 0313	Lawrence	99 0215	Bartholomew		
95 0319	Brown	99 0247	Tippecanoe		
95 0384	Grant	99 0248	Franklin		
95 0386	out of state	99 0296	Lawrence		
95 0400	Lawrence	99 0299	Lawrence		
95 0421	Monroe	99 0320	Lake		
95 0428	Tippecanoe	99 0347	Porter		
95 0429	Elkhart	99 0348	Grant		
95 0441	Morgan	99 0358	Delaware		
95 0446	Lawrence	99 0375	Bartholomew		
95 0463	Monroe	99 0377	Bartholomew		
95 0499	Vanderburg	99 0384	Brown		
95 0505	Spencer	99 0407	Dearborn		
95 0508	Porter	99 0408	Lake		
96 0074	La Porte	99 0420	Grant		
96 0096	Franklin	99 0447	Tippecanoe		
96 0190	Madison	99 0525	Vigo		
		99 0627	Elkhart		

Chapter III

Results

PCR Analysis

Figure 3 illustrates the results of the PCR amplifications using primers targeted to

the rOmpA gene of SFG rickettsiae. Secondary amplification yielded thirteen positive

pools.

Figure 3: Agarose Gel Electrophoresis of Samples 34,131,137,177,298,302 showing a 532 bp amplification product of PCR

Substrate for Restriction Enzyme Assay

The clean PCR products were subjected to digestion with *Pst*I.. Figure 4 compares

the bands of cut and uncut PCR products from the restriction enzyme digestions. Two

bands, one at 280 bp and one at 260 bp, were obtained following the digestion. This

pattern of digestion is comparable to the pattern associated with *R. montana* or *R.*

rhipicephali. Following digestion with *Rsa*I, a positive control digestion product of *R.*

rhipacephali was compared to that of sample 220. These results are presented in Figure 5.

208U 208C 220U 220C 298U 298C +C +U M

280 bp
260 bp
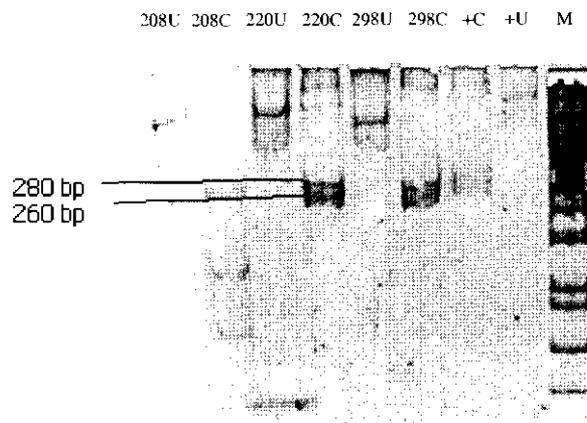
Fig 4. Polyacrylamide Gel Electrophoresis of Samples 208, 220 and 298 following digestion with restriction enzyme *Pst*I.

+U   220C   +C   M

Fig 5. Comparison of positive sample digestion *Rsa*I of *R. rhipicephali* with that of sample 220. When cut with *Rsa*I, *R. Montana* demonstrates a doublet band at 229bp and also a single band at 106bp, as illustrated above. The single band was lost in the electrophoresis due to low molecular weight. The doublet appearance of sample 220 confirms that it is, in fact, *R. Montana*, when compared to the positive control.

Gimenez Positive Survey

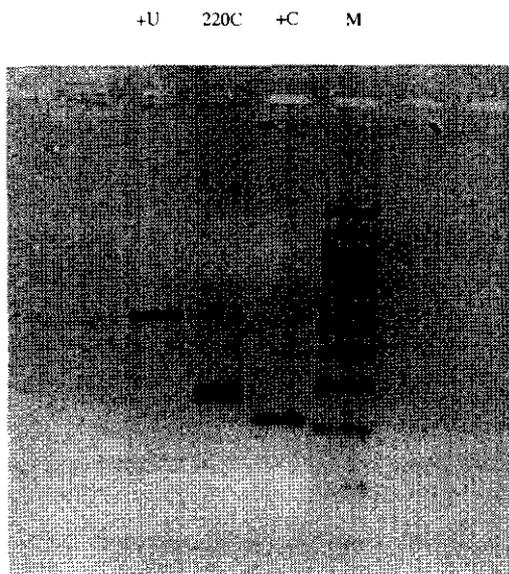Surveys began with the Gimenez positive ticks from 1995-1996. All results were negative for the rOmpA gene. PCR using primers targeted to the citrate synthase gene of *Rickettsia* yielded positive results for samples 95 0319 and 95 0051. These results may be observed in Figure 6. Tick number 95 0051 was found to be positive by immunofluorescent assay for the virulent *R. rickettsii*. Attempts to clean the PCR product and analyze it using RFLP failed.

95 0051

381 bp
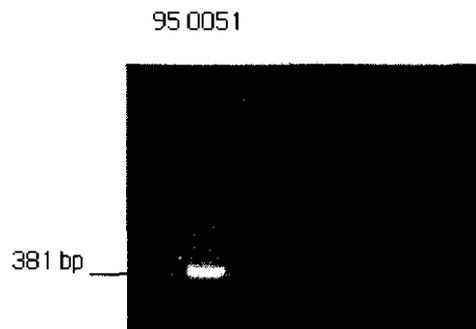
Fig 6. Gel Electrophoresis of PCR reamplification of sample 95 0051.

Summary of Results

Twenty-eight of the original 343 pools collected from southern Indiana were positive for the genus *Rickettsia* via PCR using primers targeted to the citrate synthase gene. Thirteen of the 28 pools were positive when targeted with primers for the rOmpA protein of SFG rickettsiae. RFLP yielded results that are comparable to those of *R. montana*. Of the 71 G+ ticks surveyed, only 2 ticks yielded positive results for citrate synthase. Only one, 95 0051 (Vanderburg county), showed positive rOmpA results. Tick 95 0051 was found to harbor *R. rickettsii* when subjected to immunofluorescent assay, however, positive RFLP results could not be confirmed.

Chapter IV

Discussion

Previous studies suggest that *R. montana, R. rhipicephali,* and *R. rickettsii* are the predominant species of spotted fever group rickettsiae in Indiana (Stauffer 1988). In this study the results of RFLP suggest that the most prevalent species of SFG rickettsiae in Indiana is *R. montana.* These results could be confirmed using sequencing analysis.

It was originally hypothesized that there would be a larger population of nonpathogenic rickettsiae when compared to pathogenic rickettsiae in Indiana. This belief was based on previous finding of the lethal effects of pathogens on their vectors. For example, the agents of Rocky Mountain spotted fever (*R. rickettsii*), epidemic typhus (*R. prowazekii*), and bubonic plague (*Yersinia pestis*) have been reported to reduce the survival, fecundity, or fitness of their host vectors (Niebylski et al. 1999). The effects of the agents of tularemia (*Francisella tularensis*) and Lyme disease (*Borrelia burgdorferi*) on their tick vectors are less clear, although pathological effects have been reported (Balashov 1972; Burgdorfer et al. 1989; Niebylski et al. 1999).

Currently, there is no effective vaccination against any of the SFG rickettsiae, and despite the availability of effective antimicrobial therapy, mortality occurs among patients hospitalized with Rocky Mountain spotted fever (Raoult et al. 1986; Dalton et al. 1995; Billings et al. 1998; Montero et al. 2001). However, recent advances in molecular biology as well as the knowledge that the rOmpA and rOmpB outer membrane proteins are major immunogenic antigens has led to the creation of a possible DNA vaccine (Montero et al. 2001). The first report of the use of a DNA vaccine for rickettsiae was published in 2001 by Montero et al. with quite promising results. Perhaps with further

study into the immunogenic properties of the rickettsial outer membrane proteins and the rickettsial slime layer, appropriate chemotherapies and even vaccinations can be created.

It has been demonstrated that the introduction of an avirulent pathogen into a host elicits an immune response that creates memory cells against that pathogen and those pathogens with similar structure (Tortora et al. 1998). Future research into the creation of a vaccine against nonpathogenic SFG rickettsiae, such as those identified in this survey may yield information significant to the creation of a vaccination against all rickettsiae.

The advent of new culture techniques, such as the shell vial-centrifugation technique, and the detection of rickettsial DNA have dramatically increased the number of rickettsial species detected (Raoult & Roux 1997). Although these techniques were not included in this particular survey, it is possible that they may be used to identify new species of rickettsiae in Indiana.

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