Quantitative Real-Time PCR Assay for Detection of *Ehrlichia chaffeensis*

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A real-time PCR assay was developed for the detection of *Ehrlichia chaffeensis*. The assay is species specific and provides quantitative results in the range 10 to 10¹⁰ gene copies. The assay is not inhibited by the presence of tick, human, or mouse DNA and is compatible with high sample throughput. The assay was compared with previously described assays for *E. chaffeensis*.

Ehrlichia chaffeensis is the causative agent of human monocytic ehrlichiosis, a potentially fatal disease (11). The bacterium, first isolated in 1991 (4), is a tick-borne zoonotic pathogen classified within the order *Rickettsiales*, family *Anaplasmataceae* (6). The pathogen is maintained in a natural transmission cycle between the Lone Star tick (*Amblyomma americanum*) and mammalian hosts (1, 7, 9). Experimental studies of the acquisition and transmission of *E. chaffeensis* between mammals and ticks require a quantitative assay for the organism that is compatible with high sample throughput.

Existing PCR assays for *E. chaffeensis* include a direct PCR assay for the 16S rRNA gene (2) and a nested PCR assay that amplifies the variable-length PCR target (VLPT) (13). These PCR assays can detect the presence of pathogen DNA but do not provide quantitative data. Nested PCR is typically more sensitive than direct PCR but requires more handling of amplified PCR products, decreasing throughput capacity and increasing the risk of sample cross-contamination.

Real-time (TaqMan) PCR. The single-copy 16S rRNA gene of *E. chaffeensis* was selected for the development of the real-time TaqMan PCR assay (10). Primers that amplify an 81-bp region of the gene (bases 17 to 97, GenBank accession no. U86665) were selected. The assay was optimized with a Brilliant quantitative-PCR core reagent kit (Stratagene, La Jolla, Calif.), with a final reaction volume of 25 μ l. The reaction mixture contained a 200 nM concentration of the forward primer ECH16S-17 (5'-GCGGCAAGCCTAACACATG-3'), an 800 nM concentration of the reverse primer ECH16S-97 (5'-CCCGTCTGCCACTAACAAATTATT-3'), a 100 nM concentration of the probe ECH16S-38 (5'-6-carboxyfluorescein-AGTCGAACGGACAATTGCTTATAACCTTTTGGT-3'), and 3.0 μ M magnesium chloride. Real-time PCRs and fluorescence detection were performed using an iCycler thermal cycler and iQ software (Bio-Rad Laboratories, Hercules, Calif.). The optimized thermal cycler program was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 57°C for 1 min.

Sensitivity and specificity. Quantitative results were based on a 10-fold dilution series of a plasmid encoding the 16S

rRNA gene of *E. chaffeensis*. The specificity of the assay was determined by testing genomic DNA from three strains of *E. chaffeensis*, several closely related organisms, uninfected ticks, and uninfected mammalian blood and tissues. *E. chaffeensis* isolates (Arkansas, St. Vincent, and Jax) were obtained from clinical samples, as described previously (4, 12). All three strains of *E. chaffeensis* were detected by the TaqMan PCR, and agarose gel electrophoresis revealed a single band of the appropriate size. The TaqMan assay did not amplify genomic DNA from *Ehrlichia muris* (AS145 strain) (15), *Ehrlichia canis* (Oklahoma strain) (5), *Neorickettsia sennetsu* (formerly *Ehrlichia sennetsu*, Miyayama strain) (14), or *Rickettsia prowazekii* (Breinl strain). As revealed by agarose gel electrophoresis, the primers used for the TaqMan PCR assay amplified the 16S rRNA genes from *Ehrlichia ewingii* (human clinical sample) (3) and *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*, USG3 strain) (16). However, neither *A. phagocytophilum* nor *E. ewingii* was detected during real-time amplification and fluorescence detection, indicating that the TaqMan probe is species specific for *E. chaffeensis* (data not shown).

Genomic DNA was extracted from uninfected ticks, blood, and tissue samples using the IsoQuick nucleic acid extraction kit (Orca Research, Inc., Bothell, Wash.). The TaqMan PCR assay did not amplify genomic DNA from mouse blood, spleen, or liver (BALB/c and C57BL/6 strains), human or guinea pig blood, or uninfected ticks (*Amblyomma americanum*, *Dermacentor variabilis*, or *Ixodes scapularis*).

The specificity of the TaqMan PCR assay was further tested using DNA extracted from human clinical samples submitted to the Centers for Disease Control and Prevention (Atlanta, Ga.). Three *E. chaffeensis*-positive samples and three *E. chaffeensis*-negative samples (one *A. phagocytophilum*-positive blood sample, one negative blood sample, and one negative lymph node sample) were tested. The TaqMan PCR was positive for all three samples containing *E. chaffeensis* DNA but was negative for two samples lacking *E. chaffeensis* DNA and for the sample containing *A. phagocytophilum* DNA.

Effect of background DNA. The effect of background DNA on the efficiency and sensitivity of the TaqMan PCR assay was determined. Genomic DNA was pooled from 10 adult female *Amblyomma americanum* ticks (35.3 μ g of DNA/ml) and 10 uninfected BALB/C mice (19.9 μ g of DNA/ml), and human

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TABLE 1. Effect of host DNA on the efficiency and sensitivity of the real-time TaqMan PCR assay for *E. chaffeensis*^a

Diluent	% Reaction efficiency		Reaction sensitivity	
	Avg	95% CI	Avg <i>C_T</i>	95% CI
Water	89.3	85.5–93.1	23.34	22.94–23.74
Tick DNA	95.8	90.9–100.6	23.08	23.03–23.12
Mouse DNA	104.5	96.1–112.9	22.91	22.76–23.06
Human DNA	105.1	104.4–105.9	23.06	23.03–23.09

^a Results are reported as the averages and 95% confidence intervals (CI) of results from three separate amplifications. *C_T*, threshold cycle.

genomic DNA (25 µg of DNA/ml) was obtained from Promega (Madison, Wis.). Reaction efficiencies were compared using a fivefold dilution series of genomic *E. chaffeensis* DNA in water, tick DNA, mouse DNA, or human DNA. As summarized in Table 1, the presence of background DNA did not decrease the efficiency of the TaqMan PCR. The effect of host DNA on sensitivity was determined by comparing the threshold cycles in which genomic *E. chaffeensis* DNA was detected in the presence of water, tick DNA, mouse DNA, or human DNA. *E. chaffeensis* DNA was tested at a dilution of approximately 620 bacteria per µl of blood, corresponding with a moderate degree of ehrlichemia (unpublished results). The sensitivity of the assay in the presence of background DNA was not significantly different from that in water (Table 1).

Comparison with other assays. The analytical sensitivity and specificity of the TaqMan PCR were compared with those of three previously described PCR assays for *E. chaffeensis*: a direct PCR assay for the 16S rRNA gene using primers HE1 and HE3 (2), a nested PCR assay for the VLPT of *E. chaffeensis* (13), and a real-time PCR assay for the 16S rRNA gene, based on the SYBR Green detection system (8). All PCR assays were performed in a 25-µl final volume. For the SYBR Green assay, real-time fluorescence detection was performed by use of an ABI 7900HT sequence detection system (Applied Biosystems, Foster City, Calif.).

The sensitivities of all four PCR assays were compared by testing a fivefold dilution series of *E. chaffeensis* genomic DNA (Table 2). All assays were repeated to ensure reproducible results. The sensitivity of the TaqMan assay was significantly greater than that of the direct (HE1/HE3) assay and comparable to those of the real-time SYBR Green and nested VLPT assays.

TABLE 2. Relative sensitivities of four different PCR assays for *E. chaffeensis* DNA

Dilution ^a	Test result with:			
	TaqMan PCR	HE1/HE3 PCR	VLPT nested PCR	SYBR Green PCR
1/50	+	+	+	+
1/250	+	+	+	+
1/1,250	+	+	+	+
1/6,250	+	–	+	+
1/31,250	+	–	+	+
1/156,250	+	–	+	+
1/781,250	–	–	± ^b	–

^a The 1/156,250 dilution corresponds to approximately 10 genomes of *E. chaffeensis*.

^b The VLPT assay was positive in 7 of 10 replicates.

TABLE 3. Specificities of four different PCR assays for *E. chaffeensis* DNA

DNA sample	Test result with:			
	TaqMan PCR	HE1/HE3 PCR ^a	VLPT nested PCR ^a	SYBR Green PCR
<i>E. chaffeensis</i>	+	+	+	+
<i>E. muris</i>	–	ND ^b	–	+
<i>E. canis</i>	–	–	–	+
<i>E. ewingii</i>	–	–	–	+
<i>A. phagocytophilum</i>	–	–	–	+
<i>N. sennetsu</i>	–	–	ND	–
<i>R. prowazekii</i>	–	–	ND	–
<i>Amblyomma americanum</i> ticks	–	–	–	–
Mouse DNA	–	ND	ND	–
Human DNA	–	–	–	–

^a Data derived from published results (2, 13).

^b ND, not done.

The specificity of the TaqMan PCR was compared with those of the other three PCR assays for *E. chaffeensis* (Table 3). The HE1/HE3 direct and VLPT nested PCR assays have been evaluated by using genomic DNA from several species of *Ehrlichia* and *Anaplasma* and have been demonstrated to be species specific for *E. chaffeensis* (2, 13). As reported above, the TaqMan PCR is species specific for *E. chaffeensis*. However, the SYBR Green assay detected *E. canis*, *E. ewingii*, *E. muris*, and *A. phagocytophilum*, as well as *E. chaffeensis*.

Conclusions. In conclusion, the real-time PCR assay that we have developed for *E. chaffeensis* is very sensitive (10 gene copies), is species specific, is suitable for high-throughput applications, and is not inhibited by the presence of human, mouse, or tick DNA. The assay was found to be superior to three assays previously described for the detection of *E. chaffeensis* based on one or more criteria. It is more sensitive than the HE1/HE3 direct PCR assay, more specific than the SYBR Green real-time PCR assay, and has a higher throughput capacity and less sample handling than the VLPT nested PCR assay. Most importantly, the real-time TaqMan PCR assay provides quantitative data, allowing for simultaneous detection of the pathogen and determination of the infectious loads in ticks and mammals. This assay provides a powerful tool for examining the kinetics of infection with *E. chaffeensis* and the transmission of the pathogen between mammalian hosts and arthropod vectors.

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