Research Article

Loop-Mediated Isothermal Amplification Assay for Detection of Generic and Verocytotoxin-Producing Escherichia coli among Indigenous Individuals in Malaysia

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1. Introduction

The human body is inhabited by a vast number of microorganisms. It has been reported that there are $10^{14}$ bacterial cells present in our body [1]. Besides fulfilling their physiological functions, the microbiota has a direct impact on human health [2]. Basically, the microbiota plays important roles in combating aggression from other microorganisms [3] as well as maintaining the balance of the intestinal mucosa and providing an innate defence [4].

Escherichia coli (E. coli) is an inhabitant of the intestines and faeces of humans and animals. It is among the first bacterial species to colonize the intestine during infancy. Commensal E. coli strains found mainly in the caecum and the colon provide some benefits to their host by inducing colonization resistance in the host through the production of bacteriocins and through other mechanisms [5]. Most of the E. coli strains are harmless, but they can also cause extraintestinal E. coli (ExPEC) infections in urinary tract, meninges, and bloodstream.

Nevertheless, there are certain serotypes that can cause diseases in humans and animals [6, 7]. Verocytotoxin-producing Escherichia coli (VTEC) is a serious public health concern as it has been responsible for outbreaks in European countries [8, 9] and has been associated with haemolytic-uraemic syndrome (HUS), the leading cause of acute renal failure in children [7, 10]. On the other hand, VTEC could also colonize the human intestine without causing any symptoms [6, 11]. This infection might not be harmful to the host but remains a threat to the public as asymptomatic carriers are...
the potential source for transmission of VTEC; hence, prompt
detection of carrier is important.

Given that instantaneous detection of the infectious
agents is important, hence molecular approaches have been
utilized as the detection is more rapid than conventional
biochemical tests. Compared to PCR, loop-mediated isother-
al amplification (LAMP) produces more specific results
as there are 4 primers targeting 6 regions within a gene.
Besides, LAMP is also more rapid (reaction occurs at constant
temperature) and eliminates the need for electrophoresis as
the results can be monitored through real-time turbidimeter
or visually based on turbidity or with the aid of SYBR I dye
[12].

In Malaysia, studies on sanitation-related diseases such
as parasitic infections are commonly carried out among the
indigenous communities due to their poor living conditions,
inappropriate hygiene practices, and the lack of functioning
toilet facilities in their houses [13]. Asymptomatic parasitic
infections among indigenous individuals have been reported
[14, 15]; however, the study on bacterial infections is relatively
scarce. Previously, Rahim et al. [16] had conducted a study on
the prevalence of Helicobacter pylori (HP) infection among the
indigenous individuals. A total of 19% asymptomatic indigenous
individuals were tested positive for the HP infection. The limited amount of information regarding bacterial
infections may have been contributed to the lack of rapid
treatment. The limited amount of information regarding bacterial
infections may have been contributed to the lack of rapid
technique which has the potential to be applied in the field.
This is crucial as many of the indigenous communities are
situated in remote areas which is logistically challenging if
samples were to be processed within a few hours. Development
of cost-effective and rapid modern tools which have the
potential to overcome these limitations will be crucial.

In the present study, we aim to develop a LAMP assay
that can specifically detect generic E. coli. The developed assay
will be applied in combination with another VTEC-specific
LAMP assay previously developed by Hara-Kudo et al. [17] to
detect the presence of E. coli and VTEC among Orang Asli
community in Malaysia.

2. Materials and Methods

2.1. Bacteria Strains. Fifty E. coli strains (5 verocytotoxin-
producing and 45 nonverocytotoxin-producing E. coli) and
35 bacterial species other than E. coli (including 10 Shigella
spp.) were revived from the culture collections in the Lab-
oratory of Biomedical Science and Molecular Microbiology,
Institute of Graduate Studies, University of Malaya. A loopful
of colonies of overnight culture was suspended in 100 μL
ddH2O and was subjected to boiling at 99°C for 5 min.
The suspension was snapped cool and centrifuged. The
supernatant was quantified and 1.5 μL (equivalent to ~50 ng)
was used as the DNA template for LAMP assay and PCR.

2.2. LAMP Assay. mGenomeSubtractor [18] and web-based
Artemis Comparison Tool (WebACT) (http://www.webact
.org/WebACT/) were used to compare the published
genomes of E. coli and other Enterobacteriaceae, such as
Shigella spp., Salmonella, Yersinia spp., and Klebsiella spp.
A list of conserved regions for E. coli was generated and was
again blasted against the database by using NCBI BLAST
glycerate kinase (EcolC_3109, Accession number: CP000946)
was selected and used for primers design. Two pairs of
primers, including F3 (5′-GGTAGATCGACCGTGATCG-3′), B3 (5′-GGCCAGAAACGGATTACG-3′), forward inner
primer (FIP) (5′-CGCAGACTTCAAGCTCGAAGTG-3′), and backward inner
primer (BIP) (5′-CTTTAAGGCCCACTGGGAACAAA-
CTTTTCAAGCGGCAACG-3′) as well as a loop primer (LB)
(5′-TGAGATGCGGCGCAAGTG-3′) were designed by using online PrimerExplorer V4 program
(PrimerExplorer, Eiken Chemical Co., Ltd.).

The LAMP assay was optimized on E. coli strains using
Loopamp DNA amplification kit (Eiken Chemical Co., Ltd.,
Tokyo, Japan). Briefly, the reaction was carried out in a total
of 25 μL containing 40 pmol of each FIP and BIP primers,
5 pmol of each F3 and B3 primers, 10 pmol of LB primer,
12.5 μL of 2X reaction mixture, 1 μL of Bst DNA polymerase,
and 1.5 μL of DNA (approximately 100 ng). The reaction
mixtures were incubated at 62°C for 80 min, followed by
enzyme inactivation at 80°C for 2 min in the Loopamp real-
time turbidimeter (LA-320, Teramecs Co., Ltd., Kyoto, Japan).
The positive result was indicated when the turbidity reached
0.1 within 60 min at 65 mM and Tt was recorded.

2.3. PCR Validation. PCR was performed in parallel to
validate the results produced by LAMP assay. Briefly, PCR
targeting phoA gene for E. coli detection and VT1 or VT2 gene
for verocytotoxin producer was carried out as described by
Ho et al. [19].

2.4. Detection Limit of LAMP Assay on Bacterial Culture
and Spiked Faeces. Overnight cultures of E. coli were serially
diluted 10-fold. An aliquot of 100 μL of each dilution was used
for the DNA template preparation and plated on lysogeny
agar for CFU count. On the other hand, the overnight culture
was also spiked in 900 μL of stools (diluted with brain-heart
infusion broth to avoid inhibition during PCR), followed by
a 10-fold serial dilution. An aliquot of 100 μL of the dilution
was plated on the agar for viable count while another 100 μL
was used for the extraction of DNA by using crude lysate method.
The DNA templates were subjected to LAMP assay.

2.5. Field Evaluation of LAMP Assay for the Detection of VTEC
among Indigenous Individuals. A total of 50 indigenous
individuals with positive parasitic infections were recruited in
the study (Ethics committee/IRB reference number: 824.11).
Sample collection procedure has also been approved by the
Department of Orang Asli Development (JAKOA). Consent
was given by the participating indigenous individuals either
via signature or via thumbprint prior to sample collection.
Faecal samples were subjected to DNA extraction using
QIAamp DNA Stool Mini Kit (QIAGEN, The Netherlands).
The extracted total DNA was quantified and stored at ~20°C
until further use.
The rate of verocytotoxin-producing E. coli in asymptomatic human carrier in this study was considered high (16%) compared to previous studies that reported the rate of 7.6% and 4%, conducted by Hong et al. [6] and Stephan and Hoelzle [11], respectively. In the studies of Hong et al. [6] and Stephan and Hoelzle [11], workers in slaughterhouse were targeted as they were considered at higher risk for carriage or excretion of VTEC. Thus, the finding in this study has highlighted the attention on hygiene practices and living in 6 volunteers (≥15 years old) while VT2 was detected in one adult's sample. Only one sample obtained from a 5-year-old girl was detected with VT1 and VT2 producing E. coli.

4. Discussion

In this study, a LAMP assay targeting glycerate kinase of E. coli was developed. The assay demonstrated a high specificity and was fairly sensitive when applied on bacterial culture (10³) and spiked faecal sample (10⁴). None of the Shigella spp. showed positive reaction and this indicated that the developed LAMP assay could specifically differentiate E. coli from Shigella. Therefore, this assay could be potentially applied on food and animal samples.

LAMP assays targeting different strains/pathotypes of E. coli have been described previously [17, 20–22]. Among the published studies, Hill et al. [21] had developed a LAMP assay targeting common strains of E. coli and this assay was evaluated using urine samples. malB gene was used for the primer design; however, this gene was also found in Shigella. The developed assay worked well in the identification of urinary E. coli (as Shigella is rare in extraintestinal infection) but will be restricted to other applications such as detection of common E. coli strains from faecal, food, soil, and water samples.

E. coli and Shigella are closely related. The validity of Shigella genus has become a contentious issue over the past decades. Based on the conventional view, Shigella and E. coli shared greater than 90% homology by DNA-DNA reassociation analysis and Shigella are E. coli strains that undergo the convergent evolution of Shigella phenotypic properties that contribute to the Shigella pathotype [23–25]. However, in terms of clinical manifestation, Shigella spp. are frank pathogens that readily cause disease in humans while E. coli normally lives in the intestines of humans and animals (with the exception of pathogenic clones). Prompt and reliable identification of these infectious agents is important for the right choice of treatment and to avoid any complications.

Differentiation between E. coli and Shigella is difficult but achievable based on physiological, biochemical, and serological typing [26]. For a more rapid method, alkaline phosphatase (phoA) gene has been used in PCRs for common E. coli strains detection, demonstrating high specificity [27, 28]. Therefore, this PCR was selected as a tool for validation of the results obtained by using LAMP in this study. In fact, the LAMP assay showed higher specificity in detecting generic E. coli in the indigenous samples compared to PCR targeting phoA gene, where the PCR could not detect E. coli in one of the samples (PG3.1).

The LAMP assay developed in this study was used to detect generic strains of E. coli that are present in the faecal sample while VT1 and VT2 producer were determined by using LAMP assay that was previously described by Hara-Kudo et al. [17]. The reaction consisted of approximately 200–400 ng total DNA incubated at 62°C for 45 min and the positive reactions were detected based on the colour change after the addition of a SYBR green 1:10 dilution to the reaction tube.

3. Results

All the 50 E. coli strains showed positive reaction within 25 min with an average Tt value of 21.26. None of the non-E. coli strains showed positive amplification. This indicates the high specificity of the LAMP assay for E. coli detection. Furthermore, the detection limit of the LAMP assay was as low as 10³ CFU/mL and 10⁵ CFU/mL on bacterial culture (Tt = 33.30) and the spiked faeces (Tt = 31.12), respectively. For bacterial culture, the reaction with more than 10⁵ CFU/mL of cells yielded positive results within 18 min, while, for faecal samples, only those reactions which have more than 10⁴ CFU/mL of cells showed positive amplification before 18 min (Figure 1).

![Figure 1: Sensitivity of LAMP assay of bacterial culture (BC) and spiked faecal (SF) specimens on different CFU (from 10⁶ to 10⁴).](image-url)
Table 1: PCR and LAMP results for the samples obtained from indigenous individuals which were previously detected with different parasitic infections.

<table>
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conditions of the indigenous populations in Malaysia, given the fact that these communities are afflicted with parasitic infections not only as reported previously [14, 15], but also as a reservoir for VTEC.

In this study, all the volunteers were infected by different parasites such as *Giardia, Trichuris, Ascaris*, Hookworm, *Entamoeba coli*, and *Iodamoeba*. There is no statistical evidence that showed correlation between parasitic infection and VTEC colonization. Our results also did not suggest any groups (single or multiple parasitic infections) that are prone to VTEC colonization. Interestingly, we found that *E. coli* was hardly detected in the stool samples containing multiple parasites as in the first run of LAMP assay (sample PM 25.2, DB 2.1, BP 1.5, BP 14.2, DK 17.3, DK 17.6, DK 19.10, DK 27.1, UK 15.5, UL 13.2, NT 6.2, NT 6.3, and SW 16.3). Reasons that influenced the sensitivity of this LAMP assay will be further investigated. Meanwhile, the 4 samples which were not detected with *E. coli* might be due to recent antibiotic treatments.

5. Conclusion

We have successfully developed a LAMP assay that can specifically detect generic *E. coli* and further performed in combination with the LAMP assay that detect verocytotoxin-related gene. Our study suggests the high risk of indigenous community for carriage and excretion of VTEC. Due to the high specificity and sensitivity of the generic *E. coli* LAMP assay developed in this study, we proposed the application of this assay in food and animals as an alternative approach for rapid and more specific detection of *E. coli*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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