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Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Common Strains of *Escherichia coli*▽

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We developed a highly sensitive and specific LAMP assay for *Escherichia coli*. It does not require DNA extraction and can detect as few as 10 copies. It detected all 36 of 36 *E. coli* isolates and all 22 urine samples (out of 89 samples tested) that had *E. coli*. This assay is rapid, low in cost, and simple to perform.

Escherichia coli can cause infections of a variety of extraintestinal sites such as the urinary tract, meninges, and bloodstream (3, 5, 8, 10–12, 21). Diagnosis of most extraintestinal *E. coli* (ExPEC) infections requires bacterial culture, requiring 1 to 2 days of incubation, and subsequent confirmatory testing (6, 17). Guidance as to the presence or absence of an ExPEC infection at initial presentation could be useful for a patient, especially when attempting to reduce excessive use of antibiotics. Although conventional DNA amplification using PCR can provide fast results, it is not widely used partly because it requires considerable skill and expensive equipment. Recently, loop-mediated isothermal amplification (LAMP), a rapid technique for amplifying DNA has been reported (14, 15). It requires incubation at a constant temperature (60 to 66°C for 30 to 60 min) and eliminates the need for specialized equipment or expertise. We report here the development and characterization of a LAMP assay to detect urinary *E. coli*.

Three sets of primers (outer, loop, and inner) were required for the *E. coli* LAMP assay that were designed by analyzing a conserved region of the *E. coli malB* gene with Primer Explorer version 3 software (http://primerexplorer.jp/elamp3.0.0/index.html) (Fig. 1). This gene is conserved across diverse lineages of *E. coli* and is not shared by other gram-negative bacteria except *Shigella* spp., based on BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) (18). We considered this region to be appropriate because of its conservation, as well as the rarity of *Shigella* as a cause of extraintestinal infections.

The LAMP reaction was conducted using methods described previously, with minor modifications (14, 15). The final LAMP reaction (total, 25 μl) contained the three primer pairs in the following concentrations: 0.2 μM outer primers, 0.8 μM loop primers, and 1.6 μM inner primers. The reaction mix also contained 2.5 μl of 10× *Bst* DNA polymerase reaction buffer [1× containing 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_2)SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100], 1 μl of an 8-U/μl concentration of Bst DNA polymerase (New England Biolabs, Inc., MA), 2 mM $MgSO_4$ (2 μl), 5 μl of betaine (Sigma-Aldrich, St. Louis, MO), and 5 μl of sample. We generated and quantified a plasmid containing the target *E. coli* sequence to determine the sensitivity of the LAMP assay. The target sequence in *E. coli* was amplified by PCR using the primers F3 and B3 of the LAMP assay and cloned by using a StrataClone PCR cloning kit (Stratagene, La Jolla, CA). The copy number of the solution containing the *E. coli* clone was determined by UV spectrophotometry (2). The analytical sensitivity of LAMP was determined by using 10-fold serial dilutions of the *E. coli* clone.

The LAMP reaction was performed in a heating block (LabLine, Iowa). For comparison, it was also performed using a conventional thermal cycler (Bio-Rad, California), and both machines performed equally well. The cost per reaction was ca. 50¢ (U.S. currency).

A positive *E. coli* LAMP reaction typically required incubation for 60 min at 66°C to produce a ladder pattern on agarose gel (Fig. 2i). The *E. coli* LAMP products from several of the bands in the gel were excised and sequenced. The amplicons matched the *E. coli malB* gene segment spanned by F3/B3 primers. The *E. coli* LAMP products were also detected without electrophoresis using ambient light after the addition of a number of DNA dyes to the reaction tube. These dyes included propidium iodide, ethidium bromide, methylene blue, acridine orange, and Sybr green. Of the various dyes, Sybr green (1:10

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E. coli Primers	Outer forward and backward primers	Bases
F3	GCCATCTCCTGATGACGC	18
B3	ATTTACCGCAGCCAGACG	18
	Inner forward and backward primers*	
BIP (B1+B2c)	CTGGGGCGAGGTCGTGGTAT *TCCGACAAACACCACGAATT*	40
FIP (F1c+F2)	*CATTTTGCAGCTGTACGCTCG*CAGCCCATCATGAATGTTGCT	42
	Loop forward and backward primers	
Loop F	CTTTGTAACAACCTGTCATCGACA	24
Loop B	ATCAATCTCGATATCCATGAAGGTG	25

FIG. 1. LAMP primers. (i) Location of the LAMP primers in the *E. coli malB* gene. This sequence represents positions 3204 to 3407 in the *E. coli* GenBank sequence (GDB J01648). The F2 and Loop F primers overlap by one base, depicted by "*" and an italic "T". Also, two of the reverse primers, Loop B and B2, are contiguous and have no bases separating them, and the juncture is marked by "*" and an arrow. (ii) Sequences for the LAMP primers. The F1c and B2c sequences are the reverse complements of the F1 and B2 regions shown in Fig. 1 and are underlined.

dilution of a 10,000× stock solution) and propidium iodide (1:10 dilution of 10-mg/ml stock solution) produced the best visual discrimination. For both the dyes, 1 μl of the diluted dye was added to 25 μl of the reaction mix to develop the color reaction (Fig.2ii). UV transillumination of the reaction mix containing the DNA dye improved the differentiation further.

The specificity of the assay was evaluated by testing eight gram-negative bacteria (*Acinetobacter* spp., *Citrobacter freundii*, *Enterobacter cloacae*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Serratia marcescens*) and six gram-positive bacteria (*Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Streptococcus viridians*). No ladder pattern was seen with any of the other non-*E. coli* bacteria or with the negative "no-target" control (Fig. 2i). The sensitivity of *E. coli* LAMP was found to be approximately 10 copies per reaction when an incubation period of 60 min was used. Larger copy numbers required shorter incubation times (as low as 15 to 20 min for >1,000,000 copies).

We further tested a range of *E. coli* isolates by this assay. A total of 36 isolates of *E. coli* from unique patients ($n = 23$) or the *Escherichia coli* Reference (ECOR) Collection ($n = 13$) were tested. ECOR (established in 1984) contains 72 strains and five phylotypes (16). A LAMP assay was performed on an aliquot of bacterial solution without extraction of DNA. It was able to detect all of the 36 *E. coli* isolates (Table 1). Finally, 89 fresh urine samples were tested by LAMP assay, and the results were compared to cultured urine samples obtained from microbiology laboratories of the Children's Hospital of Pittsburgh and the All India Institute of Medical Sciences from patients suspected with urinary tract infections (UTI). The culture was considered "positive" if there was growth of individual bacteria in a concentration of >100,000 colonies/ml. Growth of multiple bacteria was considered contamination. The samples were stored at 4°C after plating of urine and were analyzed by LAMP assay within 1 to 3 days of collection. Initial LAMP testing, performed on three known *E. coli*-positive urine samples, showed positive results both with and without DNA extraction using a QIAmp DNA Mini-Kit (Qiagen, California). Subsequent tests were performed on unextracted urine, i.e., 5 μl of unprocessed urine was directly added to the LAMP reaction mix, followed by incubation for 60 min at 66°C. Of the 89 urine samples, 22 were positive for *E. coli*, all of which tested positive by the LAMP assay. In addition, 36 samples had no bacterial growth, and the LAMP assay results were negative in all of these samples. Finally, in 31 samples, organisms other than *E. coli* grew, including 13 samples that pro-

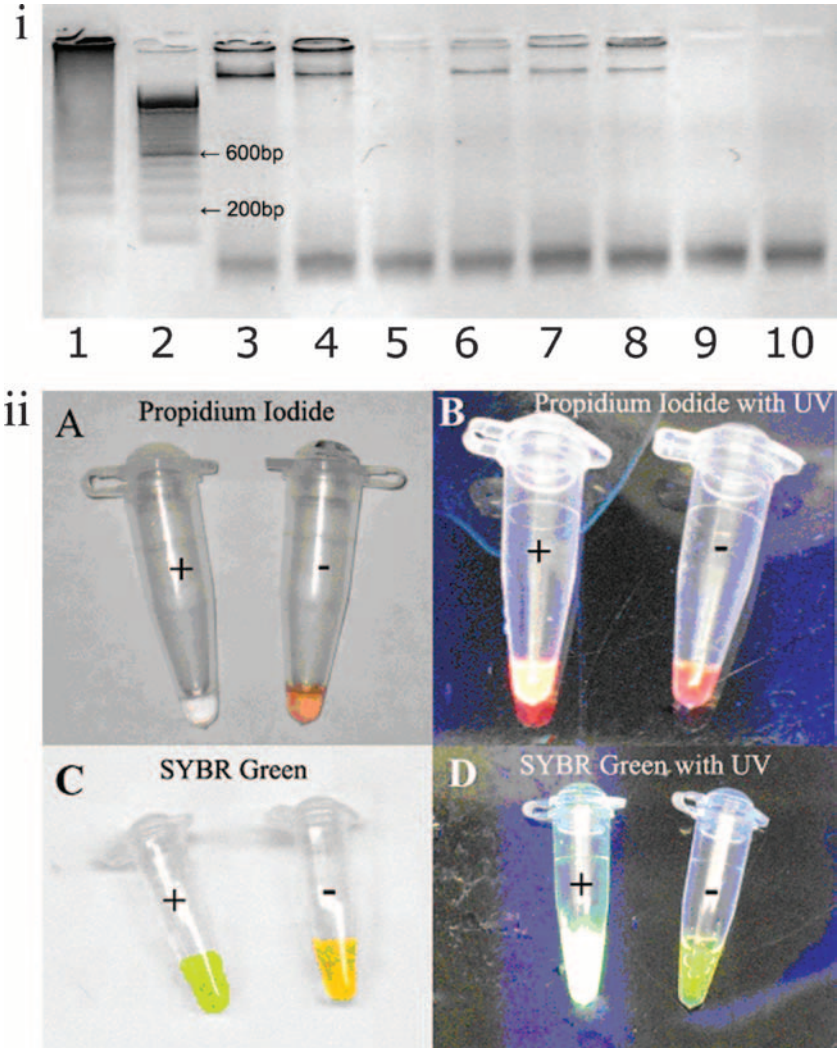

FIG. 2. LAMP assay reactions. (i) A gel image showing LAMP products from *E. coli* and other bacteria. The lane numbers correspond to the following specimens. Lanes: 1, *E. coli*; 2, 100-bp ladder; 3, *Proteus mirabilis*; 4, *Pseudomonas aeruginosa*; 5, *Enterobacter faecalis*; 6, *Staphylococcus aureus*; 7, *Streptococcus pyogenes*; 8, *Streptococcus pneumoniae*; 9, *Streptococcus viridans*; 10, no target control. (ii) Visualization of LAMP products by inspection. (A) Propidium iodide without UV illumination. The color changes from a deep red-orange in the negative reaction to a light (almost clear) pink in the positive reaction. (B) Propidium iodide with UV illumination showing bright transillumination in the positive reaction. (C) Sybr green without UV illumination. The color changes from orange in the negative to green in the positive reaction. (D) Sybr green with UV illumination showing bright transillumination in the positive reaction.

duced ≥1 bacterial species. In 29 of these 31 samples, the LAMP assay was negative. Two non-*E. coli* samples that had tested positive by the LAMP assay had grown multiple organisms and probably had *E. coli* as one of the contaminants (Tables 2 and 3).

We describe here a new, rapid, and economical assay to detect *E. coli* in human urine in 1 h (or less), which makes this assay amenable for point-of-care or outpatient settings. There are several advantages of the LAMP over PCR, the conventional method for DNA amplification. This assay only needs a heating block and obviates the need for a thermal cycler. Also, DNA extraction is not required for LAMP. In contrast, unprocessed urine normally requires DNA extraction for PCR, since urinary urea can inhibit PCR at concentrations of >50 mM and the normal concentration of urea in adult urine is >330 mM (1, 4, 9, 20). Hence, the LAMP assay, by eliminating the step of DNA extraction, is more "user friendly" than PCR. We focused on urine as the analyte for the present study and obtained consistent results for all of the culture-positive samples. Although we did not test other biological fluids (e.g., blood, cerebrospinal fluid, etc.) it is likely, based on our recent studies on BK virus LAMP, that *E. coli* LAMP would perform well with these types of specimens (2).

Another advantage of this assay is that identification of a positive reaction does not require any special processing or electrophoresis. It could be detected by looking for a color change of the reaction mix in ambient light, when a DNA-binding dye is used. This visualization can be further enhanced by UV transillumination. We also showed that a simple chemical such as propidium iodide can be used for detecting a positive reaction. To our knowledge, this is the first report of using propidium iodide to visualize LAMP products. Pro-

TABLE 1. Description of isolates of *E. coli* evaluated by LAMP

Source[a]	Sample no.	Phylogenic group	Antigen(s)	Sample[b]	Syndrome	LAMP positive
Clinical	C1	A	O147	Urine	Cystitis	Yes
	C2	A	O8	Urine	Pyelonephritis	Yes
	C3	A	O25:K2:H2	Blood	Urosepsis	Yes
	C4	A	O74	Feces	NA[c]	Yes
	C5	B1	O75	Urine	Cystitis	Yes
	C6	B1	O149	Urine	Pyelonephritis	Yes
	C7	B1	O64:H21	Blood	Urosepsis	Yes
	C8	B1	O86	Feces	NA	Yes
	C9	B2	O6	Urine	Cystitis	Yes
	C10	B2	ON	Urine	Cystitis	Yes
	C11	B2	O2	Urine	Pyelonephritis	Yes
	C12	B2	O2	Urine	Pyelonephritis	Yes
	C13	B2	O1:K1:H7	Blood	Urosepsis	Yes
	C14	B2	O6:K2:H1	Blood	Urosepsis	Yes
	C15	B2	ON	Blood	Abdominal sepsis	Yes
	C16	B2	O1	Blood	Abdominal sepsis	Yes
	C17	B2	O1	Blood	Pulmonary sepsis	Yes
	C18	B2	O1	Blood	Pulmonary sepsis	Yes
	C19	B2	O6	Feces	NA	Yes
	C20	B2	ON	Feces	NA	Yes
	C21	D	O17,77	Urine	Pyelonephritis	Yes
	C22	D	O7:K1:H−	Blood	Urosepsis	Yes
	C23	D	O21	Feces	NA	Yes
ECOR	ECOR-11	A	O6	Urine	Cystitis	Yes
	ECOR-5	A	O79	Feces	NA	Yes
	ECOR-3	A	O1	Feces	NA	Yes
	ECOR-72	B1	O144	Urine	Pyelonephritis	Yes
	ECOR-28	B1	O104	Feces	NA	Yes
	ECOR-33	B1	O79	Feces	NA	Yes
	ECOR-45	B1	ON	Feces	NA	Yes
	ECOR-55	B2	O25	Urine	Pyelonephritis	Yes
	ECOR-61	B2	O25	Feces	NA	Yes
	ECOR-57	B2	ON	Feces	NA	Yes
	ECOR-48	D	ON	Urine	Cystitis	Yes
	ECOR-39	D	O79	Feces	NA	Yes
	ECOR-37	E	ON	Feces	NA	Yes

[a] The clinical samples (C1 to C23) were collected at the Veterans Affairs Medical Center, Minneapolis, MN.
[b] Feces isolates were from an uninfected, asymptomatic host.
[c] NA, not applicable.

pidium iodide is less expensive, does not require freezing for storage, and is more easily available in developing countries, such as India, than is Sybr green. In theory, a deep color of the urine (bilirubin, myoglobin, or other chromogens) could affect the color of the final LAMP products and interfere with the assay results, but we did not see this effect in the range of samples of urine tested that had minor color variation (from clear to pale yellow).

Our assay was able to detect a large number of strains studied with a very high sensitivity. Indeed, the ability to detect 10 copies per reaction may be considered to be "too sensitive" for use as a clinical assay for UTI diagnostics. However, the assay's sensitivity can be lowered to match the conventionally accepted cutoff of >100,000 copies/ml for urine if a shorter incubation time is used (i.e., 30 to 40 min versus 60 min). It is likely that different levels of sensitivity may be required for different biological fluids. For example, it may be necessary to have a high sensitivity for blood and CSF versus a lower sensitivity for urine for the assays to be clinically meaningful. Also, an ability to detect a lower copy number may be important in partially treated infections (after empirical antibiotic doses), where the organisms are reduced in number or not culturable but the DNA is still detectable.

TABLE 2. Clinical comparison of LAMP and culture results from urine samples obtained from patients with suspected UTI

Culture result	No. of samples:		
	Tested	Positive by LAMP	Negative by LAMP
Positive for *E. coli* (pure culture and ≥100,000 CFU/ml)	22	22	0
No growth	36	0	36
Growth of bacteria other than *E. coli*, including gram-positive, gram-negative, fungus, and multiple organisms	31	0	29
Gram positive (n = 15)			
Enterococcus faecalis	1	0	1
Lactobacillus spp.	1	0	1
Staphylococcus saprophyticus	1	0	1
Streptococcus spp. (alpha-hemolytic)	2	0	2
Streptococcus spp. (group B, beta-hemolytic)	2	0	2
Gram-positive cocci (not otherwise specified)	8	0	8
Gram negative (n = 2)			
Proteus mirabilis	1	0	1
Klebsiella oxytoca	1	0	1
Fungus (n = 1)			
Candida albicans	1	0	1
Multiple organisms (n = 13)	13	2	11
Total	89	24	65

Although LAMP assays for various specific *E. coli* strains have been reported, these studies were designed to detect virulence loci or markers of specific strains, such as verotoxin (7, 19, 22). However, for detection of UTI-associated *E. coli* strains, a less discriminatory approach is intended. Our studies extend the role of LAMP to the rapid diagnosis of such infections, using this less-biased approach. In addition to the advantages described here, there are some limitations of the assay in its current format. This assay is suited for qualitative but not quantitative information. Possibly, semiquantitative assays can be developed where the time required to develop a positive reaction could be used as a surrogate for the starting copy number. Also, LAMP assays do not provide antimicrobial susceptibility information and since bacteria demonstrate increasing resistance to antimicrobial agents, this assay is more likely to be an adjunct rather than a replacement for culture. Finally, in contrast to culture, this assay detects only a single organism. However, LAMP assays for other common bacterial pathogens, including *Staphylococcus* spp., have recently been reported (13); these assays may potentially be combined with this assay to screen for common infections. Further studies involving a larger number of patients and different specimens are needed in order to evaluate the clinical utility of this assay.

TABLE 3. Statistical comparison of LAMP and culture results from urine samples obtained from patients with suspected UTI[a]

Comparison standard	% Sensitivity	% Specificity	PPV	NPV
LAMP vs culture	91.7	100	1.00	0.97
Culture vs LAMP	100	97	0.917	1.00

[a] PPV, positive predictive value; NPV, negative predictive value.

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