Novel Primers for Easy Detection of Yellow Fever Using Isothermal PCR

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Abstract: Yellow Fever is a disease caused by the Yellow Fever Virus (YFV), common in tropical areas in Africa and South America. Hundreds of Indonesian soldiers are sent on peacekeeping missions to Congo -an endemic region for YFV- every year. Therefore, an accurate technique is needed to screen those who possibly get infections. Isothermal PCR, unlike conventional PCR, uses a single temperature with 4 to 6 combinations of primers that can be performed with simple equipment such as a heat block and detected with a visual eye instead of agarose electrophoresis. Thus, it is suitable for this challenge. YFV strain 17D genome was used and particularly NS2a and NS5 genes were chosen as template for the design of primers. To check for specificity, obtained primers were checked for sequence similarity using BLASTN. Literature studies showed that various genes, including E and NS5, have been used for the target using conventional PCR. Meanwhile, for isothermal PCR, only the NS1 gene and upstream non-coding gene have been used. From the NS2a gene with length of 672 bp, 2 sets of primers were obtained. At the same time, NS5, with a longer length of 2,714 bp, gave 4 sets of primers. BLASTN results showed that all primers were specific to the YFV genome. Original primers have been successfully designed for isothermal PCR of yellow fever. Primers designed are the first step and also the most critical original works in nucleic acid testing. Therefore, towards self-sufficient diagnostic reagents, the results are significant.

1 INTRODUCTION

1.1 Yellow Fever Virus

Yellow Fever is a disease caused by the Yellow Fever Virus (YFV), which is common in tropical areas in Africa and South America. The disease primarily affects humans and nonhuman primates and is transmitted through the bite of an infected mosquito. Forty-seven countries in Africa, Central and South America are either endemic for Yellow Fever Virus (YFV) or have endemic areas. A modelling study based on African data sources estimated the incidence of Yellow Fever during 2013 was 84,000-170,000 severe cases and 29,000-60,000 deaths (Gardner et al, 2010).

Every year, hundreds of Indonesian soldiers are sent on a peacekeeping mission to Congo - an endemic region for YFV - who trained before departure and returned home first to IPSC, where The Republic of Indonesia Defense University is located. Therefore, screening for possible YFV infection among returning soldiers with an easy yet accurate technique is necessary. Isothermal PCR, unlike conventional PCR, uses single temperature and with 4 to 6 combinations of primers can be performed with simple equipment such as a heat block, and can be detected with a visual eye instead of using agarose electrophoresis. Thus, it is suitable for this challenge (Gardner et al, 2010).

1.2 Yellow Fever Virus Complete Genome

Yellow Fever Virus is a single-stranded RNA virus consisting of 10 constituent genes, namely 3 structural genes (C, prM, and E) and 7 nonstructural genes (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). A third of the YFV genome is encoded by 3 structural proteins, while two-thirds comprises 7 nonstructural proteins. One of the most targeted

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genomes for many researchers is Yellow Fever Virus-17D, which is also very effective as a vaccine to form antibodies against YFV itself. The genome was seen as the most similar to the original YFV genome, so that genome is used as the target sequence for this test. Although further research is still needed on it, and we must keep up to date with the latest information about the virus (Nunes et al, 2015).

Among the 10 genes in the entire YFV genome, NS2a is a small hydrophobic protein, one of the nonstructural proteins of YFV and plays a role in its virulence factors. Because this gene is essential for the virus, we made it one of the target genes in the test that we carried out. In addition, NS5 is another protein in the YFV genome that has been the target of molecular screening research that can be used as a primer for PCR tests. It is undoubtedly in line with our test's purpose, and is why NS5 is also one of the target genes in this paper (Voßmann et al, 2015 and Rezende et al, 2019).

1.3 Advantages of The LAMP Method

The LAMP (Loop-mediated isothermal amplification of DNA) method was designed in 1998 by a Japanese company. This technique increases the amount of DNA amplified to one billion copies in less than an hour and is very specific. Isothermal amplification can be performed without sophisticated laboratory equipment. Another innovative aspect of LAMP is its high specificity due to the use of multiple primers (from four to six), which can distinguish up to eight specific sites on the DNA template (Keikha, 2018, Wong et al, 2018, and Soroka et al, 2021).

Primary LAMP design can be done using online software such as Primer Explorer (https://primerexplorer.jp/e/), LAMP Designer Optigene (www.optigene.co.uk/lamp-designer), or Premier Biosoft (http://www.premierbiosoft.com/isothermal/lamp.html), maybe using the neb lamp (https://lamp.neb.com/#!/) can be done either which was used in this experiment (Wong et al, 2018 and Soroka et al, 2021).

The LAMP technique does not involve DNA denaturation because, due to the strand, as mentioned earlier, transfer activity of the Bst DNA polymerase, the reaction can be carried out under isothermal conditions. All LAMP steps were done at a stable temperature of 60–65 °C, eliminating the need to use a thermocycler to precisely adjust the thermal and time profiles, which are required for commonly known PCR techniques (Keikha, 2018, Wong et al, 2018, and Soroka et al, 2021).

The Loop-Mediated Isothermal Amplification method has been used to diagnose various infectious diseases and identify and differentiate pathogenic microorganisms; for example, Mycobacterium tuberculosis, Nocardia spp., Pseudomonas fluorescens, Staphylococcus aureus, Helicobacter pylori, Salmonella species, and several other bacteria and medically necessary viruses (Keikha, 2018).

2 MATERIALS AND METHODS

This study uses the insilico method, which uses software and computational techniques to test the scientific data we get from the existing literature.

First, we look for diseases prone to occur in the Garuda Contingent sent to Congo by looking for literature showing the prevalence of viral illness in that country, by looking at the possibility of this viral disease occurrence in our country. In addition, Yellow Fever Virus has the same intermediate host as the dengue virus, namely the Aedes aegypti mosquitoes. It is necessary to take precautions so our country does not become a yellow fever virus endemic area (Gardner et al, 2010).

After obtaining data on this viral disease in the country, we determined the genome of the Yellow Fever Virus, YFV strain 17D genome (Genbank entry NC_002031), which will be the target of this research through existing journals. We found that the genome is most similar to the original viral genome, primarily found in Africa, and the most used as the object of research (Nunes et al, 2015).

Next, we searched for the target gene in the genome by searching literature which states that the NS2a gene has a significant role in the virulence factor of the virus. In addition, we chose the NS5 gene because it has become the target gene in molecular screening literature using PCR (Voßmann et al, 2015 and Rezende et al, 2019).

Then, we searched for the genome sequence at https://www.ncbi.nlm.nih.gov/ using the Genbank feature. Following that procedure, we take the genome into FASTA format, obtain the complete line of the genome, and input the sequence into the Microsoft Word application.

After that, using https://lamp.neb.com/#!/, we entered the gene sequence and determined the location of the base pair sequence, which we will make into a primer. After completing the process, we obtained 6 new primers and confirmed that the base pair sequence of the reverse primer and backward primer had been reversed and complemented, which are in the viral genome sequence in Microsoft Word.

Then, the specificity of those 6 primers was tested for the viral genome at https://blast.ncbi.nlm.nih.gov/Blast.cgi, and the results were those 6 primers specific for the Yellow Fever Virus genome.

3 RESULTS

Literature studies showed that various genes, including E and NS5 (Nunes et al, 2011), have been used for targets using conventional PCR. Meanwhile for isothermal PCR, only NS1 gene (Nunes et al, 2015 and Kwallah et al, 2013) and upstream non-coding gene (Escadafal et al, 2014) have been used. From the NS2a gene with length of 672 bp, 2 sets of primers were obtained. First set, start from nc 3,636 and total region length of 207 bp. While the second set, from nc 3,956 and total region length of 207 bp. While NS5 with a longer length of 2,714 bp gave 4 sets of primers. Each starts from nc 8,159; 8,714; 9,589; 9,776 and total region length of 218 bp, 222 bp, 172 bp, and 234 bp respectively. BLASTN results showed that all primers were specific to the YFV genome. At the end, we have 6 primers that are eligible and have a high possible success rate to be the primers for the purpose of this experiment which has been mentioned before.

Table 1: Primer 1.

	len	**Tm**	
F3	20	60.22	TCGGGCAAGTAACTCTCCTT
B3	18	60.55	CCACCATGGCTGCTCCTA

Table 2: Primer 2.

	len	**Tm**	
F3	19	60.89	CCCCTCATGGCTCTGTTGA
B3	19	59.24	GGTTGCCAGAAATGCACAC

Table 3: Primer 3.

	len	Tm	
F3	20	59.70	GTTGACACCAGAGCAAAGGA
B3	20	59.17	CCAGAACTTTGGGTCTTGGA

Table 4: Primer 4.

	len	**Tm**	
F3	20	59.68	GGGGTTGACAACTTCTGTGT
B3	19	60.52	GGACGCCTCATTCTCCTCA

Table 5: Primer 5.

	len	**Tm**	
F3	20	60.58	TCCCACCACTTCCATGAACT
B3	18	59.33	CCATGAGGTGGGAACAGC

Table 6: Primer 6.

	len	**Tm**	
F3	19	59.92	CACTGAGCACGGATGTGAC
B3	20	59.10	CTCCCAATCATTCCACCCTT

4 DISCUSSION

For screening purposes, primers design is the first step and is crucial for making a diagnostic tool that can be used in the field. In its application, other reagents such as enzymes and other chemicals may use existing ones. However, with different primers, the purpose or function of the diagnostic tool will also be different. Isothermal PCR is not a common diagnostic tool, especially in the military medical environment in Indonesia. It is very suitable for the conditions of the military environment in Indonesia, so it can easily apply this diagnostic test (Keikha, 2018, Wong et al, 2018, and Soroka et al, 2021).

Primers design, as the first step, has been completed. Indeed, it will continue with the manufacture of prototypes, in vitro testing, until it is proven that screening using isothermal PCR is possible by attaching data obtained directly from all the tests. Suppose all series of tests have been carried out, and it is found that the primers we obtained computationally can be used as primers in this diagnostic tool. In that case, a new faster, easier, and cheaper way to detect the Yellow Fever Virus in Indonesia will be obtained (Nunes et al, 2015).

Indeed, it has a good impact on military medicine in Indonesia, because it proves that Indonesia can already produce its own diagnostic tool for a virus

that can quickly spread in Indonesia because of our soldiers who go directly to its endemic areas. This is a big step that can be used as an opening door for the advancement of military medicine, especially in the molecular field, so that we can become a producer independently, not just a consumer who depends on other countries.

5 CONCLUSIONS

Yellow Fever is a disease caused by the Yellow Fever Virus, which is common in tropical areas in Africa and South America. By easy yet accurate technique, there is a need to screen for possible YFV infection among returning soldiers from the YFV endemic countries such as Congo. Original primers have been successfully designed for isothermal PCR of yellow fever. Isothermal PCR/LAMP is an easy and inexpensive technology that is rarely used, but it can be used to screen for Yellow Fever disease by detecting the base pairs in the viral DNA genome. Primers designed are the first step and also the most critical original works in nucleic acid testing. From this work, 6 gene primers were obtained that could be used as primers for the planned screening. Therefore, towards self-sufficient diagnostic reagents, the results are significant.

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