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Specific and Rapid Detection of Zonula Occludens Toxin-producing *Vibrio Cholerae* Using LAMP

S.M. Mousavi, M. Zeinoddini*, A.R. Saeeidinia and N. Xodadadi

Faculty of Chemistry and Chemical Engineering, Malek Ashtar University of Technology, Iran

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ABSTRACT

Vibrio cholerae is one of the most important infectious human pathogens among the toxicogenic strains during the history of many pandemics and is a global threat to public health, especially in developing countries. Therefore, rapid and on-time detection of this infectious pathogen is necessary to prevent its outbreak. The aim of this work was the identification of zonula occludens toxin produced by V. cholerae using the LAMP technique. In this study, zot (zonula occludens toxin), one of the virulence factors of V. cholerae, was selected as the target gene and specific primers for the sequence of this gene were designed using the PrimerExplorer V5 software. The optimization of various factors affecting the LAMP reaction including Mg2+ ion, primers, temperature, and incubation time was performed in a traditional way and also by Taguchi test design. Finally, the LAMP products were visualized by agarose gel electrophoresis stained with ethidium bromide and SYBR Green I fluorescent dye. The data showed that the optimum condition for the LAMP reaction was 4-12 mM Mg^{2+} ion, 1.6-0.8 and 0.53 µM FIP/BIP, 0.4-0.2 and 0.13 µM F3/B3, temperature of 60-65 °C, and incubation time of 30-90 min. However, using Taguchi method, the optimum condition was 6 mM $MgSO_4$, incubation time of 60 min, and temperature of 65 °C. In conclusion, the results of this study showed that the LAMP method provides the rapid, sensitive, and specific detection of zonula occludens toxin-producing *V. cholerae* and can be used for the design of an identification kit of this pathogen.

Keywords: Zonula occludens toxin, Detection, Optimization, Taguchi

INTRODUCTION

Cholera is an acute gastrointestinal illness that annually allocates more than 100,000 deaths in the world. So far, there have been seven cholera pandemics more commonly in developing countries and areas that are poor in terms of providing appropriate healthy and safe drinking water [1-3]. The pathogen that causes this infectious disease is the gram-negative bacterium *Vibrio cholerae* that is found in aquatic ecosystems worldwide and enters the body through the consumption of food or contaminated water [3,4]. The bacterium is divided into several serotypes based on the diversity of its cell-wall lipopolysaccharide (O antigen), but only two serotypes including O1 and O139 are able to produce cholera enterotoxin as the leading cause of epidemics and pandemics in the world [5-7]. *V. cholerae* has several virulence factors that synergistically cause the entering and colonization of the bacterium in the small intestinal epithelium and the production of enterotoxin [8]. One of these virulence factors is zot (zonula occludens) toxin that has a synergistic role with cholera toxin. This toxin is encoded by ctxφ (cholera toxin phi) and opens the intestinal epithelial tight attachments through the cytoskeletal rearrangement and subsequently increases the permeability of the intestinal mucosa, allowing macromolecules to easily pass through the intestinal mucosal barrier [9-11]. Because of the outbreaks of cholera and the use of *V. cholerae* as a biological agent, rapid diagnostic methods are needed to prevent the outbreaks, timely treatment of patients, and disease control [12,13]. The use of molecular techniques such as polymerase chain reaction (PCR)-based techniques (simple PCR, multiplex PCR, and real-time PCR) or isothermal assay is powerful alternatives to conventional methods such as culture, microscopic, and biochemical assays [14-17]. These methods have high speed, sensitivity, and specificity than

*Corresponding author. E-mail: Zeinoddini52@mut.ac.ir

conventional methods, but the use of thermocycler machine, multi-temperature and high thermal cycles, the time required for the preparation of gel electrophoresis, and skilled personnel limit the use of these techniques. To overcome these limitations, the Loop-Mediated Isothermal Amplification (LAMP) is an isothermal technique that is used for rapid, easy, sensitive, and specific identification of *V. cholerae* [17,18]. This technique was first introduced in 2000 by Notomi *et al.* to detect the hepatitis B virus DNA. In this technique, DNA synthesis is performed with high specificity using heat-resistant Bst DNA polymerase with strand displacement activity under isothermal conditions [17-20]. The advantages of this technique include the unique temperature (isothermal), as well as high sensitivity, specificity (using 4 to 6 specific primers), and efficiency. Also, this technique is accompanied by visual colorimetric detection with unprotected eyes, which is simple and rapid without the need for electrophoresis [19-22]. The key limitation of this method is false positive control results according to carryover contamination that could be inhibited using uracil-DNA-glycosylase, UDG [23]. The purpose of this study was the molecular diagnosis of zonula occludens toxin-producing *V. cholerae* through the set-up and optimization of the LAMP technique. For this purpose, after designing the primers using the PrimerExplorer V5 online software in order to detect the gene encoding zot, all factors affecting the LAMP reaction (*e.g.* Mg^{2+} ion concentration, primers, betaine, optimum temperature, and incubation time) were analyzed. In order to simultaneously optimize the Mg^{2+} ion, temperature, and time levels, the Taguchi test design software was also examined. Eventually, agarose gel electrophoresis stained with ethidium bromide and SYBR Green I was used to confirm the reaction and identify the products.

MATERIALS AND METHODS

Design of LAMP Primers

The specific primers for the LAMP reaction (external primers: F3 and B3, internal primers: FIP and BIP) were designed using the PrimerExplorer V5 (Eiken Chemical Co.) online software for the specific detection of zot gene sequence (NC-002505.1). The specificity of primers for the zot sequence was investigated through the nucleotide blast of the NCBI database. The primers approved for the test were synthesized by Genfanavaran Co. (Tehran, Iran). The sequences of the primers used for amplification of the zot gene are shown in Table 1 and Fig. 1A.

Table 1. Designed Primers to Reproduce Zot Gene of *V. cholerae* O1 Using PrimerExplorer V5 Software

label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	874	893	20	60.99	-6.52	-4.74	0.55	CGGCTTTGTGTCCAAGATGG
B3	1100	1119	20	60.35	-7.42	-5.35	0.50	CGCTGCAAAGGTATCGAACA
FIP			40					ACCTGTCGCCCATAGACCACG-GTGATGAGCGTTATCGCCT
BIP			40					GAAACCGAGAGTGGCAGCGT-AAATCCGGTAACGGTAGCAC
F2	908	926	19	59.17	-4.56	-6.77	0.53	GTGATGAGCGTTATCGCCT
F1c	952	972	21	65.96	-5.39	-6.33	0.62	ACCTGTCGCCCATAGACCACG
B2	1066	1085	20	59.54	-4.01	-4.98	0.50	AAATCCGGTAACGGTAGCAC
B1c	1009	1028	20	64.62	-4.62	-6.57	0.60	GAAACCGAGAGTGGCAGCGT

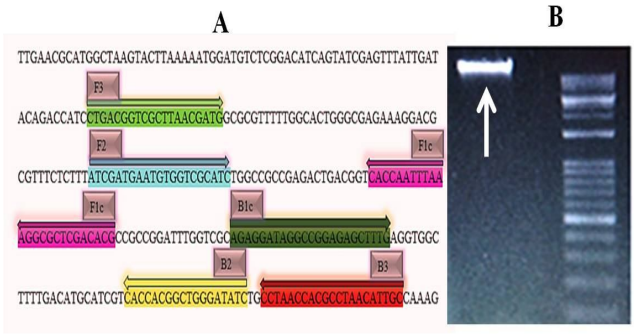

Fig. 1. (A): A schematic diagram of oligonucleotide primers designed for the zot gene sequence. (B) 1% agarose gel electrophoresis with ethidium bromide to observe the extracted genomic DNA.

Preparation of Genomic DNA

V. cholerae O1 (Inaba serotype) was received from the reference laboratory (Bu-Ali Hospital) in Tehran and aerobically cultured in LB broth and incubated in the incubator shaker (37 °C) for 24 h. After incubation, the bacterial cells were centrifuged (5000 rpm, 25 °C, 5 min) and the obtained sediment was used for the genomic DNA extraction and purification using a DNA extraction kit (DNP ™ Kit, SinaClon BioScience) according to the protocol provided in the kit. The presence of genomic DNA was confirmed with 1% agarose gel electrophoresis and the concentration was determined using the NanoDrop

instrument, then the genomic DNA was stored at -20 °C to be used for amplification in the LAMP reaction.

LAMP Assay

The LAMP reaction was performed in a final reaction mixture volume of 12.5 µl containing 1.6 µM of each of the internal primers FIP and BIP, 0.4 µM of each of the external primers F3 and B3, 1.4 mM dNTPs, 0.8 M betaine, 1.25 µl of 10X isothermal amplification buffer, 8 units of Bst DNA polymerase (New England Biolabs), 1 µl of $MgSO_4$ (6 mM), and 0.62 ng μl^{-1} of *V. cholerae* genomic DNA. A blank without DNA was included in the experiment. The reactions were incubated at 65 °C for 90 min in a heating block and at the end, the visual detection of the LAMP products was performed using 2% agarose gel electrophoresis stained with ethidium bromide (with gel documentation, Iranian company) and SYBR Green I. The ladder-like pattern and colorimetric green visual was confirmed gene amplification.

Optimization of LAMP Assay for V. cholerae Detection

In order to optimize the LAMP reaction for the specific amplification of the zot gene, several reagents affecting the LAMP reaction such as Mg^{2+} ion, primers, betaine, as well as reaction conditions including temperature and incubation time were evaluated and optimized. To achieve the optimal concentration of Mg^{2+} ion, 2-12 mM $MgSO_4$ was added to the reaction mixture (incubating at 65 °C for 90 min). The optimized amounts of primers were obtained by the addition of various concentrations including 1.6-0.016 µM of each of the internal primers FIP and BIP, 0.4-0.004 µM of each of the external primers F3 and B3 in the reaction mixture (incubating at 65 °C for 90 min). Also, the optimized concentration of betaine was obtained by the addition of various concentrations (0-1 M) of it to the reaction mixture (incubating at 65 °C for 90 min). To determine the optimum temperature, different incubation temperatures of 50-65 °C were examined. Finally, the optimum time for incubation was evaluated using reaction mixture incubation for 0-90 minutes at 65 °C. In all stages, LAMP products were visualized on 2% agarose gel electrophoresis stained with ethidium bromide, as well as the addition of SYBR Green I after amplification.

Optimization of LAMP Reaction Using Taguchi Method

The optimization of effective factors in a one-tube reaction is usually carried out by statistical methods such as Taguchi experimental design. In Taguchi methodology using software more variables and qualitative factors can be investigated. Generally, where the cost and time limitations make it difficult to perform more experiments in the optimization process and also when discrete or qualitative factors will be investigated, Taguchi is preferred in the design of experimental processes [24,25].

Nine experiments were designed with three different factors affecting the LAMP reaction including Mg^{2+} ion concentration, temperature, and incubation time at three different levels using the Qualitek-4 software, automatic design, and Taguchi software. The factors and their selected levels in the design of the Taguchi experiment are shown in Tables 2 and 3.

Table 2. The Factors and their Selected Levels in the Design of the Taguchi Experiment

Factors	Parameter	Level 1	Level 2	Level 3
A	$MgSO_4$ (mM)	2	4	6
B	Incubation temperature (°C)	45	55	65
C	Incubation time (min)	30	60	90

Table 3. Arrangement of Taguchi Experiment According to the Factors and their Selected Levels

Trial number	Factor A	Factor B	Factor C
1	1	1	1
2	1	2	2
3	1	3	3
4	2	1	2
5	2	2	3
6	2	3	1
7	3	1	3
8	3	2	1
9	3	3	2

RESULTS

Extracted DNA Genomic Electrophoresis

After bacterial culturing and genomic DNA extraction using an appropriate kit, the extracted product was examined on 1% agarose gel electrophoresis stained with ethidium bromide, which was observed as a band in the upper portion (inside the well) of the gel (Fig. 1B).

Optimization of the LAMP Reaction Products

The optimization of various factors affecting LAMP assay including the concentrations of Mg^{2+} ion, primers, and betaine, as well as incubation temperature and time were evaluated by 2% agarose gel electrophoresis and the addition of SYBR Green I after amplification. In Mg^{2+} ion optimization, the best amplification rate was observed at 4-12 (optimum: 6) mM concentrations of $MgSO_4$ (Fig. 2, Left). The optimum amounts of primers were obtained at the concentrations of 1.6, 0.8, and 0.53 µM for each of the internal primers FIP-BIP and 0.4, 0.2, and 0.13 µM for each of the external primers F3-B3 (Fig. 2, Right). In all concentrations of betaine used in the reaction mixture, the amplification of products was observed (data are not shown). The best incubation temperatures for the reaction products were 60-65 (optimum: 65) °C (Fig. 3, Left) and the best amplification times were 30-90 (optimum: 60) min (Fig. 3, Right). The results obtained from the electrophoresis were matched to the results of the SYBR Green I test.

Identification of the LAMP Reaction Products for Zot Gene-specific Amplification

Evaluation of the products obtained from the LAMP reaction was done by two methods of electrophoresis on 2% agarose gel and the addition of SYBR Green I after amplification. In the agarose gel electrophoresis, a positive control sample (containing *V.cholerae* genome) was in the form of a ladder-like pattern and the negative control sample (non-genomic) was observed as primer-dimer (Fig. 4, Left-A). After the addition of SYBR Green I and observing the products using hand dark reader (UV light), the positive control sample was brilliant green and the negative control sample had lower glow (Fig. 4, Left-B).

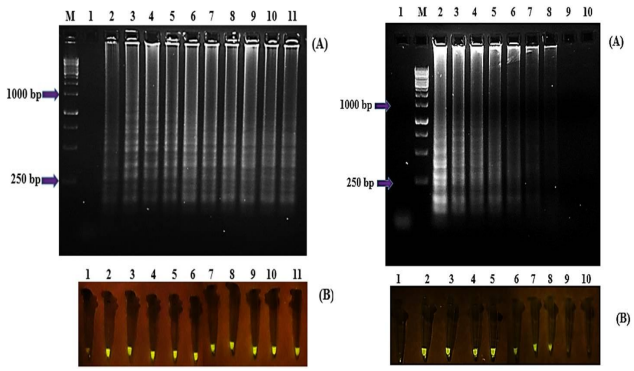

Fig. 2. Optimization of the concentration of Mg^{2+} ion and primers in the LAMP reaction: (Left) Optimization of Mg^{2+} ion concentration in the LAMP reaction. (A-Left) Analysis of the LAMP reaction products obtained from the Mg^{2+} ion concentration optimization using 2% agarose gel electrophoresis stained with ethidium bromide. Lane (M): 1 kb DNA marker, Lanes (1) to (11): dilutions of 2-12 mM from $MgSO_4$. (B- Left): Amplicon LAMP products resulting from the optimization of Mg^{2+} ion concentration using SYBR Green I fluorescent color. (Right): The optimized amount of primers in the LAMP reaction. (A- Right): Analysis of the LAMP reaction products obtained from optimizing the amount of primers using 2% agarose gel stained with ethidium bromide. Lane (1) negative control (sample without bacterial genome), Lane (M): DNA marker, 1 kb, Lane (2) 1.6 µM FIP-BIP and 0.4 µM F3-B3, Lane (3): 0.8 µM FIP-BIP and 0.2 µM F3-B3, Lane (4): 0.53 µM FIP-BIP and 0.13 µM F3-B3, Lane (5): 0.4 µM FIP-BIP and 0.1 µM F3-B3, Lane (6): 0.32 µM FIP-BIP and 0.08 µM F3-B3, Lane (7): 0.16 µM FIP-BIP and 0.04 µM F3-B3, Lane (8): 0.08 µM FIP-BIP and 0.02 µM F3-B3, Lane (9): 0.032 µM FIP-BIP and 0.008 µM F3-B3, Lane (10): 0.016 µM FIP-BIP and 0.004 µM F3-B3. (B-Right) Amplicon LAMP products resulting from the optimized amount of primers using SYBR Green I fluorescent color.

Optimization of the LAMP Reaction Using Taguchi Method

The results of the agarose gel electrophoresis for 9

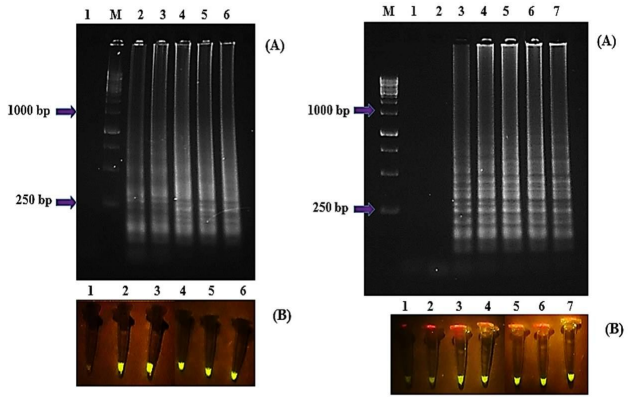

Fig. 3. Optimization of temperature and time of incubation in the LAMP reaction: (Left) Evaluation of incubation temperature optimization in LAMP reaction. (A- Left): Analysis of the LAMP reaction products obtained from the optimization of incubation temperatures using 2% agarose gel electrophoresis stained with ethidium bromide. Lane (1): negative control (sample without bacterial genome), Lane (M): 1 kb DNA marker, Lanes (2) to (6): The arrangement incubation temperatures of 50, 55, 60, 63, 65 °C. (B- Left): Amplicon LAMP products resulted from the optimization of incubation temperatures using SYBR Green I fluorescent color. (Right): Evaluation of incubation time optimization in LAMP reaction. (A- Right): Analysis of the LAMP reaction products obtained from the optimization of incubation time using 2% agarose gel electrophoresis stained with ethidium bromide. Lane (M): 1 kb DNA marker, Lane (1): negative control (sample without bacterial genome), Lanes (1) to (7): the incubation times of 0, 15, 30, 45, 60, 75, and 90 min. (B- Right): Amplicon LAMP products resulted from the optimization of incubation time using SYBR Green I fluorescent color.

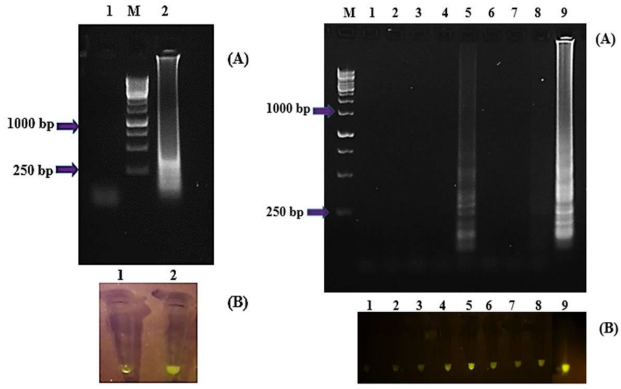

Fig. 4. Left: (A- Left): Analysis of the LAMP reaction products using 2% agarose gel electrophoresis stained with ethidium bromide. Lane (1): negative control (sample without bacterial genome), Lane (M): 1 kb DNA marker, Lane (2): positive control (sample containing *V. cholerae* genome). (B- Left): Amplicon LAMP products using SYBR Green I fluorescent color. Right: Optimization of LAMP reaction using Taguchi method. (A- Right): Analysis of the LAMP reaction products obtained from the Taguchi optimization using 2% agarose gel electrophoresis stained with ethidium bromide. Lane (M): 1 kb DNA marker, Lanes (1) to (9): Thr result of the reaction is based on experiments designed with three factors of $MgSO_4$, temperature, and time. (B- Right): Determination of amplicon LAMP products resulted from Taguchi optimization using SYBR Green I fluorescent color.

designed experiments with three different factors, Mg^{2+} ion concentration, temperature, and incubation time at three different levels determined the optimal amounts of these factors. The results showed that the best amplification was for Lane 9 [$MgSO_4$: 6 mM, temperature: 65 °C, and time: 60 min] (Fig. 4-Right). The results were also confirmed by SYBR Green I.

DISCUSSION

V.cholerae is a very infectious bacterium and one of the important bacteria that is prevalent in foodborne diseases and caused an acute gastrointestinal illness called cholera. Therefore, it is critical to achieving a precise and highly sensitive detection of *V. cholerae* from environmental samples. Today, many smart methods have been developed by researchers in order to identification of *V.cholerae* [26-29]. In this work, we set up the LAMP technique to quickly and specifically identify zonula occludens toxin-producing *V. cholerae*. In 1994, Colwell *et al.* demonstrated that zot gene is present in other *Vibrio* species such as *V.*

mimicus and *V. cholerae* O139 [30]. Also, in 2018, Castillo *et al.* reported that amongst different *Vibrio* species, 45% harbored the zot gene [31]. In turn, Xu proved that the zot gene is not present at some *V. cholerae* [32]. Therefore, this assay could be used only for zonula occludens toxin-producing *V. cholerae*. For this purpose, in the first step, we selected zot toxin as the target gene and specific primers were designed for its sequencing. In the next step, for better amplification and acceleration in the diagnostic process, various factors affecting the LAMP reaction, such as Mg^{2+} ion, primers, betaine, temperature, and incubation time were optimized. The most common method for detecting *V. cholerae* is cultivation in a specific medium, but this method requires long-term incubation and interpretation of the results by the specialist [33,34]. On the other hand, *V. cholerae* cells in stressful conditions may enter a viable but nonculturable (VBNC) state. The presence of these restrictions results in the failure of the conventional cultivar for isolation and specific detection of *V. cholerae* from other contaminated specimens [35, 36]. For this, researchers introduced the use of molecular techniques such as PCR-based techniques for reliable and rapid detection of cholera. These techniques have higher speed, sensitivity, and specificity compared to the conventional techniques such as cultivation, but the use of expensive equipment such as thermocycler, the need to prepare electrophoresis gel for detecting the reaction products, and also setting of high thermal cycles are disadvantages of these methods. To remove these limitations, in 2000, the novel isothermal in vitro amplification, named LAMP, without the need for a thermocycling device was evaluated [37,38]. Also, to overcome the limitation of *Vibrio* detection, the LAMP technique was used to identify *V. cholerae*. This nucleic acid amplification-based technique has a high advantage over PCR due to its simple reaction, high sensitivity and specificity, single temperature, and single enzyme. This method is used for rapid, sensitive, and specific detection of food pathogens such as *V. cholerae* [17-21]. The sensitivity and selectivity of the LAMP assay are more than other PCR-based techniques. In 2008, Yamazaki used a quick and sensitive LAMP method to detect the ctxA gene of *V. cholerae* and its sensitivity was 1.4 CFU in each reaction. Accordingly, the sensitivity of this assay was 10-fold more than the conventional PCR technique, and the time reaction was less than 35 min [18]. In 2009, Srisuk used the LAMP method to diagnose the ompW gene of *V. cholerae* and achieved the optimized time and temperature conditions of 75 min and 65 °C, respectively [39]. In 2010, Okada used the LAMP method as a diagnostic tool for the detection of the ctxA gene of *V. cholerae* at 64 °C for 60 min and the sensitivity of this method was 0.54 CFU in each reaction that was 10- to 100-fold more sensitive than the PCR assay [40]. In the present investigation, we used the optimization process to determine the best conditions for amplification of zot gene in the LAMP detection of zonula occludens toxin-producing *V. cholerae*. The traditional method to optimize a reaction is the effect of a variable factor at a time. To perform this method, the combination of a number of experiments that are cost, time, and labor-consuming, is necessary. One of the statistical processes for the optimization of reactions is Taguchi experimental design [24]. This robust design is an easy and simple statistical tool that has widely been applied in many industrial processes. Also, this method with orthogonal array experimental design can be used to study any given system by a set of different independent factors over a specific region of interest [24,25]. After optimization, our data was indicated that the temperature optimization for the reaction was achieved at 65 °C in which, the amplification was done even in 30 min. In most reactions, 6 mM of $MgSO_4$ was used and Betaine had no effect on the process of testing, but in all reactions, its concentration of 0.8 M was used due to its effect on the reaction specificity and increasing the efficiency of LAMP products. In follow, Taguchi experimental design was used to perform simultaneous optimization of Mg^{2+} ion concentration, temperature, and time. The results of these optimizations made it possible to diagnose *V. cholerae* in the shortest possible time.

CONCLUSIONS

Due to its high specificity, as well as the multiplicity of the other detection methods, the LAMP technique can be described as an appropriate option for the rapid diagnosis of zonula occludens toxin-producing *V. cholerae*. On the other hand, by optimizing various factors affecting the LAMP process, we can obtain the confirmation results of the amplified products in the shortest possible time. It is

expected that by using this technique, an isothermal mobile diagnostic kit can be developed to diagnose *V. cholerae*.

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