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Rapid Detection of Pathogenic Leptospires by PCR Molecular Methods

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Abstract

Leptospirosis is an animal-borne disease (zoonosis) caused by bacterial infection of the Leptospira genus. The aim of this study was to develop a rapid molecular-based method that could be applied to complement the results of frequently used antibody-based tests. The results of this study can detect leptospira DNA with primer lepto-F: 5'CCCGCGTCCGATTAG'3 and lepto-R: 5 'TCCATTGTGGCCGRACAC'3. The detected gene is the rrs 16S RNA gene. The optimum results of protocol and PCR component are optimal Annealing temperature 60 ^{0}C, Taq Polymerase 0.5 U, MgCl2 2 mM, and 150 µM dNTPs. Results from the IgM examination of 20 blood samples, all negative. PCR examination results from the 20 blood samples were also all negative.

Keyword: Leptospira, Leptospirosis ,PCR, 16S RNA gene

BACKGROUND

Leptospirosis is a disease derived from animals (zoonoses) found almost all over the world, especially in tropical climates [1]. This disease is caused by spiral bacterial infection of the genus Leptospira. Transmission of leptospirosis in humans occurs in direct contact with animals infected with leptospires or indirectly through water, soil, and contaminated food of urine infected with leptospires. These bacteria enter the body through the skin of the wound or mucous membranes [2].

Leptospirosis in humans can be mild to severe disease depending on the serovar that infects it. Patients with chronic leptospirosis can act as carriers because the bacteria can nest in the kidneys. Sphinca is excreted with urine from the first week after infection for several months. Symptoms of this disease do not show specific symptoms and often resemble other symptoms of illness, such as malaria, tuberculosis, hepatitis, thypoid fever, and other parasitic infections [3]. Therefore, to establish a diagnosis would be very difficult with clinical symptoms only, requiring rapid, precise laboratory examination, with high sensitivity and specificity.

Laboratory examination is often done by culturing Leptospira bacteria and then done visually identification using a microscope, or serologic examination on patient samples. Both checks take at least a week to get the results of the examination [4]. Currently, various molecular approaches for detecting leptospires have been developed starting from RFLP analysis (Restriction Fragment Length Polymorphism) [5] pulsed-field gel electrophoresis [6], ribotyping [7], arbitrarily primed PCR (AP-PCR) [8], low-stringency single specific primers PCR [9], and real-time quantitative PCR [2][10][11]. Molecular examination by RFLP method, pulsed-field gel electrophoresis, and ribotyping are laborious, intensive examination time, and require sufficient quantity of genomic DNA, while AP-PCR and LSSP-PCR methods produce complex banding patterns. Based on the above background it will be done research to develop Leptospira detection method using real-time quantitative PCR (qPCR). The qPCR molecular method has advantages such as high sensitivity and specificity (> 90%), easy interpretation of results, short examination time (<1 day), requires a small amount of DNA genome, and is not laborious. Based on the above background will be developed a detection method that will be made in house assay based molecular to detect leptospira in patients with leptospirosis.

MATERIALS AND METHODS

DNA Isolation Of *Leptospira*

DNA isolation was performed on a positive culture sample of Leptospira and blood samples after serology (IgM). The result of DNA culture isolation is used as the positive control used in the development stage of assay that is the optimization of protocol and PCR component. Leptospira DNA isolation for initial validation stages using blood samples from volunteers. All DNA isolation using a DNA Kit (Promega).

Primer Design

An oligonucleotide primer that acts to initiate a PCR reaction is designed with literature studies and selected specific primers that recognize specific genes for detecting pathogenic leptospires ie RRs 16S RNA genes. Then the primer was checked in NCBI and mapping was done using SnapGen software.

Primer Test

Primer that has been designed in the previous stages, then conducted primary tests that include, the percent content of GC from the primary sequence, the specific PCR product, the possibility of primary dimer, and melting temperature range (Tm). At this stage use a program like Thermo Fisher.

PCR Optimization

The next step, PCR optimization is initiated by optimizing the PCR protocol at the exact stage of annealing temperature and number of PCR cycles. Then continued optimization of component concentrations / PCR reagents, including the determination of Taq polymerase, MgCl2, and dNTPs concentrations. At this stage of PCR optimization using DNA samples positive Leptospira pathogen. PCR product results were characterized by electrophoresis and visualized with documentation gel.

PCR Validation

The last step in this research is the validation of PCR method. PCR optimization results were tested using more DNA samples derived from blood samples compared with the standard method of serologic (IgM). At this stage, the sensitivity and specificity of the PCR method was developed. The following can be seen how to validate methods:

		Standard (Serologic)	
		Positive	**Negative**
PCR	Positive	TP	FP
	Negative	FN	TN
		Sensitivity TP/(TP+FN) (94,0-98,5%)*	Specificity TN/(TN+FP) (85,4-97,0%)*

TP (*True Positive*); FN (*False Negative*); FP(*False Positive*); TN (*True Negative*)

RESULTS AND DISCUSSION

Primer Design

Primary design is done by searching the primary related literature that is widely used for detection of Leptospira with PCR technique either PCR gel base or PCR real time. After the literature study we selected one primary pair: Lepto-F: 5 'CCCGCGTCCGATTAG 3' and Lepto-R: 5 'TCCATTGTGGCCGRACAC 3'

The above primer can detect the Rrs (16S) RNA gene in leptospires. Then do the primary characterization in silicon. The first stage is done, BLAST primer using NCBI (figure 1). Results of Primer BLAST can be seen that the primers tested can recognize the 16S RNA gene on Leptospira.

Second stage, that is mapping of primer on 16S RNA gene (figure 2).

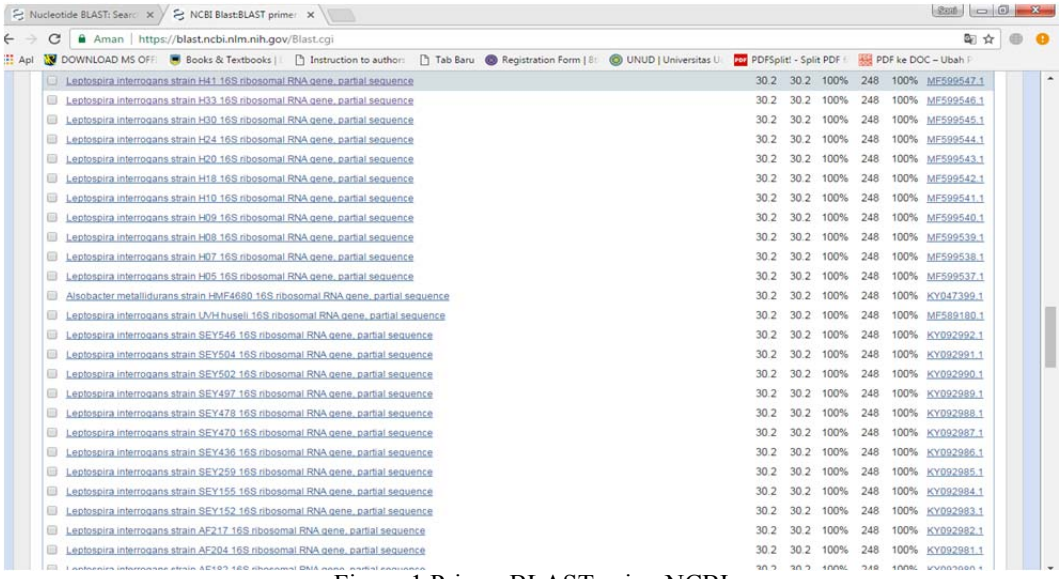

Figure 1 Primer BLAST using NCBI

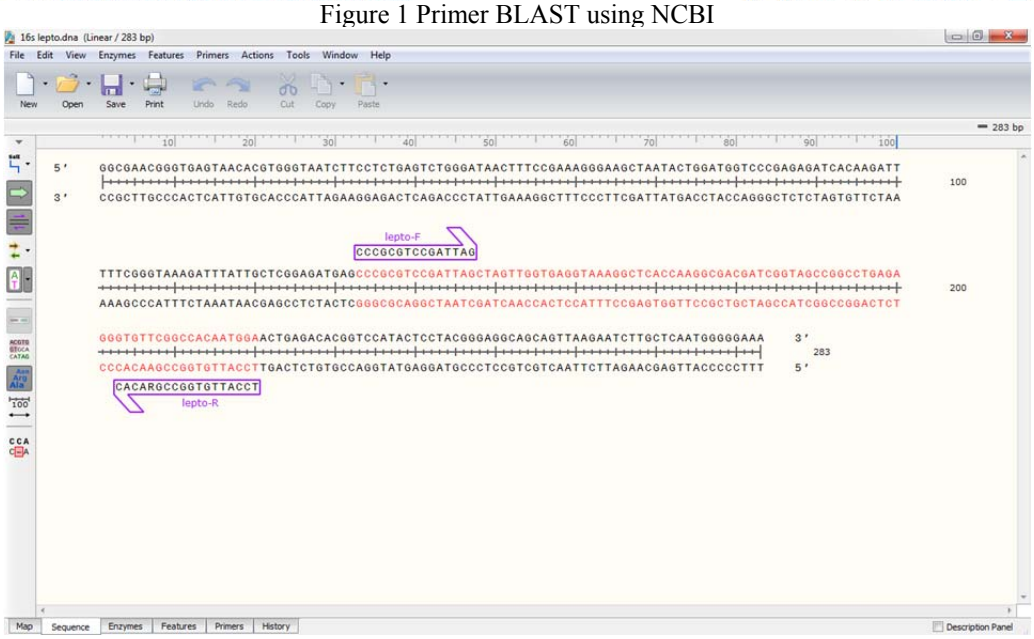

Figure 2 Primer attachment mapping on the 16s rrs RNA gene

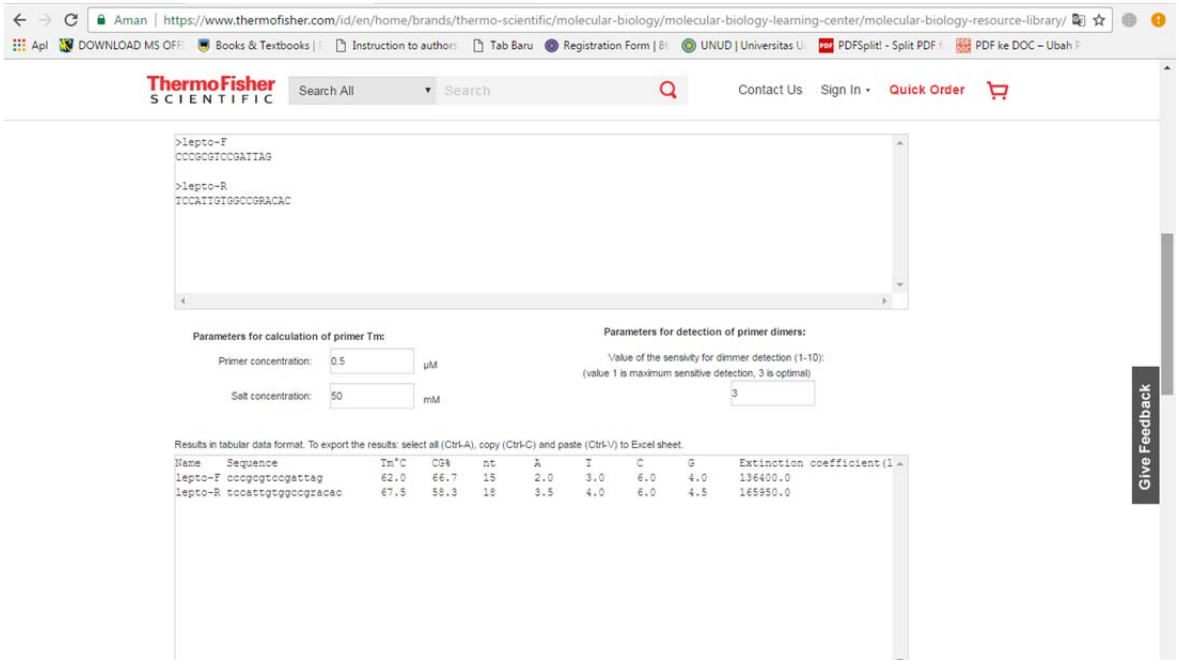

Figure 3 Primer Tm,% GC, and dimer

The third stage, checking Tm (Temperature Melting), % GC, primary dimer of primer (figure 3). The test results show the primer will be attached specifically because it has Tm above 60 ^{0}C,% GC above 55%, and no primer dimer.

DNA Isolation of *Leptospira* And DNA Concentration Measurements

The cultured leptospires for 1 week were then isolated using the Wizard (R) Genomic DNA Purification Kit. Isolation was done in Bbalivert bogor. After getting DNA leptospira, then measured DNA concentration. The tool used is nanodrop. Measurements were made at UPK UNPAD. DNA concentrations can range from 17.8 ng / ul to 20.9 ng / ul.

PCR Optimization

1. The first stage of optimization carried out primer test with annealing temperature 60 ^{0}C

This PCR begins with making MIX PCR, with the following concentrations:

Component	Final Concentration	Volume (50 µl)
Taq Poly 5 U	1 U	0.2 µl
Buffer 5x	1x	10 µl
$MgCl_2$ 25 mM	2 mM	4 µl
dNTPs 10 mM	200 µM	1 µl
Primer Lepto- F 10 µM	0.5 µM	2.5 µl
Primer Lepto-R 10 µM	0.5 µM	2.5 µl
DNA Template 10 ng/ µl	5 ng/ul	0.25 µl
Nuklease Free Water	-	Add 50 µl

The PCR Step are as follows: Initial Denaturation: 95 ^{0}C/10 Minutes; Denaturation: 95 ^{0}C/30 sec; Annealing: 60 ^{0}C/30 sec; Extensions: 72 ^{0}C/30 sec; Final Extension 72 ^{0}C/10 sec; For 35 cycles. PCR product results (Figure 4) were characterized by agarose gel electrophoresis. Gel agarose used 1% with TBE 1x Electrophoresis voltage of 100 volts for 50 minutes.

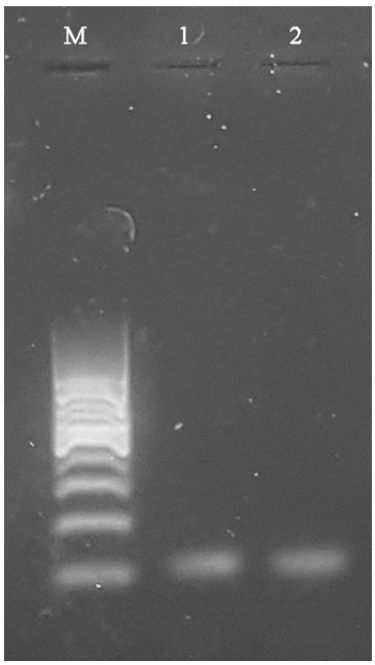

Figure 4 Electrophoregram of Primer Test
M = Marker Ledder 100 bp; 1 dan 2 = DNA Sample of *Leptospira*

2. Taq Polymerase Optimization

Taq Polymerase is varied with a final concentration of 0.5 U and 1 U. The PCR and electrophoresis concentrations are the same as in Primer test. Taq Polymerase enzyme optimization results can be seen in Figure 5. This optimization result, selected Taq polymerase 0.5 U concentration.

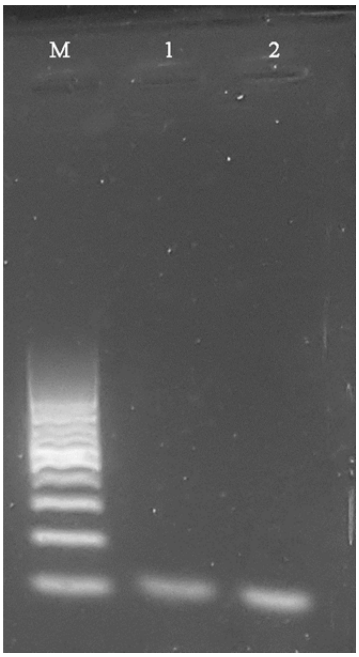

Figure 5 Electrophoregram of Taq Polymerase Optimization
M = Leadder 100 bp, 1 = 0.5 U, 2 = 1 U

3. $MgCl_2$ Optimization

$MgCl_2$ is varied with final concentration of 0 mM, 0.5 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM, and 3 mM. The PCR component is the same as the primary test, only Taq polymerase is used 0.5 U. The optimization results of $MgCl_2$ can be seen in Figure 6. The optimization results are selected $MgCl_2$ with a concentration of 2 mM.

4. dNTPs Optimization

dNTPs were varied with final concentration of 0 µM, 50 µM, 100 µM, 150 µM, 200 µM, 250 µM, and 300 µM. PCR component is the same as primary test, only Taq polymerase is used 0.5 U. The result of dNTPs Optimization can be seen in figure 7. From optimization result, dNTPs with concentration 150 µM.

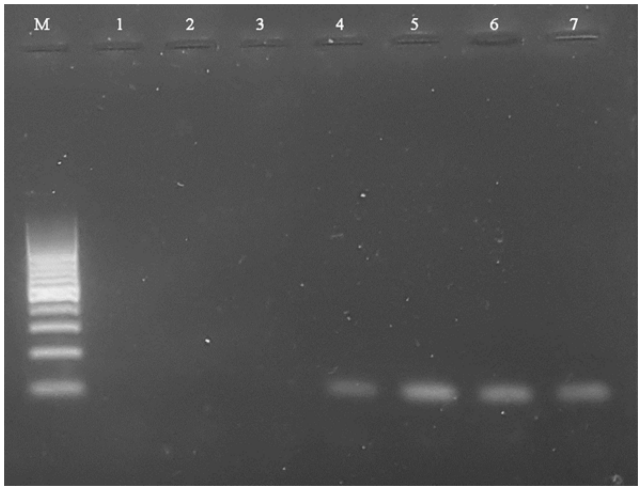

Figure 6 Electrophoregram of $MgCl_2$ Optimization
M = Leadder 100 bp, 1 = 0 mM, 2 = 0.5 mM, 3 = 1 mM, 4 = 1.5 mM, 5 = 2 mM, 6 = 2.5 mM, dan 7 = 3 mM

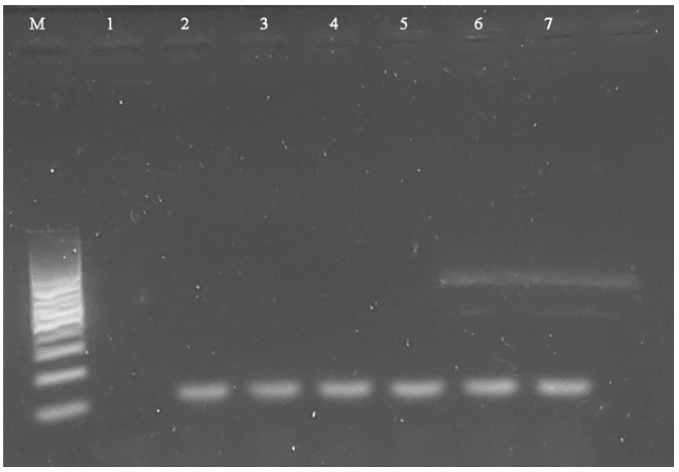

Figure 7 Electrophoregram of dNTPs Optimization
M = Leadder 100 bp, 1 = 0 µM, 2 = 50 µM, 3 = 100 µM, 4 = 150 µM, 5 = 200 µM, 6 = 250 µM, dan 7 = 300 µM

Blood Sampling and Leptospira IgM Test

Blood samples were taken from 20 volunteers willing to take blood and screened for IgM leptospira and PCR. Volunteers come from STFB students. Blood that has been taken directly checked IgM by using KIT leptospira from Biomedical Expert All the blood taken showed negative results (figure 8).

Figure 8 IgM Test

PCR Test on Blood samples

20 blood samples have been tested IgM, then DNA isolation and PCR tested using PCR optimization protocol. Test results for all samples are negative

PCR Validation

PCR Validation results from 20 blood samples, obtaining a specificity of 100%. The sensitivity value can not be calculated because it is not a positive blood sample of leptospires.

CONCLUSION

Based on the results of research that has been done, it is concluded that PCR method can be used for rapid detection in leptopira, with sensitivity up to 100%. In subsequent studies, more positive blood samples of leptospires are required, to obtain sensitivity values.

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