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Molecular and Cellular Probes 21 (2007) 17–23
Molecular and Cellular Probes
www.elsevier.com/locate/ymcpr

Development of quantitative real-time PCR assays to detect *Rickettsia typhi* and *Rickettsia felis*, the causative agents of murine typhus and flea-borne spotted fever

Katherine M. Henry[a], Ju Jiang[a], Patrick J. Rozmajzl[a], Abdu F. Azad[b], Kevin R. Macaluso[b,c], Allen L. Richards[a,*]

[a]*Rickettsial Diseases Department, Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, MD 20910-7500, USA*
[b]*University of Maryland School of Medicine, Baltimore, MD, USA*
[c]*Louisiana State University, Baton Rouge, LA, USA*

Received 16 May 2006; accepted 28 June 2006
Available online 1 July 2006

Abstract

Rickettsia typhi and *Rickettsia felis* are the etiologic agents of murine typhus and flea-borne spotted fever, respectively. We have constructed two quantitative real-time polymerase chain reaction (qPCR) assays to detect these pathogenic rickettsiae. The qPCR assays were developed utilizing unique sequences of the *R. typhi* and *R. felis* outer membrane protein B genes (*ompB*) to design the specific primers and molecular beacon probes. The assays were found to be species-specific and did not yield false-positive reactions with nucleic acid from other rickettsiae, orientiae, neorickettsiae or unrelated bacteria. In addition, the assays were sensitive enough to detect three target sequence copies per reaction and were capable of detecting *R. typhi* and *R. felis* nucleic acid in the cat flea, *Ctenocephalides felis*. These results demonstrate that two sensitive and specific qPCR assays have been successfully developed to detect and enumerate *R. typhi* and *R. felis*.
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Keywords: *Rickettsia typhi*; *Rickettsia felis*; Murine typhus; Flea-borne spotted fever; Quantitative real-time polymerase chain reaction

1. Introduction

Murine typhus (endemic typhus), caused by *Rickettsia typhi*, is characterized by headache, rash and fever. It is spread to humans through the inoculation of infected flea feces and is found throughout the world [1]. Murine typhus is particularly difficult to diagnose clinically, as its signs and symptoms are similar to those produced by many other diseases, including those caused by other rickettsiae. Moreover, serological assays to diagnose murine typhus are often not available, non-specific, insensitive and retrospective [2]. In response to these issues, polymerase chain reaction (PCR)-based assays for the detection of rickettsiae were developed. These assays successfully amplified rickettsial DNA out of nucleic acid isolated from the blood of patients suspected of having rickettsiosis and from arthropod vectors as visualized on gel electrophoresis [3–5]. Identification of this agent by conventional PCR assays requires a subsequent procedure, such as restriction fragment length polymorphism (RFLP) or nucleotide sequencing analysis [5,6]. To date there is no single-step PCR assay for the detection of *R. typhi*.

Flea-borne spotted fever (cat flea typhus), caused by *Rickettsia felis*, appears to be distributed worldwide. It has been diagnosed in humans from the USA [7], Mexico [8,9], Brazil and France [10], Spain [11,12], Germany [13], Thailand [14], Laos [15], Korea [16] and Tunisia [17]. This distribution is most likely due to the presence of its major vector: the cat flea, *Ctenocephalides felis*, which is found throughout the world. *R. felis* has been discovered in the tissues of its arthropod vectors in the USA [18,19], Mexico [20], Peru [21], Brazil [22], Spain [11,23], France [24,25], UK [26], Cyprus [27], Gabon [28], Algeria [25], Ethiopia

*Corresponding author. Tel.: +1 301 319 7668; Fax: +1 301 319 9458.
E-mail address: RichardsA@nmrc.navy.mil (A.L. Richards).

0890-8508/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.mcp.2006.06.002

[10], New Zealand [25,29], Thailand [30], Afghanistan [31] and Israel [32]. Flea-borne spotted fever presentation is similar to that of murine typhus and other rickettsial diseases making it difficult to diagnose clinically. Serologic diagnosis is similarly problematic at the time of illness due to the kinetics of the immune response [11,33]. However, PCR with RFLP and/or sequencing data has been used to detect *R. felis* in acute human samples utilizing multiple rickettsial genes such as the 17 kDa protein gene, *gltA*, *ompA*, *ompB* and *sca4*.

To overcome the difficult and time-consuming process of laboratory diagnosis of murine typhus and flea-borne spotted fever, quantitative real-time PCR (qPCR)-based assays were developed for the detection of *R. typhi* and *R. felis* DNA, and are described herein. These assays detect and discriminate *R. typhi* and *R. felis* from each other and from other disease-causing agents based on unique sequences in their outer membrane protein B gene (*ompB*). Optimal reaction conditions were determined by systematically varying the annealing temperature, as well as the magnesium, probe and primer concentrations of the assays. The optimized assays were found not only to be specific and sensitive, but capable of detecting *R. typhi* and *R. felis* DNA isolated from *C. felis* flea tissues.

2. Materials and methods

2.1. Selection of the primers and probes

Beacon Designer 2.0 software (Biosoft International, Palo Alto, CA) was used to identify *ompB* sequences suitable for use as PCR primers and molecular beacon probes. Following evaluation of the software data, optimal forward (Rt557F: 5′-TGG TAT TAC TGC TCA ACA AGC T-3′) and reverse (Rt678R: 5′-CAG TAA AGT CTA TTG ATC CTA CAC C-3′) primers and probe (Rt640BP: 5′-TET-CGC GAT CGT TAA TAG CAG CAC CAG CAT TAT CGC G-DABCYL-3′) for *R. typhi* and forward (Rf1396F: 5′-ACC CAG AAC TCG AAC TTT GGT G-3′) and reverse (Rf1524R: 5′-CAC ACC CGC AGT ATT ACC GTT-3′) primers and probe (Rf1448BP: 5′-FAM-CGC GAC TTA CAG TTC CTG ATA CTA AGG TTC TTA CAG GTC GCG-BHQ-1-3′) for *R. felis* [21] were selected and ordered from Sigma Genosys (The Woodlands, TX).

2.2. Determination of optimal real-time PCR conditions

Several real-time PCR assays were performed to determine the optimal primer, molecular beacon probe and magnesium concentrations. Master mixes containing ddH_2O, dNTPs, 10 × PCR buffer (Idaho Technology, Salt Lake City, UT), *R. typhi* or *R. felis* forward and reverse primers, *R. typhi* or *R. felis* molecular beacon probe, Platinum Taq DNA polymerase (Invitrogen, Grand Island, NY) and $MgCl_2$ (Invitrogen) were formulated and separated into 24 μl aliquots. One microliter of DNA template was added to each aliquot and subjected to real-time PCR in a SmartCycler Version 2 thermocycler (Cepheid, Sunnyvale, CA). A series of assays were performed where the $MgCl_2$ concentration varied by 0.5 mM increments and the primer and probe concentrations varied by 0.1 μM increments. Optimal PCR conditions were defined as the lowest concentration of *R. typhi* or *R. felis* DNA that produced a positive result. Using this metric, 5 mM $MgCl_2$, 0.3 μM probe and 0.2 μM primer concentrations were determined to be optimal for the *R. typhi* qPCR assay and 5 mM $MgCl_2$, 0.4 μM probe and 0.5 μM primer concentrations were optimal for the *R. felis* qPCR assay. The thermocycling parameters included an initial denaturation of 2 min at 94 °C followed by 50 cycles of denaturation (94 °C for 5 s) and annealing/elongation (60 °C for 30 s). Positive samples were defined as those that demonstrated fluorescence above background fluorescence set at a default value of 25 fluorescence units. Positive reactions were assigned a cycle threshold (Ct) value equal to the cycle number where the fluorescence observed in the experimental tube was first detectable above background. Template-free controls assayed at the same time and under the same conditions as the experimental and positive control samples were consistently negative.

2.3. Determination of assay specificity

To determine the ability of the molecular beacon probes to distinguish between *R. typhi* and *R. felis* DNA and that of other rickettsial and non-rickettsial bacteria DNA, each was used with their corresponding primers in a series of amplification reactions that were seeded with various target and non-target template DNAs (Table 1). DNA preparations from the panel of organisms representing related and unrelated bacteria were used to determine specificity of the assays as previously described [34]. The bacteria included: *R. prowazekii* Breinl and Ananiev, *R typhi* Wilmington and Museibov, *R. rickettsii* R (ATCC VR891), *R.* sp. 364-D, *R. conorii* Malish 7 (ATCC VR613), *R. montanensis* OSU 85-930, *R. parkeri* C, *R. slovaca* Arm25, *R. sibirica* 246, *R. japonica* NK, *R. akari* #29 (ATCC VR612), *R. felis* California 2, *R. canadensis* MK-29, *Orientia tsutsugamushi* Karp, *Neorickettsia risticii* Illinois, *N. sennetsu*, *Francisella persica*, *Bartonella quintana*, *B. vinsonii*, *Legionella pneumophila*, *Proteus mirabilis*, *Salmonella enterica*, *Escherichia coli*, *Corynebacterium* sp. and *Staphylococcus aureus*.

2.4. Determination of assay sensitivity

To determine the sensitivity of the *R. typhi* and *R. felis* qPCR assays, serial dilutions of known concentrations of *R. typhi* fragment A of *ompB* in plasmid pET24a and *R. felis ompB* target sequence ligated into a TOPO TA plasmid (Invitrogen), respectively, were assayed and standard curves produced (Fig. 1).

Table 1
Specificity of 17 kDa, *R. typhi* and *R. felis* quantitative real-time PCR assays

Rickettsia species	17 kDA qPCR	*R. typhi* qPCR	*R. felis* qPCR	Non-*Rickettsia* species	*17 kDA* qPCR	*R. typhi* qPCR	R. felis qPCR
R. prowazekii Breinl	+	–	–	*Orientia tsutsugamushi*	–	–	–
R. prowazekii Ananiev	+	–	–	*Neorickettsia sennetsu*	–	–	–
R. typhi Wilmington	+	+	–	*N. risticii*	–	–	–
R. typhi Museibov	+	+	–	*Francisella persica*	–	–	–
R. rickettsii R	+	–	–	*Bartonella quintana*	–	–	–
R. sp. 364-D	+	–	–	*B. vinsonii*	–	–	–
R. conorii Malish 7	+	–	–	*Legionella pneumophila*	–	–	–
R. montanensis OSU 85-930	+	–	–	*Proteus mirabilis*	–	–	–
R. parkeri C	+	–	–	*Salmonella enterica*	–	–	–
R. slovaca Arm25	+	–	–	*Escherichia coli*	–	–	–
R. sibirica 246	+	–	–	*Staphylococcus aureus*	–	–	–
R. japonica NK	+	–	–	*Corynebacterium* sp.	–	–	–
R. akari #29	+	–	–				
R. felis California 2	+	–	+				
R. canadensis MK–29	+	–	–				

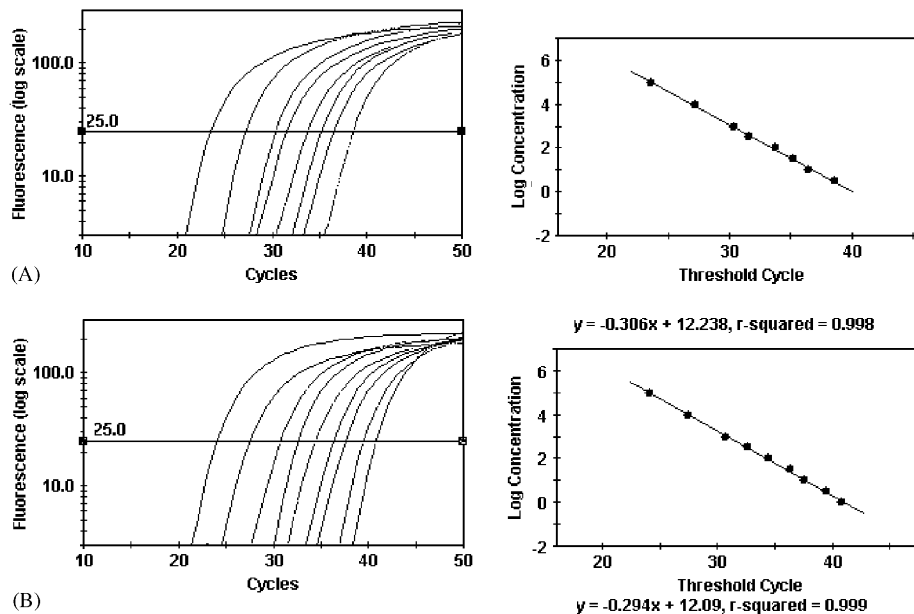

Fig. 1. (A) Standard curve for *Rickettsia typhi* qPCR assay. Assay utilized concentrations of *R. typhi* fragment A of *ompB* in plasmid pET24a target sequences ranging from 10^5 to 0.3 copies/μl. These results showed that the fluorescence above background (positive response) was seen with target DNA sequence concentrations as low as 3 copies/μl produced positive responses. (B) Standard curve for *Rickettsia felis* qPCR assay. The qPCR assay utilized concentrations of *R. felis ompB* target sequences in TOPO TA plasmid ranging from 10^5 to 0.3 copies/μl to show that fluorescence above background (positive response) was seen with target DNA sequence concentrations as low as 1 copy/μl.

2.5. Evaluation of nucleic acid extraction procedures with spiked fleas

Known uninfected *C. felis* [35] (a gift from the Heska Corp., Loveland, CO) were washed by first placing them in 70% ethanol, gently vortexing and removing the liquid by pipette. The wash was repeated with distilled water. Individual fleas were then spiked with *R. felis ompB* target sequence ligated into TOPO TA plasmid at seven final concentrations (10^4, 10^3, 10^2, $10^{1.5}$, 10^1, $10^{0.5}$ and 10^0 copies/μl). The spiked fleas were processed by each of the following extraction procedures.

2.5.1. Qiagen

Individual fleas were placed in seven separate 1.5 ml microcentrifuge tubes. Each tube contained 10 μl of a different concentration of *R. felis ompB* target sequence (10^5, 10^4, 10^3, $10^{2.5}$, 10^2, $10^{1.5}$ and 10^1 copies/μl). The fleas were crushed using the end of a pipette tip. DNA was extracted from the *R. felis* triturates using the DNeasy

Tissue Kit Protocol for Animal Tissues (QIAGEN; Valencia, CA) according to the manufacturer's instructions except that the elution was performed with 100 μl instead of 200 μl.

2.5.2. PrepMan Ultra

Aliquots were made of the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA) in order to reduce contamination from repeated opening of the stock bottle. Individual fleas were placed in five separate 1.5 ml microcentrifuge tubes each containing 5 μl of a different concentration of *R. felis* target sequence (10^5, 10^4, 10^3, $10^{2.5}$, 10^2, $10^{1.5}$, 10^1 copies/μl). The fleas were crushed in the *R. felis* solution using the end of a pipette tip. DNA was then extracted using 45 μl of the PrepMan Ultra Sample Preparation Reagent following the manufacturer's protocol, except that the elution was performed with 50 μl instead of 200 μl.

2.5.3. Water

Seven fleas were placed in separate 1.5 ml microcentrifuge tubes containing 1 μl of the same concentrations as used in the Qiagen and PrepMan Ultra methods. Nine microliters of distilled water (Invitrogen) was added, the fleas were crushed and the solution was boiled for 10 min.

Qiagen, PrepMan Ultra and water were tested three times each at the same time to compare their effectiveness in extracting rickettsial DNA without inhibitors. One microliter of template was added to 24 μl of the *R. felis* qPCR master mix in each reaction tube that had been previously prepared and stored at 4 °C while extracting the DNA. All three methods of DNA extraction were tested with *R. felis* qPCR assay using the Cepheid SmartCycler system.

2.6. Infected fleas preparations

2.6.1. Louisiana State University (LSU) fleas

Cat fleas, from laboratory colonies maintained at the LSU Agriculture Center Research Station derived from EL Labs, Soquel, CA [19,36], were obtained as DNA preparations (triturates of individual fleas in 30 μl of water were boiled for 10 min) and were then evaluated blindly for the presence of either *R. typhi* or *R. felis.* DNA was re-extracted using 50 μl of PrepMan Ultra sample preparation reagent.

2.6.2. University of Maryland at Baltimore (UMAB) fleas

C. felis whole fleas and triturates of whole fleas from a laboratory colony (Flea Data Inc., Freeville, NY) known to be positive for *R. felis* [10] were used as positive controls for the evaluation of 18 *C. felis* collected from opossums live captured in Corpus Christi, Texas [37]. Lysates from these 18 fleas plus controls were evaluated for the presence or rickettsial DNA.

2.7. qPCR assays

Reaction mixtures had a total volume of 25 μl and contained 1 μl of DNA template (described above). The reaction mixtures for both assays contained 0.2 mM dNTP, 4 mM $MgCl_2$, 1.5 U TaKaRa Taq and 25 mM HEPES buffer from OmniMix HS beads (Cepheid). In addition, the reaction mixture for *R. felis* contained 1.25 μl (10 μM) of Rf1396F (forward primer), 1.25 μl (10 μM) Rf1524R (reverse primer), 1.0 μl (10 μM) Rf1448BP (probe) and 0.5 μl (50 μM) $MgCl_2$. The reaction mixture for *R. typhi* contained 0.75 μl (10 μM) Rt557F (forward primer), 0.75 μl (10 μM) Rt678R (reverse primer), 0.5 μl (10 μM) Rt640BP (probe) and 0.5 μl (50 μM) $MgCl_2$. The master mix was prepared in a separate clean room (DNA-free). Two clean room negative controls were produced in which 1 μl of ddH_2O was added to the master mix. DNA template was added in a biosafety cabinet in a separate lab, where two additional negative controls were produced using 1 μl ddH_2O added in place of DNA template. qPCR reactions were incubated at 94 °C for 2 min followed by 50 cycles of a two-step amplification of 94 °C for 5 s and 60 °C for 30 s.

2.8. Data analysis

Fluorescence was monitored during each cycle at the annealing step and data were analyzed with SmartCycler software (ver 2.0c).

3. Results

3.1. Specificity of assays

The *R. typhi* and *R. felis* assays were initially optimized using *R. typhi* Wilmington and *R. felis* California 2, respectively. To determine the specificity of these two assays, additional rickettsial and other bacterial DNA preparations were used. Only *R. typhi* Wilmington and Museibov strains were positive by the *R. typhi* qPCR assay and only the *R. felis* California 2 strain was positive by the *R. felis* qPCR assay. All other related and unrelated non-target DNA preparations were negative (Table 1).

3.2. Sensitivity of qPCR assays

To determine the sensitivity of the *R. typhi*-specific qPCR assay, we used serial dilutions of a solution of *R. typhi* fragment A of *ompB* in plasmid pET24a ranging from 10^5 to 0.3 copies/μl. Reproducibly of the qPCR assay showed fluorescence levels above background with target sequence concentrations as low as 3 copies/μl. Similarly, we evaluated the sensitivity of the *R. felis* qPCR assay utilizing a standard curve developed with serial dilutions of a solution of the *R. felis ompB* target sequence ligated into TOPO TA plasmid. This assay routinely detected a single copy per microliter of the target sequence (Fig. 1).

3.3. DNA extraction assays

To ascertain which DNA extraction procedure would yield DNA preparations demonstrating the lowest inhibition of PCR, three procedures were assessed. Both PrepMan Ultra and the DNeasy Tissue Kit provided consistent results near that of the positive control (Table 2). However, utilizing boiling water alone was a very insensitive procedure for extracting *R.* felis DNA from spiked flea triturates. Because of the ease of use, the PrepMan Ultra extraction procedure was used in all further assays.

3.4. Evaluation of qPCR assays with flea vectors

DNA prepared from individual or pooled fleas by PrepManUltra nucleic acid sample preparation kit were screened for the presence of rickettsial DNA by the genus-specific 17 kDa common antigen gene qPCR assay [34]. Rickettsial DNA was not detected in 15 *C. felis* fleas from Heska, but was detected in four of 18 *C. felis* fleas from UMAB, and 22 of 22 *C. felis* fleas from LSU. To determine whether *R. typhi*, *R. felis* or both infected these fleas, real-time PCR assays for *R. typhi* and *R. felis* were performed. The results of these assays showed that all 15 *C. felis* fleas from Heska were negative for both *R. typhi* and *R. felis*; all 22 of the *C. felis* fleas from LSU were positive for *R. felis*, but negative for *R. typhi*; and one and three of the four 17 kDa PCR-positive *C. felis* fleas from UMAB were positive for *R. typhi* and *R. felis*, respectively (Table 3).

4. Discussion

Early studies showed the benefit of using PCR to diagnose rickettsioses [3,4]. More recently and specifically, PCR assays have been used to diagnose murine typhus and flea-borne spotted fever [7–9,12]. PCR assays have also been used alone (*Rickettsia*-specific) or with RFLP and nucleotide sequencing to detect *R. typhi* [5,35,38,39] and *R. felis* [8,9,11,16,19,20,24,27,31,32,35,40] in human tissue, rodent blood and flea vectors. Recently, a quantitative rickettsia-specific PCR assay has also been used to evaluate antibiotic susceptibility of *R. typhi*, *R. felis* and *R. conorii* [41]. In contrast to the these aforementioned assays, the assays described herein have the distinct advantage of being able to detect specifically *R. typhi* and *R. felis* in single-step procedures that are easy and quick to perform. Evaluation of these assays on fleas known to be free of [35,40] and naturally infected with [18] rickettsiae clearly demonstrates their utility. The three rickettsial assays did not recognize DNA in triturates of fleas known to be free

Table 2
Evaluation of nucleic acid extraction procedures with spiked fleas

DNA preparations	Ct values for			Average (SD)
	Exp. 1	Exp. 2	Exp. 3	Ct values
Control[a]	34.17	34.56	34.10	34.28 (+0.24)
Qiagen[b]	34.98	35.86	37.74	36.19 (+1.41)
Prepman Ultra[c]	35.79	36.07	35.57	35.81 (+0.25)
Water[d]	>50	>50	>50	>50

[a]Control DNA preparations consisted of 100 copies/reaction of the *R. felis ompB* target sequence ligated into a TOPO TA plasmid without a flea or extraction procedure.
[b]Qiagen DNA preparations consisted of 100 copies/reaction of extracted DNA from *R. felis ompB* target sequence ligated into a TOPO TA plasmid spiked into a single uninfected *C. felis* flea from Heska treated with the Qiagen DNeasy Tissue Kit protocol.
[c]Prepman Ultra DNA preparations consisted of 100 copies/reaction of extracted DNA from *R. felis ompB* target sequence ligated into a TOPO TA plasmid spiked into a single uninfected *C. felis* flea from Heska treated with the Prepman Ultra sample preparation procedure.
[d]Water DNA preparations consisted of 100 copies/reaction of extracted DNA from the *R. felis ompB* target sequence ligated into a TOPO TA plasmid spiked into a single uninfected *C. felis* flea from Heska boiled in water for 10 min.

Table 3
Detection of *R. typhi* and *R. felis* in flea samples

Source	Flea species	Number of fleas tested	Number positive		
			17 kDa qPCR	*R. typhi* qPCR	*R. felis* qPCR
Heska[a]	*C. felis*	15	0	0	0
UMAB[b]	*C. felis*	18	4	1	3
LSU[c]	*C. felis*	22	22	0	22

[a]*Rickettsia*-free laboratory-reared fleas from the Heska corporation, Loveland, CO.
[b]Fleas collected off live captured Opossums from Corpus Christi, TX.
[c]Fleas from a laboratory colony at Louisiana State University, known to be infected with *R. felis*.

of rickettsiae; whereas, the genus-specific 17 kDa and the *R. felis* qPCR assays detected rickettsial DNA in all 18 fleas assessed from a *R. felis* infected colony. Previous studies have reported that 62–100% of cat fleas from EL Labs, [18,42], 43–93% of cat fleas from eight commercial flea colonies [18] and 86–90% of cat fleas from the Louisiana colony [35] are naturally infected with *R. felis*. Moreover, in one set of experiments 75%, 86%, 94% and 100% of the LSU fleas tested were shown to be infected with *R. felis* [35]. Most recently evaluation of the 18 flea triturates at LSU by a standard PCR assay detected the *Rickettsia*-specific 17 kDa gene in 17 of 18 reactions (data not shown). These results, plus the detection of two different rickettsial genes (*ompB* and the 17 kDa gene) in the infected but not the uninfected fleas demonstrate the accuracy of the assay.

C. felis obtained from a previous epidemiological study [37] were evaluated to show the validity of using these new assays to detect *R. typhi* and *R. felis* in arthropod vectors. Of the 18 fleas collected from opossums captured in Corpus Christi, Texas four were positive for rickettsial agents and of these one and three were positive for *R. typhi* and *R. felis*, respectively. These results are similar to other studies that reported infection rates for rickettsiae in individual fleas and pooled specimens collected in nature (1–20%), which are much less than that found in commercial laboratory flea colonies [20,24,32,33,37]. It is unknown whether there is a benefit imparted to the fleas by infection with *R. felis* in artificial settings. However, it was found that *R. felis* becomes rapidly established in laboratory colonies of cat fleas [19].

Though these assays have yet to be assessed with human samples, the *R. felis* assay was able to detect *R. felis* DNA in rodent blood collected during an outbreak of febrile disease believed to be partially due to SFG rickettsiosis [21]. Similarly, developed qPCR assays have detected *O. tsutsugamushi* and SFG rickettsial DNA in human samples [43,44]. Therefore, it is believed that the assays described in this report could be eventually used to diagnose murine typhus and flea-borne spotted fever in humans.

This report describes the successful development, optimization, sensitivity, specificity and utility of two qPCR assays in detecting *R. typhi* and *R. felis*. These assays performed well with both laboratory-reared and wild-caught *C. felis*, an arthropod vector of murine typhus and flea-borne spotted fever and wild-caught *X. cheopis* [45]. Like other real-time PCR assays, they may be useful in detecting and enumerating rickettsiae in arthropod vectors and vertebrate hosts.

Acknowledgements

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the US Government. This work was supported in part by DoD work unit number: 847705.82000.25GB.A0074 and NIAID Grant# AI R37 17828 (AFA). This work was funded and completed by employees of the US Government and therefore under Title 17 USC. 105 copyright protection under this title is not available.

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