Loop Mediated Isothermal Amplification for Diagnosis of *Escherichia coli* O157:H7 and Viewpoints on its Progression into Realistic Point of Care

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***Abstract*—**A study was undertaken to develop and validate loop mediated isothermal amplification (LAMP) method for the detection of *Escherichia coli* O157:H7. PCR sensitivity was analyzed prior to LAMP application and hence, LAMP was optimized with *stx1* primers in terms of reaction conditions, analytical sensitivity and specificity. LAMP could be a useful adjunct diagnostic assay along with the conventional methods that would preclude the requirement of continuous maintenance of pure cultures. Moreover, LAMP assay is simple, rapid, specific, and sensitive for the detection of STEC O157:H7. Reaction time of the LAMP method was only one hour. The results of the LAMP reaction were also compared with routine PCR method. The amplification products of O157, which had the corresponding target genes, turned green by visual inspection and had ladder-like pattern on the gel, but products of other enterobacteria remained orange by visual examination and had no band on the gel.

***Keywords*—**Loop Mediated Isothermal Amplification Assay, Polymerase Chain Reaction, *Escherichia coli* O157:H7.

I. Introduction

MAJOR advance in diagnostic testing includes PCR for viruses, culture methods for bacteria and microscopy for parasites. Moreover, many tests that form the backbone of the "modern" microbiology laboratory are based on very old and expertise-intensive technologies such as ELISA for pathogenic antigens or its antibodies. Pressing needs include more rapid tests without sacrificing sensitivity, value-added tests, and point-of-care tests for both high- and low-resource settings.

In recent years, research has been focused on alternative methods to improve the diagnosis of viral diseases. One such method is Loop Mediated Isothermal amplification (LAMP) that amplifies a target DNA under isothermal conditions, since discovered by Notomi *et al.* in 2000 [1]. LAMP is a novel method which amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. Because LAMP recognises the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity.

Unique characteristics of LAMP includes: 1. Amplifies a target DNA under isothermal conditions. 2. Relies on autocycling strand displacement DNA synthesis performed by using the *Bst* DNA polymerase large fragment. 3. Less expensive, rapidity (results within 1 hour), low reaction temperature (60 to 65 ^{0} C), high specificity for the target and sensitivity. 4. Requires only a regular laboratory water or heat block to carry out the reaction. 5. The end product can be visualized by naked eyes.

Application of LAMP under field conditions has been limited, partly due to the infancy of the technologies associated with LAMP, such as field-based template preparation methods and product detection formats [2]. In this viewpoint, the essential technologies that require development before the LAMP platform can be progressed into a realistic point of care format for resource-poor endemic areas are highlighted.

II. Aim and Objective

To develop and validate loop mediated isothermal amplification method for the detection of a shiga--toxin producing *Escherichia coli* strain.

III. Materials and Methods

A. Preliminary work:

Since stx1 gene is a potent virulence gene of Escherichia coli O157:H7, our present study has chosen it as target gene. Selected gene was confirmed by conventional PCR. Oligonucleotide primer sequences used for PCR amplification was derived from a study conducted by J. EL-Jakee *et al.,* [3] in Egypt in 2009. After PCR amplification, PCR gel bands were cut and sequencing of stx1 gene was done. The stx1 gene was cloned in Escherichia coli BL21, since sample is not available in surplus and also, for sensitivity check to be done with serially diluted plasmid DNA.

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B. Lamp primer designing

In earlier studies, primers for LAMP test were designed by targeting the antigen coding rfbE of EHEC O157:H7, the Shiga-like toxin stx2 and the fliC encoding gene of H7 flagella antigen [4, 5]. But the present study targeted stx1 gene of EHEC O157:H7. The reaction condition and reaction system of LAMP were optimized. PrimerExplorer V3/4 software is specifically for designing the primer sets for LAMP method [6]. One primer set contains 4 primers, FIP (Forward Inner Primer), F3, BIP (Backward Inner Primer) and B3. F1, F2, F3 are about 20bp long sequences selected from the target gene, B1, B2, B3 are about 20bp long sequences selected from the complementary strand. F1c and F1, B1 and B1c are complementary regions. This software can also design the loop primers, LF and LB.

C. Lamp optimization and sensitivity check

Once Primers are ready, the concentration of MgSo4, temperature and time points need to be optimized until a ladder-like pattern observed in gel run. The end product visualized by naked eyes using SYBR green I [7] or Calcein. Conventional PCR made for the dilutions of Plasmid DNA extractions to check the sensitivity of PCR. To check the sensitivity of LAMP, LAMP too should have the same or higher sensitivity as that of Conventional PCR.

IV. RESULTS

A. Conventional PCR Amplification:

Conventional PCR amplification confirmed the presence of 614bp sized stx1 gene product when run on agarose gel electrophoresis. Primers used in PCR for STEC stx1 gene detection is tabulated in Table-I. While PCR reaction setup for stx1 gene amplification si shown in Table-II, PCR reaction conditions for stx1 gene amplification si shown in Table-III. Fig. 1 Shows the gel band of PCR gene amplification of stx1 under UV illumination.

B. LAMP Development:

Primers were successfully designed using Primer Explore V4 software and Primers used in LAMP for stx1 gene of STEC is tabulated in Table-IV. LAMP reaction mix (25 µl) as given in Table-V was employed. Optimized Conditions of LAMP for stx1 gene is given in Table-VI. Fig. 2 represents the pattern of LAMP products observed after gel run.

Fig. 3 depicts Analytical sensitivity of LAMP compared with conventional RT-PCR.

V. CONCLUSION

LAMP from research lab to clinical diagnosis: It is suggested here that the technologies associated with LAMP be considered and developed as part of a LAMP platform, rather than developing them as separate entities. To achieve these levels in resource-poor areas, specimen processing methods, production of lyophilised kits, and a closed amplification and detection system need to be developed, which will facilitate the provision of a same-day testing strategy in even the most remote rural health facilities.

A proposed three-step LAMP method for diagnosis of diseases is shown in Fig 4. As depicted in Fig 4, simultaneous development of specimen processing methods, production of lyophilised kits, a closed amplification and detection system along with LAMP optimization is the need of hour. This should be followed by rigorous evaluation of test performance to determine feasibility and acceptability under field conditions.

TABLE I
PRIMERS USED IN PCR FOR STEC STX1 GENE DETECTION

Primer Designation	Sequence (5'- 3')	Amplified product size (bp)
stx1-F	5'-ACA CTG GAT GAT CTC AGT GG-3'	614
stx1-R	5'-CTG AAT CCC CCT CCA TTA TG-3'	

TABLE II
PCR REACTION SETUP FOR STX1 GENE AMPLIFICATION

Components	Stock Concentration	Final Concentration	Volume for 20 µl setup
Milli Q Water			10.8 µl
dNTP mix	2mM	0.2mM	2 µl
Taq buffer	10X	1X	2 µl
Forward Primer	3 µm	0.3 µm	2 µl
Reverse Primer	3 µm	0.3 µm	2 µl
DNA Template	100ng/ µl	100ng	1 µl
Taq DNA Polymerase	5U/ µl	1U	0.2 µl

TABLE III
PCR REACTION CONDITIONS FOR STX1 GENE AMPLIFICATION

Step	Temperature	Time
Initial denaturation	94°C	3 min
Denaturation	94°C	1 min
Annealing	47°C	1min
Extension	72°C	1 min 20 sec
Final extension	72°C	7 min
Hold	4°C	-
Total Number of cycles: 32		

TABLE IV
PRIMERS USED IN LAMP FOR STX1 GENE OF STEC

Primers	5' pos	3' pos	Len	Sequence
F3	44	61	18	GAACTGGGGAAGGTTGAG
B3	229	246	18	CACGGACTCTTCCATCTG
FIP			44	TCCCAGAATTGCATTAATGCTTCC-GTCCTGCCTGATTATCATGG
BIP			47	AGCGTGGCATTAATACTGAATTGT-ACATAGAAGGAAACTCATCAGAT
F2	66	85	20	GTCCTGCCTGATTATCATGG
F1c	120	143	24	TCCCAGAATTGCATTAATGCTTCC
B2	200	222	23	ACATAGAAGGAAACTCATCAGAT
B1c	144	167	24	AGCGTGGCATTAATACTGAATTGT
LF	86	110	25	TCTTCCTACATGAACAGAGTCTTGT
LB	168	188	21	CATCATCATGCATCGCGAGTT

TABLE V
LAMP REACTION MIX (25 µL)

Component	Volume
F3 and B3 primers	1 µl each (10pmoles each)
FIP and BIP primers	2 µl each (40pmoles each)
LF and LR primers	1 µl each (20pmoles each)
dNTP (10mM/ml)	3.5 µl (1.4mmol/L)
Betaine	3 µl (0.8M/L)
Bst DNA polymerase large fragment	1 µl (12U/ µl)
Thermopol buffer	2.5 µl
$MgSo_4$	4mM

TABLE VI
OPTIMISED CONDITIONS OF LAMP FOR STX1 GENE

Component	Condition
F3 and B3 primers	10pmoles
FIP and BIP primers	40pmoles
LF and LR primers	20pmoles
$MgSo_4$	6mM
dNTP	2.5mM each
Temperature	62°C
Time	60 minutes

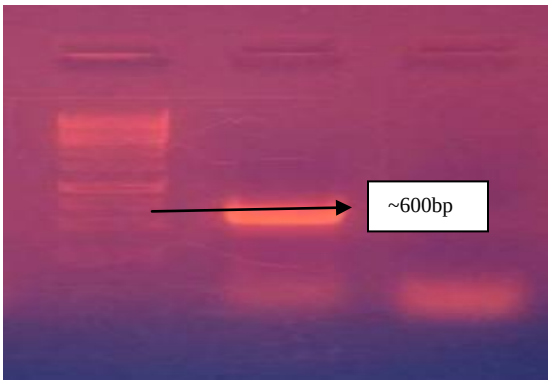

Fig. 1 Gel band of PCR gene amplification of stx1

Key of Fig. 1:
Lane 1: 1Kb DNA Ladder
Lane 2: Amplified Product of stx1 gene
Lane 3: Negative control

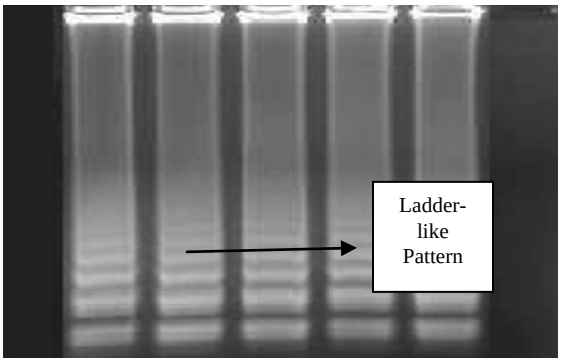

Fig. 2 LAMP products observed after gel run

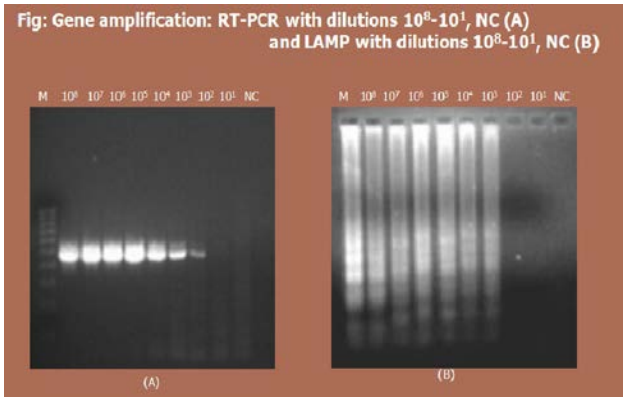

Fig. 3 Analytical sensitivity of LAMP compared with conventional RT-PCR.

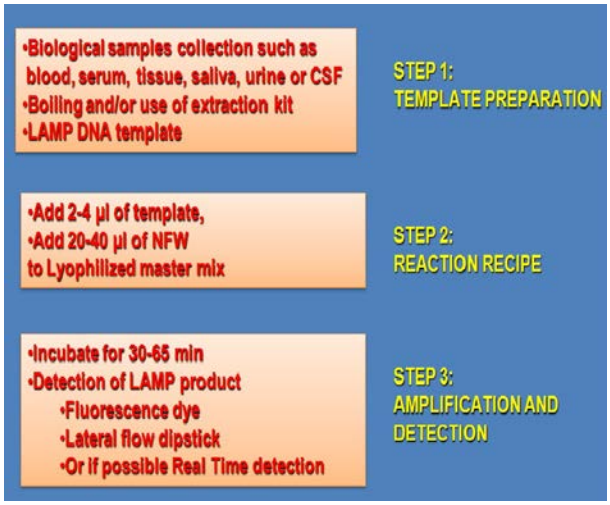

Fig. 4 A proposed three-step LAMP method for diagnosis of diseases

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