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Associated technologies ensures complete loop mediated isothermal amplification platform for pathogen diagnosis

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Loop Mediated Isothermal Amplification (LAMP) assay 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 pathogens. Having developed and validated LAMP method for the detection of an isolated pathogen, *Escherichia coli* O157:H7, an attempt was made to progress the LAMP platform to realistic point of care for resource-poor endemic areas. Reaction time of the LAMP method was only 1 h and also, the amplification products of O157, which had the corresponding target genes, turned green by visual inspection when added with Calcein/Sybr green. However, sample preparation and lyophilized master mix preparation before LAMP assay as well as developing a closed detection system for detection of LAMP amplified products remained a quest. Hence, the current study was conducted to develop a lyophilized LAMP master mix for easy platform to take LAMP to realistic point of care and Dot-Elisa based 'lateral flow dipstick' that could ease the detection of LAMP products in a closed environment. Sample preparation is another associated technology that is yet-to-be developed.

Key words: Loop mediated isothermal amplification assay, polymerase chain reaction, lyophilized master mix, and closed amplification system.

INTRODUCTION

Escherichia coli O157:H7 is an enterohemorrhagic strain of the bacterium *E. coli* and a cause of food-borne illness. Most illness has been associated with eating undercooked, contaminated ground beef, drinking unpasteurized milk, swimming in or drinking contaminated water and eating contaminated vegetables. Young children and females had an increased risk of Hemolytic Uremic Syndrome (HUS) after Shiga toxin producing *E. coli* (STEC) O157 infection. With or without HUS, elderly persons had the highest pro-portion of deaths associated with STEC O157 infection. These data support recommendations for aggressive sup-portive care of young children and the elderly early during illness due to STEC O157 (Gould et al., 2009). Haemo-lytic uremic syndrome is characterized by three features: acute renal failure, microangiopathic haemolytic anaemia and thrombocytopenia (Levin et al., 1984). *E. coli* O157:H7 was the causative agent of many out-breaks worldwide. Outbreaks of HC caused by VTEC occurred in different areas of USA in 1982 and since then outbreaks and spo-radic cases have been reported in several other coun-tries including Canada, Britain and Japan (Johnson et al.,

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1983; Pai et al., 1984). Incidence of sporadic cases of hemorrhagic colitis due to *E. coli* O157:H7 may be higher than suspected (Chik et al., 1984). An epidemiological outbreak of *E. coli* O157:H7 can be expected at any point of time and hence a constant study has to be conducted throughout the world for the early diagnosis of such pathogens.

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 pathogens. One such method is loop media-ted isothermal amplification (LAMP) that amplifies a tar-get DNA under isothermal conditions, since discovered by Tsugunori et al. (2000). LAMP is a novel method which amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. Unique characteris-tics of LAMP includes: 1) Amplifies a target DNA under isothermal conditions; 2) relies on autocycling strand dis-placement DNA synthesis performed by using the *Bst* DNA polymerase large fragment; 3) less expensive, rapidity (results within 1 h), low reaction temperature (60 to 65°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 visua-lized 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 pre-paration methods and product detection formats (Njiru, 2012). In this viewpoint, an attempt was made for the development of lyophilized LAMP master mix and lateral flow dipstick for detection of LAMP products. However, field-based sample template preparation method is yet-to-be developed for LAMP application in resource-poor endemic areas. Considering the state of art status of LAMP technique that has achieved its advancement for the diagnosis of various viruses worldwide, the current study emphasizes on its application on a bacterial patho-gen and can be extended towards parasitic and fungal pathogens in future.

MATERIALS AND METHODS

Preliminary work

An *E. coli* O157:H7 strain isolated from ground beef, India was used and stx1 gene, a potent virulence gene, was chosen as target gene. Selected gene was confirmed by conventional PCR. Oligonucleotide primer sequences used for PCR amplification of stx1 gene was derived from a study conducted by EL-Jakee et al. (2009) in Egypt, namely F1: 5'ACA CTG GAT GAT CTC AGT GG 3' and R2: 5'GGG ATA TCT TAG CTC TGT GAG AGC TCG CGG3' flanking a size of about 614 bp were used. Amplification was performed in Thermo-cycler for 32 cycles after initial denaturation for 3 min at 94°C. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 47°C for 1 min, and extension at 72°C for 1 min 20 s. The primer extension was extended to 7 min at 72°C in the final cycle. The PCR amplified products were detected by electrophoresis on 1.5% agarose gel in 1X TAE buffer at 80 v for 1 h (Sambrook et al., 1989). After PCR ampli-fication, PCR gel bands were cut, purified and sequencing of stx1 gene was done. The stx1 gene was cloned in *E. coli* BL21 using pET 32a vector, since sample is not available in surplus and also, for sensitivity check to be done with serially diluted plasmid DNA.

LAMP primer designing, optimization and sensitivity check

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 (Zhu et al., 2009; Deguo et al., 2009). But the present study targeted stx1 gene of EHEC O157:H7. The reaction condition and reaction system of LAMP were optimized. Primer Explorer V3/4 software is specifically for designing the primer sets for LAMP method (Qi-Wei et al., 2012). One primer set contains 4 primers, forward inner primer (FIP), F3, back-ward inner primer (BIP) and B3. F1, F2, F3 are about 20 bp long sequences selected from the target gene B1, B2, B3 are about 20 bp 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. LAMP primers were successfully designed using Primer Explore V4 software for stx1 gene of STEC and tabulated in Table 1. FIP and BIP alone are biotin labeled for lateral flow dipstick development in later stages. Once primers are ready, the concentration of $MgSO_4$, temperature and time points were optimized as 4 mM, 62°C and 60 min respectively, until a ladder-like pattern observed in gel run. The end product visualized by naked eyes using SYBR green I (Saleh et al., 2008) or Calcein.

Conventional PCR made for the dilutions of plasmid DNA extrac-tions to check the sensitivity of PCR (Note: Before making the dilutions, purity of plasmid DNA was checked using nanodrop spec-trophotometer and obtained as 1.9 under 260/280 absorbance; hence, serially diluted for 8 times until 10^{-8} dilution is obtained). Sensitivity of LAMP was compared with that of conventional PCR.

Lyophilized master mix and lateral flow dipstick preparation

The lyophilized isothermal master mix is an optimized master mix designed to simplify the preparation of an isothermal amplification assay. It is prepared corresponding to the optimised conditions of LAMP assay for the detection of *E. coli* O157:H7. Hence, a master reaction mix set up for 20 reactions were prepared and lyophilized and stored at -20°C. Since FIP and BIP primers were labeled with biotin, LAMP products must possess proportional biotin concentration as that of the product. Hence, lateral flow dipstick was developed involving the principle of Dot-Elisa, where LAMP products are considered equi-valent of the antigens against which anti-biotin antibody were added, secondary antibody conjugated with Horseradish peroxidase (anti-species anti-mouse HRP) added, then chromogenic substrate (DAB) added to observe for colored product as dot in positive controls. No dots obtained indicated absence of biotin labeled amplified DNA products and hence, interpreted as negatives. Positive controls were able to produce clear dots indicating the presence of biotin in the

Table 1. Primers used in LAMP for stx1 gene of STEC.

Primer	5' Pos	3' Pos	Length	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 2. Lyophilized LAMP master reaction mix components.

Component	Volume (500 µl for 20 reactions)
F3 and B3 primers	20 µl each
FIP and BIP primers	40 µl each
LF and LR primers	20 µl each
dNTP (10 mM/ml)	70 µl
Betaine	60 µl
Bst DNA polymerase large fragment	20 µl
Thermopol buffer	50 µl
$MgSo_4$	80 mM

LAMP amplified product as expected whereas negative controls had no biotin and hence, clear dots were not formed.

RESULTS AND DISCUSSION

Conventional PCR and LAMP amplification

Conventional PCR amplification confirmed the presence of 614 bp sized stx1 gene product when run on agarose gel electrophoresis. Figure 1 shows the gel band of PCR gene amplification of stx1 under UV illumination. Figure 2 represents the pattern of LAMP products observed after gel run. This ladder-like pattern as shown in this figure is unique for this technique and easily detectable as positive or negative control under gel documentation. Figure 3 depicts analytical sensitivity of LAMP compared with conventional PCR. It was observed that for both PCR as well as LAMP technique, sensitivity was up-to 10^{-2} dilution that is, amplification was possible up-to 10^{-2} dilution of plasmid DNA and could not be amplified beyond this dilution level.

Lyophilised LAMP master mix and lateral-flow dipstick development

Optimised conditions of LAMP assay for the detection of *E. coli* O157:H7 contained F3 and B3 primers 1 µl each (10 pmoles each); FIP and BIP primers 2 µl each (40 pmoles each); LF and LR primers 1 µl each (20 pmoles each); dNTP (10 mM/ml) 3.5 µl (1.4 mmol/L); betaine 3 µl (0.8 M/L); Bst DNA polymerase large fragment 1 µl (12 U/ µl); thermopol buffer 2.5 µl and $MgSO_4$ 4 mM. Hence, a master reaction mix set up for 20 reactions were prepared as shown in Table 2 and lyophilized. This master mix can be taken directly to the point of care for the addition of sample directly and has an advantage of shelf life of more than a week at -20°C. When LAMP amplified products were subjected to lateral flow dipstick assay, they were able to produce clear dots indicating the presence of biotin and hence the amplified DNA products were interpreted as positive. This assay can be further developed by coating anti-biotin antibody in latex particles (beads) and hence developing a latex agglutination kit which can give immediate results by the principle of latex agglutination of particles. Figure 4 shows the Dot-Elisa representing positive control and negative control along with the test, performed in blotting paper that can be applicable for developing lateral flow dipsticks for detection of LAMP products.

It is also to be noted that the more the concentration of product, the more the intensity of dot produced. Even though the results of Dot-Elisa were obtained consistently with repeated tests in frequent intervals of time, detection

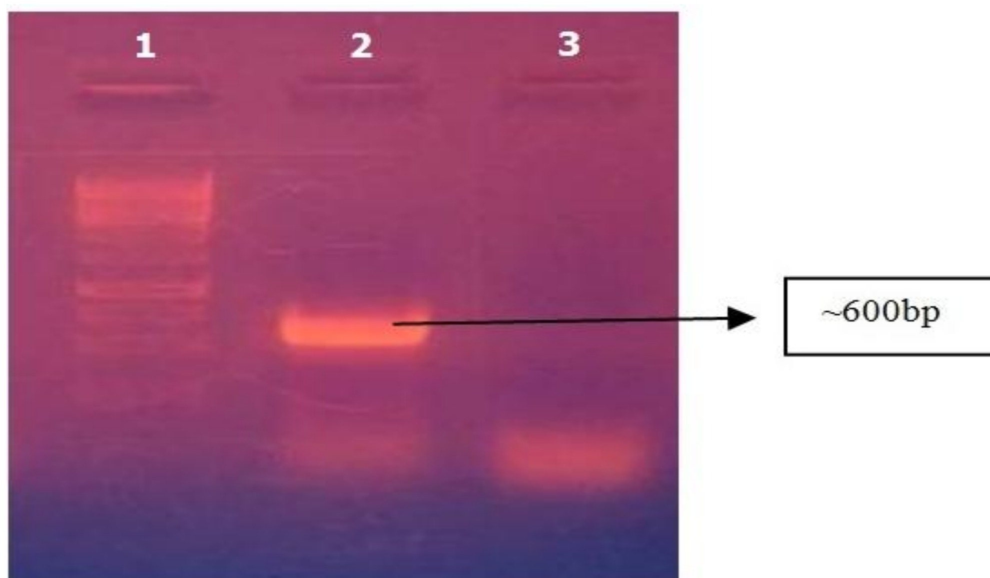

Figure 1. Agarose gel electrophoresis of amplified stx1 gene from the DNA extracted from *E. coli* O157:H7 (Lane 1: 1 Kb DNA Ladder; Lane 2: amplified product of stx1 gene; Lane 3: negative control).

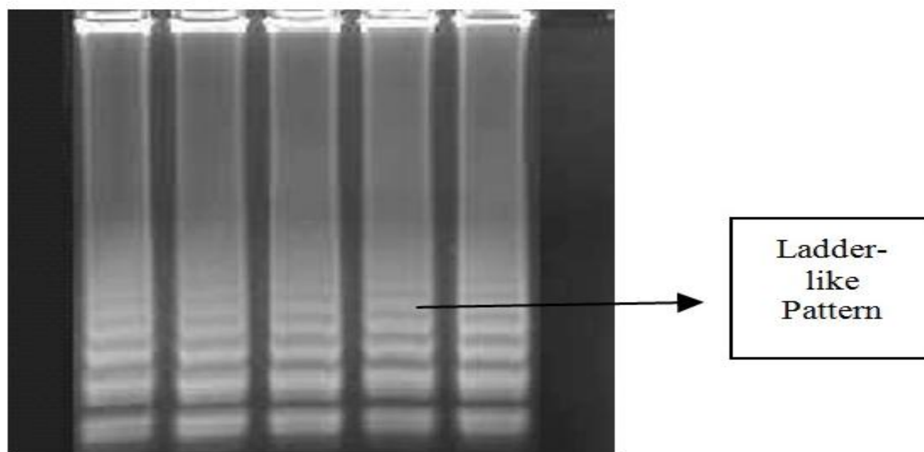

Figure 2. Ladder-like pattern developed by LAMP products (unique pattern for LAMP amplified products) when run on electrophoresis gel.

systems such as dipstick assays can be avoided in case of naked eye detection system of LAMP results using Calcein/SYBR green confirms the diagnosis.

Conclusion

LAMP from research lab to clinical diagnosis: it is suggested here that the technologies associated with LAMP can 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 lyophilized 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. Hence, this should be followed by rigorous evaluation of test performance to determine feasibility and acceptability under field conditions. However, detection systems such as dipstick assays can be avoided in case of resource unavailability, provided naked eye detection system of LAMP results is sufficiently achieved. Considering the state of art status of LAMP technique that has achieved its advancement for the diagnosis of various viruses worldwide, current study emphasizes on

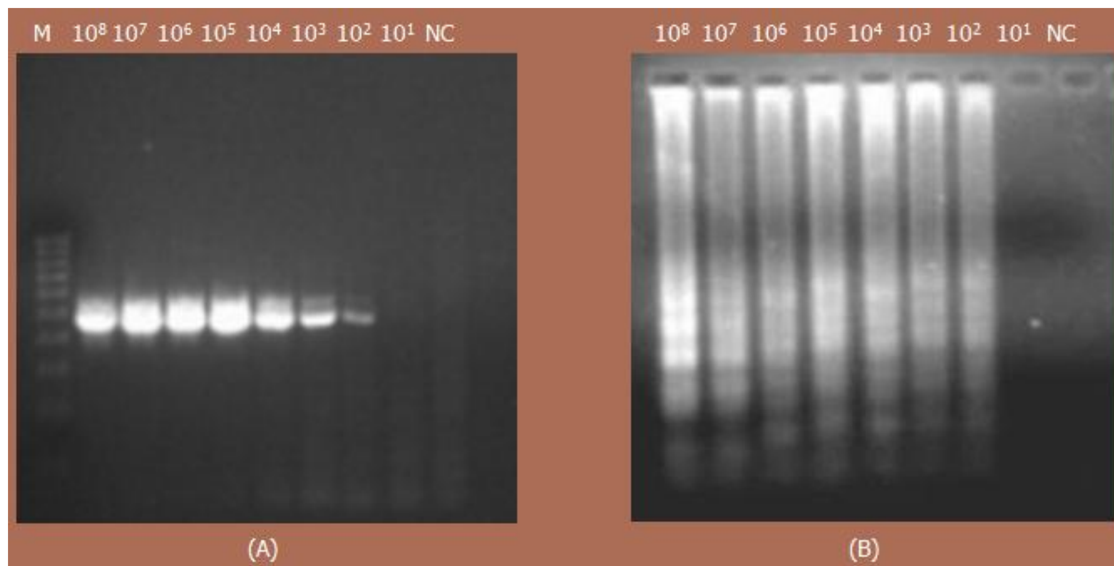

Figure 3. Analytical sensitivity of LAMP compared with conventional PCR. Gene amplification: RT-PCR with dilutions 10^8 to 10^1, NC (A) and LAMP with dilutions 10^8 to 10^1, NC (B).

Figure 4. Dot-Elisa representing positive (P), negative (N) and test (T).

its application on a bacterial pathogen and can be extended towards parasitic and fungal pathogens in future.

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