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Homogenous, real-time duplex loop-mediated isothermal amplification using a single fluorophore-labeled primer and an intercalator dye: Its application to the simultaneous detection of Shiga toxin genes 1 and 2 in Shiga toxigenic *Escherichia coli* isolates

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ABSTRACT

We developed a completely homogeneous duplex loop-mediated isothermal amplification (LAMP) method. The present LAMP method employed a combination of a 6-carboxyfluorescein (FAM)-labeled primer (donor) for one target gene, a non-labeled primer for the other, and an intercalator ethidium bromide (EtBr) dye (acceptor) on the basis of fluorescence resonance energy transfer (FRET) between the FAM donor and EtBr acceptor. Measuring changes in fluorescence of FAM enabled the LAMP method to detect two different genes simultaneously. This method was used to detect Shiga toxin genes in Shiga toxigenic *Escherichia coli* isolates, demonstrating simultaneous detection of two different genes with rapidity and accuracy.

1. Introduction

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method based on autocycling strand displacement DNA synthesis using the *Bst* DNA polymerase large fragment [1–3]. The LAMP method, which employs four to six primers and amplifies the target gene in a single temperature step, is faster and easier to perform than PCR, as well as being more specific. Unlike PCR, however, LAMP products are not easily discriminated through agarose gel electrophoresis because of the many types of structures, preventing multiplex detection from being introduced into the LAMP method. In order to overcome this drawback, some multiplex LAMP methods have been reported [4–7], although these methodologies involve heterogeneous and labor-intensive post-amplification processing steps. Furthermore, since the LAMP method produces large amounts of DNA, there is some risk of carry-over contamination in these post-amplification methods. Therefore, homogeneous methods may be a solution to compensate for the shortcomings of such post-amplification detections.

Homogeneous, fluorescence-based assays, in which product detection is concurrent with target amplification by the generation of a target-specific signal, have been utilized for real-time multiplex PCR. The intercalator-based real-time PCR can distinguish the resulting products through melting curve analysis, while melting curve analysis cannot be applied to the LAMP method because the products involve multiple types of structures. Furthermore, probe-based detections such as TaqMan [8] are also inapplicable to the LAMP method since the amplification principle of the LAMP method is based on strand displacement DNA synthesis.

Shiga toxigenic *Escherichia coli* (STEC) is a detrimental cause of gastrointestinal disease in humans, particularly since such infections may result in life-threatening sequelae such as hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura [9]. Therefore, the presence of Shiga toxin genes (*stx*1 and *stx*2) is the focus of many assays for STEC. After the isolation of suspicious colonies of STEC on selective agar plates, rapid and simple Shiga toxin (STX) typing of the organisms can serve as a useful tool in managing patients optimally or in defining the epidemiology of infections.

In this study, we developed a completely homogeneous duplex LAMP method using a single 6-carboxyfluorescein (FAM)-labeled primer and the intercalator ethidium bromide (EtBr), and applied this LAMP method to the simultaneous detection of the *stx*1 and *stx*2 genes in STEC isolates.

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Table 1
LAMP primers.

Primer	Sequence (5′–3′)
*stx*1	
FIP	TAACATCGCTCTTGCCACAGAC-CGTTGACTACTTCTTATCTGGAT
BIP[a]	TACTGTGACAGCTGAAGCTTTAC-TACATAAGAACGCCCACTG
LF	AGGTTCCGCTATGCGACAT
LB	GTTTTCGGCAAATTCAGAGGGG
F3	GATGCAGATAAATCGCCATT
B3	GATCAACATCTTCAGCAGTC
*stx*2	
FIP	TGCTGATTCGCCCCCAGTTC-CCTGTGTATACGATGACGCC
BIP	GCTTCCGGAGTATCGGGGAGA-ACAGTACCCAGTATCGCTGA
LF	GAGTGAGGTCCACGTCTCC
LB	GGATGGTGTCAGAGTGGGGAGA
F3	CGTCAGGCACTGTCTGAAAC
B3	CCCCCTGATGATGGCAATT

[a] 5′ end FAM-labeled.

2. Materials and methods

2.1. Bacterial strains

In this study, a total of 202 STEC strains were used from our own collection, and the *stx* gene types of all STEC strains were classified according to the PCR method described by Paton et al. [10]. The STEC serogroups included O157 (*stx*1: 15 isolates; *stx*2: 65 isolates; *stx*1&2: 80 isolates), O26 (*stx*1: 28 isolates; *stx*1&2: 1 isolate), O103 (*stx*1: 3 isolates), O111 (*stx*1: 1 isolate; *stx*1&2: 2 isolates), O124 and O128 (*stx*1: 2 isolates each), O115 and O145 (*stx*1: 1 isolate each); one STEC isolate carrying the *stx*2 gene was not typeable by the O serogroup. In order to acquire the optimal duplex LAMP conditions, three STEC strains, FVT120 (O157:H7; *stx*1&2), FVT20 (O157:H7; *stx*1) and FVT2 (O157:H7; *stx*2), were tested as standard strains. Six strains of other bacterial species, non-STX-producing *E. coli*, *Shigella sonnei*, *Salmonella enteritidis*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, and *Vibrio parahaemolyticus*, were used as negative controls.

2.2. DNA template preparation

A part of a colony on a Luria-Bertani plate (1% tryptone, 0.5% yeast extract, 0.5% NaCl [pH 7.0]) was transferred by a sterilized toothpick into 0.1 mL distilled water and was heated at 99 °C for 5 min. The boiled sample was centrifuged and the supernatant was used as a template. The optical density of the templates gave a reading of 0.05–0.30 at 260 nm. Prepared templates were used immediately for duplex LAMP assays or kept at 4 °C for up to one week.

2.3. Real-time duplex LAMP

Real-time duplex LAMP assay was performed using 2 μL template DNA in a final volume of 20 μL with LAMP reaction mixture. The reaction mixture consisted of 200 mM for each dNTP, 0.8 M betaine (Sigma Chemical Co., St. Louis, MO, USA), 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween 20, 2 μg/mL EtBr (Dojindo, Kumamoto, Japan) and 6.4 U of *Bst* DNA polymerase large fragment (New England Biolabs, Inc., Ipswich, MA, USA). Primer (Table 1) concentrations were as follows: 0.2 μM each of outer primers F3 and B3, 1.6 μM of inner primer FIP-*stx*1 and 0.4 μM of inner primer BIP-*stx*1 (5′ end FAM-labeled) or 1.6 μM each of inner primers FIP-*stx*2 and BIP-*stx*2, and 0.8 μM each of loop primers LF-*stx*1 and LB-*stx*1. Concentrations of loop primers LF-*stx*2 and LB-*stx*2 were optimized in order to acquire optimal FAM fluorescence signals. The LAMP reaction was performed for 60 or 90 cycles at 63 °C for 30 s (20 s + 10 s sampling time) in a CFX96

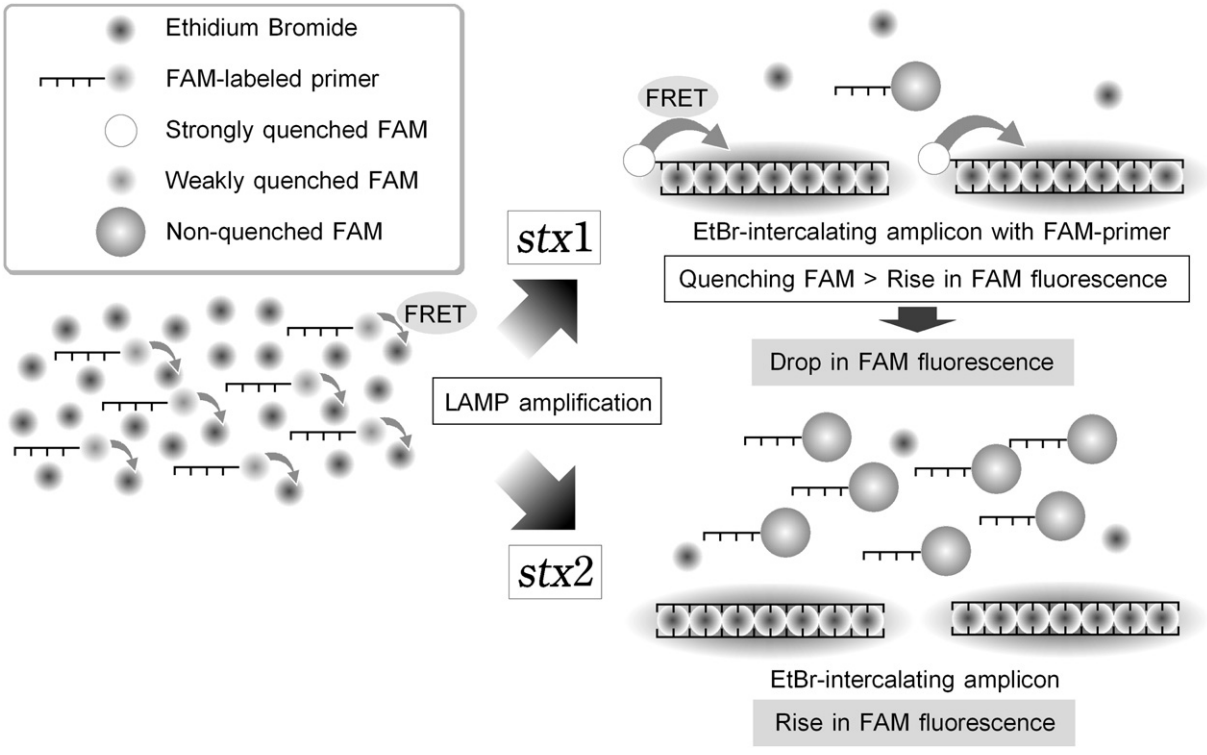

Fig. 1. Strategy of the detection scheme of duplex LAMP for the *stx*1 and *stx*2 genes. At the initial phase, the FAM-labeled primers (donor) for *stx*1 and innumerable EtBr molecules (acceptor) cause weak FRET with a weak drop in FAM fluorescence. The amplification of *stx*1 sequences brings FAM-labeled primers and the intercalating EtBr closer together, resulting in a drop in FAM fluorescence, while during the amplification of *stx*2 sequences, the decrease in free EtBr enhances FAM fluorescence with both donor and acceptor moieties further apart.