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Distribution and infection frequency of `*Candidatus* Rickettsia amblyommii' in Maryland populations of the lone star tick (*Amblyomma americanum*) and culture in an *Anopheles gambiae* mosquito cell line

Xing Zhang[a], **Xiaoxia Ren**[a,b], **Douglas E. Norris**[a,b], and **Jason L. Rasgon**[a,b,*]

[a]The W. Harry Feinstone Dept. of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland 21205, USA

[b]The Johns Hopkins Malaria Research Institute, Baltimore, Maryland 21205, USA

Abstract

Amblyomma americanum (the lone star tick) is a broadly distributed tick that transmits multiple pathogens of humans and domestic animals. `*Candidatus* Rickettsia amblyommii' is a spotted-fever group rickettsial species that is potentially associated with human disease. In 2008 and 2009, we assayed over 500 unfed adult ticks from 19 Maryland populations for the presence of `*Candidatus* R. amblyommii'. Infection frequencies ranged from 33% to 100%, with an average infection rate of 60% in 2008 and 69% in 2009. Infection frequencies did not differ statistically between sexes. To develop a system in which to study `*Candidatus* R. amblyommii' in the laboratory, we used a cell line developed from *Anopheles gambiae* mosquitoes (Sua5B) to isolate and culture `*Candidatus* R. amblyommii' from field-collected *A. americanum* ticks from 2 localities in Maryland. After infection, Sua5B cells were infected for more than 40 passages. Infection was confirmed by *Rickettsia*-specific PCR, gene sequencing, and *Rickettsia*-specific fluorescence in situ hybridization (FISH). These data show that `*Candidatus* R. amblyommii' is widespread in Maryland *A. americanum* populations and that Sua5B cells are a useful tool for culturing *Rickettsia* infections from wild ticks.

Keywords

Cell culture; Vector-borne disease; Rickettsiosis; Intracellular

Introduction

Amblyomma americanum (the lone star tick) is broadly distributed across the eastern and central United States and transmits multiple pathogens of humans and domestic animals (Childs and Paddock, 2003; Goddard and Varela-Stokes, 2009). One bacterial species associated with this tick is `*Candidatus* Rickettsia amblyommii' (which we will refer to as *R. amblyommii* in the text), which was previously reported as a non-pathogenic *Rickettsia*

*Corresponding author. Tel.: 410-502-2584. jrasgon@jhsph.edu (J.L. Rasgon).

species in several states (Burgdorfer et al., 1974, 1975, 1981; Loving et al., 1978). However, more recent studies have implicated this bacterium in human disease (Apperson et al., 2008; Sanchez et al., 1992). *R. amblyommii* infection is estimated to occur in in 40–60% of collected *A. americanum* (Clay et al., 2008; Castellaw et al., 2010; Smith et al., 2010).

R. amblyommii was previously cultured from *A. cajennense* in African green monkey kidney (Vero) cells (Labruna et al., 2004). Mosquito cells are often easier to culture than cells derived from mammals or ticks because they can be passaged rapidly (~1 week), can be grown at room temperature under ambient atmosphere, and potentially develop high bacterial titers (Rasgon et al., 2006; Sakamoto and Azad, 2007). Sakamoto and Azad (2007) identified several mosquito cell lines that seemed highly permissible to multiple *Rickettsia* species and suggested that these cell lines might be useful as screening tools to isolate and identify novel *Rickettsia* and other intracellular bacterial infections from arthropods. In particular, the *Anopheles gambiae* cell line Sua5B has been used to grow *R. felis*, *R. montanensis*, *R. peacockii*, and *R. typhi*, as well as several strains of the bacterial symbiont *Wolbachia* (Rasgon et al., 2006; Sakamoto and Azad, 2007; Hughes et al., 2011).

In this study, we examined the distribution and infection frequency of *R. amblyommii* in natural populations of *A. americanum* in Maryland. We also used Sua5B cells to isolate and culture *R. amblyommii* from wild *A. americanum* ticks collected from 2 Maryland populations.

Materials and methods

R. amblyommii infection survey

Unfed male and female adult *A. americanum* ticks were collected from 19 Maryland populations (Fig. 1) in 2008 and 2009 (Tables 1 and 2) by dragging. Ticks were brought live in collection vials to the Johns Hopkins School of Public Health (JHSPH) for processing. Ticks were bisected with a sterile razor blade and half stored at −20°C for archiving. Genomic DNA was extracted from the other half as previously described (Zhang et al., 2011).

PCR

DNA extractions were screened for spotted fever group (SFG) rickettsiae as described by Blair et al. (2004). rompA PCR was employed to further confirm *Rickettsia* infection by using primers Rr190.70p and Rr190.602n as described (Regnery et al., 1991). To further characterize the *Rickettsia* species, gltA (Regnery et al., 1991), rompB (Roux and Raoult, 2000), and GeneD genes (Sekeyova et al., 2001) were also amplified as described. PCR using template DNA from uninfected Sua5B cells was included as a negative control. All PCR products were separated on a 2.5% agarose tris-borate-EDTA (TBE) gel by electrophoresis and visualized using ethidium bromide staining under UV light. PCR products were directly sequenced.

Tick source material for culturing *Rickettsia*

Nine female ticks (collection #: 20090351-0359) were collected on June 12, 2009, at Calvert Cliffs SP, Calvert County, MD. Fifteen male ticks (collection #: 0100029-0043) were collected on May 13, 2010, at Serpentine SP, Montgomery County, MD. Ticks were brought alive to the JHSPH in collection vials and maintained until processing.

Cell line infection

Uninfected *Anopheles gambiae* Sua5B cells were cultured as previously described (Rasgon et al., 2006) in Falcon 24-well plates (Becton Dickinson). Ticks from each collection were

pooled, surface-sterilized 3 times with 70% ethanol, then rinsed with sterile water for 5 min. Ticks were homogenized by using a TissueLyser II bead mill (Qiagen, Valencia, CA) with 5-mm stainless steel beads in 300 μl sterile Schneider's insect medium (Invitrogen Corporation, CA). Debris was separated by centrifugation at 200×G for 2 min, and the supernatant collected. The supernatant was passed through a sterile 2.7-μm Whatman filter and layered into cultured Sua5B cells from which the medium had been removed. The plate was centrifuged at 2500×G for one hour. Cells were incubated at room temperature with 100 U/ml penicillin for 2 days and transferred to 25-cm^2 flasks for culturing in Schneider's medium supplemented with 10% fetal bovine serum (FBS). Cells were split and passaged once a week. Two independent cultures were attempted, one from ticks collected in 2009 and the other one from ticks collected in 2010.

Confirmation of *Rickettsia* infection of Sua5B cells

Genomic DNA from cells was isolated using the MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, WI) and eluted in 100 μl sterile water. PCR to confirm and characterize *Rickettsia* infection of cells was carried out as described above. *Rickettsia* were also visualized in Sua5B cells using fluorescence in situ hybridization (FISH) as described (Sakamoto and Azad, 2007). Infected cells were grown overnight in Labtek chamber slides (8-well Permanox slides; Nalge Nunc International). Cells were fixed in 4% formalin phosphate-buffered saline at room temperature for 20 min, and the chamber walls and gasket removed. The *Rickettsia*-specific FISH probe was 5'-conjugated with rhodamine and is specific to all known *Rickettsia* 16S rRNA genes (5'-rhodamine-TCCACGTCGCCGTCTTGC, IDT, Coralville, IA) (Sakamoto and Azad, 2007). The probe was prepared to a final concentration of 10 pmol/ml hybridization solution as previously described (Rasgon et al., 2006). Probed slides were incubated in a humid chamber at 37°C overnight, then washed as described (Rasgon et al., 2006). Slides were mounted in 50 μl of ProLong (Invitrogen) with 1 ng/ml 4',6-diamidino-2-phenylindole (DAPI) and viewed by epifluorescent microscopy on an Olympus BX-41 compound microscope fitted with epifluorescent optics. Images were taken with a 0.5–0.75 s exposure in a two-by-two-pixel bin at gamma level one using a SPOT RT digital camera (Diagnostic Instruments, Inc., MI), and images merged with SPOT advanced imaging software (Universal Imaging, PA). Uninfected mosquito Sua5B cells were visualized as a negative control.

Results

Infection survey

In 2008, we assayed a total of 198 unfed adult ticks (120 females, 78 males) from 15 Maryland populations for *R. amblyommii* infection (Table 1). In 2009, we collected 304 ticks from 10 populations (166 females, 138 males) (Table 2). Infection frequencies in populations ranged from 33% to 100%. In total, 60% and 69% of ticks were positive for infection in 2008 and 2009, respectively. Infection frequencies were not statistically different between sexes (2008: female: 65%; male: 52.6%; P=0.1, Fisher's Exact Test; 2009: female: 66.3%; male: 72.5%; P=0.26, Fisher's Exact Test). BLAST search results of sequenced PCR amplicons indicated that rompA (452 bp) was 100% identical with *R. amblyommii* (EF689733), rompB (763 bp) was 100% identical with Candidatus *R. amblyommii* isolate 85–1084 (FJ455415), gltA was 100% identical with uncultured *Rickettsia* sp. clone from *A. americanum* (GQ302944), and GeneD (579 bp) was 99% identical with Candidatus *R. amblyommii* isolate 85–1084 (FJ358549).

Isolation and culture of *R. amblyommii* in Sua5B cells

Rickettsia-specific PCR and sequencing was performed at passage number 3 post-infection to initially confirm *Rickettsia* presence in the cell line, and was repeated approximately

every 10 passages thereafter. At the time of manuscript submission, cells were PCR-positive for 45 passages for the 2009 culture and for 20 passages for the 2010 culture. Obtained sequences from PCR amplicons were identical to those described above. Fluorescence in situ hybridization to visualize *Rickettsia* in Sua5B cells was performed at passage 43 (Fig. 2) for the 2009 infection and at passage 12 for the 2010 collection. Results from both were qualitatively similar. Rickettsiae were detectable as red fluorescent punctate dots in the cytoplasm of infected cells (Fig. 1) that were not visible in *Rickettsia*-negative cells (data not shown). The cell infection rate was greater than 95%, although interestingly the infection titer in individual cells was relatively low (<10 rickettsiae per cell).

Discussion

In this study, we found that *R. amblyommii* infection is widespread in Maryland *A. amblyommii* populations, similar to previous studies in other areas of the United States. Although not confirmed, *R. amblyommii* has been implicated as a potential human pathogen (Apperson et al., 2008). Due to the extensive distribution and high frequency of infection of this bacterium in natural populations, the potential for *R. amblyommii* to cause human disease merits further study.

We were able to use a mosquito cell line to isolate *R. amblyommii* from wild specimens of *A. americanum*. Infection was stable in the cells for over 40 passages with no decrease in the cell infection rate, although the bacterial titer in individual cells remained relatively low. Our data indicate that cultured mosquito cells can be highly effective for isolating and cultivating *Rickettsia* infections from wild ticks. The Sua5B cell line, in particular, seems to be highly amenable to *Rickettsia* infection (Sakamoto and Azad, 2007; Hughes et al., 2011) as well as to infection by other obligate intracellular bacteria such as *Wolbachia* (Rasgon et al., 2006; Hughes et al., 2011). Mosquito cells have several advantages over other cell lines (tick- or mammal-derived) commonly used for *Rickettsia* culture – they can be cultured in standard commercially available media, can be grown at room temperature, and do not require additional carbon dioxide atmosphere. The permissiveness of this cell line to infection with intracellular bacteria makes it a useful tool for isolation of *Rickettsia* from wild-collected arthropods.

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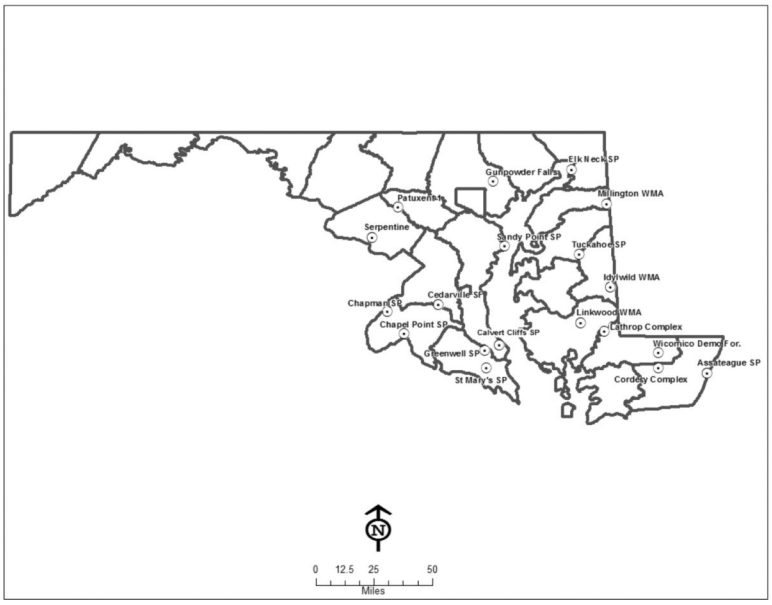

Fig. 1.
Amblyomma americanum collection locations in 2008 and 2009.

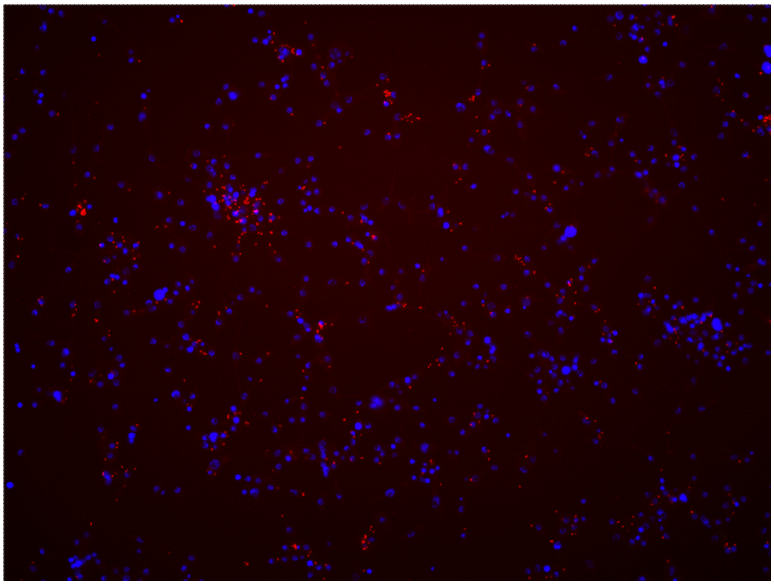

Fig. 2.

Color-merged fluorescence in situ hybridization image of *Rickettsia amblyommii*-infected Sua5B cells (passage 43 post-infection). Individual *Rickettsia* bacteria are visible as punctate red dots. Cells were counterstained with DAPI to visualize mosquito cell nuclei (blue signal).

Table 1

2008 tick collection data.

Location	County	Sex	N	# positive	% infected
Idylwild WMA	Caroline	Female	19	12	63%
		Male	10	4	40%
Linkwood WMA	Dorchester	Female	29	20	69%
		Male	19	12	63%
Assateague SP	Worchester	Female	9	8	89%
		Male	7	7	100%
Cedarville SF	PG and Charles	Female	2	1	50%
		Male	1	0	0
Chapel Point SP	Charles	Female	3	1	33%
Chapman SP	Charles	Female	17	15	88%
		Male	9	3	33%
Serpentine	Montgomery	Female	15	9	60%
		Male	17	8	47%
Patuxent 1	Montgomery	Female	1	1	100%
St Mary's SP	St Mary's	Female	4	2	50%
		Male	1	1	100%
Greenwell SP	St Mary's	Female	3	1	33%
		Male	1	0	0
Sandy Point SP	Anne Arundel	Female	8	4	50%
		Male	9	4	44%
Millington WMA	Kent	Female	2	0	0
		Male	3	1	33%
Calvert Cliffs SP	Calvert	Female	7	4	57%
Elk Neck SP	Cecil	Female	1	0	0
Tuckahoe SP	Queen Anne's	Male	1	1	100%
	Total	Female	120	78	65.0%
		Male	78	41	52.6%

Table 2

2009 tick collection data.

Location	County	Sex	N	# positive	% infected
Idylwild WMA	Caroline	Female	20	13	65%
		Male	17	15	88%
Linkwood WMA	Dorchester	Female	2	0	0
		Male	4	3	75%
Assateague SP	Worchester	Female	4	2	50%
		Male	4	4	100%
Cordery Complex (WR10)	Worchester	Female	30	26	87%
		Male	31	22	71%
Cedarville SF	PG and Charles	Female	3	0	0
		Male	3	1	33%
Serpentine	Montgomery	Female	40	32	80%
		Male	25	21	84%
Calvert Cliffs SP	Calvert	Female	24	9	38%
		Male	12	5	42%
Wicomico Demo Forest (W46)	Wicomico	Female	16	11	69%
		Male	15	10	67%
Lathrop Complex (W06)	Wicomico	Female	27	17	63%
		Male	25	18	72%
Gunpowder Falls	Baltimore	Male	2	1	50%
	Total	Female	166	110	66.3%
		Male	138	100	72.5%