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. 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. Rickettsia are found worldwide and are usually transmitted via an arthropod that is both its reservoir and vector.

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 microm immunofluorescence assay has been the most sensitive and specific, but usually it is not positive when the patient is acutely unwell. 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.

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 with later tests detecting the rickettsial citrate synthase and ompA genes. These assays, although specific for rickettsia, were not very sensitive until nested procedures were introduced. Recently, assays involving Orientia tsutsugamushi real-time PCR, Anaplasma phagocytophilum and Borrelia burgdorferi multiplex real-time PCR, and Rickettsia prowazekii and Borrelia recurrentis real-time duplex PCR 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), 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 AGC GTA CTT T-3′) and CS-R (5′-TCG TGC ATT TCT TTT CAT TGT G-3′) and the probe CS-P (5′-6-FAM-TGC AAT GGC AAC CGT AGG CTG GAT G-3′) (Biosearch Technologies Inc., Novato, CA) corresponding to nucleotides 1126–1147, 1199–1178, and 1149–1176, respectively. Tm values were calculated to be 54.4°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 MgCl2, 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 3 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 MgCl2 (0–15 mM). When a R. honei DNA stock was tested, the primer/probe/MgCl2 combination with the lowest concentrations to achieve the lowest Ct 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-
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. 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. japonica, R. massiliae, R. monacensis, R. rhipicephali, R. rickettsii, R. sibirica, R. tsihoca, and R. parkeri).

### 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 $1 \times 10^0$ to $1 \times 10^{11}$ copies per reaction) was shown to be 1 copy of the SFG gltA target. The $C_v$ value of each reaction varied from 6 for $1 \times 10^{11}$ SFG gltA copies per reaction, to a $C_v$ value of 35 for $1 \times 10^0$ SFG gltA copies per reaction. The reaction efficiency, as calculated by the real-time PCR analysis software, was 128% due to the disproportional 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 Rickettsiaceae. 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 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-
Sensitve Real-time PCR for Rickettsiae

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REFERENCES


