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Detection of Bacterial Agents in *Amblyomma americanum* (Acari: Ixodidae) From Georgia, USA, and the Use of a Multiplex Assay to Differentiate *Ehrlichia chaffeensis* and *Ehrlichia ewingii*

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Abstract

Amblyomma americanum, the lone star tick, is the most common and most aggressive human biting tick in the Southeastern United States. It is known to transmit the agents of human ehrlichioses, *Ehrlichia chaffeensis* and *Ehrlichia ewingii.* In addition, it carries agents of unspecified pathogenicity to humans, including *Rickettsia amblyommii*, *Borrelia lonestari*, and the newly emerging Panola Mountain *Ehrlichia* (PME). Surveillance of these ticks for recognized or emerging pathogens is necessary for assessing the risk of human infection. From 2005 to 2009, we surveyed *A. americanum* ticks from four locations in the state of Georgia. Ticks (1,183 adults, 2,954 nymphs, and 99 larval batches) were tested using a multiplex real-time polymerase chain reaction (PCR) assay designed to detect and discriminate DNA from *Rickettsia* spp., *E. chaffeensis*, and *E. ewingii.* This assay was capable of detecting as few as 10 gene copies of the aforementioned agents. Ticks were also tested for PME and *B. lonestari* by nested PCR. The prevalence of infection ranged from 0 to 2.5% for *E. chaffeensis*, 0 to 3.9% for *E. ewingii*, 0 to 2.2% for PME, 17 to 83.1% for *R. amblyommii*, and 0 to 3.1% for *B. lonestari*. There were 46 (4.1%) individual adults positive for two agents, and two females that were each positive for three agents. Two larval batches were positive for both *B. lonestari* and *R. amblyommii*, indicating the potential for transovarial transmission of both agents from a single female. Although infrequent in occurrence, the dynamics of coinfections in individual ticks should be explored further, given the potential implications for differential diagnosis and severity of human illness.

Keywords

Amblyomma americanum; multiplex; *Ehrlichia*; *Rickettsia*; *Borrelia*

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Amblyomma americanum, the lone star tick, is distributed widely from the Southeastern to Midwestern United States, with recent range expansion into the Northeast (Mixson et al. 2006). It is an aggressive, human-biting tick, and carries the known pathogens *Ehrlichia chaffeensis* and *Ehrlichia ewingii,* the causative agents of human monocytic ehrlichiosis and human granulocytic ehrlichiosis, respectively. In addition, lone star ticks carry agents of undetermined relevance to human health (*Rickettsia amblyommii,* Panola Mountain *Ehrlichia* (PME), and *Borrelia lonestari*). These ticks have also been implicated in the transmission of *Rickettsia parkeri* (Cohen et al. 2009) and *Rickettsia rickettsii* (Breitschwerdt et al. 2011, Berrada et al. 2011), both of which cause spotted fever rickettsioses in humans. The incidences of reported human cases of rickettsioses and ehrlichioses have both quadrupled over the past decade. In comparison, reports of Lyme disease, transmitted by the black-legged tick, *Ixodes scapularis,* have remained relatively steady (Centers for Disease Control and Prevention, http://www.cdc.gov/lyme/stats). Surveillance of questing lone star ticks should help in understanding what role the dynamics and changing distribution of these vectors play in the increased incidence of human infections.

The two ehrlichial agents of known public health significance, *E. chaffeensis* and *E. ewingii,* can sometimes be found within a single tick (Mixson et al. 2006, Schulze et al. 2011). Current procedures for the identification of these agents in ticks require multistep assays to distinguish between the two, which can lead to delayed detection and diagnosis, increasing the risk of disease progression in people and increased cost of testing. As such, a faster, single-step procedure is warranted. Here we present a single-step multiplex assay that distinguishes between these two pathogens, while also screening for *Rickettsia* spp.

Materials and Methods

Tick Collection

Questing ticks were collected from vegetation by flagging at four locations in the state of Georgia: Panola Mountain State Park (SP), Clybel Wildlife Management Area (WMA), Rum Creek WMA, and Ossabaw Island Heritage Preserve. Panola Mountain SP, Clybel WMA, and Rum Creek WMA were sampled in 2008. The Ossabaw Island field site was added and sampled in 2009. Panola Mountain was a previously established field site used for the study of the newly discovered PME. Previously untested ticks collected from this site in 2005 were included for comparison of tick prevalence data. All tick collections occurred between April and September each year. An interactive map of the collection sites can be found at http://www.arcgis.com/home/webmap/viewer.html?webmap=b35a8188c9ad49d4b426ea6f0aff4676. Larval batches were collected from the flag with masking tape, immediately after attaching to the flag. Ticks were collected under scientific collections permit #21869, issued by the Georgia Department of Natural Resources Wildlife Resources Division.

DNA Extraction

From 2005 to 2007, tick DNA was extracted, as previously described, using Isoquick kits (ORCA Research, Inc., Bothell, WA) (Levin and Fish 2000). After the discontinuation of

Isoquick reagents in 2007, ticks were extracted using the Qiagen DNEasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA), according to manufacturer's instructions. Adult ticks were extracted individually, nymphs either individually or in pools of <10 ticks, and larvae in pools of 20–100. Pools of larvae were taken from individual larval batches removed from flags with tape, so each tested larval sample is treated as the progeny of a single female tick.

Multiplex Polymerase Chain Reaction Assay

Ticks were tested using a multiplex real-time polymerase chain reaction (PCR) assay for the simultaneous detection of three *A. americanum*-associated bacteria: *Rickettsia* spp., *E. chaffeensis,* and *E. ewingii.* This assay combines genus-specific primers with species (*Ehrlichia* spp.)- or genus (*Rickettsia* spp.)-specific dual-labeled hydrolysis probes in a single tube and is performed under standardized thermocycling conditions. Primer sequences specific for a 114-bp region of the rickettsial 17-kDa antigen gene, and probe, are as previously described (Loftis et al. 2006). The ehrlichial forward primer and *E. chaffeensis* probe, targeting 16s rRNA, were also taken from a previously described assay (Loftis et al. 2003). The ehrlichial reverse primer from the aforementioned assay was replaced with one designed to be less specific, and is identified as Ece16S-99 (5′-CACCCGTCTGCCACT AAC AAY TAT-3′). These primers amplify an 82-bp fragment of DNA for both ehrlichial agents, while probes are species-specific with no cross-reactivity between species. In addition, a probe for *E. ewingii* was included. This probe sequence is identified as EEW16S HEX (5′-TC GAA CGA ACA AT"T" CCT AAA TAG TCT CTG ACT ATT-3′) and labeled at the 5′ and 3′ ends with hexachloro-6-carboxy-fluorescein (HEX) and BHQ2, respectively. Oligonucleotides were synthesized by the Biotechnology Core Facility Branch at CDC. Primer and probe sequences, and concentrations are summarized in Table 1.

The multiplex assay was performed using Brilliant qPCR Core Reagent Kit (Agilent Technologies, LA Jolla, CA). Concentrations of the reagents from the previously described assays were modified to achieve optimum results when run as a multiplex reaction. Each 20-µl reaction contained 0.2 µl of SureStart *Taq* (5 U/µl), 2.0 µl of 10× core PCR buffer, 2.0 µl of $MgCl_2$ (50 mM), 0.8 µl of dNTP mix (20 mM), 0.2 µl of each primer and probe, 11.6 µl of PCR-grade water, and 2 µl of sample DNA. All multiplex PCR assays were run on an I-Cycler (Bio-Rad Laboratories, Hercules, CA) using the following program: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing–extension at 57°C for 1 min. Cloned DNA from positive tick samples were used as positive controls and distilled water was used for negative controls. All samples were run in duplicate. Samples with a fluorescence level exceeding the threshold before cycle 40 were considered as positive. In addition to the multiplex, ticks were tested by previously described nested PCR assays for *B. lonestari* (Barbour et al. 1996) and PME (Loftis et al. 2008).

Ten *Rickettsia* spp.-positive and 10% of the positive samples for all other agents were randomly selected for sequencing to confirm identity. Positive PCR products were purified with QIAquick-Spin PCR columns (QIAGEN) or the Wizard SV gel and PCR clean-up kit (Promega, Madison, WI). Sequencing reactions were performed using primers from the multiplex assay (for *Rickettsia* spp., *E. ewingii,* and *E. chaffeensis*), or with secondary primers for the nested assays. Amplicons were sequenced using an ABI PRISM 3.0 BigDye

Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA.) and analyzed using an Applied Biosystem model 3100 automated DNA sequencer. Nucleotide sequences were edited and assembled with the Pregap and Gap programs of the STADEN sequence analysis package (Staden, Cambridge, UK) (Staden et al. 2003), or with the DNASTAR Lasergene 8 software package. Nucleotide comparisons were made using the Netblast function of the GCG package (Wisconsin Package version 10.3, Accelrys, San Diego, CA), or with the National Center for Biotechnology Information (NCBI) Basic Local Alignment Sequence Tool (BLAST).

Statistical Analysis

Minimum infection prevalences (MIPs) given for pooled nymphs are calculated by assuming one infected tick per pool. Predicted incidences of coinfection (not shown in results) were calculated according to the following formula:

$$\Pr(A \text{ and } B) = \Pr(A) \times \Pr(B)$$

where the Pr (A and B) is the predicted incidence (probability) of coinfection with agents A and B, Pr(A) is the overall prevalence of agent A, and Pr(B) is the overall prevalence of agent B (Whitlock and Schluter 2009).

The predicted number of ticks coinfected with agents a and b (n_{a+b}) was calculated by:

$$n_{\mathrm{a+b}} = \Pr(A \text{ and } B) \times n$$

where “n” is the total number of ticks tested. Differences between the number of observed and expected coinfections were assessed by χ^2 or Fisher exact analysis. χ^2 analysis was also used to compare prevalence results between field sites and to assess potential differences in prevalence data from 2005 and 2008 at Panola Mountain SP.

Results

Multiplex Assay

The multiplex PCR assay was successful in differentiating *E. chaffeensis* and *E. ewingii,* with a sensitivity of 10 gene copies for both agents. *Anaplasma phagocytophilum,* PME, and *Neoehrlichia lotoris* DNA were not detected by the ehrlichial primers and probes. The Rickettsial primers amplified both spotted fever group *Rickettsia* (SFGR) and typhus group *Rickettsia* (TGR), including *R. rickettsii, R. amblyommii, R. parkeri, Candidatus Rickettsia tuberculatum, Rickettsia typhi, and Rickettsia prowazekii.* It is expected the assay will amplify all SFGR and TGR, but not the ancestral group of *Rickettsia canadensis* and *Rickettsia bellii,* due to a 3-bp mismatch in the forward primer, and a 4-bp mismatch in the probe. The sensitivity is 10 gene copies, as previously described (Loftis et al. 2006). The sensitivity of this assay for any agent was not diminished by a 100-fold excess of either of the other two agents.

PCR Results

From 2005 to 2009, 4,236 ticks were collected and tested from all four locations, including 1,183 adults, 2,954 nymphs, and 99 larval batches. The prevalence of infection of each life stage from each location is shown in Table 2, with MIP given for pooled nymphs. All agents were detected at three of the field sites; PME was not identified in ticks from Clybel WMA, possibly due to insufficient sample size. Otherwise, the prevalence of infection ranged from 0 to 2.5% for *E. chaffeensis,* 0 to 3.9% for *E. ewingii,* 0 to 2.2% for PME, 17 to 83.1% for *Rickettsia spp.,* and 0 to 3.1% for *B. lonestari.* Prevalences of infection did not differ significantly among the different field sites ($P < 0.05$). There were also no differences noted between prevalences of agents in ticks collected from Panola Mountain in 2005 and those collected in 2008 ($P < 0.05$). All 10 *Rickettsia*-positive samples that were sequenced were identified as *R. amblyommii,* and therefore, *Rickettsia* spp.-positive samples are hereafter referred to as *R. amblyommii.*

Coinfections

Pooled nymphs were excluded from the analysis of coinfections. Forty-eight adult ticks were positive for two or more agents: 46 dual infections and two triple infections. This accounts for 6.5% of the total number of infected adults (48/737) and 4.1% of the total number of adult ticks tested (48/1,183). Dual infections are represented in Table 3, and the number of single infections versus the number of coinfections for each agent can be seen in Table 4. The two ticks carrying three agents were both collected from Ossabaw Island. One was positive for *R. amblyommii, B. lonestari, and E. chaffeensis;* the other had *R. amblyommii, E. ewingii,* and PME. Dual-infected ticks were collected from three locations: Panola Mountain SP, Ossabaw Island, and Rum Creek WMA. Coinfections in ticks from Clybel likely went undetected due to the small number of ticks collected at this site. The majority of coinfections occurred with *R. amblyommii* and another agent (45/48). In addition to these 48 adults, 2 of the 99 larval pools from Panola Mountain were coinfected with *B. lonestari* and *R. amblyommii.* There was no significant difference between the observed frequency of coinfections and that which would be expected given the independent mixing of agents, indicating no positive or negative correlations between agents. The detection of the two triple-infected ticks did, however, show significant deviation from the expected values ($P < 0.05$).

Discussion

Here we present a multiplex assay that detects both SFGR and TGR, as well as detecting and discriminating between two common ehrlichial pathogens, *E. ewingii* and *E. chaffeensis,* eliminating the need for additional testing and/or sequencing steps. The sensitivity and specificity of the assay were compared with, and found similar to, those of conventional previously published single-target assays (Roux et al. 1996, Gusa et al. 2001, Loftis et al. 2003). Although this assay has the capacity to be used for quantification purposes, the main focus in the development was for differentiation of *E. ewingii* and *E. chaffeensis.* For our purposes, it is used primarily for the screening of large numbers of wild ticks for the specified agents, and not for enumeration of bacterial loads.

The prevalences of infection with each agent in ticks were similar across all sites and fell within previously reported ranges (Steiert and Gilfoy 2002, Mixson et al. 2004, Cohen et al. 2010). For nymphal pools, the MIPs given for the ehrlichial agents likely represent the true prevalence, given their relative rarity in reported tick populations from this and other regions (Steiert and Gilfoy 2002, Mixson et al. 2004, Cohen et al. 2010). For *R. amblyommii,* 519 of 598 (87%) nymphal pools were positive, with a MIP of 17.6%. Previous reports of this agent, in addition to prevalences found in adults, vary widely depending on location, but estimates in adults and larvae from our study areas indicate that the MIP underestimates the true prevalence of the agent in questing nymphs (Schulze et al. 2005, Mixson et al. 2006, Moncayo et al. 2010). Although this multiplex assay is capable of detecting both SFGR and TGR, it is unable to distinguish between different species of *Rickettsia.* With the high prevalence of *R. amblyommii* reported in lone star ticks, it is not feasible to sequence all positive samples. A restriction fragment length polymorphism assay that distinguishes different species of SFGR is available (Eremeeva et al. 1994). Testing of other populations of ticks, using the restriction fragment length polymorphism assay, found *R. amblyommii* to be the only SFGR infecting *A. americanum* from South Georgia (G.E.Z., unpublished data). Based on our experience, published literature, and selective sequencing of samples in this study, we are confident that the majority, if not all, of the *Rickettsia*-positive samples presented here are *R. amblyommii.* Still, this does not eliminate the possibility that other species of *Rickettsia* may be harbored and transmitted by the lone star tick, and that these other SFGR may go unnoticed and underreported. In addition, the restriction fragment-length polymorphism assay, although less expensive than sequencing, is time-consuming and laborious. A single-step assay that either excludes *R. amblyommii* or distinguishes it from other SFGR would be beneficial to determining the extent to which *A. americanum* may be contributing to the increasing numbers of SFGR cases reported in the Southeastern United States.

Forty-eight (4.1%) adult ticks were infected with more than one bacterium, with the majority of coinfections involving *R. amblyommii,* the most prevalent agent. There were only two occurrences where the prevalence of coinfection was higher than expected, and both of these were single ticks with triple infections. In our opinion, any conclusions based on finding such coinfections in a single tick would be of limited value, at best. It is likely that the statistical significance of these coinfections is an artifact of sample size, especially because there was no indication of positive association between agents in ticks with double infections. Given the number of ticks tested, the expected numbers for the triple infections with *R. amblyommii/E. chaffeensis/B. lonestari* and *R. amblyommii/E. ewingii*/PME were 0.32 and 0.18, respectively. The two larval batches infected with *B. lonestari* and *R. amblyommii* indicate the transovarial transmission of both of these agents in the lone star tick, which has been previously reported (Stromdahl et al. 2008).

A. americanum are indiscriminate feeders and use many host species, increasing the possibility of coming into contact with multiple pathogens. Also, their main host, white-tailed deer, is fed upon by multiple tick species and, consequently, is a potential reservoir for many different tick-borne diseases. The presence of more than one human pathogen in a single tick increases the risk of human exposure to multiple agents in a single bite. Among the five tick-borne agents detected in our study, *R. amblyommii* and *B. lonestari* are not yet

proven to cause illness in people, and there has been a single case report of PME in a human (Reeves et al. 2008). *E. chaffeensis* and *E. ewingii* are, however, well-known human pathogens. In the case of *E. chaffeensis,* coinfections with other agents occurred as often as single infections. For ticks infected with *E. ewingii,* coinfections occurred twice as often as single infections. These data indicate that patients diagnosed with *A. americanum*-borne ehrlichial infections can reasonably be expected to have been exposed to another agent, most often *R. amblyommii.* Although studies have yet to be conducted with *A. americanum,* previous laboratory experiments with *Ix. scapularis* have indicated that transmission of two pathogens by coinfected ticks can occur independently of each other, and with similar efficiency to that of singly infected ticks (Levin and Fish 2000). Infections with more than one agent can lead to both complications in diagnosis and increased severity of disease (Swanson et al. 2006). Although several of the agents detected here are not known to be human pathogens, further studies regarding coinfections with these bacteria could help decipher the extent of their contributions to the aforementioned complications and lead to more precise diagnoses.

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Table 1

Multiplex assay primer and probe sequences

	Target	Sequence 5′ →3′	Working concentration	References
Primers				
R17K135F	*Rickettsia* 17-kDa	ATG AAT AAA CAA GGK CAN GGH ACA C	80 μM	(Loftis et al. 2006)
R17K249R	*Rickettsia* 17-kDa	AAG TAA TGC RCC TAC ACC TAC TC	80 μM	(Loftis et al. 2006)
ECH 16s-17	*Ehrlichia* 16s	GCG GCA AGC CTA ACA CAT G	80 μM	(Loftis et al. 2003)
ECE 16s-99	*Ehrlichia* 16s	CAC CCG TCT GCC ACT AAC AAY TAT	80 μM	This paper
Probes				
R17KBC Cal Rd	*Rickettsia spp.*	TT GGT TCT CAA TTC GGT AAG GGT AAA GG	10 μM	(Loftis et al. 2006)
EEW 16s-40–74 HEX	*Ehrlichia ewingii*	TC GAA CGA ACA ATT CCT AAA TAG TCT CTG ACT ATT	10 μM	This paper
ECH 16s-38–70 FAM	*Ehrlichia chaffeensis*	AG TCG AAC GGA CAA TTG CTT ATA ACC TTT TGG T	10 μM	(Loftis et al. 2003)

Table 2

Prevalence of infection in ticks collected from field sites in Georgia, USA

Field site	Life stage[a]	No. ticks	No. pools[b]	No. infected (% infected)				
				E. ewingii	*E. chaffeensis*	*R. amblyommii*	PME	*B. lonestari*
CLYBEL WMA	N	100	20	0 (0)	0 (0)	19 (19)	0 (0)	2 (2)
	A	42		1 (2.4)	1 (2.4)	25 (60)	0 (0)	0 (0)
Ossabaw Island	N	333	67	3 (0.9)	3 (0.9)	59 (17.7)	1 (0.3)	1 (0.3)
	A	326		10 (3.1)	8 (2.5)	271 (83.1)	1 (0.3)	4 (1.2)
Panola Mountain State Park	L		99	0 (0)	0 (0)	61 (61.6)	0 (0)	2 (2)
	N	2421	491	21 (0.9)	17 (0.7)	421 (17.4)	15 (0.6)	25 (1.0)
	A	764		15 (2)	10 (1.3)	327 (42.8)	15 (2)	24 (3.1)
Rum Creek WMA	N	100	20	0 (0)	0 (0)	20 (20)	2 (2.2)[c]	0 (0)
	A	51		2 (3.9)	1 (2)	21 (41.2)	0 (0)	1 (2)
Total	N	2954	598	24 (0.8)	20 (0.7)	519 (17.6)	18 (0.6)	28 (0.9)
	A	1183		25 (2.1)	23 (1.9)	644 (54.4)	16 (1.4)	29 (2.5)

[a] N, nymphs; A, adults; L, larvae.

[b] For pooled nymphs, minimum infection prevalence is given.

[c] Not enough extracted DNA for two of the samples, prevalence is given from 18 pools.

Table 3

Prevalence of dual infections in adult *A. americanum*

Agent	Dual infections detected (%)			
	B. lonestari	***E. chaffeensis***	***E. ewingii***	**PME**
R. amblyommii	14 (1.2%)	9 (0.8%)	15 (1.3%)	5 (0.4%)
B. lonestari		1 (<0.1%)	0	0
E. chaffeensis			1 (<0.1%)	0
E. ewingii				1 (<0.1%)

Table 4

Comparison of single infections with dual infections inadult *A. americanum*

Agent	No. single infections	No. coinfections	Total positive
B. lonestari	14	15	29
E. chaffeensis	12	11	23
E. ewingii	7	18	25
PME	9	7	16
R. amblyommii	599	45	644