Jpn. J. Infect. Dis., 70, 119–123, 2017

Method

Development of a Novel Loop-Mediated Isothermal Amplification (LAMP) Assay for the Detection of *Rickettsia* spp.

Nozomu Hanaoka[1,2], Minenosuke Matsutani[3], Masaaki Satoh[1], Motohiko Ogawa[1], Mutsunori Shirai[3], and Shuji Ando[1]*

[1]*Department of Virology-1;* [2]*Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo 162-8640; and* [3]*Department of Microbiology and Immunology, Yamaguchi University School of Medicine, Yamaguchi 755-8505, Japan*

SUMMARY: We developed a novel loop-mediated isothermal amplification (LAMP) method to detect *Rickettsia* spp., including *Rickettsia prowazekii* and *R. typhi*. Species-specific LAMP primers were developed for orthologous genes conserved among *Rickettsia* spp. The selected modified primers could detect all the *Rickettsia* spp. tested. The LAMP method was successfully used to detect 100 DNA copies of *Rickettsia* spp. within approximately 60 min at 63°C. Therefore, this method may be an excellent tool for the early diagnosis of rickettsiosis in a laboratory or in the field.

INTRODUCTION

Members of the genus *Rickettsia*, which includes several virulent species, are obligate intracellular bacteria that are endemic worldwide (1) and are known to cause rickettsiosis. Rickettsiosis can be cured by appropriate antibiotic treatment when administered at early stages (2). Therefore, the early determination of an appropriate therapeutic strategy for rickettsiosis is important and this requires rapid diagnosis. Although the genus *Rickettsia* comprises several virulent groups, such as spotted fever group and typhus fever group, the therapeutic strategy against infection caused by all these *Rickettsiae* spp. is the same, as they are resistant to macrolides and sensitive to tetracycline antibiotics (2). DNA can be used for the detection of *Rickettsiae* spp. effectively. To increase the specificity of DNA detection, the best target might be a specific DNA sequence that is conserved only within a certain strain. However, several confirmed virulent and novel virulent *Rickettsia* strains have been reported in the last decade (1,3); in addition, the detailed mechanisms of virulence expression of these strains in the host are poorly understood (1). Hence, a detection assay that is highly specific for a certain strain would not be suitable for the rapid screening of a Rickettsial infection. Therefore, in this study, we aimed to develop a system that can be easily used to detect all *Rickettsia* spp., and which would hence serve as a useful diagnostic tool for Rickettsial infection. Here, we focused on orthologous genes, which are conserved genes in different species that originate from a common ancestor (4). A genus-specific orthologous gene is more effective than other genes for the purpose of DNA detection because of its specificity and high level of conservation at the genus level; the 16S ribosomal RNA gene sequence is one such highly conserved sequence.

In clinical laboratories in Japan, diagnostic systems using real-time polymerase chain reaction (qPCR), loop-mediated isothermal amplification (LAMP) (5), or both, have already been implemented. These 2 types of detection assays have wide clinical applications with unique advantages over other types of diagnostic systems. In the LAMP assay, amplification and detection can be completed in one isothermal step (5). Thus, the LAMP assay is a simpler method for molecular diagnosis than the qPCR assay. In our previous study, we have developed a *Rickettsia japonica*-specific detection assay (6). For the detection of *Rickettsia* spp. other than *R. japonica*, qPCR (7) and LAMP assays for *Rickettsia* causing spotted fever (8) and murine typhus (9), respectively, have also been reported. However, simple LAMP assay suitable for the simultaneous detection of all the *Rickettsia* spp. including *R. japonica*, *R. typhi*, and *R. prowazekii* is unavailable.

Therefore, here, we searched for orthologous sequences that are conserved among various *Rickettsia* spp. as targets for the development of a genus-specific detection assay using a LAMP system.

MATERIALS AND METHODS

Identification of orthologous genes: The following published, complete, or draft assembly genome sequences of *Rickettsia* spp. (as of 2006) were downloaded from the NCBI FTP site (ftp://ftp.ncbi.nih.gov/genomes/Bacteria): *R. akari* Hartford (BioProject ID: PRJNA12953), *R. bellii* OSU 85-389 (BioProject ID: PRJNA17237), *R. bellii* RML369-C (BioProject ID: PRJNA13996), *R. canadensis* McKiel (BioProject ID: PRJNA12952), *R. conorii* Malish 7 (BioProject ID: PRJNA42), *R. felis* URRWXCal2 (BioProject ID: PRJNA13884), *R. massiliae* MTU5 (BioProject ID: PRJNA18271), *R. prowazekii* str. Madrid E (BioProject ID: PRJNA43), *R. rickettsii* Iowa

Received November 13, 2015. Accepted May 31, 2016.
J-STAGE Advance Publication June 30, 2016.
DOI: 10.7883/yoken.JJID.2015.579

*Corresponding author: Mailing address: Department of Virology-I, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111, Fax: +81-3-5285-1208, E-mail address: shuando@nih.go.jp

(BioProject ID: PRJNA19943), *R. rickettsii* Sheila Smith (BioProject ID: PRJNA9636), *R. sibirica* 246 (BioProject ID: PRJNA1414), and *R. typhi* Wilmington (BioProject ID: PRJNA10679).

Each orthologous gene was identified by homology searches of amino acid sequences using BLASTP filtering, with an expectation value (e-value) $\leq e^{-10}$ and sequence overlap $\geq 80\%$ (10). All the open reading frames of each species were searched against one another, and the reciprocal best hit was regarded as an orthologous gene. Only protein-coding genes of the chromosomal sequences were used for the identification of the orthologous groups. Of these, 10 orthologous sets were selected and defined as an orthologous group conserved among *Rickettsia* spp. Multiple sequence alignment of each of these orthologous groups was performed using ClustalW, and the conserved region was extracted (11).

Preparation of the DNA template: The DNA templates used in this study are summarized in Table 1. The genomic DNA from all the *Rickettsia* spp. was extracted as described in our previous study (6). Tris-EDTA buffer containing 100 μg/ml salmon sperm was used as the DNA dilution buffer to eliminate the absorption of DNA fragment in the microtube for the evaluation of sensitivity. Human genomic DNA and mouse genomic DNA were used as negative controls for this examination (Table 1).

To calculate the detection limit of the LAMP assay, PCR standards were generated by PCR amplification. The PCR mixture was prepared based on the manufacturer's instructions. In brief, each target DNA sequence (Table 1) was amplified in a 50-μl mixture containing 0.5 μl Ex Taq (5 U/μl; Takara Bio Inc., Shiga, Japan), 5 μl Ex Taq buffer, 4 μl dNTP mixture (2.5 mM), 0.5 μl 5′-primer: 164_3detection5 (100 μM), 0.5 μl 3′-primer: 164_3detection3 (100 μM) (Table 2), 1 μl template DNA, and distilled water. The reaction conditions for PCR were as follows: 30 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s, followed by an incubation step at 94°C for 3 min. The final cycle was followed by an extension step at 72°C for 3 min. The amplified PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The concentrations of the PCR products were determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher, Waltham, MA, USA).

Primers for LAMP: The oligonucleotide primers for LAMP (Table 2) were designed using Primer Explore ver. 4 (Fujitsu Ltd. Tokyo, Japan). BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to verify whether similar sequences of the candidate primers exist in the genome database.

LAMP assay: The LAMP assay was performed using the RT-160C Loopamp turbidimeter (Eiken Chemical Co., Tokyo, Japan), which can monitor optical density in real time. All the reagents used for the LAMP assay, except the oligonucleotides, were obtained from Eiken. The reaction mixture was prepared according to the manufacturer's instructions. Each 25-μl sample per Loopamp reaction tube (Eiken) contained 12.5 μl reaction mixture, 1 μl 40 pmol FIP primer, 1 μl 40 pmol BIP primer, 1 μl 5 pmol F3 primer, 1 μl 5 pmol B3 primer, 1 μl *Bst* DNA polymerase, 1 μl template DNA, and distilled water. In the LAMP assay with Loop primers, 2 primers, 1 μl each of Loop-F (20 pmol) and Loop-B (20 pmol) primers, were added to the above mixture. The amplification reaction was performed as follows—the reaction tubes were placed in the RT-160C system with a Hot Bonnet and incubated at 63°C for 60 min. For the inactivation and termination of the Loopamp reaction, the mixture was incubated for 2 min at 95°C. The LAMP parameters were set as follows—the average turbidity from 0 to 7 min after the initiation of LAMP reaction was used as the correction base line. The differential value of detection was fixed at 0.01. The decision of positive or negative judgment in the assay was automatically determined by the system at specific time intervals. For versatility, such as in cases where other LAMP machines that do not allow real-time monitoring are used, the endpoint was determined based on the turbidity in this study.

Table 1. DNA templates used in this study

Sample No.	Species	Strain	Reference
1	*Rickettsia asiatica*	IO-46	(12)
2	*Rickettsia canadensis*	Sai-109	(12)
3	*Rickettsia conorii*	Malish 7	ATCC VR 613T
4	*Rickettsia helvetica*	IC-1	(12)
5	*Rickettsia honei*	RH-1	(12)
6	*Rickettsia japonica*	YH	ATCC VR-1363
7	*Rickettsia prowazekii*	breinl	(13)
8	*Rickettsia rickettsii*	Sheila Smith	(14)
9	*Rickettsia sibirica*	246	ATCC VR-151
10	*Rickettsia tamurae*	HM-1	(12)
11	*Rickettsia typhi*	Wilmington	ATCC VR-144
12	*Rickettsia heilongjiangensis*	CH8-1	(12)
13	Human		Clontech Laboratories, Inc. (Takara Bio Inc, Japan)
14	Mouse (L929 NTCC Clone)		ATCC CCL-1

Table 2. LAMP primers used in this study

Set ID	name	Nucleotide sequence (5′–3′)	Purpose
164_1	164_1_F3	TGTTGCATCCATATGTATGGTA	LAMP for *Rickettsia spp.*
	164_1_B3	GCCGCTCCTAAATAACCA	
	164_1_FIP	GCCCTGAAACAAAAAAGCCTATTAATTAGTCTTGTTTTGGAAACTTCC	
	164_1_BIP	CTCAGCTCCTTGTAGGTATAGCAATAAACCTGATAATCCATTAGCC	
164_2	164_2_F3	GCAACTCCTGCTATAAGAGAA	LAMP for *Rickettsia spp.*
	164_2_B3	TCCATAAAATTCCTATAAGCCAT	
	164_2_FIP	CGTTACACGCTTTACTAACTCCATATATTTTGGGGTTACTAAAACACA	
	164_2_BIP	ATGCTCGGATATTTATGGTTTTGGGGAATCTGAAAAACCTATTAGCATAG	
164_3	164_3_F3	GGATCATTACTATGCTAATAGGTT	LAMP for *Rickettsia spp.*
	164_3_B3	TCGATTAAATAACCGCAGCT	
	164_3_FIP	TCTTTGTTGCAGGAGGCCAGGGCTTATAGGAATTTTATGGATAGC	
	164_3_BIP	TACTCACTGGTTCGCTTCAAAAGAATAGCAAGAGCACCACCT	
	164_3_F3_cana	GGATCATTACTATTCTAATAGGCT	
	164_3_B3_cana	TCGACACCAATAAATTAACT	
	164_LF	CCCATAGATTGAAACCAGTTACTG	
	164_LB	TGGGCTATGGGTGCAACTTCTAATC	
	164_3detection5	TCGTCAGAATTTTAATATAGC	Copy number detection
	164_3detection3	TGGAACAAAAAAGGCAGCTCTCC	
164_4	164_4_F3	CCTCAGCTCCTTGTAGGT	LAMP for *Rickettsia spp.*
	164_4_B3	AATCCTCCTCCTAAAAGAGC	
	164_4_FIP	CCGCTCCTAAATAACCAAATAAACCCAGCTGCTGATTTTAGTACC	
	164_4_BIP	GGAGTCGGCGTAGGATGGATCTGAAAAATATAAATACCCCATTCC	
164_5	164_5_F3	ATAGCCAGTAACTGGTTTCA	LAMP for *Rickettsia spp.*
	164_5_B3	AAAAGCGATACTACACAAGC	
	164_5_FIP	ACCCATAGCCCATTTAGTTCCAAGCTCCTGCAACAAAGATGC	
	164_5_BIP	TAGGTGGTGCTCTTGCTATGAACCTGGAACAAAAAAGGCA	

RESULTS

Orthologs in *Rickettsia* spp.: We determined 211 orthologous groups among the genomes of 13 *Rickettsia* spp. (data not shown). The BLASTN search for any known nucleotide sequences revealed 10 Rickettsial-specific orthologous groups (Table 3). Rickettsial-specific LAMP primers could be constructed from only 7 orthologs: ortholog IDs 3, 43, 77, 158, 164, 167, and 175. The most suitable ortholog was then screened out manually by visual inspection, and BLASTN analysis was then performed to align the sequences corresponding to the region containing the LAMP primer sequences. Ultimately, ortholog ID164 was selected for the LAMP assay (data not shown).

Primer selection: Five primer sets (Set ID 164_1 to 164_5, Table 2) were constructed and selected using Primer Explore for ortholog ID 164. The primer sets were tested with various DNA templates in the respective LAMP assays, and the results are summarized in Table 4. Among the primer sets, Set ID 164_3 showed the best reactivity for the DNA detection of several *Rickettsia* spp. Non-specific reactions were not observed with genomic DNA from the human or murine fibroblasts (negative controls) in the LAMP assay (Table 4).

Optimization and calibration of LAMP assay: The detection limit of the LAMP assay was determined using the PCR products produced previously. The copy numbers of DNA fragments from all *Rickettsia* spp. tested in this study were calculated based on the optical density at 260 nm and used for 10-fold serial dilutions (10 to 100,000 copies/reaction). The results showed that the limit of detection for almost all the DNA fragments was 100 copies, except for the DNA fragment of *R. canadensis* (data not shown). Thus, the specific primers for *R. canadensis*, B3_cana and F3_cana, were added to the LAMP reaction mixture to improve the reactivity for this species. The assay with *R. canadensis*-specific primers added could detect 100 copies of the target DNA fragments within 90 min. To accelerate the reaction, 164_LF and 164_BF primers (15) were constructed using Primer Explore and added to the LAMP mixture. Thus, 8 primers were used in this LAMP assay, which showed rapid reactivity where 100 copies of DNA could be detected within 60 min.

DISCUSSION

Several methods for the rapid molecular diagnosis of rickettsiosis based on genomic sequence data have been reported (3,6–9). However, a method that can simultaneously detect the DNA of several *Rickettsia* spp. is not available. In this study, with an aim to develop a method that can be used to easily detect the DNA of several *Rickettsia* spp., we focused on orthologs conserved among members of the *Rickettsia* genus, i.e., gene groups that are homologously conserved in different species originating from a common ancestor (4). We identified 10 *Rickettsia*-specific orthologs using a

Table 3. *Rickettsia* spp.-specific orthologous groups

Ortholog ID	Putative function	Lengh (AA)	Locus tag in Rickettsia											
			R. akari Hartford	*R. bellii* OSU 85-389	*R. bellii* RML369	*R. canadensis* McKiel	*R. conorii* Malish 7	*R. felis* URRWXCal2	*R. massiliae* MTU5	*R. prowazekii* Madrid E	*R. rickettsii* Iowa	*R. rickettsii* Sheila Smith	*R. sibirica* 246	*R. typhi* Wilmington
3	lipid A biosynthesis lauroyl acyltransferase	300	A1C_05570	A1I_06940	RBE_0186	A1E_04690	RC1091	RF_0197	RMA_1125	RP718	RrIowa_1302	A1G_06065	rsib_orf1006	RT0704
43	VacJ lipoprotein precursor	300	A1C_00660	A1I_00895	RBE_1331	A1E_00445	RC0123	RF_0076	RMA_0131	RP093	RrIowa_0157	A1G_00725	rsib_orf591	RT0044
77	DNA uptake lipoprotein	300	A1C_01315	A1I_01815	RBE_1102	A1E_00915	RC0230	RF_1088	RMA_0243	RP183	RrIowa_0285	A1G_01315	rsib_orf474	RT0174
115	exodeoxyribonuclease VII small subunit	80	A1C_02625	A1I_04940	RBE_0766	A1E_03715	RC0475	RF_0557	RMA_0491	RP350	RrIowa_0567	A1G_02690	rsib_orf227	RT0339
125	intracellular septation protein A	180	A1C_02940	A1I_06175	RBE_0324	A1E_03430	RC0539	RF_0613	RMA_0556	RP392	RrIowa_0644	A1G_03060	rsib_orf161	RT0380
126	FTR1 family protein	280	A1C_02945	A1I_06170	RBE_0325	A1E_03425	RC0540	RF_0614	RMA_0557	RP393	RrIowa_0645	A1G_03065	rsib_orf160	RT0381
158	Iron-sulfur cluster assembly accessory protein	110	A1C_03950	A1I_03940	RBE_0945	A1E_02345	RC0728	RF_0843	RMA_0821	RP484	RrIowa_0866	A1G_04115	rsib_orf1356	RT0471
164	glycerol-3-phosphate transporter	430	A1C_00445	A1I_00780	RBE_1354	A1E_00255	RC0082	RF_0121	RMA_0089	RP054	RrIowa_0107	A1G_00520	rsib_orf631	RT0078
167	Penicillin-binding protein	594	A1C_04385	A1I_01180	RBE_1223	A1E_02085	RC0852	RF_0900	RMA_0891	RP565	RrIowa_1014	A1G_04730	rsib_orf1247	RT0553
175	magnesium transporter	456	A1C_04520	A1I_03650	RBE_0999	A1E_01940	RC0886	RF_0951	RMA_0919	RP583	RrIowa_1054	A1G_04890	rsib_orf1214	RT0572

Table 4. Overview of the LAMP results

Primer set ID[2)]	Template ID[1)] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Decision[3)]
164_1	−	+	−	+	−	−	+	+	−	−	+	−	−	−	−
164_2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
164_3	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+
164_4	+	+	−	+	+	−	+	−	−	−	−	+	−	−	−
164_5	−	+	−	+	+	+	+	+	+	+	+	+	−	−	−

[1)]: Consist with sample ID in Table 1.
[2)]: ID numbers are consistent with the ortholog ID in Table 3.
[3)]: + means ''The best Primer set using for *Rickettia* spp assay.''

BLASTN search from 211 orthologous groups in several *Rickettsia* spp. with published genome sequences. Currently, further genome sequencing has been completed for *R. africae* (BioProject ID: PRJNA18269) and *R. peacockii* (BioProject ID: PRJNA59301), and these sequences are now available in GenBank.

LAMP is a unique gene amplification method that can detect the target in approximately 60 min at about 63°C. Although we did not compare the sensitivity of our LAMP assay with that of qPCR, with respect to its performance as a diagnostic technique, our assay shows superior ability with respect to the procedures and equipment required, and thus may be better adapted for fieldwork than for laboratory diagnosis.

The LAMP primers developed for each *Rickettsia* spp. will be useful for detecting Rickettsial infection at an early stage in the field or in the context of bioterrorism. The combination of probes and primers makes LAMP a more powerful tool for rapid DNA detection, not only in clinical laboratories but also in other settings such as in a consulting room and/or a patient's bedside. Our LAMP assay can also detect several specific types of *Rickettsia* spp. simultaneously, such as typhus group, *R. prowazekii*, *R. typhi*, *R. rickettsii*, and *R. japonica*. Furthermore, these assays could be completed within 1 h. These results demonstrate the potential of the new LAMP assay for the specific detection of *Rickettsia* spp. One of the aims of this study was to provide an easy molecular diagnosis technique that possesses versatility. Therefore, we used the development of turbidity in the reaction as an endpoint.

Thus, we identified 10 orthologous genes that are highly conserved among *Rickettsia* spp. and then developed a novel LAMP assay with a high level of sensitivity and specificity targeted to select these orthologous genes for *Rickettsia* spp. Unfortunately, there are no homologous orthologs for *Orientia tsutsugamushi* and members of the family Anaplasmataceae, which are genetically closely related outgroups. Nevertheless, we believe that our novel LAMP assay will be a powerful and useful tool for laboratory and field diagnosis of rickettsiosis.

Acknowledgments This work was supported by a Health Science Research Grant for Research on Emerging and Reemerging Infectious Diseases from the Japan Health Sciences Foundation, the grants of the Japanese Ministry of Health, Labour and Welfare and the Research Program on Emerging and Re-emerging Infectious Diseases from Japan Agency for Medical Research and development, AMED. The funders had no role in this study design, data collection and anal-

ysis, decision to publish, or preparation of the manuscript.

Conflict of interest None to declare.

REFERENCES

1. Walker DH. Rickettsiae and rickettsial infections: the current state of knowledge. Clin Infect Dis. 2007;45 Suppl 1:S39-44.
2. Raoult D. Antibiotic treatment of rickettsiosis, recent advances and current concepts. Eur J Epidemiol. 1991;7:276-81.
3. Fournier PE, Dumler JS, Greub G, et al. Gene sequence-based criteria for identification of new *rickettsia* isolates and description of *Rickettsia heilongjiangensis* sp. nov. J Clin Microbiol. 2003;41:5456-65.
4. Fitch WM. Distinguishing homologous from analogous proteins. Syst Zool. 1970;19:99-113.
5. Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000;28: E63.
6. Hanaoka N, Matsutani M, Kawabata H, et al. Diagnostic assay for *Rickettsia japonica*. Emerg Infect Dis. 2009;15:1994-7.
7. Kato CY, Chung IH, Robinson LK, et al. Assessment of real-time PCR assay for detection of *Rickettsia* spp. and *Rickettsia rickettsii* in banked clinical samples. J Clin Microbiol. 2013;51: 314-7.
8. Pan L, Zhang L, Wang G, et al. Rapid, simple, and sensitive detection of the *ompB* gene of spotted fever group rickettsiae by loop-mediated isothermal amplification. BMC Infect Dis. 2012; 12:254.
9. Dittrich S, Castonguay-Vanier J, Moore CE, et al. Loop-mediated isothermal amplification for *Rickettsia typhi* (the causal agent of murine typhus): problems with diagnosis at the limit of detection. J Clin Microbiol. 2014;52:832-8.
10. Altschul SF, Madden TL, Schaffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25:3389-402.
11. Larkin MA, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23:2947-8.
12. Fujita H. Cell culture system for isolation of disease agents: 15 years of experience in Ohara Research Laboratory. Annu Rep Ohara Hosp. 2008;48:21-42. Japanese.
13. Perez Gallardo F, Fox JP. Infection of guinea pigs with massive doses of rickettsiae of epidemic and murine typhus. J Immunol. 1948;60:455-63.
14. Dantas-Torres F. Rocky Mountain spotted fever. The Lancet Infect Dis. 2007;7:724-32.
15. Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes. 2002;16:223-9.