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Real-time multiplex PCR assay for detection and differentiation of rickettsiae and orientiae

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Summary The high incidence of rickettsial diseases in Southeast Asia necessitates rapid and accurate diagnostic tools for a broad range of rickettsial agents, including *Orientia tsutsugamushi* (scrub typhus) and *Rickettsia typhi* (murine typhus), but also spotted fever group infections, which are increasingly reported. We present an SYBR-Green-based, real-time multiplex PCR assay for rapid identification and differentiation of scrub typhus group, typhus group and spotted fever group *rickettsiae* using 47 kDa, gltA and ompB gene targets. Detection limits for amplification of these genes in reference strains ranged from 24 copies/μl, 5 copies/μl and 1 copy/μl in multiplex and 2 copies/μl, 1 copy/μl and 1 copy/μl in single template format, respectively. Differentiation by melt-curve analysis led to distinct melt temperatures for each group-specific amplicon. The assay was subjected to 54 samples, of which all cell-culture and 75% of characterised clinical buffy coat samples were correctly identified. Real-time PCR has the advantage of reliably detecting and differentiating rickettsial and oriential cell-culture isolates in a single-template assay, compared with the more time-consuming and laborious immunofluorescence assay. However, further optimisation and validation on samples taken directly from patients to assess its clinical diagnostic utility is required.

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1. Introduction

The high incidence of rickettsial diseases in Southeast Asia, coupled with the problems of interpreting conventional diagnostic serology results and the low sensitivity of in vitro culture, necessitates the need for new rapid and accurate diagnostic tools for a broad range of rickettsial agents. Rickettsioses are responsible for a serious burden of disease worldwide, and knowledge about the extent of morbidity and associated mortality in warmer climate zones, such as Asia and the Western Pacific region, is limited. In these highly populous areas, *Orientia tsutsugamushi* (scrub typhus) and *Rickettsia typhi* (murine typhus) infections are the dominant rickettsial pathogens (Phongmany et al., 2006; Suttinont et al., 2006), although spotted fever group infections are increasingly reported (Choi et al., 2005; Jiang et al., 2005; Parola et al., 2003a).

These epidemiological data are based on the current diagnostic gold standard of serology, which unfortunately lacks sensitivity and specificity (Blacksell et al., 2007). Recent serological results suggest that dual infections with scrub typhus are not uncommon in tropical fever patients, often treated empirically for malaria (Singhsilarak et al., 2006). However, these findings need confirmation by prospective studies aimed at directly identifying the causative organism. Current gold standard serology requires specially trained personnel and infrastructure, and culture of *rickettsiae* from blood in the early phase of infection is desirable but difficult, as cell-culture facilities are required. The majority of infections go undiagnosed through a lack of effective diagnostic tools (Watt and Parola, 2003). Genomic or antigenic detection methods for *rickettsiae*, including PCR-based methods, are attractive as they not only circumvent the need for culture, but also because they offer potentially higher sensitivity and specificity and allow for direct detection of rickettsial DNA in samples as diverse as patient's blood (Jiang et al., 2004; Stenos et al., 2005), skin biopsies (Fournier and Raoult, 2004; Lee et al., 2006) and in arthropods (Hirunkanokpun et al., 2003; Kelly et al., 1994). PCR amplification of eschar/skin or blood samples also has the advantage of being able to detect infection before cell culture is positive or seroconversion has occurred, especially important in endemic areas where high levels of background antibodies pose a challenge for serology.

Here we describe an efficient and rapid single-tube, multiplex real-time PCR method for the detection of clinical and in vitro cultivated rickettsial samples and differentiation into the conventional groupings of scrub typhus (STG), typhus (TG) and spotted fever (SFG) groups.

2. Materials and methods

2.1. Real-time PCR

A pan-rickettsial PCR assay to detect and differentiate all known rickettsiae would optimally target a single gene, producing amplicons diverse enough to allow differentiation into antigenic groups or even species. Unfortunately, a single rickettsial gene with sufficient conservation to permit universal amplification, but enough variation to fulfil these conditions, remains to be discovered. To cover a sufficiently broad spectrum of pathogens and to allow for differentiation into subgroups, a multiplexed combination of three target genes was chosen: the citrate synthase gene (gltA) (Roux et al., 1997; Stenos et al., 2005) to detect TG and SFG rickettsiae; the outer membrane protein B (ompB) (Blair et al., 2004; Parola et al., 2003a,b) to detect SFG rickettsiae; and the 47 kDa outer surface protein/antigen, also known as the 'high temperature requirement A gene' (47 kDa) (Jiang et al., 2004) to detect STG orientiae. The primer sets of two previously described real-time PCR assays, based on the use of fluorescent probes (Table 1) specific for gltA (Stenos et al., 2005) and 47 kDa (Jiang et al., 2004) genes were used, but without the specific probes (Taq-Man probe for 47 kDa and FAM-BHQ probe for gltA). Detection was instead based on intercalating SYBR Green, with differentiation of the products using fluorescence-change of dissociating dsDNA amplicons at characteristic temperatures (melt-curve analysis). An additional set of primers specific for the ompB gene of SFG was designed for this study using PrimerSelect Version 6.1 software (DNAStar, Madison, WI, USA). The in-silico design of the ompB primers included coverage for

Table 1 Description of primers used in this study

Primer[a]	Gene length (bp)	Primer (5′ to 3′)	bp	T_m (°C)	dT_m (°C)	GC content (%)	Product size (bp)
gltA—F	approx. 1300	TCGCAAATGTTCACGG TACTTT	22	54.0	1.1	40.9	74
gltA—R		TCGTGCATTTCTTTCC ATTGTG	22	55.1		40.9	
ompB—F	approx. 4900	CGACGTTAACGGTTT CTCATTCT	23	54.3	0.5	43.5	252
ompB—R		ACCGGTTTCTTTGTAG TTTTCGTC	24	54.8		41.7	
47 kDa—F	approx. 1400	AACTGATTTTATTCAAA CTAATGCTGCT	28	54.8	1.0	28.6	118
47 kDa—R		TATGCCTGAGTAAGATA CRTGAATRGAATT	30	55.8		36.7	

T_m: melt temperature of the product amplicon; dT_m: delta or difference of melt temperatures within the primer pairs; GC: guanine and cytosine.
[a] Name of the gene with either forward (F) or reverse (R) primer.

19 different SFG rickettsiae (*R. rickettsii*, *R. conorii*, *R. honei*, *R. australis*, *R. felis*, *R. heilongjiangensis*, *R. helvetica*, *R. akari*, *R. amblyommii*, *R. hulinensis*, *R. Israeli TT*, *R. japonica*, *R. massiliae*, *R. mongolotimonae*, *R. parkeri*, *R. rhipicephali*, *R. sibirica*, *R. slovaca*, Astrakhan spotted fever and *R. africae*). *Rickettsia aeschlimanii*, *R. peacockii* and *R. bellii* could not be included in the target spectrum. The amplicon sizes were 74 bp for gltA, 118 bp for 47 kDa and 252 bp for ompB genes. Each PCR reaction contained 7.2 μl of a prepared primer mix (with final concentrations of each primer being 70 nM for 47 kDa, 60 nM for gltA and 50 nM for ompB), 2 μl extracted DNA, 10 μl Mastermix (QuantiMix Easy; Biotools, Madrid, Spain) containing SYBR Green, Taq polymerase, $MgCl_2$ 4 mM, dNTPs and water to a final volume of 20 μl. The PCR reactions were performed and analysed using a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) real-time thermocycler, with an initial holding temperature of 95 °C for 5 min, followed by 37 cycles of 95 °C for 10 s, 54 °C for 15 s and 72 °C for 20 s with fluorescence monitoring at the 54 °C annealing step on a predetermined SYBR/FAM channel. Melt-curve analysis was performed with increments of 1 °C/30 s (72–95 °C) to determine peak fluorescence change over time (dF/dT). Positive results were confirmed by electrophoresis of the product on a 3% (w/v) agarose gel in TAE buffer and stained with ethidium bromide (BioRad, Hercules, CA, USA).

2.2. Clinical samples

Twelve clinical buffy coat samples were prospectively collected, nine from patients with a positive scrub typhus IgM and IgG cassette-based rapid immunochromatographic test (PanBio, Queensland, Australia) (Wilkinson et al., 2003) on admission in northeast Thailand ($n = 6$) and Laos ($n = 3$) and three from Australia, provided by the Australian Rickettsial Reference Laboratory (ARRL), Geelong Hospital, Melbourne. On admission, patients gave written informed consent and a total of 5 ml full blood was collected into EDTA, the buffy coat immediately separated, aliquotted and the tubes stored at −80 °C until DNA extraction was performed. The samples were characterised by using real-time PCR detec-

Table 2 Differentiation and range of rickettsial group-specific melt temperatures derived from melt-curve analysis (SYBR Green first derivative plot) of resulting DNA amplicons from the multiplex real-time PCR assay with templates derived from cell-culture isolates

Reference organism	Product (range) T_m (°C)	Overall average (range) T_m (°C)
O. tsutsugamushi UT76	78.3 (78.2–78.4)	79.0 (78.2–79.8)
O. tsutsugamushi UT125	79.0 (78.8–79.3)	
O. tsutsugamushi UT144	78.3 (78.2–78.5)	
O. tsutsugamushi UT150	79.2 (78.9–79.4)	
O. tsutsugamushi UT167	79.0 (78.9–79.1)	
O. tsutsugamushi UT169	79.0 (79.0–79.1)	
O. tsutsugamushi UT176	79.0 (78.9–79.1)	
O. tsutsugamushi UT177	79.1 (79.0–79.2)	
O. tsutsugamushi UT196	79.0 (78.9–79.2)	
O. tsutsugamushi UT213	79.7 (79.5–79.8)	
O. tsutsugamushi UT219	79.2 (79.0–79.3)	
O. tsutsugamushi UT302	79.0 (78.9–79.2)	
O. tsutsugamushi UT316	79.0 (78.8–79.1)	
O. tsutsugamushi UT332	79.0 (78.9–79.1)	
O. tsutsugamushi UT336	79.0 (78.9–79.2)	
O. tsutsugamushi UT340	79.0 (78.8–79.1)	
O. tsutsugamushi UT395	79.6 (79.5–79.8)	
O. tsutsugamushi UT418	79.0 (78.8–79.2)	
O. tsutsugamushi UT497	78.3 (78.2–78.5)	
O. tsutsugamushi FPW2016	78.5 (78.4–78.7)	
O. tsutsugamushi FPW2031	79.7 (79.6–79.8)	
O. tsutsugamushi FPW2049	78.7 (78.6–78.9)	
R. typhi – Wilmington strain	81.4 (81.3–81.5)	81.5 (81.3–81.5)
R. prowazekii – Breinl strain	81.5 (81.4–81.5)	
R. conorii – Malish strain	84.8 (84.6–84.9)	84.7 (84.4–85.0)
R. honei – RB strain	84.6 (84.4–84.7)	
R. akari – Kaplan strain	84.9 (84.7–85)	
R. australis – JC strain	84.4 (84.4–84.5)	
R. rickettsii – Bitterroot strain	84.8 (84.6–84.9)	
R. sibirica – 246 strain	84.9 (84.7–85.0)	
R. bellii	–[a]	

T_m: melt temperature of the product amplicon.
[a] *Rickettsia belli* not included in target spectrum of spotted fever group primer set.

tion of the 47 kDa and gltA genes as well as conventional PCR amplification of the 56 kDa gene for scrub typhus (Horinouchi et al., 1996). The cell-culture isolates underwent 16S rRNA gene amplification (Fournier et al., 2003), and commercial DNA sequencing (Macrogen, Seoul, Korea) was performed on the 16S rRNA amplicons, using BigDyeTM terminator cycling conditions on an automated nucleotide sequencer model 3730xl (Applied Biosystems, Foster City, CA, USA), followed by BLAST analysis (sequences of approx. 1350 bp) to confirm *O. tsutsugamushi* as the infecting agent in all these cases. Paired serum samples from these patients were assessed using the gold standard immunofluorescence assay (IFA), for the detection of IgM antibodies (Luksameetanasan et al., 2007). The criterion for serological positivity in STG and TG patients was a four-fold rise in paired samples where available, or a single titre $\geq$25 600 (Brown et al., 1983) using slides provided by the ARRL. For SFG patients, gold standard IFA for the detection of IgM antibodies was performed by the ARRL using a 1:128 cut-off level on admission samples and positive real-time PCR targeting the gltA gene.

2.3. Samples from cell culture

The 22 *O. tsutsugamushi* isolates, cultured from patient samples in VERO cells were confirmed as *O. tsutsugamushi* by positive IFA as well as positive PCR detection of the 47 and 56 kDa genes. The TG and SFG *rickettsiae* were characterised reference strains (Table 2) provided by the ARRL.

2.4. Negative controls

Members of the families Anaplasmataceae, Bartonellaceae and Coxiellaceae (*Anaplasma phagocytophilum*, *Bartonella bacilliformis*, *B. henselae*, *B. vinsonii*, *Ehrlichia chaffeensis* and *Coxiella burnetii*), kindly provided by the ARRL as well as non-rickettsial agents (*Burkholderia pseudomallei*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Salmonella typhi*), served as negative controls.

2.5. Sensitivity of the assay

To determine the detection limits of the assay, plasmids were generated from the amplified regions of gltA (*R. typhi* Wilmington strain), 47 kDa (*O. tsutsugamushi* UT76 strain) and ompB (*R. conorii* Malish strain) genes by ligation and transformation into chemically competent *E. coli* (pGEM-T Easy Vectors; Promega, Madison, WI, USA) grown in a shaking incubator at 37 °C in Luria-Bertani broth, supplemented with ampicillin (100 μg/ml) and extracted with QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). The DNA was purified and linearised by restriction enzyme digestion with *Pst*I (Promega, USA). DNA from the linearised plasmids was quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Molecular Probes, Eugene, OR, USA). Ten-fold dilution series served as external controls and the theoretical number of plasmid copies and corresponding reaction efficiencies were calculated (Rotor-Gene Software Version 6.0, Corbett Research, Australia). Duplicates of each serial dilution underwent real-time PCR to create a standard curve. All assays were validated and optimised as single-template PCR reactions before combination into a multiplex assay, followed by optimisation of primer ratios. As the gltA gene is present in both TG and SFG rickettsiae (Roux et al., 1997), the issue of relative sensitivity was addressed. The detection levels of all tested SFG rickettsiae were lower using the ompB assay than using the gltA assay, as quantified by dilution series of plasmids. Those assays including a template of an SFG member led to a melt-temperature peak corresponding to the ompB amplicon and no peak corresponding to the gltA amplicon. Thus, the ompB target gene demonstrated an increased sensitivity over the gltA gene for detection of spotted fever rickettsiae in both single-template and multiplex PCR assay. In other words, the dominant amplicon in the presence of SFG rickettsiae is ompB, whereas gltA is the main amplicon with TG rickettsiae. Dual infections were assessed using the cell-culture isolates UT76 (STG), *R. typhi* (TG) and *R. conorii*, *R. honei* and *R. australis* (SFG). The assays including STG with SFG or TG demonstrated two clear peaks, corresponding to the target gene amplicons; the assays including TG and SFG demonstrated a double peak both for gltA and ompB amplicons.

2.6. Specificity of the assay

Specificity of the real-time PCR was determined in triplicate by assessment of reference rickettsial strains extracted from IFA-positive VERO cell cultures: SFG members (*R. conorii* — Malish strain, *R. rickettsii* — Bitterroot strain, *R. honei* — RB strain, *R. australis* — JC strain, *R. sibirica* — 246 strain, *R. akari* — Kaplan strain); STG members (*O. tsutsugamushi* isolates comprising 22 Karp, Gilliam and TA716-like strains isolated in Thailand during 2003—2005); TG members (*R. prowazekii* — Breinl strain, *R. typhi* — Wilmington strain) and ancestral group (*R. bellii*). In addition, 11 non-rickettsial and non-oriential controls were included to assess specificity.

3. Results

3.1. Reference strains

All STG, TG and SFG species and positive controls (plasmid preparations for 47 kDa, gltA and ompB) produced a positive real-time PCR result. All samples underwent single-template PCR detection in duplicate and multiplex PCR detection in triplicate. *Rickettsia bellii* and other members of the orders *Anaplasmataceae*, *Bartonellaceae* and *Coxiellaceae* were negative, as anticipated during primer design. Non-rickettsial and non-oriential, as well as non-template, controls gave negative results in all runs performed. In summary, 22 cell-culture isolates from the genus *Orientia*, nine isolates from the genus *Rickettsiae* (Figure 1) and nine of 12 characterised clinical buffy coat samples (Table 3) were reliably and repeatedly detected and differentiated by the multiplex PCR assay.

3.2. Limits of detection

Using SYBR Green for detection of the stated organisms, limits of detection were similar to the previously reported probe-based PCR assays: STG detection limit by single template using SYBR Green was 3 (3—12) copies/μl of *O.*

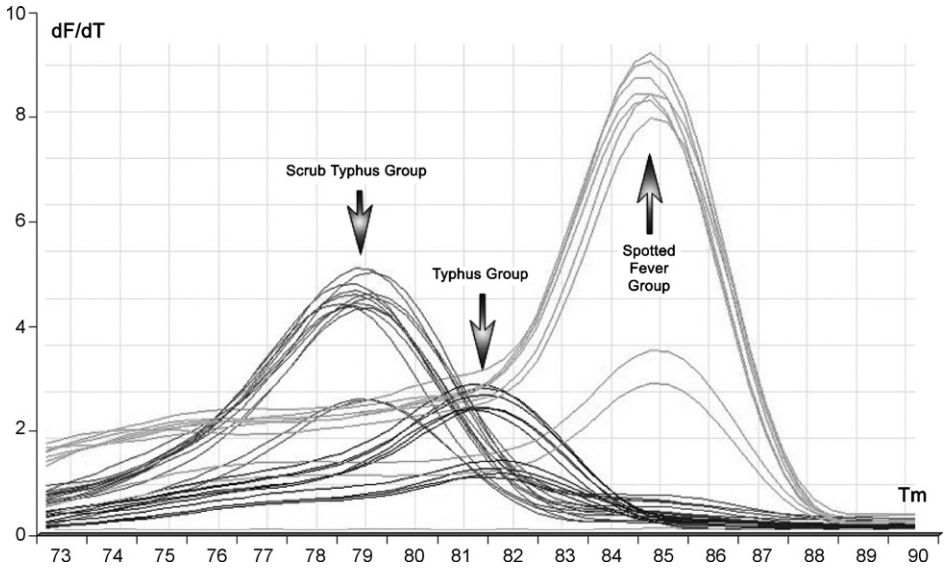

Figure 1 Melt-curve analysis (SYBR Green first derivative plot) of resulting DNA amplicons from the multiplex real-time PCR assay with templates derived from rickettsial cell-culture isolates. Differentiation of products is based on intercalating SYBR Green by dissociation of dsDNA amplicons at characteristic temperatures using fluorescence-change over time (dF/dT), leading to the following melt temperatures (T_m; range in °C): scrub typhus group 78.2—79.8 °C, typhus group 81.3—81.5 °C and spotted fever group 84.4—85.0 °C.

tsutsugamushi UT76 and by multiplex 24 (24—72) copies/μl; for *R. typhi* — Wilmington strain the detection limit by single-template PCR using SYBR Green was 1 (1—5) copy/μl and by multiplex 5 (5—26) copies/μl; and for SFG *R. conorii* — Malish strain for both single-template 1 (1—2) copy/μl and multiplex 1 (1—5) copy/μl SYBR-Green-based assays.

Melt-curve analysis of post-PCR amplicons of cell-culture and clinical buffy coat samples were clearly differentiated by their unique melt temperatures; the means and ranges for all 54 samples were: STG (mean 79.0 °C; range 78.9—79.8 °C), TG (mean 81.5 °C; range 81.4—81.5 °C) and SFG (mean 84.7 °C; range 84.4—85.0 °C) (Table 2; Figure 1).

3.3. Clinical samples

Nine of 12 admission buffy coat samples (75%) gave positive results, with melt-temperature profiles ranging between 78.9 and 79.8 °C for the scrub typhus group, 81.4 and 81.5 °C for the typhus group and 84.4 and 85.0 °C for the spotted fever group. Isolation in VERO cell culture was successful in all *O. tsutsugamushi* samples and one *R. typhi* sample. Two of the other four samples came from pre-treated patients; all clinical samples demonstrated clear serological and positive PCR evidence of acute infection (Table 3).

4. Discussion

The problems of interpreting conventional diagnostic serology results and the relatively low sensitivity of in-vitro culture highlight the need for diagnostics with genetic and/or antigenic targets. To be clinically useful as a diagnostic tool, sensitivity needs to be sufficiently high in the early stage of disease to support specific diagnosis-driven treatment. The utility of the genes targeted in this assay have been demonstrated previously in single-template reactions (Jiang et al., 2004; Parola et al., 2003b; Stenos et al., 2005). The combination of all three target genes into an adequately sensitive multiplex real-time PCR assay, providing broad coverage of clinically relevant rickettsial agents in Southeast Asia, is a novel approach. In comparison to single-template assays, multiplexing reduces sensitivity by a factor of 10 or more, although with our assay the limits of detection remain below the range of available quantitative data for bacterial loads in cell-culture samples and within the range for full blood samples (Eremeeva et al., 2003; Jiang et al., 2004). Possible reasons for this reduction in sensitivity for multiplex assays using intercalating dyes, include the interference with high-levels of background human genomic DNA, and possible variance of primer affinity within the initial reaction mix as well as potential formation of primer dimers limiting the amplification cycle numbers required for detection of low-level bacteraemia. In buffy coat templates, DNA extraction will always yield a substantial level of genomic DNA due to the intracellular nature of rickettsiae and orientiae. Using a more selective method for extraction of specific target DNA could improve this negative effect.

The benefit of a multiplex assay over a single-template assay conducted for each group of organisms is the increased savings in cost and time. However, in the case of *O. tsutsugamushi* and TG rickettsiae detection, the loss in sensitivity (approximately ten- and five-fold, respectively) in conducting a multiplex versus a single-template assay may not be appropriate, as the number of organisms found in the blood can be quite low in scrub and murine typhus patients. This is probably why the multiplex assay only worked on two-thirds of buffy coat samples tested from scrub typhus (5 of 7) and murine typhus (1 of 2) patients when compared with nested conventional PCR and single-template real-time PCR assays. Thus, until multiplex assays can obtain the level of sensitiv-

Table 3 Overview of clinical buffy coat samples directly tested by the multiplex real-time PCR assay[a]

Code	Patient serology (IgM and/or IgG values)		Buffy coat DNA extracts			Cell-culture DNA extracts		
	Admission	Convalescent	T_m (range) (°C)	56 kDa	gltA	T_m (range) (°C)	16S rRNA	56 kDa RFLP
TM1084	ST ≥1:25 600	ST ≥1:25 600	–	+	–	79.3 (79.2–79.5)	OT Kawasaki	OT Gilliam
TM1055	ST 1:12 800	ST ≥1:25 600	79.2 (79.1–79.4)	+	–	79.2 (79–79.4)	OT Kawasaki	OT Gilliam
UT512	ST ≥1:25 600	ST ≥1:25 600	79.6 (79.5–79.7)	+	–	79.5 (79.4–79.7)	OT Karp	OT Karp
UT559	ST ≥1:25 600	ST ≥1:25 600	79.5 (79.5–79.6)	+	–	79.7 (79.6–79.9)	OT Karp	OT Karp
UT601	ST ≥1:25 600	ST ≥1:25 600	79.3 (79.2–79.4)	+	–	79.8 (79.7–79.8)	OT Karp	OT Karp
UT530	NA	ST ≥1:25 600	79 (78.9–79)	+	–	79.5 (79.4–79.7)	OT Kawasaki	OT NP[d]
UT528	ST ≥1:25 600	ST ≥1:25 600	–	+	–	79.6 (79.4–79.7)	OT Karp	OT Karp
TM 962	MT 1:12 800	MT ≥1:25 600	81.5 (81.4–81.5)	–	81.5 (81.4–81.6)	[b]		
TM1041	MT 1:6 400	MT ≥1:25 600	–	–	81.4 (81.3–81.5)	81.3 (81.3–81.4)	*R. typhi* (Wilm.)	NA
ARRL10333	SFG 1:128	NA	84.5 (84.4–84.6)	–	81.6 (81.5–81.6)	[c]		
ARRL10366	SFG 1:256	NA	84.8 (84.7–85)	–	81.4 (81.3–81.5)	[c]		
ARRL10492	SFG 1:128	NA	84.6 (84.5–84.7)	–	81.5 (81.4–81.5)	[b]		

T_m: melt temperature; ST: scrub typhus; MT: murine typhus; SFG: Spotted Fever Group; –: negative; +: positive; NA: not available; OT: *Orientia tsutsugamushi*.

[a] Scrub typhus group samples were characterised by serology, cell culture isolation, 56 kDa RFLP analysis and 16S rRNA sequencing. Typhus group and spotted fever group samples were characterised by serology, isolation in cell culture and gltA real-time PCR. The multiplex assay was able to detect 9/12 samples (75%) from clinical buffy coat and all cell-culture isolates (100%) related to these clinical samples.

[b] No isolate available.

[c] Pre-treated patients.

[d] NP: new pattern.

ity of single-template assays, they should not be suggested for use with clinical samples where an individual's health depends upon appropriate treatment, which again depends upon proper laboratory diagnosis.

In relatively resource-poor settings such as rural South-east Asia, where rickettsioses are a very important but under-diagnosed group of diseases (Phongmany et al., 2006; Suttinont et al., 2006), antibiotic treatment of acute febrile illness is usually empirical, and there is a requirement to define more accurately the spectrum of organisms causing disease. Our approach to this problem is to define the clinical epidemiology by conducting studies of the causes of unexplained fever, including cell culture for rickettsiae and orientiae as one of the central tools. The method described provides a reliable, simple and efficient approach for the early assessment of in-vitro cultures. In its current state of validation this represents the main immediate use for this assay. The method has not yet been extensively validated on clinical samples taken directly from patients, and the limited data presented here on buffy coat samples suggests that, at least for *O. tsutsugamushi*, sensitivity may be a problem, although there are grounds for believing that sensitivity would be less of a problem for SFG and TG samples. Prospective evaluation of the clinical diagnostic utility of this assay in the field is currently underway.

The real-time PCR with melt-curve analysis using SYBR Green described here is a promising tool for the early detection and characterisation of cell-culture isolates for 'difficult-to-diagnose' and 'easy-to-treat' tropical rickettsial diseases, but requires further optimisation before it can be used for patient samples in the clinical setting.

Authors' contributions: DHP, SDB, JS and NPJD conceived and designed the study; SDB performed isolation and cell culture of all rickettsial isolates from Thailand; SRG generously provided all non-*O. tsutsugamushi* isolates from cell culture; JS provided the negative control specimens tested; NBU performed isolation and cell culture of all Australian isolates involved, including DNA extractions and template preparation; RP and PNN collected clinical samples; DHP conducted all the molecular work, analysed the data and prepared the first draft of the manuscript; SDB, SRG, RP, PNN and NPJD revised the manuscript for intellectual content. All authors read and approved the final manuscript. NPJD is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: Clinical samples and *O. tsutsugamushi* strains termed UT were collected in Udon Thani, north-east Thailand; strains termed FPW were collected on the Thai—Burmese border in West Thailand; and strains termed TM were collected in Vientiane, Laos PDR during 2002—2006. All samples collected were intended for diagnostic purposes within studies having granted ethical approval by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (Thai—Burma border); The Ministry of Public Health, Royal Government of Thailand, Bangkok, Thailand (NE Thailand); the Faculty of Medical Sciences Ethical Review Committee, National University of Laos, Vientiane, Lao PDR (Laos); and the Oxford Tropical Research Ethics Committee, Oxford, UK (OXTREC, UK).

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