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CHLAMYDIOLOGY AND RICKETTSIOLOGY

A Novel TaqMan Assay for Detection of *Rickettsia* 364D, the Etiologic Agent of Pacific Coast Tick Fever

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ABSTRACT Pacific Coast tick fever is a febrile illness associated with the bite of *Dermacentor occidentalis* and results from an infection due to the intracellular pathogen *Rickettsia* 364D (also known by the proposed name "*Rickettsia philipii*"). Current molecular methods for the detection of this pathogen rely on the amplification of a conserved spotted fever group rickettsial gene (*omp*A) followed by DNA sequencing of the amplicon to identify the species. This work describes the development of a *Rickettsia* 364D-specific TaqMan assay to simplify and accelerate the detection and identification processes. The assay demonstrated a sensitivity of 1 genomic copy per 4-μl sample and is highly specific for *Rickettsia* 364D. The utility of this assay for ecological and diagnostic samples was evaluated using banked specimens collected in a single-blind manner and yielded a clinical sensitivity and specificity of 100%. In conclusion, we describe the development and evaluation of a novel TaqMan real-time PCR assay for the detection and identification of *Rickettsia* 364D suitable for ecological and diagnostic applications.

KEYWORDS *Rickettsia* 364D, *Rickettsia philipii*, Pacific Coast tick fever, real-time PCR, *Rickettsia*, TaqMan

The original strain of *Rickettsia* 364D (strain 364-D) was isolated from *Dermacentor occidentalis* (the Pacific Coast tick) collected from southern California in 1966 (1). It was not until 2008 that this agent was confirmed to be a human pathogen (2). Fourteen cases of *Rickettsia* 364D infection, now called Pacific Coast tick fever (PCTF), have been identified in California (2, 3). *Rickettsia* 364D is most closely genetically related to *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF). Mouse serotyping classification schemes recognize the type strain (strain 364-D) as a unique serotype (1, 4), and the name "*Rickettsia philipii*" has been proposed (5).

Pacific Coast ticks are found throughout much of California, with a range that extends from parts of Washington and southwestern Oregon to northern Baja California, Mexico. All three life stages of this mammophilic tick bite humans. *Dermacentor occidentalis* is a potential vector of several pathogens of human and veterinary importance, including *Francisella tularensis*, Colorado tick fever virus, *Anaplasma bovis*, *R. rickettsii*, and *Rickettsia* 364D (2, 6, 7). The estimated prevalence of *Rickettsia* 364D among *D. occidentalis* in California is approximately 2% to 8% (3, 5, 8, 9). Additional studies are required to better identify cases of PCTF and to understand the ecological factors involved in the maintenance of this pathogen in nature. Current molecular assays to detect *Rickettsia* 364D use conventional PCR amplification of the *omp*A gene, followed by DNA sequencing of the amplicon (2, 3, 5, 10). However, *D. occidentalis* ticks are more commonly infected with *Rickettsia* species other than *Rickettsia* 364D (5, 8), so this represents an inefficient method to screen large numbers of ticks for the pathogen. From a clinical perspective, evaluation of specimens using conventional PCR with sequence analysis can delay diagnostic confirmation of PCTF. The aim of this work was

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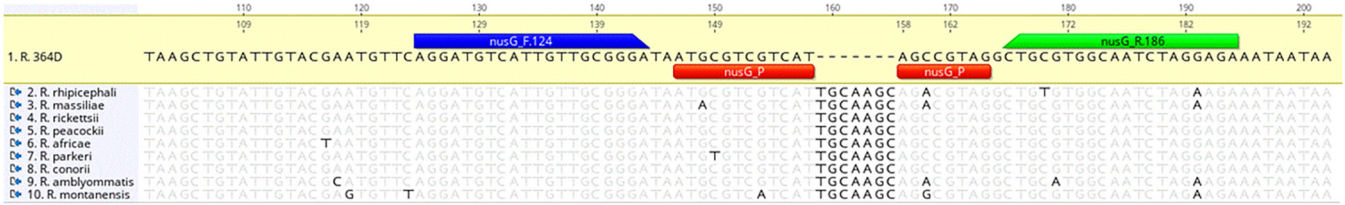

FIG 1 Primer and probe design. The TaqMan probe is designed to overlap a 7-bp gap in *Rickettsia* 364D compared with other closely related *Rickettsia* species. Blue, forward primer; green, reverse primer; red, FAM-labeled probe.

to develop a highly sensitive and specific assay that provides rapid and accurate diagnosis of PCTF in patients and facilitates large-scale entomological surveys to better define the occurrence and distribution of *Rickettsia* 364D in nature.

MATERIALS AND METHODS

Primer and probe design. During a previous investigation to examine intergenic regions as a method to differentiate isolates of *R. rickettsii*, a 7-bp deletion in the intergenic region between *nus*G and *rpl*K was identified in *Rickettsia* 364D that did not occur in any of the prototypical isolates of *R. rickettsii* (4). A comparison of this locus to nine other related spotted fever group (SFG) *Rickettsia* genomes (*Rickettsia rhipicephali*, *Rickettsia massiliae*, *R. rickettsii*, *Rickettsia peacockii*, *Rickettsia africae*, *Rickettsia parkeri*, *Rickettsia conorii*, *Rickettsia amblyommatis*, and *Rickettsia montanensis*) revealed that this deletion is unique to *Rickettsia* 364D. Geneious v10.2.2 (Biomatters Ltd.) was used to design primers nusG_F.124 (5′-AGG ATG TCA TTG TTG CGG GA-3′) and nusG_R.186 (5′-TCT CCT AGA TTG CCA CGC AG-3′), which produced a 62-bp amplicon spanning the deletion region. Probe nusG_P_FAM (6-carboxyfluorescein [FAM]-5′-ATG CGT CGT CAT AGC CGT AG-3′-BHQ1) was designed to overlap the deletion site (Fig. 1). All oligonucleotides were synthesized by the Biotechnology Core Facility Branch at the Centers for Disease Control and Prevention.

Assay optimization. DNA was extracted from Vero E6 cells infected with *Rickettsia* 364D (strain 364-D) using a KingFisher mL magnetic particle purification system and a KingFisher cell and tissue DNA kit (Thermo Fisher Scientific, Waltham, MA) and eluted in 150 μl elution buffer. The PCRs were run on a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA), and each reaction consisted of 400 nM (each) each primer, 200 nM probe, 10 μl QuantiTect probe master mix (Qiagen, Valencia, CA), 4.4 μl sterile PCR-grade water, and 4 μl template DNA in a final reaction volume of 20 μl. To determine the optimum annealing temperature of the assay, a gradient real-time PCR was performed using annealing temperatures from 55 to 65°C based on the estimated melting temperatures of the primers and probe.

Sensitivity and specificity testing. A control plasmid was constructed using an Invitrogen TOPO TA Cloning kit for sequencing (Thermo Fisher Scientific, Waltham, MA) and an amplicon from strain 364-D. To confirm the sequence of the clone, the insertion site was sequenced in both directions using primers T7 Promoter and M13 Reverse. Sequencing reads were assembled using Geneious v10.2 (Biomatters Ltd., Auckland, New Zealand). The plasmid concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The limit of detection of the assay was assessed using a 10-fold dilution series of the control plasmid, with concentrations ranging from 1,000,000 copies/4 μl to 1 copy/4 μl. To test the specificity of the assay, 92 individual DNA samples comprising 10 additional isolates of *Rickettsia* 364D (5), 27 isolates of *R. rickettsii*, 26 isolates of 24 other *Rickettsia* species, 10 strains of closely related bacteria and *Escherichia coli*, 9 tick species, and 8 cell lines were used (Table 1). All evaluations included a nontemplate control (sterile, PCR-grade water) and a positive control comprising the control plasmid.

To assess the clinical sensitivity of the assay, we evaluated sterile collection swabs containing whole blood spiked with live *Rickettsia* 364D bacteria to reproduce the most commonly used specimen collection technique for PCR (i.e., eschar swab). An aliquot of whole blood, obtained from an anonymous human donor from the Tennessee Blood Services was initially tested by the TaqMan assay to confirm that the sample was negative for *Rickettsia* 364D. Dilutions of cultured *Rickettsia* 364D (strain Lake) were inoculated into 100-μl aliquots of blood to achieve final concentrations of 1×10^6 to 1 organism/4 μl (10-fold dilution series), assuming a 100% recovery rate from the DNA extraction procedure. Each aliquot was then absorbed onto a Fisherbrand bacteriology culture collection and transport system swab (Thermo Fisher Scientific) and allowed to dry overnight. DNA was extracted from the swabs using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) as follows. The dried swabs were placed into vials containing 400 μl phosphate-buffered saline (PBS), 180 μl buffer ATL, and 20 μl proteinase K and incubated at 56°C for 3 hours with intermittent vortexing. The swabs were then removed from the vials, 400 μl buffer AL was added, and the samples were incubated at 70°C for 10 minutes. Four hundred microliters of 100% ethanol was then added to the samples, and the samples were put into spin columns and extracted following the manufacturer's directions, with the samples eluted in 200 μl buffer AE. The samples were tested in duplicate along with an extraction blank (whole blood alone), a no-template control, and a plasmid positive control.

An additional 61 DNA extracts, comprising 30 *D. occidentalis* ticks (Table 2) and 31 clinical specimens (Table 3), which were previously tested at the California Department of Public Health Viral and Rickettsial

TABLE 1 Reference DNAs used to test assay specificity

DNA sample
Rickettsia 364D 364-D^T
Rickettsia 364D Cache Creek Ridge
Rickettsia 364D Crystal Cove
Rickettsia 364D El Moro Canyon
Rickettsia 364D Highland Creek
Rickettsia 364D Lake
Rickettsia 364D Mt. Konocti
Rickettsia 364D Orange
Rickettsia 364D Pierce Canyon
Rickettsia 364D Pine Mountain
Rickettsia 364D Wright Peak
Rickettsia rickettsii 78-RL
Rickettsia rickettsii AZ-3
Rickettsia rickettsii Bitterroot
Rickettsia rickettsii Brazil
Rickettsia rickettsii BSF Bell
Rickettsia rickettsii BSF Rab1
Rickettsia rickettsii BSF-Si7
Rickettsia rickettsii Colombia
Rickettsia rickettsii Costa Rica
Rickettsia rickettsii Domino
Rickettsia rickettsii Gila
Rickettsia rickettsii Hauke
Rickettsia rickettsii Hino
Rickettsia rickettsii HLP-2
Rickettsia rickettsii Iowa
Rickettsia rickettsii Lost Horse Canyon
Rickettsia rickettsii Morgan
Rickettsia rickettsii Panama1
Rickettsia rickettsii PriceT
Rickettsia rickettsii Ripley
Rickettsia rickettsii RSMG
Rickettsia rickettsii Salt River
Rickettsia rickettsii São Paulo
Rickettsia rickettsii Sawtooth
Rickettsia rickettsii Sheila Smith^T
Rickettsia rickettsii Tarheel
Rickettsia rickettsii Taiaçu
Rickettsia amblyommatis WB-8-2^T
Rickettsia africae Z9-Hu^T
Rickettsia akari Kaplan^T
Rickettsia asemboensis NMRCii^T
Rickettsia asiatica IO-1^T
Rickettsia australis Cutlack
Rickettsia bellii 369-C^T
Rickettsia canadensis McKiel 24
Rickettsia conorii subsp. *indica*
Rickettsia conorii Malish 7^T
Rickettsia felis Marseille-URRFXCal2^T
Rickettsia honei TT118
Rickettsia massiliae Mtu1^T
Rickettsia montanensis OSU 85-930
Rickettsia monteiroi Intervales^T
Rickettsia parkeri Portsmouth
Rickettsia peacockii Rustic
Rickettsia rhipicephali Burgdorfer3-7-Female 6^T
Rickettsia rhipicephali Ect
Rickettsia sibirica 246^T
Rickettsia slovaca B^T
Rickettsia vini Breclav^T
Rickettsia typhi Wilmington^T
Rickettsia sp. strain SF143
Rickettsia sp. strain SF145
Rickettsia sp. Tillamook str. 23
Anaplasma phagocytophilum USG3
Ehrlichia canis Oklahoma^T

(Continued on next page)

TABLE 1 (Continued)

DNA sample
Ehrlichia chaffeensis Arkansas^T
Ehrlichia muris eauclairensis Wisconsin^T
Ehrilichia muris muris AS145^T
Orientia tsutsugamushi Karp^T
Bartonella elizabethae
Bartonella henselae Houston-1^T
Bartonella quintana
Bartonella vinsonii Berkofii
Amblyomma americanum
Amblyomma maculatum
Dermacentor andersoni
Dermacentor occidentalis
Dermacentor variabilis
Haemaphysalis leporispalustris
Ixodes pacificus
Ixodes scapularis
Rhipicephalus sanguineus
DH82 (canine macrophage cells)
EA.hy926 (human endothelial cells)
Escherichia coli
Human lung endothelium cells (HULECs)
Human dermal endothelium cells (HMECs)
Human aorta cells
ISE6 (*Ixodes scapularis* tick cells)
Vero E6 (African green monkey epithelial cells)
XTC-2 (African clawed frog cells)

Disease Laboratory (VRDL), were similarly evaluated using the same amplification parameters on an Applied Biosystems (AB) 7500 fast real-time PCR system (Thermo Fisher Scientific). Clinical specimens originated from 12 confirmed cases of PCTF, 2 confirmed cases of murine typhus, 1 confirmed case of RMSF, and 10 samples submitted for varicella-zoster virus screening. All specimens were tested using a

TABLE 2 *Dermacentor occidentalis* samples used to test assay specificity[a]

Specimen no.	Pan-*Rickettsia* real-time PCR result	Previous species identification	364D real-time PCR result
1	Detected	*Rickettsia* 364D	Detected
2	Detected	*Rickettsia* 364D	Detected
3	Detected	*Rickettsia* 364D	Detected
4	Detected	*Rickettsia* 364D	Detected
5	Detected	*Rickettsia* 364D	Detected
6	Detected	*Rickettsia* 364D	Detected
7	Detected	*Rickettsia* 364D	Detected
8	Detected	*Rickettsia* 364D	Detected
9	Detected	*Rickettsia* 364D	Detected
10	Detected	*Rickettsia* 364D	Detected
11	Detected	*R. rhipicephali*	Not detected
12	Detected	*R. rhipicephali*	Not detected
13	Detected	*R. rhipicephali*	Not detected
14	Detected	*R. rhipicephali*	Not detected
15	Detected	*R. rhipicephali*	Not detected
16	Detected	*R. rhipicephali*	Not detected
17	Detected	*R. rhipicephali*	Not detected
18	Detected	*R. rhipicephali*	Not detected
19	Detected	*R. rhipicephali*	Not detected
20	Detected	*R. rhipicephali*	Not detected
21	Detected	*R. bellii*	Not detected
22	Detected	*R. bellii*	Not detected
23	Detected	*R. bellii*	Not detected
24	Not detected	Not done	Not detected
25	Not detected	Not done	Not detected
26	Not detected	Not done	Not detected
27	Not detected	Not done	Not detected
28	Not detected	Not done	Not detected
29	Not detected	Not done	Not detected
30	Not detected	Not done	Not detected

[a]Shading indicates samples positive for *Rickettsia* 364D.

TABLE 3 Human DNA samples used to test assay specificity[a]

Sample no.	Specimen type	Diagnosis	Rickettsial real-time PCR result	Previous species identification	364D real-time PCR result
1	Lesion swab/scab	*Rickettsia* 364D	SYBR SFGR detected	*Rickettsia* 364D	Detected
2	Lesion swab	*Rickettsia* 364D	SYBR SFGR detected	*Rickettsia* 364D	Detected
3	Eschar	*Rickettsia* 364D	SYBR SFGR detected	*Rickettsia* 364D	Detected
4	Eschar	*Rickettsia* 364D	SYBR SFGR detected	*Rickettsia* 364D	Detected
5	Eschar	*Rickettsia* 364D	SYBR SFGR detected	Unable to determine	Detected
6	Lesion swab	*Rickettsia* 364D	PanR detected	*Rickettsia* 364D	Detected
7	Eschar	*Rickettsia* 364D	PanR detected	*Rickettsia* 364D	Detected
8	Eschar	*Rickettsia* 364D	PanR detected	N/A	Detected
9	Lesion swab	Varicella-zoster virus	Not done	N/A	Not detected
10	Skin scraping	Varicella-zoster virus	Not done	N/A	Not detected
11	Lesion swab	Varicella-zoster virus	Not done	N/A	Not detected
12	Lesion swab	Varicella-zoster virus	Not done	N/A	Not detected
13	Lesion swab	Varicella-zoster virus	Not done	N/A	Not detected
14	Lesion swab	Varicella-zoster virus	Not done	N/A	Not detected
15	Lesion swab	Varicella-zoster virus	Not done	N/A	Not detected
16	Lesion swab	Varicella-zoster virus	Not done	N/A	Not detected
17	Lesion swab	Varicella-zoster virus	Not done	N/A	Not detected
18	Lesion swab	Varicella-zoster virus	Not done	N/A	Not detected
19	Serum	*Rickettsia* 364D	Not done	N/A	Not detected
20	Serum	*Rickettsia* 364D	Not done	N/A	Not detected
21	Serum	*Rickettsia* 364D	Not done	N/A	Not detected
22	Serum	*Rickettsia* 364D	Not done	N/A	Not detected
23	Serum	*Rickettsia* 364D	Not done	N/A	Not detected
24	Serum	*Rickettsia* 364D	Not done	N/A	Not detected
25	Serum	*Rickettsia* 364D	Not done	N/A	Not detected
26	Serum	*Rickettsia* 364D	Not done	N/A	Not detected
27	Serum	*Rickettsia* 364D	Not done	N/A	Not detected
28	Serum	*Rickettsia* 364D	Not done	N/A	Not detected
29	Plasma	*R. typhi* (PCR)	PanR detected	*R. typhi*	Not detected
30	Serum	*R. typhi* (PCR)	PanR detected	*R. typhi*	Not detected
31	Whole blood	*R. rickettsii* (PCR confirmed at CDC)	PanR detected	Unable to determine	Not detected

[a]Shading indicates samples positive for *Rickettsia* 364D. N/A, not applicable; SFGR, spotted fever group *Rickettsia*; PanR, pan-*Rickettsia* real-time PCR.

two-assay algorithm that consisted of an initial screening using either a pan-*Rickettsia* real-time assay or an SFG *Rickettsia* real-time assay (11, 12). Species identification was then accomplished using a nested PCR assay and DNA sequencing of a segment of the *omp*A gene (2, 13, 14). An assay targeting the human RNAse P gene (15) was used to evaluate clinical specimens for the presence of substances that may interfere with the *Rickettsia* 364D PCR assay.

RESULTS

The positive-control plasmid was used to determine the optimal reaction conditions. Based on C_q values, the optimal annealing temperature was determined to be 61°C. The limit of detection was determined to be 1 copy per 4 μl, with an R^2 value of 0.992 and an amplification efficiency of 98.3% (Fig. 2).

To assess the specificity and accuracy of the real-time PCR assay, a total of 153 positive- and negative-control DNA samples were evaluated (Tables 1 and 2). The assay correctly detected all 21 *Rickettsia* 364D-positive samples comprising 11 unique isolates and 10 *Rickettsia* 364D-positive specimens of *D. occidentalis*. None of the 53 other rickettsial isolates (25 distinct species) and none of the 13 *D. occidentalis* ticks infected with either *R. rhipicephali* ($n = 10$) or *Rickettsia bellii* ($n = 3$) were detected. In a similar manner, the assay did not detect DNA from any of the 10 species in closely related genera, including *Ehrlichia*, *Anaplasma*, *Orientia*, and *Bartonella*; 9 species of hard ticks endemic to the United States; 8 uninfected *D. occidentalis*; 8 cell lines of human, animal, or tick origin; or *E. coli*.

To assess the clinical sensitivity of the assay, human whole blood was spiked with known concentrations of *Rickettsia* 364D and absorbed onto specimen collection swabs. The DNA was extracted, and the samples screened for the detection of rickettsial DNA. The clinical sensitivity was found to be between 100 and 1,000 genomic copies

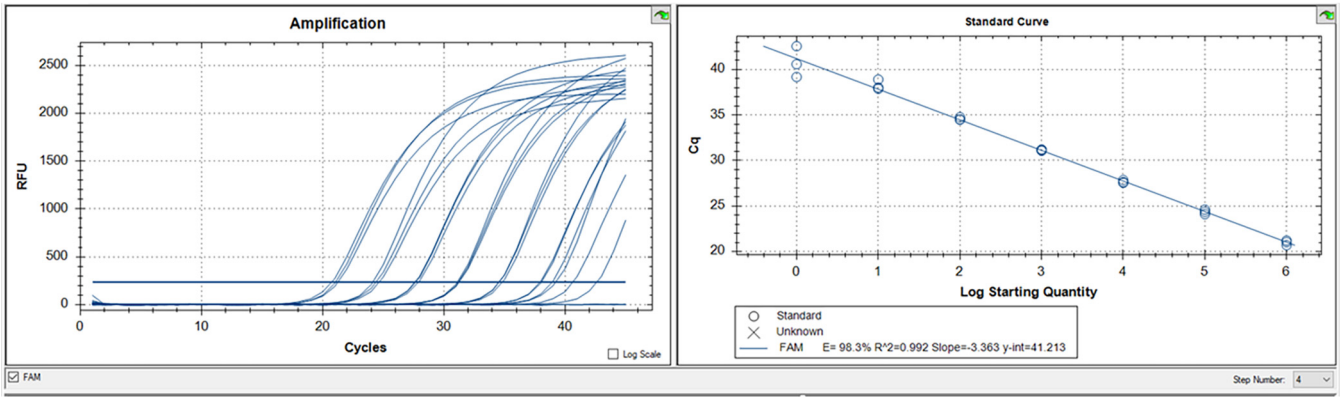

FIG 2 Evaluation of *Rickettsia* 364D-specific TaqMan assay sensitivity and efficiency. Standard curve produced using serial 10-fold dilutions of the control plasmid (1×10^6 copies/4 μl to 1 copy/4 μl). All samples were run in triplicate. The sensitivity of the assay was found to be 1 copy/4 μl, the R^2 value was 0.992, and the PCR efficiency was 98.3%.

per 4 μl, with an R^2 value of 0.992 and an amplification efficiency of 110.1% (results not shown).

To assess the clinical accuracy and specificity of the assay, DNA extracts of 31 clinical specimens were also evaluated. These included specimens from 8 cases of previously diagnosed PCTF (skin biopsy specimens or swabs) and an additional 13 serum, plasma, or whole-blood specimens from 13 cases of suspected or confirmed PCTF, endemic typhus, or RMSF. Eleven of these samples were tested with the 364D assay and a pan *Rickettsia* or an SFG *Rickettsia* real-time assay (the 10 PCTF serum samples were not previously tested using the pan *Rickettsia* assays). Of these 11 samples, all were positive for a *Rickettsia* species, including the 8 eschar swabs and biopsy specimens from 8 cases of PCTF, 2 blood specimens from confirmed cases of murine typhus, and 1 of RMSF. Using the *Rickettsia* 364D assay, only the eight eschar swabs and biopsy samples tested positive. Six of these samples were previously shown to be positive for *Rickettsia* 364D by DNA sequencing (sample 8 did not undergo DNA sequencing). However, a seventh sample, which was known to be positive for unidentified rickettsial DNA, was from a patient in whom the 2-assay algorithm had confirmed PCTF using another lesion swab. The remaining three samples tested negative with the new assay and were also negative for *Rickettsia* 364D using the 2-assay algorithm. Although *Rickettsia* 364D DNA has never been detected in patient blood, 10 serum samples from confirmed PCTF patients were screened using the new assay. Additionally, 10 lesion swabs obtained for varicella-zoster virus testing were also tested. All 20 samples were negative using the *Rickettsia* 364D assay. The RNase P target was detected in all 30 clinical specimens tested. Overall, both the clinical accuracy and specificity of the assay were 100% compared with previous results.

DISCUSSION

This work describes the development and application of a highly sensitive and specific TaqMan real-time PCR assay for *Rickettsia* 364D that provides a simplified molecular method to more rapidly evaluate samples for ecological studies and provide more timely and accurate results for the diagnosis of persons suspected to have PCTF. As tested, the clinical sensitivity of the assay (100 to 1,000 genomic copies per 4 μl) did not match the limit of detection of the assay (1 copy per 4 μl) when tested using the control plasmid. However, the extraction procedure was not optimized for the recovery of DNA from collection swabs, and no extraction procedure is 100% effective at recovering the entire input DNA quantity. Therefore, it is unknown what the actual concentration of rickettsial DNA was in each of the eluted samples and how these concentrations relate to the input concentration. Nevertheless, the novel TaqMan assay showed complete clinical concordance with the two-assay algorithm currently used at the VRDL when evaluated against a panel of 31 archival specimens of blood, serum,

plasma, eschar, and swab biopsy specimens. Current molecular methods for the detection of *Rickettsia* 364D rely on DNA sequencing of PCR amplicons (2, 3, 10); this new assay will facilitate the detection of *Rickettsia* 364D by providing a more rapid and cost-effective method than is currently available.

Much about the ecology of *Rickettsia* 364D remains unknown. To date, *D. occidentalis* is the only tick species in which *Rickettsia* 364D has been detected (5). Other human-biting tick species coexist with *D. occidentalis* in areas where *Rickettsia* 364D has been identified, and some of these species have been shown to be competent vectors of other, closely related rickettsial species. Additionally, more work needs to be conducted to determine which vertebrate hosts are associated with the transmission or maintenance of *Rickettsia* 364D in nature. A more thorough understanding of the natural history of *Rickettsia* 364D is needed to better define the public health significance posed by this pathogen. This novel assay provides a new tool to help answer these important ecological questions.

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