HHS Public Access

Author manuscript

J Med Entomol. Author manuscript; available in PMC 2024 August 27.

Published in final edited form as:

J Med Entomol. 2016 November ; 53(6): 1492–1495. doi:10.1093/jme/tjw111.

Development of a *Rickettsia bellii*-Specific TaqMan Assay Targeting the Citrate Synthase Gene

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Abstract

Rickettsia bellii is a rickettsial species of unknown pathogenicity that infects argasid and ixodid ticks throughout the Americas. Many molecular assays used to detect spotted fever group (SFG) *Rickettsia* species do not detect *R. bellii*, so that infection with this bacterium may be concealed in tick populations when assays are used that screen specifically for SFG rickettsiae. We describe the development and validation of a *R. bellii*-specific, quantitative, real-time PCR TaqMan assay that targets a segment of the citrate synthase (*glt*A) gene. The specificity of this assay was validated against a panel of DNA samples that included 26 species of *Rickettsia*, *Orientia*, *Ehrlichia*, *Anaplasma*, and *Bartonella*, five samples of tick and human DNA, and DNA from 20 isolates of *R. bellii*, including 11 from North America and nine from South America. A *R. bellii* control plasmid was constructed, and serial dilutions of the plasmid were used to determine the limit of detection of the assay to be one copy per 4 µl of template DNA. This assay can be used to better determine the role of *R. bellii* in the epidemiology of tick-borne rickettsioses in the Western Hemisphere.

Keywords

Rickettsia bellii ; spotted fever group *Rickettsia*; real-time PCR; citrate synthase gene

First isolated in 1966 from a pool of *Dermacentor variabilis* ticks collected near Fayetteville, AR, *Rickettsia bellii* is an obligate intracellular, Gram-negative coccobacillus of unknown pathogenicity. *R. bellii* has been found in tick populations throughout the Western Hemisphere where it infects both argasid and ixodid ticks including *Argas*, *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes*, and *Ornithodoros* species (Philip et al. 1983, Horta et al. 2006, Labruna et al. 2007b, Miranda and Mattar 2014). While *R. bellii* is not known to be pathogenic to humans, it might play a role in the maintenance and distribution of other pathogenic tick-borne *Rickettsia* species. For example, in *Amblyomma dubitatum* ticks, a primary *R. bellii* infection has been shown to markedly diminish the transovarial transmission of *R. rickettsii* when secondarily infected with this pathogenic SFG *Rickettsia*

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species (Sakai et al. 2014). Considering the wide variety and collectively expansive range of tick species that are infected with *R. bellii*, inhibition of transovarial transmission resulting from a primary *R. bellii* infection could play an important role in the ecology of several pathogenic rickettsial species.

Many contemporary surveys for *Rickettsia* species in ticks have relied primarily or exclusively on assays that target the rickettsial outer membrane protein A (*omp*A) gene (Moncayo et al. 2010, Fritzen et al. 2011, Stromdahl et al. 2011, Venzal et al. 2012, Goddard et al. 2014, Henning et al. 2014, Trout Fryxell et al. 2015). Because *R. bellii* lacks this gene (Ogata et al. 2006), *omp*A-based PCR assays will not detect *R. bellii*. As previous culture-based assessments have identified infection rates as high as 80% in some tick populations (Philip et al. 1983), it is possible that many current molecular surveys underestimate the prevalence of this *Rickettsia*. A *R. bellii*-specific assay would be instrumental in determining whether or not the prevalence of *R. bellii* has truly fallen, or if the decrease in prevalence is a result of the limitations of the assays being used to screen for SFG *Rickettsia*.

We describe the development and validation of a *R. bellii*-specific real-time TaqMan assay that targets a 338-bp segment of the *R. bellii glt*A gene, using previously published primers (Szabó et al. 2013) and a novel probe. With the addition of a specific probe, this assay provides a highly sensitive and quantitative assay for detecting *R. bellii*.

Materials and Methods

Primer Verification

Primers targeting a 338-bp region of *glt*A were previously reported to be *R. bellii* specific (forward: 5′-ATCCTGATTTGCTGAATTTTTT-3′; reverse: 5′-TGCAATACCAGTACTGACG-3′); however, the reaction conditions and supporting specificity data were not provided (Szabó et al. 2013). Using *R. bellii* DNA, we determined the optimal cycling conditions for these primers to be a 5 min melt at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 52°C, and 1 min at 72°C, followed by a final extension step of 10 min at 72°C. Primer specificity was tested against a panel of 51 different DNA samples; 31 samples of various *Rickettsia*, *Orientia*, *Ehrlichia*, *Anaplasma*, *Bartonella* species, several tick species, human cells, and several other bacterial species (Table 1). Primer sensitivity was evaluated against a panel of DNA samples extracted from 20 distinct *R. bellii* isolates from North and South America (Table 2). Each reaction consisted of 10 µl Taq PCR Master Mix (QIAGEN, Valencia, CA), 1 µM each of the forward and reverse primers, 2 µl of template DNA and PCR-grade water to bring the final volume to 20 µl. All PCR reactions were run in an Eppendorf Mastercycler nexus gradient thermal cycler (Eppendorf AG, Hamburg, Germany) and the amplified PCR products were visualized by UV lamp in a 1.5% agarose gel containing 0.1 µg/ml ethidium bromide. Positive control DNA and no template control samples were included with each set of reactions.

Probe Design

The *glt*A gene sequences from eight different strains of *R. bellii* were aligned with the primer set and the *glt*A gene sequences of other rickettsial species (Fig. 1) using MEGA

version 6 software (Tamura et al. 2013) to identify areas within the 338-bp target region that differed among *R. bellii* and the other ricketsial species. A 26-bp FAM-labeled probe was designed (5′FAM-ATGATGTTTGCCACACCTTGTGAAAA-BHQ1–3′) that is identical to all of the *R. bellii* sequences in the alignment.

Real-Time PCR Optimization

All real-time PCR reactions were run in a BioRad CFX 96 thermal cycler using the QuantiTect Multiplex PCR Kit (QIAGEN, Valencia, CA). Each reaction consisted of 12.5 µl Quantitect Multiplex PCR Master Mix, 0.2 µM of the forward and reverse primers as well as the probe, 4 µl of template DNA, and PCR grade water to bring the final volume to 25 µl. Samples were run in duplicate, with positive control DNA and no template control samples added to every plate. The efficacy of the probe was tested using three different *R. bellii* samples. Gradient PCR was used to determine the optimal cycling conditions of 15 min at 95°C followed by 45 cycles of 30 s at 93°C, 30 s at 57°C, and 1 min at 72°C. This was followed by a final extension step of 10 min at 72°C. Fluorescence data were collected at the end of the annealing step of every cycle.

Cloning and Sequencing

A control plasmid was constructed by ligating the 338-bp *R. bellii* amplicon into pCR2.1 using the TOPO TA cloning kit (Life Technologies, Grand Island, NY). The integrity of the insertion site and the sequence of the *R. bellii* fragment were verified via PCR and DNA sequencing using primers M13 reverse and T7 promoter. The plasmid was subsequently used to determine the limit of detection of the assay and as a positive control for the real-time assay. As the purified plasmid may contain a small amount of *Escherichia coli* DNA carried over from the competent cells used to replicate the plasmid, the assay was tested to ensure that it would not amplify *E. coli* DNA.

Specificity and Sensitivity of Real-time Assay

To determine the limit of detection of the assay, the concentration of the *R. bellii glt*A control plasmid was ascertained using a Qubit 2.0 fluorometer (Life Technologies) and serial dilutions of 10^5, 10^4, 10^3, 10^2, 10, 5, 2.5, and 1 copy number per 4 µl of DNA were used as template DNA. The specificity of the combined primer-probe set was evaluated using the same panel of 51 DNA samples previously mentioned (Tables 1 and 2) in order to verify that the addition of the probe did not result in nonspecific binding.

Results and Discussion

A dilution series was run in duplicate to determine the limit of detection of the assay to be 1 copy number per 4 µl of DNA, with an R^2 value of the standard curve for the dilution series of 0.993, establishing the cutoff for positive samples at 40 cycles for future screenings. All 31 of the non-*R. bellii* DNA samples used to assess assay specificity were negative using the TaqMan assay. Additionally, each of the 20 *R. bellii* DNA samples, obtained from nine different tick species collected from North and South America, were positive by this assay. This panel of 20 *R. bellii* isolates represents a geographically and genetically (F.S.K., unpublished data) diverse group, which demonstrates that this assay provides a sensitive

technique to detect *R. bellii*. Application of this assay may facilitate efforts to better understand the role of *R. bellii* in the ecology and epidemiology of tick-borne rickettsioses in the Western Hemisphere.

Acknowledgments

The research reported here was supported in part by an appointment of J. Hecht to the Research Participation Program at the Centers for Disease Control and Prevention administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the CDC. The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Probe	A	T	G	A	T	G	T	T	T	G	C	C	A	C	A	C	C	T	T	G	T	G	A	A	A	A
R. bellii (DQ146481.1)	A	T	G	A	T	G	T	T	T	G	C	C	A	C	A	C	C	T	T	G	T	G	A	A	A	A
R. bellii (AY375161.1)	A	T	G	A	T	G	T	T	T	G	C	C	A	C	A	C	C	T	T	G	T	G	A	A	A	A
R. bellii (DQ517288.1)	A	T	G	A	T	G	T	T	T	G	C	C	A	C	A	C	C	T	T	G	T	G	A	A	A	A
R. bellii (DQ865204.1)	A	T	G	A	T	G	T	T	T	G	C	C	A	C	A	C	C	T	T	G	T	G	A	A	A	A
R. bellii (JQ906786.1)	A	T	G	A	T	G	T	T	T	G	C	C	A	C	A	C	C	T	T	G	T	G	A	A	A	A
R. bellii (AY362703.1)	A	T	G	A	T	G	T	T	T	G	C	C	A	C	A	C	C	T	T	G	T	G	A	A	A	A
R. bellii (U59716.1)	A	T	G	A	T	G	T	T	T	G	C	C	A	C	A	C	C	T	T	G	T	G	A	A	A	A
R. bellii (EU567181.1)	A	T	G	A	T	G	T	T	T	G	C	C	A	C	A	C	C	T	T	G	T	G	A	A	A	A
R. bellii (CP000849.1)	A	T	G	A	T	G	T	T	T	G	C	C	A	C	A	C	C	T	T	G	T	G	A	A	A	A
R. africae (CP001612.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A
R. amblyommii (CP012420.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A
R. conorii (AE006914.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A
R. massiliae(CP003319.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A
R. montanensis (CP003340.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A
R. parkeri (CP003341.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A
R. peacockii (CP001227.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A
R. prowazekii (U59715.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	T	C	C	T	T	G	T	A	C	T	A	A
R. rhipicelhali (CP003342.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A
R. rickettsii (CP000848.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A
R. rickettsii (CP003305.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A
R. slovaca (CP003375.1)	A	T	G	A	T	G	T	T	T	G	C	A	A	C	G	C	C	T	T	G	T	A	C	G	A	A

Fig. 1.

Probe sequence aligned with *glt*A sequences of various *Rickettsia*. The boxes highlight the bases that differ between *R. bellii* and the other *Rickettsia* in the alignment.

Table 1.

Bacterial isolates evaluated to verify specificity of *R. bellii* real-time PCR assay primer set and probe

Isolate designation	Source	Geographic origin
Rickettsia akari Toger	Human	Ukraine
Rickettsia amblyommii Darkwater	*Amblyomma americanum*	Florida, USA
Rickettsia australis Cutlack	Human	Australia
Rickettsia canadensis McKiel 24	*Hemaphysalis leporispalustris*	Ontario, Canada
Rickettsia conorii Malish 7^{T}	Human	Johannesburg, South Africa
Rickettsia helvetica C3	*Ixodes ricinus*	Switzerland
Rickettsia honei RB^{T}	Human	Australia
Rickettsia massiliae Mtu1^{T}	*Rhipicephalus turanicus*	Camargue, France
Rickettsia parkeri CWPP	*Amblyomma maculatum*	USA
Rickettsia peacockii Rustic	*Dermacentor andersoni*	Colorado, USA
“Candidatus Rickettsia philipii” 364D	*Dermacentor occidentalis*	California, USA
Rickettsia prowazekii Madrid II	Human	Madrid, Spain
Rickettsia rhipicephali 12T	*Rhipicephalus sanguineus*	Mississippi, USA
Rickettsia rickettsii Hlp#2	*Hemaphysalis leporispalustris*	Montana, USA
Rickettsia rickettsii Sheila Smith^{T}	Human	Montana, USA
Rickettsia sibirica 246^{T}	*Dermacentor nuttali*	Siberia, former USSR
Rickettsia slovaca B^{T}	*Dermacentor marginatus*	Banská Bystrica, Slovakia
Rickettsia typhi Wilmington^{T}	Human	North Carolina, USA
Ehrlichia canis Oklahoma^{T}	Canine	Oklahoma, USA
Ehrlichia chaffeensis Arkansas^{T}	Human	Arkansas, USA
Ehrlichia muris AS145^{T}	*Eothenomys kageus*	Aichi Prefecture, Japan
Neoehrlichia mikurensis	*I. ricinus*	Netherlands
Orientia tsutsugamushi Gilliam	Human	Japan
Bartonella elizabethae F9251^{T}	Human	Massachusetts, USA
Bartonella henselae Houston-1^{T}	Human	Texas, USA
Bartonella vinsonii subsp. berkhoffii	Canine	North Carolina, USA

Table 2.

Rickettsia bellii isolates evaluated to verify specificity of the *R. bellii* real-time PCR assay primer set and probe

Isolate designation	Tick species from which isolate was obtained	Geographical origin, year	Reference
369C^{T}	*Dermacentor variabilis*	Washington County, Arkansas, USA, 1966	Philip et al. 1983
CA13–1	*D. variabilis*	Yolo County, California, USA, 2013	C.D. P., unpublished data
CA13–9	*D. variabilis*	Yolo County, California, USA, 2013	C.D. P., unpublished data
CA13–17	*D. variabilis*	Yolo County, California, USA, 2013	C.D. P., unpublished data
Putah Creek	*D. variabilis*	Solano County, California, USA, 2015	C.D. P., unpublished data
Stevenson Bridge	*D. variabilis*	Yolo County, California, USA, 2015	C.D. P., unpublished data
Yolo	*D. variabilis*	Yolo County, California, USA, 2015	C.D. P., unpublished data
UT 13–26	*Dermacentor parumapertus*	Tooele County, Utah, USA, 2013	C.D. P., unpublished data
UT 13–34	*D. parumapertus*	Tooele County, Utah, USA, 2013	C.D. P., Unpublished data
UT 13–17	*D. parumapertus*	Tooele County, Utah, USA, 2013	C.D. P., unpublished data
UT13–9	*D. parumapertus*	Tooele County, Utah, USA, 2013	C.D. P., unpublished data
Mogi	*Amblyomma aureolatum*	Mogi das Cruzes, São Paulo State, Brazil, 2006	Pinter and Labruna 2006
Cord	*Amblyomma dubitatum*	Cordeirópolis, São Paulo State, Brazil, 2009	Pacheco et al. 2009
Ad 25	*A. dubitatum*	Ribeirão Grande, São Paulo State, Brazil, 2009	Pacheco et al. 2009
PNSM	*Amblyomma incisum*	Cubatão, São Paulo State, Brazil, 2010	Sabatini et al. 2010
AO	*Amblyomma ovale*	Ribeirão Grande, São Paulo State, Brazil, 2008	Pacheco et al. 2008
HJ-04	*Haemaphysalis juxtakochi*	Ribeirao Grande, São Paulo State, Brazil, 2007	Labruna et al. 2007a
IL-Mogi	*Ixodes loricatus*	Mogi das Cruzes, São Paulo State, Brazil, 2006	Horta et al. 2006
HJ-1	*H. juxtakochi*	São Paulo City, São Paulo State, Brazil, 2007	Labruna et al. 2007a
A. ovale 51	*A. ovale*	Peruíbe, São Paulo State, Brazil, 2013	Szabó et al. 2013