JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2004, p. 5349–5352 Vol. 42, No. 11
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.11.5349–5352.2004

Sensitive and Specific Detection of *Yersinia pseudotuberculosis* by Loop-Mediated Isothermal Amplification

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Received 8 March 2004/Returned for modification 23 April 2004/Accepted 5 July 2004

We developed a loop-mediated isothermal amplification method able to detect *Yersinia pseudotuberculosis* strains in 30 min by using six primers designed by targeting the *inv* gene. This method is more sensitive than PCR and might be a useful tool for detecting and identifying *Y. pseudotuberculosis*.

Yersinia pseudotuberculosis is known to be an important causal agent of zoonosis. *Y. pseudotuberculosis* infection in humans causes several diseases, such as enteritis, mesenteric lymphadenitis, reactive arthritis, erythema nodosum, and septicemia (1, 14, 15). This bacterium has been isolated from many animals, including monkeys, dogs, pigs, rodents, rabbits, deer, and birds, and is sometimes fatal to them (1, 3, 4).

Of several molecular genetic methods, PCR is the most widely used for specific amplification of a target gene, and it has also been reported to be able to detect pathogenic *Yersinia* species from foods and environmental samples (7, 16, 17, 19). Recently, a novel nucleic acid amplification method, named loop-mediated isothermal amplification (LAMP), that amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions has been developed (2, 9, 12). This method simply consists of incubating a mixture of the target gene, four different primers, DNA polymerase with strand displacement activity, and substrates at a constant temperature between 60 and 65°C. The target gene is detected by the increase in the turbidity of the reaction mixture that coincides with the production of precipitate correlated with the amount of target DNA synthesized, i.e., the amplicons. The aim of this study was to develop a *Y. pseudotuberculosis* detection method, more sensitive and specific than PCR, based on the LAMP method, and to evaluate the performance of this method for detection of *Y. pseudotuberculosis* in clinical samples.

Thirty-one *Yersinia* species comprising 21 strains of *Y. pseudotuberculosis*, 4 strains of pathogenic *Y. enterocolitica*, and 6 strains of nonpathogenic *Yersinia* species strains, as well as 10 other gram-negative bacilli, were tested (Table 1). Template DNAs used for LAMP were prepared as follows. Bacterial cells of each strain from colonies on trypticase soy agar (TSA; BBL) were suspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) to achieve a concentration of approximately 10^8 CFU/ml. In order to examine the detection limit for LAMP and PCR, a series of 10-fold dilutions of *Y. pseudotuberculosis* serovar 1b with TE buffer was made. The cells were heat treated in a boiling water bath for 10 min and were centrifuged for 10 min at 9,000 × *g*. The resulting supernatant was used as the template for LAMP and PCR. The LAMP reaction requires four oligonucleotide primers recognizing six distinct regions (F1, F2, F3, B1, B2, and B3) on the target DNA: the forward inner primer (FIP), back inner primer (BIP), and two outer primers (F3 and B3) (12). FIP consists of a complementary sequence of F1 and a sense sequence of F2. BIP consists of a sense sequence of B1 and a complementary sequence of B2. LAMP primers targeting the *inv* gene of *Y. pseudotuberculosis*, the chromosomal virulence gene (8), were designed based on the gene sequence of *inv* (accession no. M17448) obtained from the DNA Data Bank of Japan by using the software program Primer Explorer V2 (Fujitsu, Tokyo, Japan). The sequences of the designed primers are shown in Table 2. Those four primers are sufficient to carry out the amplification reaction; however, the LAMP reaction can be accelerated by using additional primers termed loop primers (10), so loop primers LF and LB targeting the *inv* gene of *Y. pseudotuberculosis* were designed (Table 2). These loop primers were used in the reactions through which the amplification data were collected. However, the designed loop primers react with the restriction site of restriction enzyme BssHII (New England BioLabs, Beverly, Mass.), which was used to digest the obtained amplicons so as to confirm that the amplicons are of the target genes. Therefore, the loop primers were not used in the reactions involving BssHII. The LAMP reaction was carried out with the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). A reaction mixture (25 μl) containing 1.6 μM each inner primer (FIP and BIP), 0.2 μM each outer primer (F3 and B3), 0.8 μM each loop primer (LF and LB), *Bst* DNA polymerase (0.5 μl), 2× reaction mix (12.5 μl), and template DNA (2 μl) was incubated at 63°C for 50 min and then heated at 80°C for 2 min to terminate the reaction. A DNA-omitted reaction mixture was used as a negative control. The amplification of the gene was confirmed by real-time monitoring of the increase of turbidity by using LA-200 (Teramecs, Kyoto, Japan), which sequentially measured the absorbance of the reaction mixture at 650 nm. To determine the detection

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TABLE 1. Bacterial strains subjected to LAMP and results

Species	Serotype	Strain	LAMP result[a]
Y. pseudotuberculosis	1a	3384	0.49
Y. pseudotuberculosis	1b	NYP95001	0.50
Y. pseudotuberculosis	1c	Kuratani	0.46
Y. pseudotuberculosis	2a	49	0.49
Y. pseudotuberculosis	2b	1799	0.49
Y. pseudotuberculosis	2c	274	0.49
Y. pseudotuberculosis	3	T-312	0.46
Y. pseudotuberculosis	4a	51	0.44
Y. pseudotuberculosis	4b	NYP01001	0.48
Y. pseudotuberculosis	5a	204	0.47
Y. pseudotuberculosis	5b	197	0.50
Y. pseudotuberculosis	6	#14	0.55
Y. pseudotuberculosis	7	141	0.50
Y. pseudotuberculosis	8	151	0.50
Y. pseudotuberculosis	9	R708Ly	0.49
Y. pseudotuberculosis	10	6088	0.48
Y. pseudotuberculosis	11	R80	0.43
Y. pseudotuberculosis	12	MW900-3	0.42
Y. pseudotuberculosis	13	N916	0.47
Y. pseudotuberculosis	14	CN7	0.45
Y. pseudotuberculosis	15	93422	0.47
Y. enterocolitica	O:3	8	0.00
Y. enterocolitica	O:5,27	S203	0.00
Y. enterocolitica	O:8	NY9306089	0.00
Y. enterocolitica	O:9	314-2	0.00
Y. enterocolitica	O:8,19	NY8904001	0.00
Y. aldovae		JCM 5892	0.00
Y. intermedia		JCM 7579	0.00
Y. cristensenii		JCM 7576	0.00
Y. bercovieri		NY8704001	0.00
Y. rohdei		JCM 7376	0.00
Campylobacter jejuni		ATCC33560	0.00
Campylobacter coli		JCM2529	0.00
Campylobacter lari		JCM2530	0.00
Citrobacter freundii		JCM1657	0.00
Enterobacter cloacae		JCM1232	0.00
Escherichia coli		JCM5431	0.00
Pasteurella haemolytica		NP8507001	0.00
Pseudomonas fluorescens		JCM 5963	0.00
Salmonella enterica serovar Typhimurium		NMJS1	0.00
Salmonella enterica serovar Enteritidis		NS9506003	0.00

[a] Turbidity after 30 min of incubation.

limit, 1 μl of the LAMP products was submitted to electrophoresis, and, to confirm the amplicon structure, the LAMP products were digested with restriction enzyme BssHII and submitted to electrophoresis. The electrophoresis was carried out in 2% Tris-acetic acid-EDTA (TAE) agarose gel, and staining was performed with ethidium bromide to confirm the presence of the expected DNA fragments. One kilobase of

TABLE 2. LAMP primers

Primer	Sequence (5′-3′)
F3	CTCGTCGCGTGATTTCTCC
B3	GATCTACCCCGACAGTGAGT
FIP	CCAGTTGTGGGAGTGCAGGTAACTATAAAG AGCGCCCAGCC
BIP	CACCGGTGAGCGTGTTGCTTTGTGTAATTGA TCCCGGCAGT
LF	CATTCGCGCGCAAATCC
LB	GCAACGCAACCCTTATGC

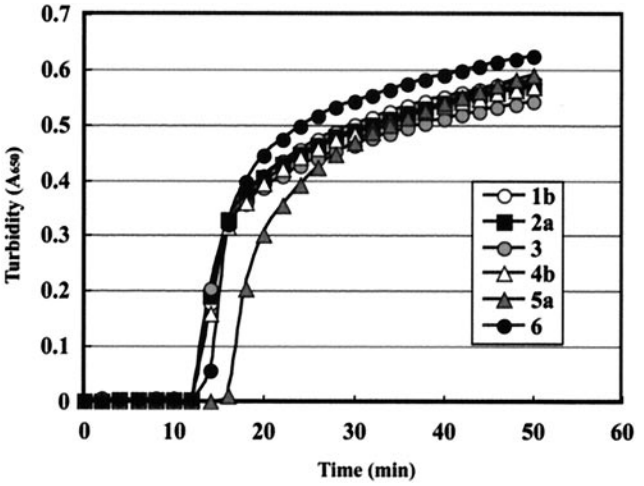

FIG. 1. Detection of the LAMP amplification signals. A total of 10^5 CFU of template DNA of *Y. pseudotuberculosis* 1b, 2a, 3, 4b, 5a, and 6 was used for the LAMP reaction.

Plus DNA ladder (Invitrogen) was used as a molecular weight standard. The PCR was carried out with the primers for the *inv* gene designed by Nakajima et al. (11). PCR was performed with a Program Temperature Control System PC-701 (ASTEC, Fukuoka, Japan) at 94°C for 1 min as an initial denaturation step and then was subjected to 30 cycles consisting of 30 s at 94°C, 1 min at 55°C, and 2 min at 70°C, followed by a single 5-min extension step at 70°C. The PCR mixture (50 μl) contained 4 μl of template DNA, 0.1 mM each of the four deoxynucleoside triphosphates, 5 μl of 10× PCR buffer (Applied Biosystems Japan Ltd., Tokyo, Japan), 0.1 μM each primer, and 0.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.). Ten microliters of the PCR amplification products was subjected to electrophoresis under the same protocol of the LAMP products in a 1.5% agarose gel.

The specificity of LAMP using the newly designed primers was examined by carrying out reactions with DNAs from the *Yersinia* species and other gram-negative bacilli. The results of turbidity measurements for the LAMP reaction for 30 min at 63°C are shown in Table 1, and the representative curves are shown in Fig. 1. Turbidities derived from the LAMP reaction of *Y. pseudotuberculosis* strains began to increase after approximately 15 min of incubation, and they continued to increase as the LAMP progressed. All *Y. pseudotuberculosis* strains examined showed turbidities above 0.4 at 650 nm after 30 min of incubation. In contrast, turbidities were not observed even after 50 min of incubation when template DNA from *Y. enterocolitica*, a nonpathogenic *Yersinia* species, and other gram-negative bacilli were tested. This result proved the specificity of the developed primers. The differences among the turbidities of *Y. pseudotuberculosis* and all of the other samples became evident after 20 to 25 min of incubation. The use of loop primers shortened the reaction time for amplification by about one-half compared to that of amplification performed without loop primers (data not shown). These results showed that the LAMP method using these newly designed primers is able to detect *Y. pseudotuberculosis* specifically.

The sensitivity of LAMP and PCR for *Y. pseudotuberculosis* was determined by determining the detection limit as de-

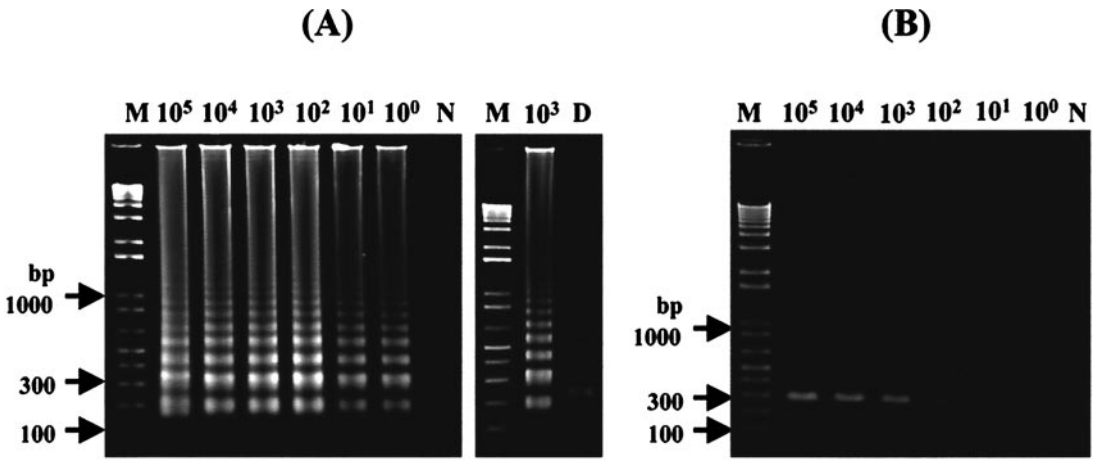

FIG. 2. Electrophoretic analysis of LAMP (A) and PCR (B) products. The numbers above each lane represent 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU per reaction tube of template DNA of *Y. pseudotuberculosis* 1b. Lane D, LAMP product after digestion with BssHII; lane N, LAMP or PCR in the absence of template DNA; lane M, 1-kb ladder DNA size marker.

scribed above. The results showed that the LAMP method is able to detect the target gene even with 10^0 CFU of bacteria present in the tube (Fig. 2). In contrast, the detection limit of PCR was 10^2 CFU. Thus, LAMP was 100 times more sensitive than PCR. The products of LAMP from *Y. pseudotuberculosis* that were submitted for confirmation by digestion with restriction endonuclease, with cleavage sites within the amplicon, showed the expected size band of 246 bp (Fig. 2).

It is known that PCR inhibitors in samples reduce the sensitivity of PCR when attempting to detect a target gene (6, 13, 17, 18). Notomi et al. (12) reported that the sensitivity of LAMP is not influenced by the copresence of nontarget DNA in samples, and Enosawa et al. (2) reported that LAMP was not inhibited by blood serum and plasma heparin, which are known to inhibit PCR. Therefore, we evaluated the performance of this method in clinical specimens. A total of 15 livers from dead monkeys were used. Of the 15 monkeys, 9 squirrel monkeys (*Saimiri sciureus*) and 1 orangutan (*Pongo pygmaeus*) died by natural *Y. pseudotuberculosis* infection, 2 squirrel monkeys and 1 dark-handed gibbon (*Hylobates agilis*) died by natural *Y. enterocolitica* O:8 infection, and 2 other squirrel monkeys died by other causes and no *Yersinia* species was isolated. Isolation of *Yersinia* from those monkeys was carried out as described previously (5). The number of bacteria in the *Y. pseudotuberculosis*-positive samples ranged from 2.2 to 6.8 log CFU/g. DNA for LAMP from liver samples was extracted by using the Wizard Genomic DNA Purification kit (Promega). The LAMP reaction was positive only for those samples from

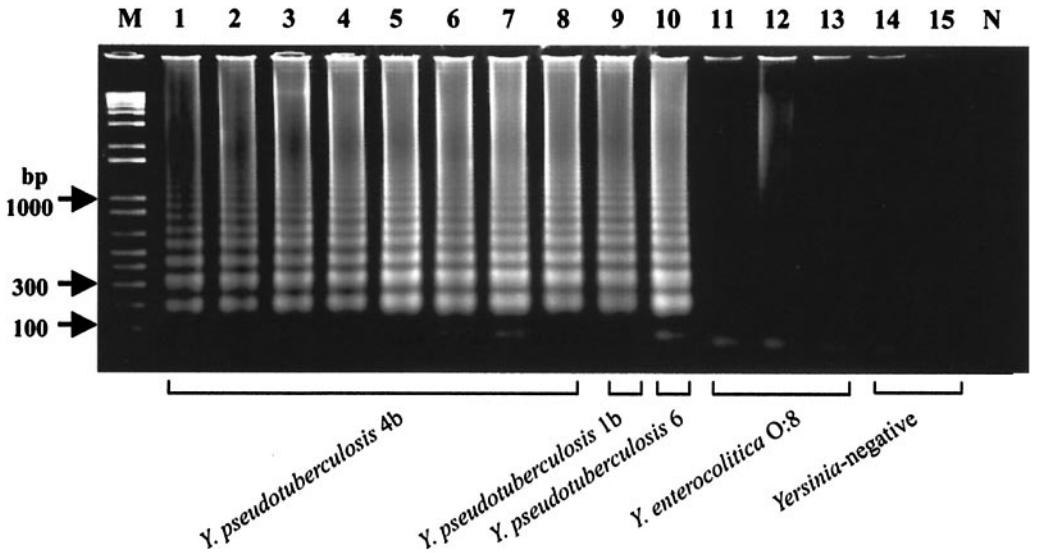

FIG. 3. LAMP detection of the *inv* gene in liver samples from *Y. pseudotuberculosis*-infected monkeys and uninfected monkeys. The samples of each lane and the number of bacteria isolated, in log CFU/gram, from each sample are the following: lanes 1 to 7, squirrel monkey, 5.1, 6.8, 6.4, 6.8, 5.1, 2.2, and 5.0, respectively; lane 8, orangutan, 5.2; lanes 9 to 12, squirrel monkey, 4.9, 6.3, 6.7, and 5.6, respectively; lane 13, dark-handed gibbon, 5.2. Lanes 14 and 15, squirrel monkeys from which no *Yersinia* species were isolated. Lane N, LAMP in the absence of template DNA. Lane M, 1-kb ladder DNA size marker.

the monkeys infected by *Y. pseudotuberculosis* and was negative for the other samples, even after 60 min of incubation (Fig. 3). Thus, this result shows the high specificity of this method for detection of *Y. pseudotuberculosis* in clinical specimens.

Furthermore, as complicated thermoregulators are not needed to carry out the reactions and LAMP amplicons can be detected by visually confirming a white precipitate of magnesium pyrophosphate, this method might also be a useful and powerful tool for the screening and detection of *Y. pseudotuberculosis* in the field. Thus, further studies applying this LAMP method to detect this bacterium in food and environmental samples should be carried out.

We thank Hiroshi Fukushima (The Shimane Prefectural Institute of Public Health and Environmental Science, Shimane, Japan) for kindly providing us with *Y. pseudotuberculosis* strains. We also thank Keiko Watanabe (Eiken Chemical Co., Ltd.) for technical assistance.

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