REVIEW ARTICLE

The Utilization of the LAMP Method in SARS-CoV2 Detection as an Alternative Diagnostic

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ABSTRACT

SARS-CoV2 requires full attention from all countries because this disease can cause death in patients with comorbid diseases. Though some countries already put the endemic status for COVID19, the emerging cases due to new variants should be prevented by mass tracing. Extraction test RT-qPCR is still the gold standard in declaring someone infected with COVID-19. However, the high cost of financing in RT-qPCR is the biggest problem in the diagnostic test process. Loop-mediated Isothermal Amplification (LAMP) is an inexpensive, accurate, and fast method that amplifies the nucleic acid by using four or six different primers. By doing some modification and enhancement, LAMP method assay and its modified technique could replace RT-qPCR as a rapid, sensitive, low-cost assay when there is a need for increased sample throughput by semi-quantitative visual detection. The LAMP method could promote faster and more economic tracing methods in the dense population.

Keywords: COVID-19, LAMP assay, Humans, RNA, Diagnostic tests

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INTRODUCTION

As we all know, at the end of 2019, the world was shocked by information about the emergence of respiratory diseases caused by viruses from the Corona family in Wuhan – China. This disease spreads very quickly throughout the world and has been declared by WHO as an epidemic condition of SARS-CoV2, and requires full attention from all countries because this disease can cause death in patients with comorbid diseases, as well as the elderly. In Indonesia, the presence of this disease was first announced in March 2020 and cases increased in December 2020 and peaked in June-July 2021, where the number of deaths from the SARS-CoV2 virus was higher (1-4).

The reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) of extracted virus is a standard diagnostic test to determine if a person has COVID-19 or is infected with the SARS-CoV2 virus. In addition to these tests, there are other methods that are also used to detect the presence of the SARS-CoV2 virus in a person's body, namely antibody tests and antigen tests. Although antibody and antigen tests can be used in diagnostic tests, the virus extraction test – RT-qPCR is a test that has become the gold standard in declaring someone infected with COVID-19. This is because for the antigen test, a longer incubation period of the virus is required so that the amount of virus can be detected by the test material as well as the antibody test which requires a longer time so that the body can produce an adequate number of SARS-CoV2 antibodies that can be detected by the test material. Virus extraction test – RT-qPCR is the best test, because in this test, we will extract the viral genome that is in the sample, and certain gene areas in the viral genome will be propagated by the heating cooling system and polymerase enzyme action. Subsequently, the engine sensor will detect the presence of the virus gene. Because in this method, we extract the viral genome and reproduce it at the same time, the presence of a small amount of virus can still be detected compared to other methods. Therefore, the virus extraction method – RT-qPCR is the best method to determine the presence or absence of a virus in a person's body (2,5-10).

Although the virus extraction method – RT-qPCR is the gold standard method in conducting diagnostics, the high cost of financing is the biggest problem in the diagnostic test process. The qPCR machine is different from the usual PCR machine or conventional PCR, although the stages of the PCR process for these two tools are the same. This is because the qPCR tool is added to a lamp with a channel filter of different light colours and the light will be captured by a sensor that is sensitive to the resulting fluorescence colour, to be processed into a reading value (6,11,12). In addition to the equipment, another factor that causes the high cost of testing for SARS-CoV2 with RT-qPCR is the test material. For the

extraction of genetic material or viral genomes, RNA or DNA, in principle it is no different from extraction for other organisms, but generally in the virus extraction method certain materials are added so that more viral genomes are obtained than other organisms' genomic materials. In addition to the material for virus extraction, the thing that also causes the qPCR to be expensive is the use of materials that convert RNA genetic material into DNA. SARS-CoV2 is an RNA virus, in order to reproduce genetic material using PCR, the RNA material must first be converted into DNA. Test material in converting RNA material into DNA, requires special enzymes. After changing the test material from RNA to DNA, in order to detect the presence of SARS-CoV2 virus material in qPCR, another material called a probe or synthetic oligonucleotide fragment that fluorescence is obtained. The price of the probe depends on the type of fluorescence marker used (6,12).

The high cost of RT-qPCR examination, requires diagnostic technology developers to look for other methods in examining the genomic material of an organism that is cheaper but has a sensitivity and specificity that is close to or equal to RT-qPCR. One of the methods currently attracting the attention of experts in developing diagnostic technology is the Loop-mediated Isothermal Amplification (LAMP) method. There are three essential features applied in the LAMP method including: (1) all reactions in the LAMP method can be done under isothermal conditions; (2) the amplification is high and a large number of amplification products can be obtained; and (3) the reaction is very specific. Furthermore, the standard LAMP method was developed into a fast LAMP method, by adding a primer loop, so as to reduce the time of amplification from 1 hour to less than 30 minutes. Additionally, the presence of magnesium pyrophosphate precipitate as a by-product of the amplification process helps visual detection be possible even without the use of reaction indicators or detection equipment. However, the use of reaction indicators has also been developed to facilitate development. We believe the LAMP method, with the integration of these features, can be appropriately applied to clinical genetic testing, food and environmental analysis.

The LAMP method has been reported in several articles and is used to detect DNA from many different organisms, from viruses to humans. Meanwhile, the LAMP reverse transcriptase (RT) method has been developed to detect the presence of RNA in an organism, such as a virus, and will be used as a diagnostic test in the health sector (13-19). This review article aimed to elaborate all evidence related to the LAMP and RT-LAMP method from basic to applied as an alternative diagnostic for COVID-19.

BASIC OF THE LAMP METHOD

The LAMP method is an inexpensive, fast, accurate but simple method that amplifies nucleic acid by using four or six different primers to recognize four to six different areas of the target gene. All the LAMP reaction processes can be done at a constant temperature. In brief, the amplification and detection process of the gene can be done through one process by incubating the primer, sample mixture, sample DNA substrate and DNA polymerase together at an isothermal temperature. This condition will provide high amplification efficiency since DNA can be amplified around 100 times in the first 15 minutes to one hour. Due to the high specificity of the primers, the presence of the amplified product at the end of the process will indicate the presence of the target gene (13,14,19).

In the LAMP method, no denaturation step separates the double-stranded DNA into its single form. All multiplication or amplification reactions occur under isothermal conditions or the reactions take place at a constant system temperature. The LAMP method can be combined with the RT method so that it can be used to detect RNA genetic material in an organism. The presence of 4-6 primers where one of the pairs is a LOOP primer, will increase the recognition of target genes compared to using only 1-2 pairs of target gene primers (14-20).

PRIMER DESIGN OF THE LAMP METHOD

One of the successes in conducting the LAMP test is the proper use of primers. In the LAMP method, using a different polymerase enzyme from the polymerase enzyme used in common PCR. Here, the polymerase was isolated from Bacillus stearothermophilus and four to six primers were used to specifically recognize six distinct regions of the target gene. The primer consists of an outer primer pair, encoded F3 (Forward) and B3 (Backward); a pair of inner or internal primers, coded Forward Inner Primer (FIP) and Backward Inner Primer (BIP). As depicted in Figure 1, FIP and BIP, each has two different sequences, namely F2 and F1c for FIP; B2 and B1c for BIP, all of which correspond to the sense and antisense sequences of the target DNA template (21, 22, 23).

To speed up the reaction of the LAMP method, it is possible to use additional primers including Loop Primers. Loop Primers Loop Forward (LF) or Loop Primer Loop Backward (LB) contain sequences that complete the single-stranded loop region (either between regions F1 and F2 or between regions B1 and B2) at the 5' end which will support the starting points for DNA synthesis. The existence of this primer loop will provide a "dumbbell"-like structure that will increase the sensitivity and specificity of the target gene being tested (17,19,21-24).

To get the best results in the LAMP method, a primer that has good specificity and sensitivity is needed. In principle, there is no difference between the

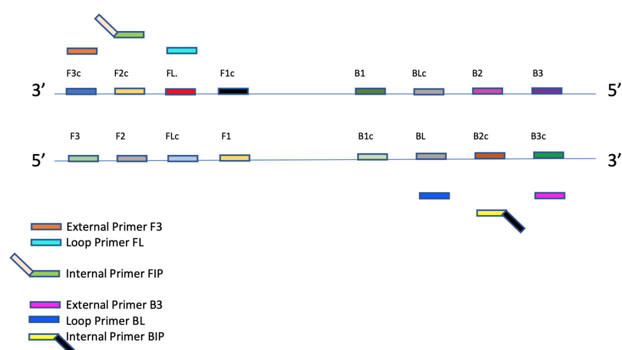

Figure 1: There are 4 primers F3, FIP, B3 and BIP which recognize the sense and antisense areas of target genes. FIP and BIP are primary internals while F3 and B3 are primary externals. Modification LAMP primer design and assay optimization. Figure is available at the commercial website of Eiken Chemical Co.Ltd in the https://www.eiken.co.jp/en/products/lamp/covid19test/

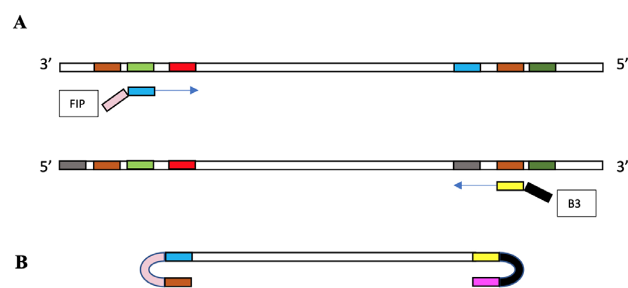

Figure 2: Schematic of the two loop structures generated during the LAMP reaction: the FIP and LB primers will hybridize to one type of dumbbell, while the BIP and LF primers will hybridize to the other type of dumbbell. (A) Primer Loop Attachment; (B) The explanation for each LAMP primer attachment

manufacture of LAMP primers and primers in general. There are 4 primary types that are based on the target gene in different regions, namely the 3′ area of F3c, F2c and F1c; and the 5′ side areas of B1, B2 and B3 (Fig. 1). Important points in designing LAMP primers are (17,19,21-24):

1. The distance between each primary area. The distance from the 5′ ends of F2 to B2 is ranging from 120 to 180 base pairs, while the distance from F2 to F3; and from B2 to B3 should not exceed of 20 base pairs. Additionally, the distance for forming the loop between 5′ F2 to 3′ F1, and 5′ B2 to 3′ B1 is around 40 to 60 base pairs.
2. The Tm value or melting temperature ranges from 60-65ºC for GC rich primers, with GC content of 50-60%. For primers that are AT rich, the Tm is 55-60% with a GC amount of around 40-50%.
3. Primers must not easily form a secondary structure, and the sequence of the 3′ end must not have many ATs or be complementary to other primers

To find out how the basic process of attaching LAMP primers to the target gene, can be seen in Figure 2A and B. Primer Loop plays a role in the catalysis of the amplification reaction so that it will increase the rate of DNA production which in turn will reduce the reaction time compared to using the LAMP method without loop primers. The existence of amplification dynamics between the Bst polymerase enzyme from the strand that has been produced without the need for denaturation and the primer complex, allows for DNA amplification with high specificity up to more than 1000 x in less than 60 minutes (17,19,21-24). Many software and applications can assist in designing primers for LAMP such as: Primerexplorer V5, NEB, LAVA or other applications. There are free and paid applications.

THE LAMP METHOD POSITIVE RESULT ANALYSIS

Two widely used methods to detect the LAMP amplification products and to differentiate between positive and negative samples are turbidimetry and fluorescence. Turbidimetric detection method consists of measuring the turbidity of all reaction mixture caused by magnesium pyrophosphate precipitation. This insoluble salt comes from the interaction of the Mg2+ present in solution and the inorganic pyrophosphate from the incorporation of dNTPs in the growing DNA strand. Thus, the amount of amplified DNA is proportional to magnesium pyrophosphate precipitation. Turbidity is visible to the naked eye and can be measured on a turbidimeter as a transmission signal either at the endpoint for qualitative analysis or in real-time, enabling quantitative applications (25).

Another approach for detecting LAMP reaction products is the use of fluorescent intercalation dyes that bind to double-stranded DNA. This method allows direct real-time visualization of isothermal amplification by measuring the fluorescence increment in the total reaction that is correlated with exponential synthesis of LAMP amplicons and reverse transcription amplification, RT-LAMP. However, the turbidimetric method and fluorescent intercalation dye are not very specific detection methods, because it is possible to detect other double-stranded DNA so that there is a bias between the specific product and the specific product (25-29). However, by testing the DNA target sample by triplication, it will be able to minimize the bias results.

THE LAMP METHOD IN DIAGNOSTIC APPLICATION

The LAMP method is pertinent for detecting many pathogens in various condition, including food safety, bioterrorism, veterinary medicine, environmental monitoring, agriculture and also for the clinical diagnostics (30,31). The LAMP method give beneficial support most for a relatively unskilled person since they can easily perform the LAMP method for the entire sample and conclude the diagnostic process. In brief, sample management from collection to analysis

will match the ease and speed of the nucleic acid amplification technique used (32). The simplicity of the LAMP method is also a good match for easily accessible samples including serum, plasma, cerebrospinal fluid saliva, saliva, capillary whole blood as opposed to venous whole blood. Recently, outbreaks of several viral disease including Ebola (33), Zika (34) and now SARS-CoV2 (35-37) have generated interest in urine and saliva as alternative specimens for non-invasive diagnostics.

THE LAMP METHOD FOR PORTABLE AND VISUAL DIAGNOSTIC OF COVID-19

Considering the ability of the LAMP method in visually identifying SARS-CoV2, the LAMP method protocol then could be proposed as a diagnostic test at least in the context of fast tracing the cases. The LAMP method could be done without RNA extraction and RNA amplification could be started directly from the sample with the sensitivity being low as 80 copies of viral RNA (38). Various sensitivity of the LAMP method had been reported ranging from 60% to 100% depending on the viral copies and cut off the CT value (39,40). Fowler et al reported sensitivity and specificity of RT-LAMP could reach 97% and 99%, respectively, for COVID-19 diagnosis. However, these results depend on the cut-off of the CT value. In brief, when CT value set to 33 the sensitivity increased up to 100% and when CT value set to 25 to 33 the sensitivity decreased to 75% (39). Therefore, sensitivity does matter in the application of LAMP or RT-LAMP assay. Many techniques and modifications have been applied to overcome the obstacles. El-Tholoth et al increased the sensitivity by enhancing LAMP protocol through the Penn-RAMP strategy (41,42). Through the amplification process of recombinase polymerase, Penn-RAMP uses outer LAMP primers to intensify all RNA targets (41,42). As a result, PCR methods need 700 viral RNA copies per reaction for 100% success rate while Penn-RAMP provided 100% success rate at 7 -10 copies of viral RNA (41,43). Additionally, understanding the LAMP as a useful method but below that of the standard RT-qPCR, Zhang et al increased the sensitivity and speed of colorimetric LAMP by using guanidine hydrochloride and combining primer sets for detection of target DNA and RNA (44). The addition of guanidine chloride at around 40-50mM in the colorimetric LAMP master mix stimulated the greatest amplification speed nearly twofold in detecting the RNA of SARS-CoV2 (43). Interestingly, the addition of guanidine chloride reduced the time for reaction even in small template input. As a result, positive detection in the 100 copies of RNA significantly increased from below 50% without guanidine hydrochloride to over 90% with guanidine hydrochloride (44). Furthermore, positive detection in the 50 copies of the template improved from 30% to 70%. Conclusively, the addition of guanidine hydrochloride can increase the reaction sensitivity at nearly fourfold (44). Therefore, adding guanidine hydrochloride not only shortened the reaction time by increasing amplification speed but also increased the sensitivity by enhancing the positive detection even at low input or less RNA copies of the template. In the more general context, Ding et al had shown the probe-enhanced LAMP (PE-LAMP) method to diagnose SNP typing (45). Ding et al merged conventional LAMP with the oligonucleotide probe to apprehend the SNP discrimination by investigating the great difference in the efficiency of amplification process. The merged technique successfully detects SNP at 1000 copies of target DNA or RNA (46). Thus, compared to RT-qPCR that needs high-cost expenses, needs specific biosafety level 2 (BSL2) centralized laboratories, and requires sophisticated skills and process in extracting the RNA, the LAMP method assay and its modified technique could replace RT-qPCR. RT-qPCR not only offers a rapid, sensitive, low-cost semi-quantitative visual detection assay but also overcomes most of the RT-qPCR limitations. Therefore, the LAMP method assay could promote faster low-cost tracing methods in the dense population. It can be easily developed in the public health centers, schools, airports as an integral effort to overcome the COVID19 spread as a point-of-care (POC) diagnostic tool as mentioned by recent evidence (40,47,48).

CONCLUSION

In conclusion, the LAMP method can be utilized to detect SARS-CoV2 virus in this pandemic outbreak for lower cost and for a sensitivity that close to the results using RT-qPCR, in areas that lack facilities for real-time PCR examinations.

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