

## A HIGHLY SENSITIVE AND SPECIFIC REAL-TIME PCR ASSAY FOR THE DETECTION OF SPOTTED FEVER AND TYPHUS GROUP RICKETTSIAE

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**Abstract.** A highly specific real-time polymerase chain reaction (PCR) assay was developed to detect spotted fever and typhus group rickettsiae using the citrate synthase gene as the target. The assay amplified rickettsial members of the spotted fever and typhus group including *Rickettsia akari*, *R. australis*, *R. conorii*, *R. honei*, “*R. marmionii*,” *R. sibirica*, *R. rickettsii*, *R. typhi*, and *R. prowazekii*. The ancestral group rickettsia, *R. bellii*, did not produce a positive reaction, nor did other members of the order *Rickettsiales* or any non-rickettsial bacteria. The assay had a sensitivity of one target copy number per reaction as determined by serial dilutions of a plasmid containing a spotted fever group target sequence. This quantitative assay is useful for the enumeration of rickettsiae in clinical specimens and the diagnosis of rickettsial illnesses, when rickettsial numbers are very low.

### INTRODUCTION

*Rickettsia* are obligate intracellular bacteria, usually pathogenic to humans and closely related to mitochondria.<sup>1</sup> There are three biotypes of *Rickettsia*; the spotted fever group (SFG) and typhus group (TG) and the ancestral group, consisting solely of *Rickettsia bellii*, a rickettsia thought not to be pathogenic to humans.<sup>2</sup> *Rickettsia* are found worldwide and are usually transmitted via an arthropod that is both its reservoir and vector.<sup>3</sup>

The rapid diagnosis of a rickettsial illness is important for appropriate antibiotic treatment to be given promptly. Traditionally, the diagnosis of a rickettsial illness has been based on serological tests. Of the serological tests, the indirect microimmunofluorescence assay has been the most sensitive and specific, but usually it is not positive when the patient is acutely unwell.<sup>4</sup> Culture techniques can be used for diagnosis and are very sensitive but can require up to 60 days to yield a positive result, limiting their clinical usefulness.<sup>5</sup>

In the late 1980s, polymerase chain reaction (PCR) detection of rickettsial nucleic acid became available as a quick and reliable method for the diagnosis of rickettsioses. The first of these assays detected the rickettsial 17-kDa gene<sup>6</sup> with later tests detecting the rickettsial citrate synthase and *ompA* genes.<sup>7</sup> These assays, although specific for rickettsia, were not very sensitive until nested procedures were introduced.<sup>8</sup> Recently, assays involving *Orientia tsutsugamushi* real-time PCR,<sup>9</sup> *Anaplasma phagocytophilum* and *Borrelia burgdorferi* multiplex real-time PCR,<sup>10</sup> and *Rickettsia prowazekii* and *Borrelia recurrentis* real-time duplex PCR<sup>11</sup> have been developed. They are highly sensitive and specific, with the potential for quantifying DNA copy numbers.

In this manuscript, we describe a real-time assay based on the rickettsial citrate synthase gene. This assay is both highly specific and extremely sensitive for the diagnosis of rickettsioses and can also be used to quantify rickettsial DNA copy numbers.

### MATERIALS AND METHODS

Rickettsial real-time PCR was performed using in-house designed primers to the *Rickettsia rickettsii* citrate synthase gene, *gltA* (GenBank accession no. U59729),<sup>12</sup> using the Primer Express program (Applied Biosystems, Foster City, CA). The oligos designed amplified a 74 base pair fragment and were designated CS-F (5'-TCG CAA ATG TTC ACG GTA CTT T-3') and CS-R (5'-TCG TGC ATT TCT TTC CAT TGT G-3') and the probe CS-P (5'-6-FAM-TGC AAT AGC AAG AAC CGT AGG CTG GAT G-BHQ-1-3') (Biosearch Technologies Inc., Novato, CA) corresponding to nucleotides 1126–1147, 1199–1178, and 1149–1176, respectively. Tm values were calculated to be 54.0°C, 55.1°C, and 65.6°C for CS-F, CS-R, and CS-P, respectively. This region is highly conserved with a majority of the SFG sequences completely homologous to the primers and probes and has a maximum of 9 nucleotide substitutions, with the ancestral groups *Rickettsia bellii* (Table 1).

Each reaction contained 200 nM of each primer and probe, 2 × Platinum qPCR SuperMix-UDG Mastermix (Invitrogen, Melbourne, Australia), 5 mM MgCl<sub>2</sub>, and extracted DNA to a total reaction volume of 25 μL. The reactions were performed and analyzed using the Rotor-Gene 3000 (Corbett Research, Sydney, Australia) with a *Rickettsia honei* positive control and a “no template control,” with an initial holding temperature of 50°C for 3 minutes, followed by 95°C for 5 minutes and 60 cycles of 95°C for 20 seconds and 60°C for 40 seconds. Emission was monitored at the end of every 60°C annealing step on a predetermined FAM channel. Positive results were confirmed by repetition and by visualizing the product on a 3% Tris acetate EDTA (TAE) agarose gel (Amresco, Solon, OH) stained with ethidium bromide (Sigma, Sydney, Australia). Optimization was performed by performing varying concentrations of each primer and probe (50–400 nM) with variable concentrations of MgCl<sub>2</sub> (0–15 mM). When a *R. honei* DNA stock was tested, the primer/probe/MgCl<sub>2</sub> combination with the lowest concentrations to achieve the lowest C<sub>t</sub> was considered to be optimal (concentrations previously stated).

A portion of the “*Rickettsia marmionii*” *gltA* was amplified using the above protocol without probe. The resulting 74 base pair fragment was ligated into a pCR 2.1 plasmid (ligated plasmid size; 4,003 base pairs) and subsequently cloned into One Shot Top10 chemically competent *Escherichia coli* with a TA Cloning kit (Invitrogen, Australia) using the manufactur-

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TABLE 1  
Primers and probe used in this assay and their nucleotide substitutions when compared with rickettsial sequences

Name	Biogroup	Sequence (5'→3')										
Consensus		TCGCAAATGTTTCACGGTACTTTTGTGCAATAGCAAGAACCGTAGGCTGGATGGCACAATGGAAGAAATGCACGA										
CS-F		TCGCAAATGTTTCACGGTACTTT										
CS-R		CACAATGGAAGAAATGCACGA										
CS-P		TGCAATAGCAAGAACCGTAGGCTGGATG										
Most SFG*	SFG	(No substitutions)										
<i>R. africae</i>	SFG					T						
<i>R. aeschlimannii</i>	SFG				A							
<i>R. heilongjiangensis</i>	SFG				T							
<i>R. felis</i>	SFG		T							G		
<i>R. helvetica</i>	SFG						C	A			G	
<i>R. prowazekii</i>	TG				T				T			
<i>R. typhi</i>	TG				T				T	G		
<i>R. canadensis</i>	TG		A		A	G				G		
<i>R. bellii</i>	Ancestral	C	G		A	G	C	C	A		G	T

\* Including *R. akari*, *R. australis*, *R. conorii*, *R. honei*, "*R. marmionii*," *R. japonica*, *R. massiliae*, *R. montanensis*, *R. rhipicephali*, *R. rickettsii*, *R. sibirica*, *R. slovacica*, and *R. parkeri*.

er's instructions. Transformed *E. coli* were grown overnight in a shaking water bath at 37°C in Luria Bertani broth (Oxoid, Hampshire, England) supplemented with ampicillin (CSL, Melbourne, Australia). The *E. coli* were pelleted and had plasmids extracted and purified with the FastPlasmid Mini kit (Eppendorf, Hamburg, Germany) using the manufacturer's instructions. Five microliters of purified plasmid solution was diluted (1:100) and the DNA quantified in a scanning spectrophotometer.<sup>13</sup> A theoretical number of plasmid copies and reaction efficiency were calculated and serial 10-fold dilutions of the plasmid solution made. Duplicates of each serial dilution underwent real-time PCR. Results enabled the sensitivity and efficiency of the assay to be determined.

DNA was extracted from cell cultures of members of the order *Rickettsiales* (*Anaplasma phagocytophilum*, *Bartonella bacilliformis*, *B. henselae*, *B. vinsonii*, *Ehrlichia chaffeensis*, *Orientia tsutsugamushi*, *Rickettsia akari*, *R. australis*, *R. conorii*, *R. honei*, "*R. marmionii*," *R. prowazekii*, *R. rickettsii*, *R. sibirica*, *R. typhi*, and *R. bellii*) and other medically important bacteria (*Escherichia coli*, *Enterococcus faecalis*, *Coxiella burnetii*, *Haemophilus influenzae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, and *Streptococcus pneumoniae*) with a DNA extraction kit (Gentra, Minneapolis, MN) using the manufacturer's instructions. A real-time PCR was conducted on each specimen to determine the specificity of the assay. A conventional 16S rRNA PCR was used to determine the presence of bacterial DNA.<sup>14</sup> All reactions that involved testing the specificity and sensitivity of this assay were also spiked with human DNA.

## RESULTS

Initial real-time PCR performed on a *R. honei* positive control produced a positive result. An amplicon of 74 base pairs was confirmed by gel analysis. The "no template control" reaction was negative.

The sensitivity of the assay (determined by performing real-time PCR reactions on plasmid DNA solutions ranging from  $1 \times 10^9$  to  $1 \times 10^{11}$  copies per reaction) was shown to be 1 copy of the SFG *gltA* target. The  $C_t$  value of each reaction varied from 6 for  $1 \times 10^{11}$  SFG *gltA* copies per reaction, to a

$C_t$  value of 35 for  $1 \times 10^9$  SFG *gltA* copies per reaction. The reaction efficiency, as calculated by the real-time PCR analysis software, was 128% due to the unproportional digestion of the probe compared with the amplicon produced.

All tested species of the SFG and TG including *R. akari*, *R. australis*, *R. conorii*, *R. honei*, "*R. marmionii*," *R. prowazekii*, *R. rickettsii*, *R. sibirica*, and *R. typhi* produced a positive real-time PCR result with the exception of *R. bellii*, an ancestral group *Rickettsia*. All other members of the *Rickettsiales* and medically important bacteria tested were real-time negative. All bacteria tested produced a positive result for the conventional 16S rRNA PCR. All PCR reactions that were spiked with human DNA had no effect on the sensitivity or specificity of the assay.

## DISCUSSION

The rapid diagnosis of rickettsial disease is crucial for effective treatment of the illness. This new assay is highly specific for members of the genus *Rickettsia*, with the exception of the ancestral *R. bellii*. It produces negative results for all other bacteria tested including *O. tsutsugamushi*, also of the tribe *Rickettsieae*. The assay amplified all tested species of the SFG and TG. Conventional 16S rRNA PCR confirmed the presence of prokaryotic DNA in all negative assays. The assay is very sensitive and capable of detecting 1 SFG rickettsia per PCR reaction. This is very important, as the concentration of rickettsia in a rickettsiemic patient may be very low.<sup>15</sup>

The citrate synthase gene, *gltA*, is a highly conserved gene among the genus *Rickettsia*.<sup>12</sup> The highly conserved nature of the *gltA* gene makes it an ideal target for real-time PCR. The section of the genome that the probe and primers span is relatively homogeneous in the genus *Rickettsia*. The number of substitutions in this section of the *gltA* gene varies from 0 in most SFG rickettsiae to 4 in the TG. Nine substitutions in the ancestral group's sequence (Table 1) probably explains why the assay does not detect *R. bellii*. Although individual sensitivity assays were not performed for many of the rickettsial species tested, including the TG, it may be assumed that the assay will amplify, with similarly high sensitivities, SFG/TG rickettsiae (except *R. bellii*), due to the highly conserved nature of this region of the gene (Table 1). An excep-

tion may be *R. canadensis*, as it has three nucleotide substitutions in the primer CS-F. This new assay will detect all rickettsial species of the SFG and TG, but not the ancestral group, with very high sensitivities.

The ability to quantify rickettsiae is useful. Traditionally, enumeration of rickettsiae has been done via the plaque assay.<sup>16</sup> These assays are time consuming, rely on the rickettsia being capable of infecting a cell monolayer, and are not that sensitive.<sup>17</sup> More recent methodologies in enumerating rickettsia have been PCR based, with sensitivities of five copies per reaction.<sup>18</sup> The recent development of a real-time assay to detect *O. tsutsugamushi* has demonstrated that high specificity and a sensitivity of one copy number is achievable.<sup>9</sup> The development of a real-time PCR assay for *R. prowazekii* using the *ompB* gene has also been published,<sup>11</sup> although it cannot detect other members of the genus *Rickettsia* and has a sensitivity of approximately 10 copies. Enumeration of living rickettsia inoculated into an animal or tissue culture is important for the study of rickettsial pathogenicity,<sup>19</sup> host susceptibility,<sup>20</sup> and vaccine efficacy.<sup>21</sup> Due to its high sensitivity, the new real-time assay would be useful in the quantification of rickettsia within animal and human organs and tissues, including blood, where a very high sensitivity is needed.

The ability to quickly diagnose an acute rickettsial illness is important for the rapid administration of appropriate antibiotics. Serology is no longer considered an adequate marker of rickettsial illness even when used as a retrospective test. Cases of confirmed rickettsioses have now been described where no increase in rickettsial antibody titer was detected or where a positive serum titer was not detected at any stage during or after the illnesses.<sup>22</sup> Traditional PCR techniques lack the sensitivity to diagnose infection when there are low numbers of rickettsiae in peripheral blood mononuclear cells.<sup>18</sup> The development of a real-time PCR specific for SFG and TG rickettsiae is useful in overcoming the defects of serology and conventional PCR. The ability of this new real-time PCR to detect 1 copy number of the SFG citrate synthase gene target and the highly specific nature of the assay makes it a valuable tool for the diagnoses of acute rickettsial infection.

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