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Molecular assay development: detecting pathogenic *Rickettsia* in the tick vector *Amblyomma americanum*

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2012

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Abstract

Molecular assay development: detecting pathogenic *Rickettsia* in the tick vector *Amblyomma americanum*

By Ellen Dugan

Purpose: *Rickettsia amblyommii* is a common and abundant member of the bacterial community of the tick vector *Amblyomma americanum*. While *R. amblyommii* is thought to cause only mild illness, if any, in humans, its presence makes the detection of rare and clinically more severe *Rickettsia* difficult to detect with available assays. A conventional PCR assay was developed to improve the detection of *Rickettsia* species of known or potential pathogenicity in the presence of *R. amblyommii*, in the tick vector *Amblyomma americanum*.

Methods: Homologous gene sequences from *Rickettsia* were aligned to detect polymorphisms unique to *R. amblyommii*. These polymorphisms were used to design 14 primer pairs. One primer pair was chosen for assay creation based on its ability to detect target *Rickttsia* DNA and to not detect *R. amblyommii* DNA. Further testing was conducted to validate the assay's sensitivity, specificity, and accuracy. Tests included annealing temperature optimization, determination of the LOD for *Rickettsia*, and testing of wild-caught tick samples with known and unknown *Rickettsia* infection.

Results: One hundred percent of 20 target *Rickettsia* species tested with the assay were detected and 0% of *A. americanum* DNA with known *R. amblyommii* infection detected *R. amblyommii*. Both results demonstrate assay specificity for the target *Rickettsia* species. Sensitivity testing indicated that *R. parkeri* could be detected in the presence of tick and *R. amblyommii* DNA in 92.8% of samples at a dilution as low as 1:6000 from the stock concentration. *Rickettsia rhipicephali* CWPP and *Rickettsia* 364D were detected in 100% of wild-caught naturally infected *Dermacentor occidentalis* ticks, indicating high accuracy of the assay to detect natural *Rickettsia* infection in ticks.

Conclusions: This simple, easily replicable, inexpensive, and rapid testing method detects *Rickettsia* other than *R. amblyommii* in ticks. It eliminates the need for sequencing to differentiate target *Rickettsia* from *R. amblyommii,* allowing for resources to be focused on less prevalent and abundant *Rickettsia* that cause more severe clinical disease.

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Background

Amblyomma americanum

Amblyomma americanum, also known as the lone star tick, is a hard bodied (ixodid) tick species described in the United States in 1754 (Childs & Paddock, 2003). The silvery-white spot on the dorsum distinguishes females from males who display nondescript streaks around the margins of the body. *Amblyomma americanum* is the most abundant and aggressive human-biting tick species in the southeastern United States at the larval, nymphal, and adult life stages (Felz, Durden, & Oliver, 1996). Of approximately 10,000 ticks collected in 2008 and 2010 in North Carolina, this species comprised about 99% (Apperson et al., 2008; Smith et al., 2010). The lone star tick has also been increasing in abundance and range throughout the United States over the past 20-30 years (Cohen et al., 2009). *Amblyomma americanum* was considered to be a nuisance species until the demonstration of disease transmission in the 1990s: laboratories in the U.S. such as the Ohio Department of Health did not even screen *Amblyomma* ticks for tick-borne pathogens until 2003 (Childs & Paddock, 2003; Kelly, 2005).

In addition to definitively vectoring bacteria that cause ehrlichiosis and tularemia, *A. americanum* has been associated with the pathogens that cause Rocky Mountain Spotted Fever (RMSF) and Southern Tick Associated Rash Illness (STARI) (Childs & Paddock, 2003). Firstly, the pathogen for RMSF, *R. rickettsii,* is predominately found in *Dermacentor* species but has recently been described in 4 of 870 *A. americanum* samples. *A. americanum* is not directly associated with clinical disease but may play an integral role in RMSF transmission due to its ability to harbor the infectious agent (Berrada, Goethert, Cunningham, & Telford, 2011).

Amblyomma americanum has also been associated with STARI, a Lyme-like illness, according to physician-diagnosis following lone star tick bites (Master 1998). *Borrelia lonestari* is a suspected pathogen, but more than 14 studies have failed to provide a consistent, causative agent of STARI (James et al., 2001; Masters, Grigery, & Masters, 2008; Wormser et al., 2005). Uncertainty about *A. americanum* disease potential lies in the lack of knowledge about pathogenic bacteria in these ticks.

Rickettsia species

Rickettsia is a dominant genera of tick endobacteria that can be divided into two categories: the typhus group (TG) and spotted fever group (SFG) (Didier Raoult & Philippe Parola, 2007). Rickettsial isolates can be classified by genus, group, and species using a combination of the gltA, ompA, ompB genes, the16S rRNA (rrs) gene and gene D. The isolate must exhibit homology with rrs and gltA at ≥ 98.1 and 86.5% respectively to be categorized in the genus *Rickettsia* (Fournier et al., 2003). There are 24 recognized species of *Rickettsia* in addition to several dozen strains that can be vectored by ticks, fleas, and/or mites and cause human disease in the U. S. and internationally (Didier Raoult & Philippe Parola, 2007).

Rickettsia amblyommii

Rickettsia amblyommii is an endobacteria in the SFG initially discovered in a 1974 collection of *A. americanum* and is also referred to as strains 85-1034, WB-8-2, and MOAa (Didier Raoult & Philippe Parola, 2007; Smith et al., 2010). *Rickettsia amblyommii* is found to infect individual *A. americanum* at a high density and a high frequency in comparison to other SFGR. Studies have detected about 1,300 to 250,000 *R. amblyommii* per tick and recorded a 60- 69% frequency of positive tests for *R. amblyommii* in *A.*

americanum (n=500) (Zanetti, Pornwiroon, Kearney, & Macaluso, 2008; Zhang, Norris, & Rasgon, 2011).

Furthermore, the infection frequency of *Rickettsia amblyommii* in *A. americanum* is much higher than infection of Rickettsiales and other bacteria in the lone star tick. *Rickettsia amblyommii* infection ranges from about 40-90% in *A. americanum* compared to infection prevalences of less than 1% to 4.5% for *Ehrlichia chaffensis*, *E. ewingii, Borrelia lonestari*, and *R. rickettsii* (Table 1) (Apperson et al., 2008; Kelly, 2005; Mixson et al., 2006; Smith et al., 2010).

Despite its ubiquity in *A. americanum, R. amblyommii* rarely, if at all, causes clinical disease. Laboratory studies have shown *R. amblyommii* as non-pathogenic to animals (Burgdorfer, SF Hayes, & AJ Mavros, 1981). In two isolated cases, *R. amblyommii* was detected in a tick removed from one patient and was also found in one patients' sera. These patients experienced mild fever and macular rash respectively (Billeter, Blanton, Little, Levy, & Breitschwerdt, 2007; Dasch, D.J. Kelly, A.L. Richards, J.L. Sanchez, & C.C. Rives, 1993). Overall, *R. amblyommii* is found at a high frequency and in a high percentage of *A. americanum* but is not of significant clinical concern, thus detection of this species is not desired.

***Rickettsia amblyommii* masking effect**

The study hypothesis is that *R. amblyommii* interferes with the detection of other *Rickettsia* species. Support for this statement includes previously described and newly discussed evidence. As mentioned, *R. amblyommii* infects a large proportion of *A. americanum* and in a high frequency (Zanetti et al., 2008; Zhang et al., 2011). Regression analysis results showed that a high proportion of *R. amblyommii* correlate with a low

proportion of *Rickettsia* species in *A. americanum* ($p<0.001$). Since co-infection of *R. amblyommii*-positive ticks with *Rickettsia* spp. was not determined in this study, the relationship may be explained by interference of *R. amblyommii* infection in detecting other *Rickettsia* species (Smith 2010). Infection interference of rickettsial organisms has been reported for *D. variablis* and postulated for *A. americanum* (Fuente, Blouin, & Kocan, 2003; Macaluso, Sonenshine, Ceraul, & Azad, 2002; Smith et al., 2010; Stromdahl, Vince, Billingsley, Dobbs, & Williamson, 2008).

Rickettsia species identification and detection

Current methods of *Rickettsia* detection in ticks involves either xeno-diagnositcs or molecular methods based on polymerase chain reaction (PCR). PCR assays are preferred because they provide more sensitive, specific, and rapid detection and identification of species. Unfortunately, there are few *Rickettsia* species-specific assays (Didier Raoult & Philippe Parola, 2007). Real-time PCR assays have been developed for select species of known pathogenesis such as strains of *R. rickettsia* (strains R and Sheila Smith) (Kato et al., 2013). One barrier to the production of species-specific tests is that the most common method used, creating plasmid DNA for real-time PCR analysis, is tedious and costly (Kim et al., 2011). Resources are therefore used in testing known pathogens rather than exploring the disease potential of lesser-known species and their interaction with prominent vectors.

A second reason for the lack of *Rickettsia* testing methods is the fact that all *Rickettsia* citrate synthase PCR-amplified products are similar in base pair length (Regnery, Spruill, & Plikaytis, 1991). Using gel electrophoresis, all species are amplified at the same marker and products must be further sequenced to differentiate species, which

is a costly and time consuming process (Didier Raoult & Philippe Parola, 2007). Even new real-time PCR assays do not differentiate between *R. amblyommii* and other species (Kato et al., 2013). The inability to analyze the distribution of bacteria in ticks hinders knowledge about the potential for disease transmission. For example, circa 1986, *A. americanum* was a proven vector of STARI but in over 20 years of testing, the etiologic agent has not been identified (Burkot et al., 2001; Levine, Sonenshine, Nicholson, & Turner, 1991; Schulze et al., 1986; Suei, 2013). Findings from both new and old studies support the need for enhanced testing capacity and *Rickettsia* species distinction.

Relevance to environmental public health

Currently, there are 16 known infections caused by *Rickettsia* isolates (rickettsioses) but the pathogenicity of many *Rickettsia* species is unknown (Didier Raoult & Philippe Parola, 2007; Regnery et al., 1991). Studies by Warrell et al. (2012) determined that the disease-causing potential of at least five *Rickettsia* species including *R. helvetica*, *R. parkeri*, *R raoultii*, *R. canadensis,* and *R. amblyommii,* is inconclusive. Likewise, new *Rickettsia* isolates with undetermined pathogenicity are constantly being discovered and existing species are being found on a larger geographical scale. Furthermore, species including *R. asiatica, R. helongjangesis, R. raoultii,* and *R. tamurae* along with several dozen isolates await classification and disease characterization. This is partly due to disagreements between Rickettsiologists on the classification of a 'species' versus a 'strain' (Hechemy, Brouqui, & Samuel, 2009). Of additional concern is that some rickettsiosis, such as diseases caused by *R. slovaca* and *R. africae,* display similar characteristics of infection which complicates diagnosis (Walker, 2007). In all situations, further characterization of the ecology and movement of *Rickettsia* bacteria would

increase understanding of tick-borne disease transmission and facilitate disease prevention (Walker, 2007; Warrell, Cox, Firth, & Török, 2012).

A conventional PCR assay was therefore developed to detect *Rickettsia* species but exclude *R. amblyommii*. Further sequencing is still required to determine species type after use of the novel assay, but testing is conducted on a much smaller number of samples because *R. amblyommii* is not detected.

Methods

Rickettsia samples

All 21 *Rickettsia* DNA samples were acquired through collaborators at the Centers for Disease Control and Prevention in Atlanta, GA. *Rickettsia* samples include *R. amblyommii* GAT30V, *R. parkeri* Portsmouth, *R. sibirica* JC 65, *PSF* JC685 V4, *R. typhi* Wilmington, *R. canadensis* McKiel, *R. conorii* VR613 Malish, *R. rhipicephali* CWPP, *R. montanensis* OSU 85-930, *Rickettsia* 364D, *R. akari* HtCWPP, *R. massiliae* AZT80, *R. honei* HS, *R. slovaca* DCWPP, *R. africae* ETHSF2500, and *R. rickettsii* Sheila Smith. All *Rickettsia* were grown in cell culture and were used at a 1:10 DNA dilution from the extraction concentration unless otherwise noted. Five samples, *R. japonica* TH VR1363, *R. heilongjiangii* VR1524, *R. asiatica* VR1593, *R. raoultii* DnS14, and *R. helvetica* C3 VR1375 were acquired from the same collaborators but were not diluted prior to use. All 20 aforementioned *Rickettsia* species not including *R. amblyommii* will be subsequently referred to as 'target *Rickettsia*'.

Tick samples

Three collections of tick DNA with known or unknown infection status were obtained from laboratories at the Centers for Disease Control and Prevention (CDC) and Rollins School of Public Health in Atlanta, GA including wild *A. americanum* nymphs collected in GA in 2011 by the flagging method with unknown bacterial infection status (AmerU), wild adult *A. americanum* collected by flagging in NY, NC, SC, GA, NC, and NJ from 1998 to 2002 with known *R. amblyommii* infection status (AmerK), and wild-caught adult *Dermacentor occidentalis* collected by flagging in California. Collection sites were depicted pictorially using the software R version 3.0.0 ((Figure 1).

Primer design and selection

Rickettsia sequence data was collected from publicly available sources and from unpublished datasets provided by collaborators in the Rickettsial Zoonoses Branch at the CDC. Eight fragments of the atpE, pyrH, tipA and P34 genes and the intergenic region (IGR) 1577 for *R. amblyommii* were chosen from these datasets and aligned. Primers from the IGR/gene whose lengths were conserved across all available species of *Rickettsia* but differed in *R. amblyommii* was chosen for further testing. The software Geneious Pro 5.5.6 was then used to align homologous gene sequences of this fragment in order to detect polymorphisms (particularly insertion-deletion events (INDELs)) that were unique to *R. amblyommii*. Subsequently, polymorphisms were used to design primer pairs that were chosen based on strict parameters of functionality using the online program Oligo Analyzer 3.1. (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Optimal parameters included 18-30 bases in length, a melting temperature of 50-60°C, approximately 50% GC content and a lack of function-inhibiting secondary structure formation.

Conventional PCR screening and gel electrophoresis

Candidate primer sets were tested with a 1:10 dilution of *R. parkeri* and a 1:1000 dilution of *R. amblyommii* DNA using conventional PCR in a Fisher Scientific PCR Workstation (Labconco Corporation, Kansas City, Missouri, USA) and gel electrophoresis in a Horizontal gel electrophoresis system (Apogee Horizon58, Baltimore, MD, USA). The primer pairs chosen for further testing and assay creation displayed amplification of *R. parkeri* DNA and the absence of *R. amblyommii* DNA amplification at the anticipated amplicon size of 250 bp (Table 2).

Primers tested though PCR screening targeted a fragment of IGR 1577 of *Rickettsia amblyommii* GATV30 and were designed to detect the target *Rickettsia.* All reactions throughout the experimentation process unless otherwise specified contained reagent concentrations of 10 µL Taq polymerase High Fidelity (AccuPrime, Grand Island, NY, USA). 1 µL forward primer (20 µM), 1 µL reverse primer (20 µM), 2 µL DNA sample, and 6 µL deionized water per 20 µL reaction. Positive and negative controls of *R. parkeri* diluted 1:10 and deionized water were used for each reaction. Reactions were amplified in a Eppendorf AG Master Gradient thermocycler (Hamburg, Germany) under the following program: 95°C for 5:30 minutes, 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 1:30 minutes, and 72°C for 10 minutes. Following annealing temperature optimization, the program was revised to run at 55°C in place of the 50°C segment. Reactions were stored at 4°C prior to gel electrophoresis. PCR products were electrophoresed for 30 minutes at 80 volts in a 1% agarose gel with 1.26 µL of ethidium bromide (EtBr) or for 50 minutes at 80 volts for a 2% agarose gel throughout the experimentation process unless otherwise specified. When completed, gels were assessed

using a Bio-Rad Quality 1 4.6.3 Basic computer system (Hercules, CA, USA) under trans UV light. Amplification of DNA samples at 250 bp relative to a standard denoted presence of *Rickettsia* DNA.

CFR performance measures applied to assay design

Assay validation standards and procedures are created and regulated by the Food and Drug Administration. This DNA assay is categorized as a analyte[1]-specific reagent (ASR) under the Code of Federal Regulations (CFR) because it involves 'nucleic acid sequences (a type of analyte)…which, through specific binding…with a substance in a sample, are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens (Food and Drug Administration, Department of Health and Human Services, 2010a).' The qualitative assay is further classified as a class I device because it is not intended for use in blood banking or donor screening for high-risk infectious disease such as HIV or tuberculosis. This classification is exempt from FDA premarket notification requirements (Food and Drug Administration, Department of Health and Human Services, 2010b). A systematic experimentation process guided by the CFR was followed to maximize assay validity. The following CFR-recommended performance measures were addressed when testing the novel assay; reportable range, analytical sensitivity, precision, analytical specificity, accuracy, and reference intervals (*Protocols for determination for limits of detection and limits of quantitation; approved guideline*, 2004a).

Reportable range. *CFR guideline:* Reportable range refers to the range of detectable values of an analyte. *Testing of novel assay:* This measure is not applicable to

[1] Any material or chemical substance subjected to analysis (e.g. antibody, proteins, nucleic acid sequences) (dictionary.com)

qualitative tests and thus exempt in this study (Burd, 2010).

Analytical sensitivity. *CFR guideline:* Analytical sensitivity measures the 'ability of the assay to detect very low concentrations of a given substance in a biological specimen (Burd, 2010).' The limit of detection (LOD) is a consistently used and valid measure for analytic sensitivity that indicates the lowest detectable concentration of analyte in a specimen. Lower LODs indicate increased sensitivity. To determine the LOD, empirical testing methods using serial dilutions of samples with known concentrations of the target substance is optimal (Protocols for determination for limits of detection and limits of quantitation; approved guidelin*e*, 2004b). CFR recommends that at least 12 samples be tested at the LOD for sufficient validity. This measure also provides information on the concentration of positive control to be used in standardized testing. *Testing of novel assay:* To determine a detection range for *Rickettsia* species, 10-fold dilutions of *R. parkeri* DNA from 1:100 to 1:10000 were tested for amplification at the 250 bp amplicon. Serial dilutions of *R. parkeri* at 1:2000, 1:4000, 1:6000, 1:8000 were then used to specify the LOD. DNA from fourteen AmerK were then inoculated with *R. parkeri* at the LOD and tested for amplification at the 250 bp amplicon.

Precision. *CFR guideline:* Replicates conducted throughout an experimentation process increases precision. Using a positive control within each run (PCR test followed by gel electrophoresis test) also increases precision. The Clinical and Laboratory Standards Institute (CLSI) recommends performing experiments over at least a 20-day working period to ensure long-term functionality of operating conditions (Evaluation of precision performance of quantitative measurement methods. Approved guideline, 2nd ed., 2004). *Testing of novel assay:* Replicates ranged from 5 to 50 samples per experiment

depending on sample availability. *Rickettsia parkeri* DNA was used as a positive control for each test and the experiment was conducted over a 12-month period.

Analytical specificity. *CFR guideline:* Analytical specificity denotes the ability of the assay to detect only the intended target such that this target is not affected by cross-reactivity of specimen or by interfering substances (Burd, 2010). *Testing of novel assay*: Analytical specificity was tested through three steps. First, cross-reactivity was addressed by identifying organisms with nucleotide sequences homologous to each primer using the Basic Local Alignment Search Tool (BLAST) (http://www .ncbi.nlm.nih.gov) to search the online genetic sequence database, GenBank (maintained by the National Center for Biotechnology Information (NCBI)). Second, annealing temperature optimization was conducted to reduce amplification of non-target DNA while maintaining detection of *Rickettsia* DNA. This was accomplished through testing of concentration of 1:100 and 1:1000 of *A. americanum* and *R. parkeri*, across a gradient of primer annealing temperatures (48-58°C). Thirdly, AmerK were tested for the absence of *R. amblyommii* amplification at the 250 bp amplicon. The optimized annealing temperature was used in all reactions. Samples showing potential amplification of *Rickettsia* at the target 250 bp region were further tested using a 2% agarose gel solution. The 2% gel was used to increase clarity of results. Additionally, 108 samples of AmerU were tested for the amplification of *Rickettsia* at the 250 bp region.

Accuracy. *CFR guideline:* Accuracy tests measure the assay's ability to detect a true value or known substance. *Testing of novel assay:* This experiment employed the recovery study design in which *Rickettsia* DNA was either known to be present in or added to tick DNA samples. Subsequently, the primer set was tested for amplification of

20 *Rickettsia* DNA samples. Further testing for amplification at the 250 bp amplicon was conducted with two *D. occidentalis* DNA samples of confirmed natural *Rickettsia* 364D infection along with three samples of wild-caught *D. occidentalis* with confirmed natural *R. rhipicephali* CWPP infection.

Reference interval. *CFR guideline:* This measure is used for clinical testing to determine normal and abnormal ranges of values in a diagnostic test. *Testing of novel assay:* Since the assay is a qualitative ASR rather than a diagnostic test, experimentation to determine an interval was not performed and the reference interval can be denoted as *Rickettsia* 'detected' or 'not detected' (Burd, 2010).

Controls. *CFR guideline:* The Clinical Laboratory Improvement Amendments of 1988 state that laboratories can determine their own protocols for control samples (Health Care Financing Administration, 2009). A typical qualitative study uses positive and negative controls and should be included in each run. *Testing of novel assay:* The positive control in this experiment, a 1:10 dilution of *R. parkeri* DNA, served as a reference for desired amplification of all *Rickettsia* species at the 250 bp ladder indicator. The negative control, deionized water, was used to determine contamination of reagents and included in each PCR run.

Results

RP819F and RP819R were the chosen primers to create the assay primer set RP819-FR, which was used for all final testing (Table 2).

CFR performance measures applied to assay results

Analytical sensitivity. Using serial dilutions, the LOD of *R. parkeri* was

determined to be 1:6000 from the concentration of the cell culture DNA extraction. Fourteen AmerK were then inoculated with *R. parkeri* at the LOD concentration 1:6000 to mimic natural infection in wild-caught ticks. 100% of samples displayed amplification of DNA bands at the 250 bp amplicon (Figure 2).

Analytical specificity. In terms of cross-reactivity of similar Rickettsiales, the RP819F and RP819R nucleotide sequences were 100% homologous with the bacterial symbionts *Midichloria* and *Wolbachia*. Additionally, *Coxiella* bacterial species were not homologous with either nucleotide sequence. Twenty *Rickettsia* whose alignment indicated compatibility with the primer pair but whose in-vitro detection could not be demonstrated due to lack of samples include: *R. canadensis* CA410, *R. australis* Cutlack, *R. rickettsii* Hauke, *R. rickettsii* Iowa, *R. rickettsii* Columbia, *R. rickettsii* Hino, *R. rickettsii* Brazil, *R. rickettsii* Hlp#2, *R. peacockii*, *R. felis*, *R. typhi* TH1527, *R. typhi* B9991CWPP, *R. prowazekii* BuV67CWPP, *R. prowazekii* Chernikova, *R. prowazekii* Dachau, *R. prowazekii* GvV250, *R. prowazekii* GW257, *R. prowazekii* Katsinyian, *R. prowazekii* Madrid E, *R. prowazekii* RP22. Furthermore, in order to reduce amplification of interfering substances, the assay annealing temperature was optimized. An annealing temperature of 55.8°C in the thermocycler program resulted in decreased spurious bands of DNA of AmerK in addition to amplification of *R. parkeri* DNA (Figure 3).

Additionally, 50 AmerK were tested for amplification of *R. amblyommii* DNA (Figure 4a). Seven of 50 samples (14%) displayed spurious bands at the 250 bp mark, indicating potential *R. amblyommii* amplification. When samples were run in a 2% gel for increased resolution, 0% (0/7) displayed amplification, confirming absence of *R. amblyommii* DNA in all 50 samples (Figure 4b). In order to determine the infection

prevalence of target *Rickettsia*, 108 AmerU were tested for DNA amplification at the 250 bp amplicon. Zero percent (0/108) were positive for *Rickettsia* species, indicating an absence of target *Rickettsia* in this sample population (not shown).

Accuracy. Using RP819-FR, 100% of target *Rickettsia* DNA was amplified at 250 bp (Figure 5). Additionally, tick samples with known natural *Rickettsia* infection were tested in order to determine accuracy of the DNA assay: 100% (2/2) of wild-caught *D. occidentalis* naturally infected with *Rickettsia* 364D displayed DNA amplification and 100% (3/3) of wild-caught *D. occidentalis* with known natural *R. rhipicephali* infection were detected (Figure 6). Results indicate high accuracy of the assay in detecting *Rickettsia* species naturally infecting wild-caught ixodid ticks.

Discussion

A novel PCR-based assay is described here for the detection of spotted fever group *Rickettsia* species excluding *R. amblyommii*. Assay validity was maximized by following CFR recommendations to evaluate the following performance measures; reportable range, analytical sensitivity, precision, analytical specificity, accuracy, and reference intervals.

Discussion of the CFR performance measures applied to the assay

Analytical sensitivity. A dilution series was tested to determine the LOD of *Rickettsia* DNA. Comparison data on detectable concentrations of *Rickettsia* is unavailable because most studies determine an absolute quantitation of DNA copy numbers. This is done by constructing a plasmid, determining DNA concentration, then conducting serial dilutions (Baldridge et al., 2010; Eremeeva, Dasch, & Silverman, 2003). Such testing is

needed to determine absolute quantitation of detectable *Rickettsia* DNA but was beyond the resources of the laboratory during the study.

Regardless of testing methodology, detection of *Rickettsia* spiked into AmerK at the LOD indicates the assay's ability to detect target *Rickettsia* in the presence of background isolates such as tick DNA or DNA from other bacteria found naturally in ticks.

Analytical specificity. Results of gene alignment indicated that 20 additional *Rickettsia* nucleotide sequences were compatible with the primer pair. Further laboratory testing was not feasible but future research potential exists with these species. *Midichloria* and *Wolbachia* were the only non-target bacteria known to infect *A. americanum* that had high identity with both nucleotide sequences RP819F and RP819R. This was unsurprising given that both genera are members of the Rickettsiales. *Midichloria mitochondrii* and *Wolbachia* are recently designated endosymbionts of *Ixodes ricinus* and *A. americanum,* respectively, with various novel effects and consequences on tick biology (Plantard et al., 2012; Rounds et al., 2012). Detection of these organisms provides additional research utility to the assay (Sassera et al., 2008). The fact that *Coxiella* sp. were not homologous with either primer is important due to a high abundance (100%, 89%) detected in *A. americanum* samples (Heise, Elshahed, & Little, 2010; Plantard et al., 2012).

When determining *Rickettsia* prevalence in wild ticks, 0% of samples were positive. This is an expected result due to the small proportion of non-*R. amblyommii* *Rickettsia* commonly found in *A. americanum*. According to Smith et al. (2010), *A. americanum* infection prevalence of *Rickettsia* species other than *R. amblyommii* ranges

from 4.4% to 30.2%. Thus, a sample size of 67 would result in 1- 9 samples positive for *Rickettsia*. With a sample size of 217in this study, a range of 4 to 30 positive samples is projected. Despite expected values, bacterial infection prevalence is highly variable between sites and within populations. For example, the site prevalence of *B. lonestari* found in *A. americanum* ranged from 0-12.2 with an overall site prevalence of 3.5 (n=29) (Mixson et al., 2006). In sum, zero positives for *Rickettsia* in 217 samples is a reasonable result.

Accuracy. In terms of assay accuracy, there was a 100% detection rate of *Rickettsia* in ticks with known natural infection. Band strength was inconsistent in terms of DNA amplification due to natural variation in the number of bacteria present in wild-caught ticks.

Conclusion

A novel assay has been validated for sensitivity, specificity, and accuracy in detecting *Rickettsia* species other than *R. amblyommii* in ticks. This eliminates the need for sequencing to differentiate target *Rickettsia* from *R. amblyommii;* allowing resources to be focused on less prevalent and abundant *Rickettsia* that cause more severe clinical disease.

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Tables

Table 1: Prevalence of *R. amblyommii* in comparison to the prevalence of other bacterial species in *A. americanum*

Studies were aggregated to show that the detection prevalence of *R. amblyommii* (40-90%) is high in comparison to the prevalence of other bacterial species (1%to 4.5%) in *A. americanum* ticks.

Bacterial species	*Bacterial detection prevalence (%)*	*R. amblyommii detection prevalence (%)*	*No. of A. americanum sampled*	*Reference*
E. chaffensis E. ewingii B. lonestari	4.7% 3.5% 2.5%	41%	2038	Mixon et al. 2006
R. rickettsii	<1%	44%	25	Apperson 2008
E. chaffensis B. lonestari	1.6% 0.4%	54.8%	1590	Smith 2010
R. rickettsii	0.46%	90%	870	Berrada et al. 2011

Table 2. Nucleotide sequences created as potential candidates for the assay

Seven nucleotide sequences were designed from polymorphisms that were unique to *R. amblyommii* in the intergenic region 1577 (IGR) of *R. amblyommii* GATV30 DNA. Sequences used for the final primer pair RP819-FR are highlighted in gray.

Primer designation	**Nucleotide sequence 5' → 3'**	**Direction**	**Base pair Length**	**%GC content**	**T_m (°C)**
RP 330	CTATAACCGAGATAAATAMAAAAAT	F	25	22	45.8-47.3
RP 329	GAGTACCGATCCAYGTAGTAT	R	21	45.2	50.7-53
RP 329	GCAGGATAACGTGAAAG	R2	17	47.1	47.7
RP 819	**CACAAGCGCGAGGAGTAA**	**F**	**18**	**55.6**	**55**
RP 819	**TGCTAGCTCCRGATCTTCT**	**R**	**19**	**50**	**52.4-55.2**

RP 819	CTG CAC TTA TTA ATG CAC ARR YG	F2	23	41.3	51-56.8
RP 819	GGC AGC AGC CGT GAA ATT AAA	R2	21	47.6	56.6

Figures

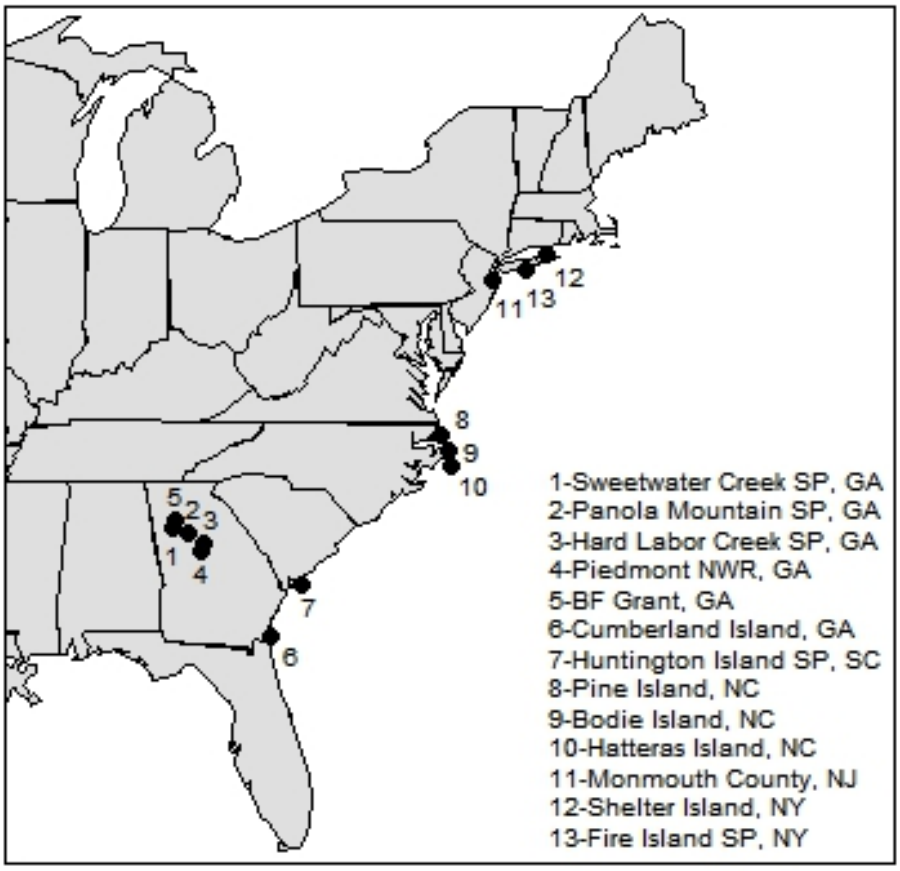

Figure 1: Distribution of tick samples across the United States

Amblyomma americanum and D. *occidentalis* were collected from sampling sites across the U.S. Adult *A. americanum* nymphs were collected by flagging in 2011 [sites 1,2] and bacterial infection status was unknown. Samples of adult *A. americanum* were also collected by flagging from 1998 to 2002 [sites 3-13] *R. amblyommii* infection status was known at the time of testing in this study. Additionally, adult *Dermacentor occidentalis* were collected by flagging in California (not shown).

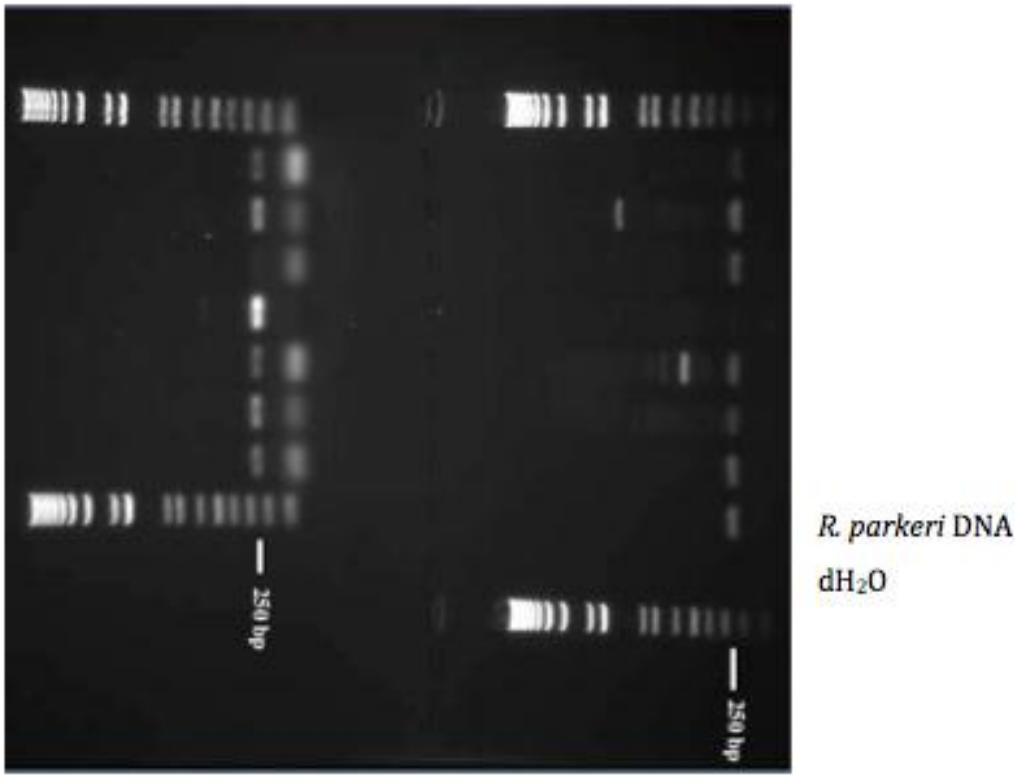

Figure 2: Assay sensitivity: Detection of *R. parkeri* inoculated into wild *A. americanum* at the LOD concentration of 1:6000

Fourteen *R. amblyommii* positive *A. americanum* were inoculated *with R. parkeri* DNA in a 1:6000 concentration to mimic natural infection in wild-caught ticks. One hundred percent of samples run in a 1% gel showed bands at the 250 bp amplicon when tested with primers RP819-FR. Results indicate an LOD of 1:6000 and the assay's ability to detect target *Rickettsia* in the presence of background isolates such as tick DNA or DNA from other bacteria found naturally in ticks.

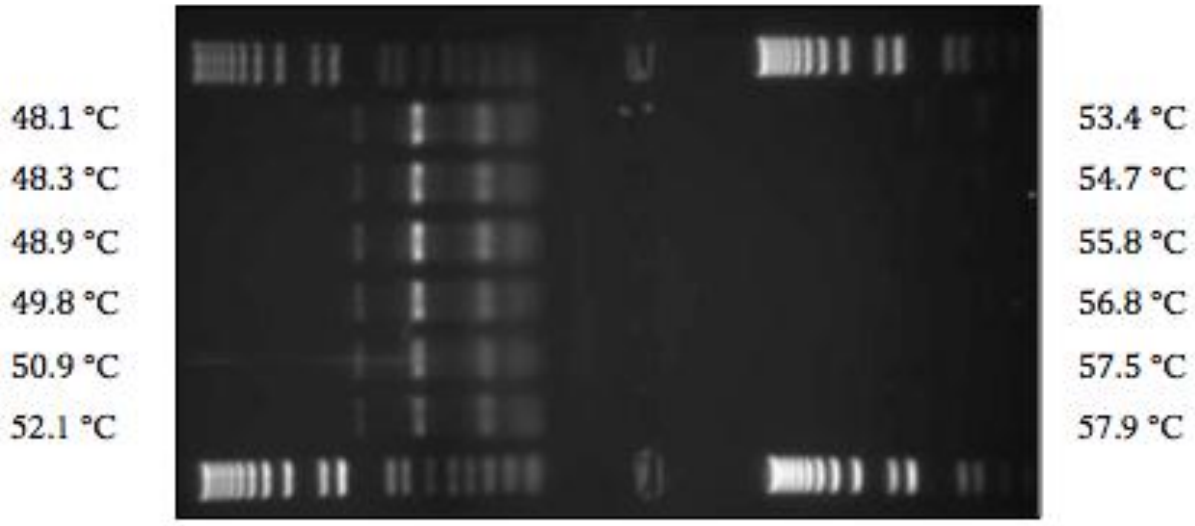

Figure 3: Assay specificity: annealing temperature optimization for *A. americanum* DNA

A 1% gel was conducted on an annealing temperature gradient of 48-58°C for wild-caught *A. americanum* DNA with the primer pair RP819-FR. No amplification was seen at 55.8°C, 56.8°C, 57.5°C and 57.9°C. The temperature 55.8°C was chosen as the optimal annealing temperature and used for all final tests. Assay specificity is high due to reduced amplification of non-target DNA while maintaining detection of *Rickettsia* DNA.

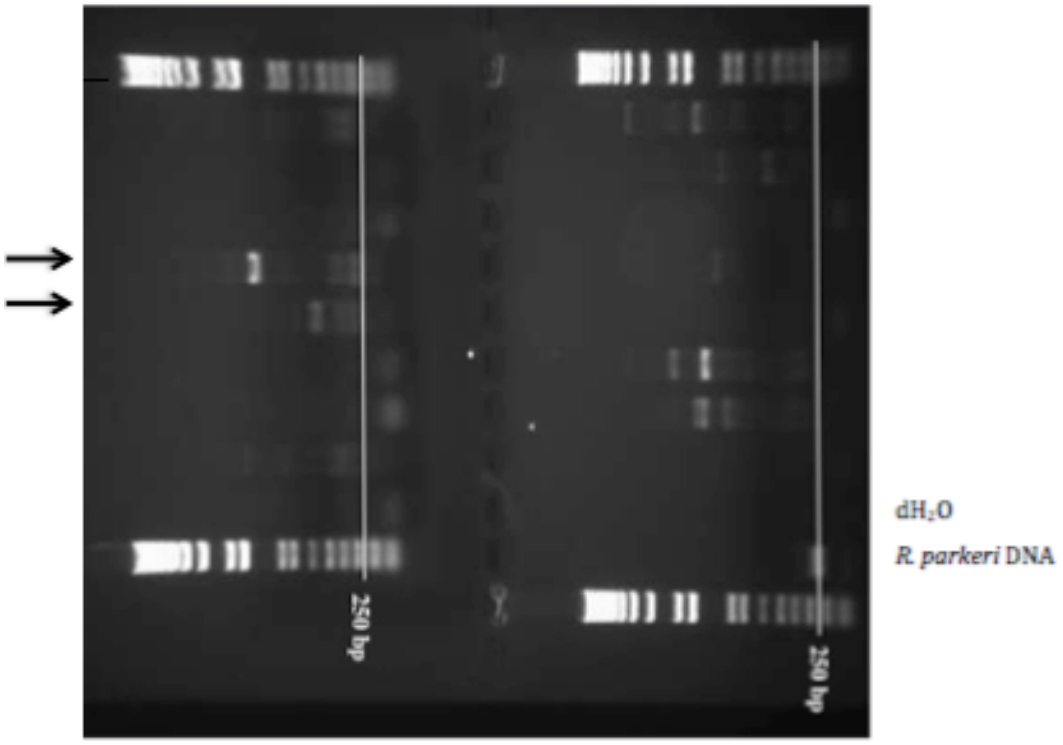

Figure 4a: Assay specificity: Spurious amplification of bands in *A. americanum* with known *R. amblyommii* infection

A 1% gel was conducted with RP819-FR and 50 DNA samples (19 of 50 shown) from wild caught *A. americanum* with known *R. amblyommii* infection. Seven of 50 samples (2 shown here) displayed spurious amplification (indicated by arrows) at the 250bp amplicon size indicating potential *R. amblyommii* infection.

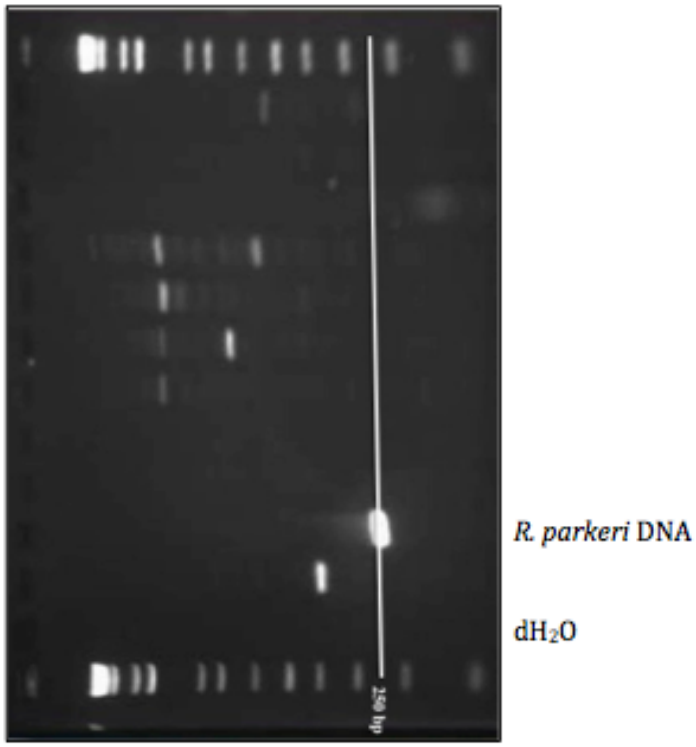

Figure 4b: Assay specificity: High resolution testing of ambiguous bands for the detection of *R. amblyommii*

Twelve of the 50 *A. americanum* samples with known *R. amblyommii* infection that displayed ambiguous amplification of bands were re-tested using a high-resolution 2% gel with the primer set RP819-FR. No DNA bands were detected in the 250 bp region denoting the absence of *R. amblyommii* species.

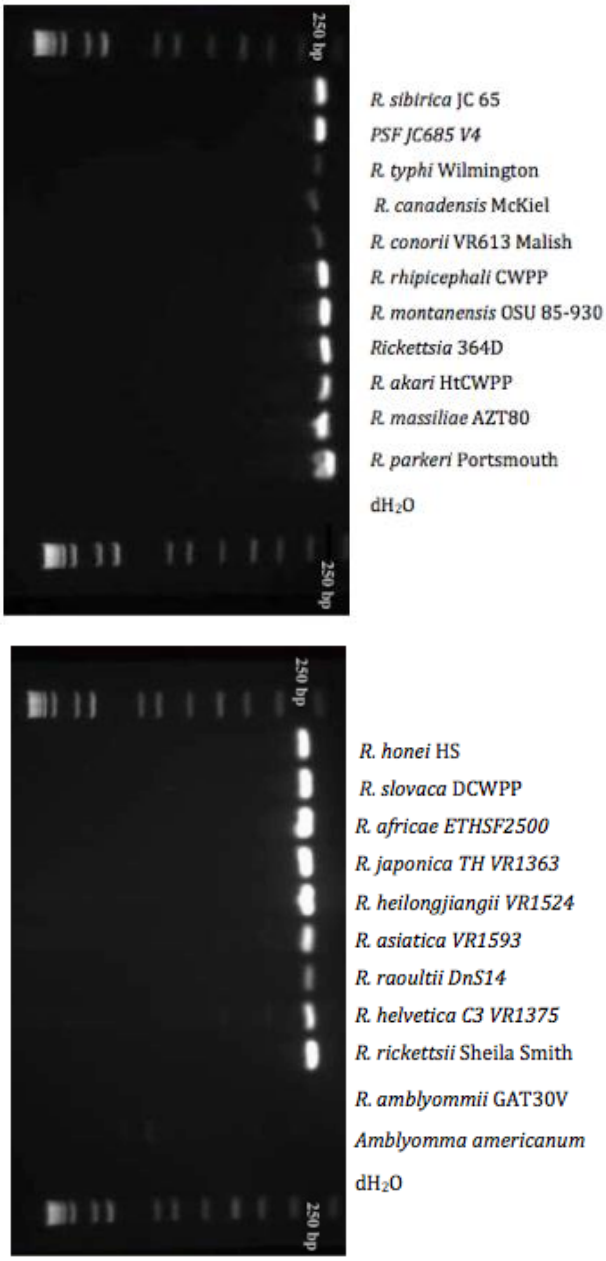

Figure 5: Assay accuracy: detection of 20 target *Rickettsia* species and the absence of *R. amblyommii* and *A. americanum* detection

A 2% gel was conducted with RP819-FR primers, and DNA from 20 target *Rickettsia*, *R. amblyommii*, *A. americanum*, and *R. parkeri* (positive control) along with a sample of dH_2O (negative control). Amplification was seen at 250 bp for all target *Rickettsia* species while *R. amblyommii* and A. *americanum* DNA were not detected. This indicates accuracy of the assay in detecting target *Rickettsia* while not detecting *R. amblyommii* or tick DNA.

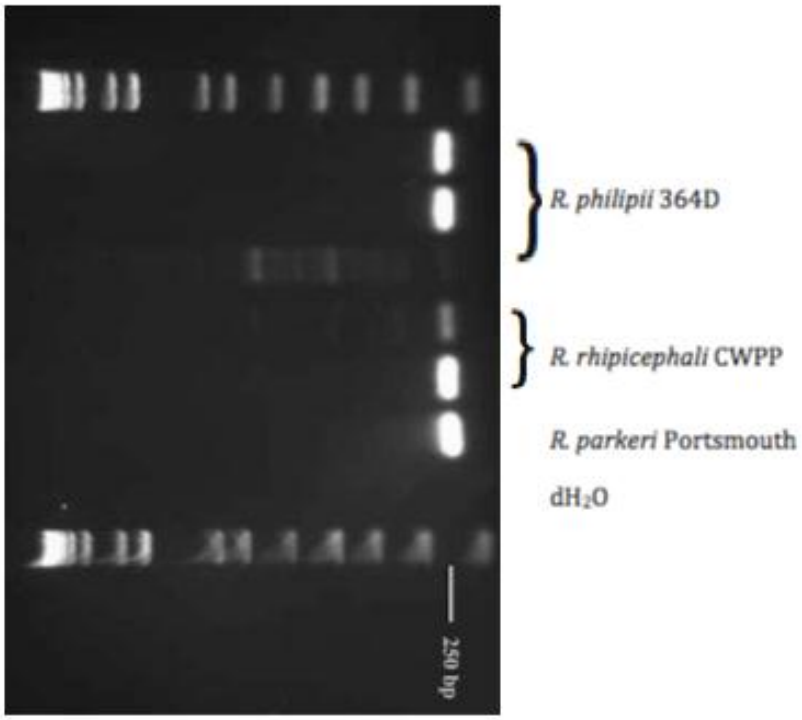

Figure 6: Assay accuracy: detection of natural *Rickettsia* infection in *D. occidentalis*

Three wild-caught *D. occidentalis* of confirmed *R. rhipicephali* CWPP natural infection and 2 wild-caught *D. occidentalis* of confirmed *Rickettsia* 364D natural infection were tested with RP819-FR in a 2% gel. One hundred percent amplification indicated the assay's accuracy in detecting *Rickettsia* species.

Appendix

Non-print references

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