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Loop-Mediated Isothermal Amplification Assay Targeting the 47-Kda Gene of *Orientia tsutsugamushi:* A Rapid and Sensitive Alternative to Real-Time PCR

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Abstract

A sensitive, specific and rapid diagnostic test for the detection of *Orientia tsutsugamushi,* the causative agent of scrub typhus, is necessary to accurately and promptly diagnose patients and ensure that they receive proper treatment. A Loop-Mediated Isothermal Amplification (LAMP) assay targeting the 47-kDa gene of *Orientia tsutsugamushi* was developed. The LAMP assay was capable of detecting eleven different strains of *Orientia* at levels comparable to that of the quantitative PCR based method of detection. Ten patient specimens, confirmed to be positive for *Orientia* by two different PCR methods, were tested and nine out of ten were determined to be positive by LAMP. In terms of specificity, the assay was able to differentiate between *Orientia* and other phylogenetically similar bacteria as well as mouse and human host DNA. In addition to being sensitive and specific, the LAMP reaction was completed in 1 hour, demonstrating that it is a highly time-efficient method of diagnosing scrub typhus.

Introduction

Scrub typhus, a febrile illness caused by *Orientia tsutsugamushi,* is endemic to the Asia-Pacific region. The bacteria are transmitted to humans by the bite of larval trombiculid mites, commonly known as chiggers [1]. Approximately 1 million people are infected annually with many more at risk of acquiring infection [2]. The disease can account for up to 23% of febrile illnesses in the endemic region and can cause up to a 35% mortality rate if left untreated [3,4]. Scrub typhus has been re-emerging and occurring in new areas of many countries including Japan, Korea, Sri Lanka, China, Maldives, India, Palau, Malaysia, Taiwan, and Australia [5-14] and evidence of antibiotic resistance has been shown [15,16]. More recently, a new species of Orientia, *Orientia chuto* was identified in Dubai, where scrub typhus has not previously been described [17]. Furthermore, an endemic scrub typhus-like illness has been documented in Chile recently [18]. These observations clearly show that the disease is gradually expanding to various parts of the world and that there is an immediate need for an accurate method of detection that can cover a broad range of *Orientia* strains in different geographic regions.

The symptoms of scrub typhus, including fever, headache, and rash, are similar to those of other febrile diseases, making it difficult to differentiate between infections. In order to effectively deliver treatment to patients it is crucial for clinicians to have the ability to accurately distinguish between these symptomatically similar infections. Several methods are currently available for diagnosing scrub typhus including nucleic acid based detection and serological techniques. Although these methods are useful, they have limitations, especially in areas without sufficient resources. For example, quantitative real-time PCR or nested PCR targeting different genes, including the 56 kDa [19], 47kDa [20], GroEL [21], requires the use of an expensive, complex thermocycler which can be very difficult if not impossible to obtain and maintain in resource poor settings. Serological techniques are limited because they require a wait period for antibodies to be produced to a detectable level, and sometimes even healthy individuals in endemic areas can test positive due to the persistence of antibodies from previous scrub typhus infections [22]. Furthermore, the requirement for consistent and high quality antigen preparation has been an obstacle for the development of these assays. Therefore, there is an urgent need to develop a sensitive and specific assay that can be used in endemic areas where there are limited resources.

A Loop-Mediated Isothermal Amplification (LAMP) method has been extensively used as an alternative nucleic acid test to PCR or real-time PCR, and has demonstrated similar sensitivity levels for various infectious diseases [23-29]. Here we describe the detection of *Orientia tsutsugamushi* DNA by targeting the 47-kDa gene using LAMP. The 47-kDa gene sequence is highly conserved with >97% identity in 25 different strains of *Orientia* [30]. Therefore, the gene is an excellent target in achieving broad phylogenetic and geographic coverage. The LAMP assay was shown to be capable of detecting multiple strains of Orientia with high sensitivity levels similar to that obtained by real-time PCR using the same target. The presence of excess host genomic DNA or inclusion of near-neighbor rickettsial DNA did not interfere with the assay. Furthermore, when only near-neighbor rickettsial DNA was included in the reaction without *Orientia* template, no false positives were seen. In addition, the LAMP assay successfully identified PCR-confirmed, positive patient samples as positive for *Orientia*. These results suggest that the LAMP assay can substitute real-time PCR for the sensitive detection of *Orientia* DNA in resource-limited endemic areas.

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Materials and Methods

Design of primers

Oligonucleotide primers used for the LAMP and quantitative PCR assays were designed based on the 47 kDa gene sequence from the Karp strain of *O. tsutsugamushi*. Two sets of primers, each consisting of two outer (F3 and B3) and two inner (FIP and BIP) primers, were used for the LAMP assay. All LAMP primers were designed using PrimerExplorer V4 (http://primerexplorer.jp/e/). The primers used for the quantitative PCR assay were designed based on previously described assays [21,29]. Two bases were changed from the reverse primer described by Paris et al. [29] to match the Karp strain sequence. All primers were synthesized by Eurofins MWG Operon (Huntsville, AL) and are described in Table 1.

Plasmid and genomic DNA template

The 47 kDa gene sequence of *O. tsutsugamushi* Karp strain was cloned into a VR1012 vector, the closed circular plasmid was purified using standard Qiagen plasmid mini kit following manufacturer's instruction. The pure plasmid was quantified spectrophotometrically and used as a standard to determine the best primer combination used in the LAMP assay. The optimized assay was then evaluated using genomic DNA material for sensitivity and specificity reported in the results. The genomic DNA template from multiple strains of *O. tsutsugamushi* including Karp, Gilliam, Kato, TA763, TH1811, TH1812, TH1814, AFC-1, Garton, Ikeda, and MAK119 were used as template in both LAMP and quantitative-PCR as described below. In addition to pure genomic DNA, genomic DNA in the presence of an abundance of non-specific mouse or human DNA was also used. All genomic DNA was extracted as previously described [31]. Due to the limited amounts of starting materials (i.e., genomic DNA), select strains were amplified using the Illustra GenomiPhiV2 DNA Amplification Kit according to the manufacturer's instructions (GE Healthcare, Waukesha, WI) prior to performing the LAMP reaction [32].

Loop-mediated isothermal amplification reaction

LAMP reactions were carried out as described previously [33]. Briefly, the reactions were a total volume of 25 µL containing 1.6 µM of each FIP and BIP primer, 0.4 µM of each F3 and B3 primer, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.8 M betaine (Sigma-Aldrich, St Louis, MO), 1.4 mM dNTP mixture (New England Biolabs, Beverly, MA), 8U Bst DNA polymerase (New England Biolabs, Beverly, MA), and DNA template. The reaction mixture, excluding Bst enzyme, was heated to 95°C for 5 min to denature the DNA template and then incubated on ice for 5 min. After denaturation of the DNA, the Bst DNA polymerase was added to the reaction mixture and the mixture was incubated at 60°C for 60 min. Each reaction was terminated by adding 5 µL of 10X BlueJuice™ (Invitrogen, Carlsbad, CA). The reaction product was electrophoresed on a 2% agarose gel stained with a 1:10,000 dilution of GelRed (Phenix Research Products, Asheville, NC) to visualize results.

Primer Name	Length (bp)	Tm(°C)	Sequence 5'- 3'
LAMP			
Primer Set 1 (#3)			
F3	22	58.9	CTATTCATATGGGTAGCTTTGG
B3	25	58.0	CCTAAATTCTCATTTAATTCTGGAG
FIP	51	69.6	AGCAAAACTTATGCCTGAGTAAGAT-TTTT-GACCGATGTTTAATCTTGAAGG
BIP	50	69.7	CTGTACTTGAAGCAGTTGAATGCT-TTTTA-CATTTAACATACCACGACGAA
Primer Set 2 (#5)			
F3	24	57.7	TGAAGTTATAAAAGAAGGATCTGC
B3	23	57.4	GCTTTGATCATTACTTTGTTGCT
FIP	47	70.0	ACCTGTAAATCTCTTCCTGTTTTGAT-TTTT-GGAATTGCTCCTGGAGA
BIP	49	70.6	CTGAAAGAGAAGTTGAGCTTTTACG-TTT-TACTATCCTCACCTTTGTTGG
qPCR			
47kDa-F	28	58.8	AACTGATTTTATTCAAACTAATGCTGCT
47kDa-R	30	63.3	TATGCCTGAGTAAGATACGTGAATGGAATT

Table 1: Description of LAMP and qPCR primers.

kDa – based quantitative PCR

Quantitative PCR was performed to compare and to confirm the sensitivity of the LAMP assay. The 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA) was used to perform the qPCR reactions and analyze the results. Primers designed against the 47-kDa gene sequence of the Karp strain of *Orientia*, described in previous studies were used (Table 1) [20]. The total volume of each reaction was 20 µL. Each reaction mixture contained 6 µM of the forward primer, 6 µM of the reverse primer, 1X RT2 SYBR Green qPCR Mastermix (SA-Biosciences, Frederick, MD), and DNA template. An initial 5 minute activation step at 95°C was followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and a melting curve determination cycle.

Results

The LAMP assay targeting the 47 kDa gene sequence of *O. tsutsugamushi* demonstrated high sensitivity and specificity. The sensitivity of the assay was first determined using plasmid DNA and pure Karp genomic DNA. The assay was able to detect down to 20 copies for both plasmid and genomic DNA template (data not shown). After the initial determination of sensitivity, 10 other strains of *Orientia* were tested to demonstrate the ability of the assay to detect a broad range of isolates. These are representative strains from different geographic areas within the Orientia triangle [34]. The LAMP assay was capable of detecting all other tested strains of *Orientia*, which included Kato, Gilliam, TA763, TH1811, TH1812, TH1814, AFC-1, Garton, Ikeda, and MAK119, with similar sensitivity. The sensitivity of the LAMP assay was comparable to that of qPCR (Table 2). Among the eleven strains tested, the detection limit for LAMP compared to qPCR was the same or lower for five strains. Among the remaining six strains, LAMP was able to detect between 16-50 copies/reaction.

DNA was extracted from the liver/spleen homogenate of mice challenged with different strains of *O. tsutsugamushi* in order to test the

Strain of *Orientia*	qPCR detection limit	LAMP detection limit
Karp (WGA)	11	17
Gilliam	25	50
Kato	13	28
TA763	11	43
TH1811	4	4
TH1812	20	20
TH1814	1	1
AFC-1	5	5
Garton (WGA)	1	16
MAK119	2	20
Ikeda	9	9

Table 2: Detection limit of the LAMP assay compared to quantitative PCR.

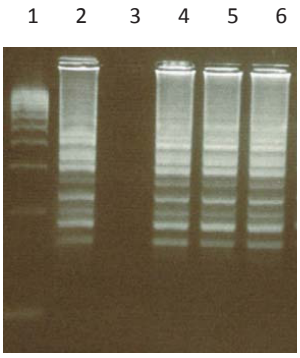

Figure 1: LAMP assay performed using total DNA purified from mouse liver/spleen homogenate, known to contain the Karp strain of *Orientia*. Lane 1: 100bp DNA ladder, Lane 2: positive control, Lane 3: negative control, Lane 4-6: products from LAMP reaction containing different copies (lane 4-6 has 20,000, 2,000, and 200 copies, respectively) of *Orientia* DNA and an over-abundance (1,000 fold excess) of mouse DNA.

Sample	47 kDa gene (accession #)	Geographic location	Diagnosis* (strain)
L1	+ (L31934)	New Guinea	ST (KARP)[1]
L2	+ (L31933)	Burma	ST (GILLIAM)[2]
L3	+ (L31933)	Burma	ST (GILLIAM)
L4	+ (L31934)	New Guinea	ST (KARP)
L5	+ (L31934)	New Guinea	ST (KARP)
L6	+ (L11697)	Japan	ST (KATO)[3]
L7	+ (HM595490)	Thailand	ST (TA763)[4]
L8	- (L11697)	Japan	ST (KATO)
L9	+ (L11697)	Japan	ST (KATO)
L10	+ (L31934)	New Guinea	ST (KARP)
L11	(-)		MT
L12	(-)		MT
L13	(-)		LEPTO.
L14	(-)		LEPTO.
L15	(+)		MALARIA(PF)
L16	(-)		MALARIA(PF)
L17	(-)		DENGUE
L18	(-)		DENGUE
L19	(-)		HEALTHY
L20	(+)		HEALTHY

*. ST: scrub typhus; MT: murine typhus.
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[4]. Jiang,J., Newton,P.N., Phetsouvanh,R., Izzard,L., Stenos,J., Graves,S.R., Hu,X., Wallqvist,A., Ge,H., Day,N.P.J., Richards,A.L.,Blacksell,S.D., Paris,D.H. and Aukkanit,N. Direct deposited to NCBI database Jul 25, 2011.

Table 3: Evaluation of LAMP assay using the 47 kDa gene as the target with PCR confirmed positive and negative samples.

ability of the LAMP assay to detect *Orientia* in the presence of host DNA (at 1 *Orientia* DNA/1000 host DNA). The assay was able to detect the target DNA despite the abundance of mouse DNA in the sample (Figure 1). Additionally, in order to simulate the use of this assay in a true clinical setting, the LAMP assay was performed using DNA extracted from patient serum that was determined by two well-validated PCR analyses targeting 16s rRNA and the 56 kDa gene to be positive or negative for *O. tsutsugamushi* [35,36]. These DNA materials were kindly provided to us by Dr. Yupin Suputtamongkol (Siriraj Hospital, Mahidol University, Bangkok, Thailand). A total of 20 samples were tested, ten positive and ten negative. Of the ten positive patient samples that were

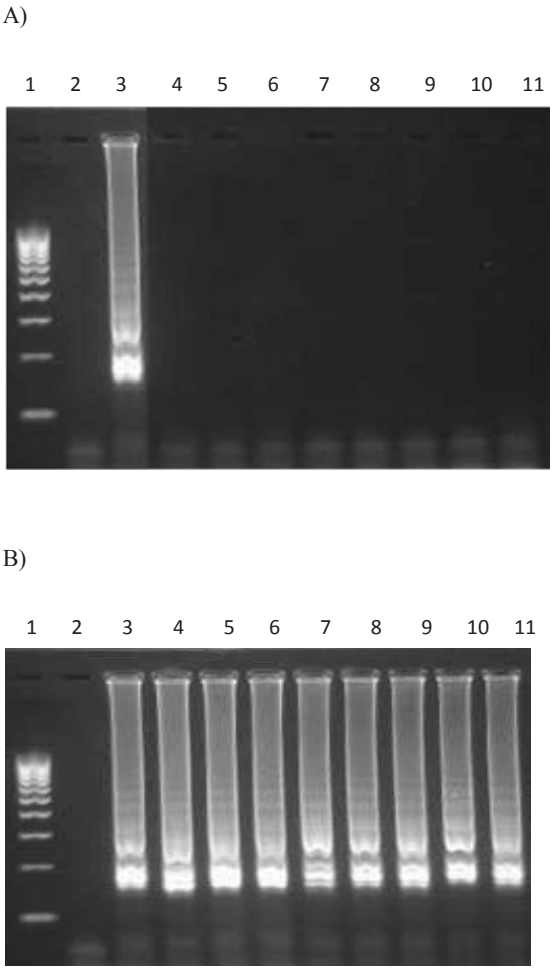

Figure 2: Demonstration of specificity of the LAMP assay. **A)** Lane 1: 100bp ladder, Lane 2: negative control, Lane 3: positive control, Lane 4-11: products from LAMP reactions containing genomic DNA from bacteria which are phylogenetically similar to *Orientia* including *R. conorii R. prowazakii, R. belli, R. rickettsii, R. typhi, R. felli, C. burnetii*, and *L. interrogans*, respectively. **B)** LAMP assay performed using a combination of Orientia genomic DNA and other bacterial genomic DNA. Lane 1: 100 bp ladder, Lane 2: negative control, Lane 3: positive control, Lane 4-11: *O.tsu + R. conorii, O.tsu + R. prowazakii, O.tsu + R. belli, O.tsu + R. rickettsii, O.tsu + R. typhi, O.tsu + R. felli, O.tsu + C. burnetii,* and *O.tsu + L. interrogans*, respectively.

confirmed positive by PCR, the LAMP assay detected nine out of the ten as being positive. Among these samples the Karp, Gilliam, Kato, and TA763 strains of *Orientia* were represented. The false negative was determined to be of the Kato strain of *Orientia*. Of the ten PCR-confirmed negative patient samples, the LAMP assay determined eight out of the ten samples to be negative. The other two samples showed a positive signal with a similar banding pattern to the *Orientia* positive samples (Table 3).

The specificity of the LAMP assay was also demonstrated by using purified DNA from bacteria that are phylogenetically close to *Orientia* as the template. LAMP reactions containing *R. conorii, R. prowazakii, R. belli, R. rickettsii, R. typhi, R. felis, C. burnetii,* or *L. interrogans* DNA as template all tested negative (Figure 2A). Addition of the Karp strain of *Orientia* at 1/10 of DNA copy of other DNA resulted in positive results for all reactions, indicating that the assay is specifically detecting *Orientia* and sensitive enough to detect the desired target in

the presence of other phylogenetically close genomic DNA (Figure 2B).

Discussion

The 47 kDa gene sequence was selected as a target for the LAMP assay because it is very conserved among the different strains of *Orientia* with the exception of *O. chuto* [18]. As stated previously, the 47 kDa gene has >97% identity in 25 different strains of *Orientia* [5,22]. The ability of this LAMP assay to cover a broad range of isolates was shown. This indicates that the assay could be utilized in various geographic settings. The strains able to be detected by the LAMP assay are endemic to multiple countries including Thailand, Taiwan, Japan, New Guinea, Australia and Burma [34].

During the optimization process of the development of the LAMP assay, several sets of primers were screened individually and in combination. Combining two sets of primers without loop primers was proved to be more sensitive in amplifying the target DNA than using one set alone. It should be noted that while an incorrect loop primer was mistakenly added to LAMP reactions during some trials with the Karp and Gilliam strains initially, follow up experiments conducted confirmed that this error did not interfere with the reaction (the LAMP detection limit with the incorrect loop primer was 6 copies and 55 copies for Karp and Gilliam, respectively, Table 2). This is consistent with the notion that the primary function of loop primers is to accelerate the reaction and not to improve sensitivity or specificity [37]. To further increase the sensitivity of the assay, a TTTT-linker was added to the inner primer sequences, which allows the primers to have more physical flexibility in binding to the target DNA. Temperatures ranging between 59 and 65°C were tested to determine the optimal incubation temperature for the LAMP reaction. Sixty degrees was the temperature at which the assay performed with the highest sensitivity. Also in an effort to increase the sensitivity of the assay, the DNA template was denatured to increase the amount of exposed single stranded DNA to which the primers could bind to. In addition to the method used, denaturing the DNA after adding it to the reaction mixture, a second method was tested in which the DNA was denatured prior to adding it to the reaction mixture. The latter method was found to be less efficient as the method that was used for this study. The primers being present in the mixture when the DNA is denatured likely increases the amount of primer that will anneal to the DNA.

Whole genome amplification (WGA) was performed using the Illustra GenomiPhiV2 DNA Amplification Kit with the Karp and Garton strains of *Orientia* to obtain more genomic material prior to running the LAMP assay. This was due to the limited amount of pure DNA material from these two strains. Kato et al. [32] had shown that WGA of low amounts of rickettsia DNA in the presence or absence of host DNA material could lead to an increased amount of rickettsial DNA available as template for downstream qPCR amplification. The WGA reaction used in this study also showed amplification of Orientia DNA and subsequent detection by LAMP and qPCR. It was not clear whether there was a difference in efficiency of amplification of *Orientia* DNA with or without the presence of host DNA material. Nevertheless, it appeared that both genomic materials were amplified when WGA was applied. The use of WGA posed an uncertainty as to whether the 47 gene sequence would be over-amplified during this procedure. Therefore, quantitative PCR was performed and it was confirmed that the 47 gene sequence was not over-amplified and that the amplification process did not alter the genomic DNA or the interpretation of the results (data not shown). This observation suggests that WGA can be incorporated in the sample preparation step to increase the amount of target DNA for a subsequent PCR or real-time PCR assay. Alternatively, WGA can be applied to hard-to-get DNA samples in order to preserve the precious material for downstream applications.

The one confirmed-positive clinical sample that was not detected as positive by LAMP was characterized and found to be of the Kato strain. This is the only one among the three Kato strain positives that was not detected by LAMP. It is not clear why this particular Kato strain infected patient sample was not detected by LAMP. One possibility is that there was a low amount of DNA extracted from this sample, or that the target region of the LAMP primers, the 47 kDa gene sequence, was not available in the purified DNA material, as the PCR assays originally determining positive or negative status targeted the 56 kDa and 16s rRNA genes. We performed additional experiments to confirm the presence of the 56 kDa gene using nest PCR [30] and were not able to detect the gene. Even after whole genome amplification of the original DNA material followed by nest PCR, we were not able to detect the 56 kDa gene. The WGA amplified material was not positive by LMAP assay either (data not shown). Therefore, it is possible that this sample was misdiagnosed as scrub typhus patient serum.

The assay demonstrated high specificity when tested with DNA template that was phylogenetically close to that of *Orientia*. However, two out of ten clinical samples that were PCR-confirmed negatives were tested positive by the LAMP assay. Given that the banding pattern for these two samples was similar to that of *Orientia* positive, it is reasonable to think that these samples were positive due to contamination by *Orientia* genomic material and not because the primers were non-specifically binding to the host or other DNA. Further experiments to demonstrate the specificity of the current LAMP assay by eliminating contamination are desirable and more clinical samples shall be tested to evaluate the clinical usage of the assay. Two recent publications by Blacksell et al. [38] and Paris et al. [39] have examined the clinical usage of the LAMP assay targeting the groEL gene individually or in combination with serological assays using well-characterized scrub typhus patient samples. It was concluded that the use of either a serological assay or LAMP assay alone would not provide desired levels of sensitivity and specificity. The combination of both serological assay and nucleic acid detection covering a complete time frame during the progression of disease is the best clinical practice for accurate diagnosis of the disease. However, the cost of performing both serological assay and nucleic acid test (i.e. qPCR or PCR) in low resource, endemic areas can be substantial. With the development of an immunochromatographic test for serological testing and the less expensive, qPCR comparable LAMP assay, it is possible to perform both assays in parallel at an affordable price to provide timely and accurate diagnosis for proper treatment.

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