Pilot Clinical Evaluation of Real-Time PCR Assays for Detection of *Rickettsia* spp. and *Orientia tsutsugamushi*

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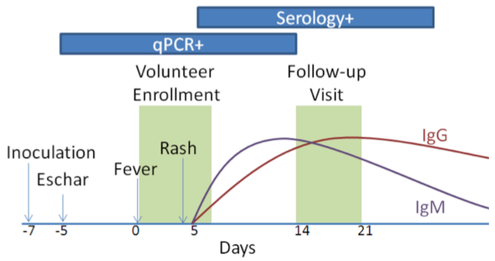
INTRODUCTION

Though easily treatable, illness caused by infection with *O. tsutsugamushi* (causative agent of scrub typhus) and *Rickettsia* spp. (causative agents of typhus and spotted fevers) are very difficult to diagnose because symptoms of early disease are identical to other diseases of similar epidemiology (e.g. leptospirosis, malaria, typhoid, dengue). Current diagnosis methods are serology-based and compare acute and convalescent phase antibody titers to the pathogens of interest. As such, they require sufficient time for seroconversion (See Figure 1); thus they are not always reliable to detect disease in the early stages. A delayed diagnosis (and treatment) leads to a higher mortality rate.

Molecular-based methods, such as real-time PCR, offer rapid, sensitive, and specific detection of analytes during the acute phase of disease when rapid and appropriate treatment is critical. Idaho Technology, Inc. (Salt Lake City, UT), in collaboration with the Naval Medical Research Center (Silver Spring, MD), developed PCR assays for the detection of *Orientia tsutsugamushi* and *Rickettsia* species, infectious agents responsible for a variety of spotted fever and typhus illnesses. The purpose of this study was to conduct a preliminary evaluation of the clinical sensitivity and specificity of these assays when compared to the gold-standard reference method of paired IgM IFA serology. A comparison between sample matrices for PCR detection (whole blood, buffy coat, serum) was also investigated.

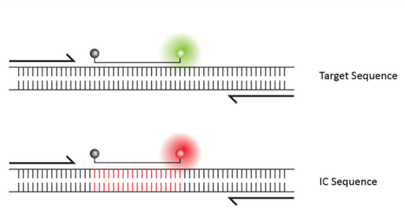
Figure 1. Rickettsial Infection Timeline



ASSAY DESIGN AND TEST SYSTEM

PCR assays for the detection of *O. tsutsugamushi* and *Rickettsia* spp. are described elsewhere (Jiang et. al. Am. J. Trop. Med. Hyg. (2004) 70, 351–356) and were used here with slight modifications. Both assays were multiplexed with an internal inhibition control (IC) that consists of a synthetic DNA construct containing the primer binding sequences of the target assay and a heterologous intervening sequence to which a novel probe binds. The target assay is read in Channel 1 (530 nm); the IC is read in Channel 3 (705 nm; Figure 2).

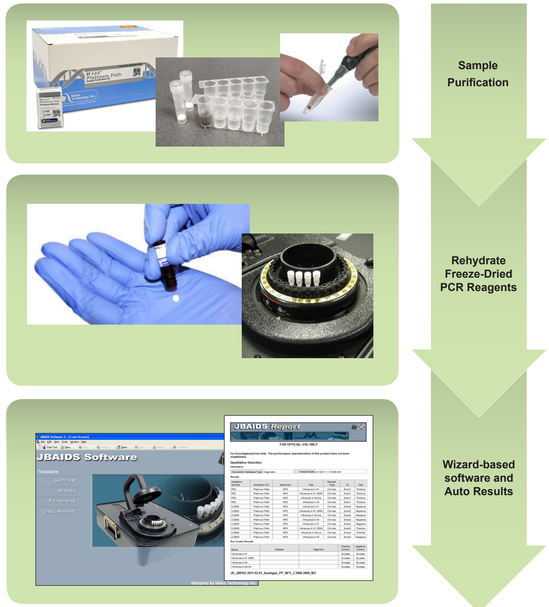
Figure 2. Multiplexed Inhibition Control (IC) Strategy



Assays conditions were then optimized for use in a ruggedized, freeze-dried chemistry format in order to be used with the Joint Biological Agent Identification and Diagnostic System (JBAIDS). The JBAIDS system includes a ruggedized real-time PCR instrument, custom software, and specialized nucleic acid extraction kits.

Nucleic acids were isolated from specimens for analysis by purification using the IT *1-2-3*™ Platinum Path™ sample purification kit (Idaho Technology, Inc.). In this simple-to-use kit, bead-beaten specimens were incubated with magnetic beads which bind nucleic acids. The beads were then moved through a series of washes using a magnetic tool and finally eluted in buffer. Purified sample was combined with reconstitution buffer to rehydrate the freeze-dried PCR reagents, which were then loaded into glass capillaries in duplicate and run on the JBAIDS instrument. The JBAIDS instrument is controlled by a simple-to-use Wizard-based interface. The instrument automatically calculated crossing point (Cp) values for each sample and made a Positive (both capillaries positive), Negative (both capillaries negative), Uncertain (one capillary positive and one negative), or Inhibited (IC negative) call (Figure 3).

Figure 3. JBAIDS System Workflow



ASSAY LIMIT OF DETECTION

The assays used here were previously tested for reactivity against panels of organisms and found to be both specific for the organisms of interest and inclusive of multiple strains/species of interest (Jiang et. al. Am. J. Trop. Med. Hyg. (2004) 70, 351–356). Following optimization for the freeze-dried format of the JBAIDS platform and addition of the multiplexed internal inhibition control, nucleic acid limit of detection (naLoD) was determined for each assay. The LoD was defined as the lowest level of synthetic target that could be detected in at least 19 out of 20 replicate reactions using a purified whole blood background (to mimic any inhibitory effect of the sample matrix). The LoD was found to be 25 copies of target template per reaction. The assay extinction point (defined as the lowest level of template detectable in any of 6 replicate reactions) was found to be 5 copies of synthetic template per reaction for both assays.

Next, naLoD was determined using purified genomic DNA from *R. typhi* and *O. tsutsugamushi* (calculated in genomic equivalents, GE) and is listed in Table 1.

Table 1. naLoD in Copies per Reaction

Assay	Assay Extinction[a]	naLoD[b]	
		Synthetic Template	Genomic DNA
pan-*Rickettsia*	5	25	50[c]
O. tsutsugamushi	5	25	250[d]

[a] Lowest concentration resulting in at least 1 out of 6 positive reactions
[b] Lowest concentration resulting in at least 19 out of 20 positive reactions
[c] *Rickettsia typhi* strain Wilmington
[d] *O. tsutsugamushi* strain Karp

FOOTNOTES
This work was performed under DoD contract number DASG60-03-0094. The clinical evaluation at SMRU was supported by the Li Ka Shing Foundation (Grant SM08).

CLINICAL EVALUATION DESIGN

The clinical study was conducted with oversight from the Ethics Committee of the Faculty of Tropical Medicine at Mahidol University, the Oxford Tropical Research Ethics Committee, and the Human Research Protection Office of the U.S. Army Medical Research and Materiel Command.

Two hundred (200) patients were recruited from clinics operated by the Shoklo Malaria Research Unit at Mawker Thai village, Wang Pha village, and Maela refugee camp, in Northwestern Thailand near the Myanmar border between March and August of 2011.

The study included any patient aged at least 5 years presenting to the clinic with a documented fever of at least 38°C of up to seven days duration.

Exclusion criteria included:

- Pregnancy
- Patients with a clear clinical diagnosis (except clinically suspected dengue, leptospirosis, rickettsial infection, or typhoid), for example:
  - Measles
  - Chickenpox
  - Pneumonia (based on current clinical criteria)
  - Skin/soft tissue infection (e.g. cellulitis)
  - Urinary tract infection
- Currently taking antimicrobials, or have taken antimicrobials in the seven days prior to presentation
- A positive malaria smear/ rapid diagnostic test
- Unable to give informed consent (assent for pediatric patients)

Whole blood and serum specimens were collected from each volunteer during the first visit (acute phase). A serum specimen was obtained during a follow up visit 14 days after the initial collection (convalescent phase; see Figure 1).

Specimens collected during acute phase illness were tested with the assays. For adult patients, an aliquot of whole blood was extracted with the Platinum Path kit and a buffy coat fraction was then isolated from the remaining whole blood (approximately 4 mL) and extracted with the Qiagen QIAamp® DNA Mini Blood Kit. Extracted specimens were tested with both assays. If a patient tested positive for either assay in whole blood or buffy coat, the corresponding serum sample was also tested after extraction with the Platinum Path kit. Due to limited sample volume, only buffy coat fractions (extracted with the Qiagen kit) were tested for children (Table 2).

Table 2. Nucleic Acid Purification Methods

Purification Method	Specimen Volume	Elution Volume	Specimen Type	Patient Population
Platinum Path	800 µl	200 µl	Whole Blood, Serum	Adults
Qiagen	200 µl	100 µl	Buffy Coat	Adults & Children

Diagnosis of patient illness was determined using the gold-standard method of seroconversion or a ≥4-fold rise in IgM antibodies to typhus group or scrub typhus group antigens between paired acute and convalescent sera using screening slides from the Australian Rickettsial Reference Laboratory. System performance was calculated by comparing the PCR test results to those obtained with reference testing for each specimen.

CLINICAL PERFORMANCE DATA

Of the 200 patients enrolled during the study period, 181 returned to the clinic to donate a convalescent specimen (90%). Nineteen (19) gave an acute specimen only and could not be analyzed by the reference serology method and were excluded from the study. One (1) additional patient had inconclusive serology (equal rise in titer to both typhus and scrub typhus group antigens) and was excluded from analysis.

SCRUB TYPHUS

Patients testing positive for scrub typhus by IgM IFA serology or PCR are listed in Table 3. A total of 7 patients had serological evidence of acute scrub typhus infection via IgM IFA (7/180; 3.9%). Three (3) of these patients were children and only the buffy coat was tested with PCR. Of the four (4) adults with serological evidence of scrub typhus, the *O. tsutsugamushi* PCR assay was positive for two samples in both whole blood and buffy coat fractions (2/4, 50%), but negative in the serum fractions. One additional patient that was negative for scrub typhus via IgM IFA was positive via PCR in all blood fractions. Notably, the *O. tsutsugamushi* assay gave a negative result for all of the children with serological evidence of infection.

As seen in Table 3, there was perfect concordance between results obtained with whole blood purified by Platinum Path and buffy coat purified with the Qiagen extraction kit, though Cp values were 1 to 3 cycles later in whole blood, suggesting a higher titer of organism in buffy coat fractions. The serum fraction was only positive by PCR for one patient, FDS-3057.

Table 3. Patients positive for scrub typhus by either serology or PCR.

Patient ID	*O. tsutsugamushi* PCR									Serology Interpretation
	Blood Platinum Path			Buffy coat Qiagen			Serum Platinum Path			
	Result	Cp	F_{max}	Result	Cp	F_{max}	Result	Cp	F_{max}	
FDS-3039	Negative	-	-	Negative	-	-	N/D	N/D	N/D	Acute scrub typhus
FDS-3040	Positive	35.7	5.1	Positive	32.5	8.23	Negative	-	-	Acute scrub typhus
FDS-3062	N/A	N/A	N/A	Negative	-	-	N/A	N/A	N/A	Acute scrub typhus
FDS-3112	N/A	N/A	N/A	Negative	-	-	N/A	N/A	N/A	Acute scrub typhus
FDS-4046	Negative	-	-	Negative	-	-	N/D	N/D	N/D	Acute scrub typhus
FDS-1044	N/A	N/A	N/A	Negative	-	-	N/A	N/A	N/A	Acute scrub typhus
FDS-3074	Positive	33.7	7.3	Positive	31.5	8.6	Negative	-	-	Acute scrub typhus
False Positive										
FDS-3057*	Positive	32.0	8.8	Positive	30.6	9.8	Positive	32.9	7.7	Negative

*Patient FDS-3075 was negative for scrub typhus via IgM IFA, but discrepancy investigation using IgM ELISA demonstrated serological evidence of scrub typhus infection. N/A = not applicable N/D = not determined

MURINE TYPHUS

Patients testing positive for *Rickettsia* (murine typhus) infection by IgM IFA serology or PCR are listed in Table 4. Out of 180 patients with complete and interpretable serology, 11 patients had serological evidence of *R. typhi* (murine typhus) infection (11/180, 6.1%). One (1) patient was a child and only the buffy coat fraction was tested with PCR. Of the 10 adult patients with positive murine typhus serology whose blood fractions were analyzed with the *Rickettsia* PCR assay, three (3) were positive and one (1) additional tested uncertain two times in a row and was considered positive (4/10; 40%). Of the 11 patients whose buffy coats were tested with the *Rickettsia* PCR assay, 3 were positive (3/11; 27.3%). Two additional samples initially tested uncertain but re-tested negative and were considered negative, though serological data indicated acute murine typhus. Another patient tested uncertain twice and was considered positive, but was negative for murine typhus using IgM IFA. Two additional patients tested positive by PCR but were excluded from the study for un-interpretable serology (one patient did not donate a convalescent specimen and the other had equivalent titers to both murine and scrub typhus and could not be interpreted).

Data are presented in Table 4. There was poor concordance between results obtained with whole blood purified by Platinum Path and buffy coat purified with the Qiagen extraction kit. In general, *Rickettsia* target was detected in buffy coats with the same frequency as whole blood, but not all patients with *Rickettsia* detected in whole blood had organism detected in the buffy coat. Additionally, the serum fraction was not often positive. Perfect concordance between all fractions tested was only observed for two patients, FDS-3015 and FDS-3055. All uncertain test results were due to split calls between replicate capillaries (one capillary positive, one negative) and all were retested. Of the 5 initially uncertain samples, 2 retested as uncertain and 3 retested as negative.

Table 4. Patients positive for *Rickettsia* infection by either serology or PCR

Patient ID	*Rickettsia* PCR									Serology Interpretation
	Blood Platinum Path			Buffy coat Qiagen			Serum Platinum Path			
	Result	Cp	F_{max}	Result	Cp	F_{max}	Result	Cp	F_{max}	
FDS-1012	Negative	-	-	Negative	-	-	N/D	N/D	N/D	Acute murine typhus
FDS-3005	Negative	-	-	Negative	-	-	N/D	N/D	N/D	Acute murine typhus
FDS-3015	Positive	32.8	2.3	Positive	32.8	1.8	Positive	35.2	4.0	Acute murine typhus
FDS-3016	Negative	-	-	Negative	-	-	N/D	N/D	N/D	Acute murine typhus
FDS-3025	Positive	33.5	1.3	Negative	-	-	Negative	-	-	Acute murine typhus
FDS-3035	Negative	-	-	Uncertain/ Negative	34.0	0.7	Negative	-	-	Acute murine typhus
FDS-3054	Uncertain/ Uncertain	33.8	1.2	Negative	-	-	N/D	-	-	Acute murine typhus
FDS-3063	Negative	-	-	Negative	-	-	N/D	N/D	N/D	Acute murine typhus
FDS-3067	N/A	N/A	N/A	Uncertain/ Negative	33.8	1.0	N/A	N/A	N/A	Acute murine typhus
FDS-3107	Positive	33.4	1.9	Positive	31.9	9.0	Positive	36.2	3.7	Acute murine typhus
FDS-4023	Negative	-	-	Positive	28.2	1.3	Uncertain/ Negative	36.5	1.8	Acute murine typhus
False Positive										
FDS-3037	Negative	-	-	Uncertain/ Uncertain	32.3	1.1	Negative	-	-	Negative
Uninterpretable										
FDS-3055	Positive	31.8	5.9	Positive	31.1	6.2	Positive	34.2	4.9	No convalescent serology
FDS-3017	N/A	N/A	N/A	Positive	32.5	5.1	N/A	N/A	N/A	Equivocal Serology

CONCLUSION

In summary, 3.8% of patients were diagnosed with scrub typhus and 6.1% were diagnosed with murine typhus using paired serology during the study period. The PCR assays had a sensitivity of 27-50% compared to the reference method. Specificity was >99% with only 1 false positive for each assay out of 180 patients tested. The PCR assays tested here demonstrated similar performance in both buffy coat and whole blood fractions for both assays. However, the serum fraction was rarely positive using the PCR assay suggesting this matrix is a poor choice for PCR diagnostic testing.

While a negative PCR result is not definitive for the absence of scrub typhus or *Rickettsia* infection, a positive test result is an excellent indication of these diseases, particularly early in the course of the disease when medical intervention is critical and serological diagnostic methods are not reliable.

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