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OPTIMIZING MOLECULAR TECHNIQUES FOR ACCURATE EXAMINATION OF LEPTOSPIRA SPECIES: A COMPREHENSIVE PRIMER FOR RESEARCHERS

OPTIMISASI TEKNIK MOLEKULER UNTUK PEMERIKSAAN AKURAT SPESIES LEPTOSPIRA: PANDUAN KOMPREHENSIF BAGI PENELITI

Original Research Article
Penelitian

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A B S T R A C T

Background: *Leptospirosis is a potentially life-threatening disease caused by bacteria of the genus Leptospira. The accurate identification and characterization of Leptospira species are critical for disease surveillance, outbreak investigation, and treatment strategies. Molecular techniques, such as Polymerase Chain Reaction (PCR) and Deoxyribonucleic acid (DNA) sequencing, have revolutionized the field of microbiology, providing rapid and accurate identification of Leptospira strains. However, optimizing these molecular techniques for accurate examination of Leptospira species can be challenging due to the genetic diversity and complexity of these bacteria.* ***Purpose:*** *This research aims to identify the most suitable primers for the precise identification of pathogenic Leptospira strains.* ***Method:*** *This research used the PCR method, using LipL32, rrs2, seqY, LipL41, IcdA, and Adk primers. A total of 17 isolates of pathogenic Leptospira bacteria were cultured from Institute of Vector Control and Reservoir Disease (IVRCD) in Salatiga, Indonesia.* ***Result:*** *The results of the research showed that the LipL41 and IcdA primers were found to be effective in distinguishing pathogenic strains, while the seqY, LipL32, Adk, and rrs2 primers required further refinement. The suitable Melting Temperature (TM) or annealing temperature is 58°C with 35 cycles of amplification. DNA concentration and purity had an A260/A280 ratio ranging between 1.8 and 2.8.* ***Conclusion:*** *LipL41 (500 bp) and IcdA (700 bp) are suitable primers for identifying pathogenic Leptospira.*

A R T I C L E I N F O

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A B S T R A K

Latar belakang: Leptospirosis adalah penyakit potensial yang mengancam nyawa yang disebabkan oleh bakteri dari genus *Leptospira*. Identifikasi dan karakterisasi yang akurat terhadap spesies *Leptospira* sangat penting untuk pengawasan penyakit, investigasi wabah, dan strategi pengobatan. Teknik molekuler seperti reaksi berantai polimerase atau *Polymerase Chain Reaction* (PCR) dan sekuensing DNA telah merevolusi bidang mikrobiologi, memberikan identifikasi cepat dan akurat terhadap *Leptospira*. Namun, mengoptimalkan teknik molekuler ini untuk pemeriksaan yang akurat terhadap spesies *Leptospira* dapat menjadi tantangan karena keragaman genetik dan kompleksitas bakteri ini. **Tujuan:** Penelitian ini bertujuan untuk mengidentifikasi primer yang paling sesuai untuk mengidentifikasi bakteri *Leptospira* patogen yang tepat. **Metode:** Penelitian ini menggunakan metode PCR dengan menggunakan primer *LipL32*, *rrs2*, *seqY*, *LipL41*, *IcdA*, dan *Adk*. Sebanyak 17 isolat bakteri *Leptospira* patogen yang dikultur dari Institute of *Vector Control and Reservoir Disease* (IVRCD) Salatiga, Indonesia. **Hasil:** Hasil penelitian menunjukkan bahwa primer *LipL41* dan *IcdA* terbukti efektif dalam membedakan spesies patogen, sedangkan primer *seqY*, *LipL32*, *Adk*, dan *rrs2* memerlukan pemurnian lebih lanjut. Suhu peleburan (TM) atau suhu annealing yang sesuai adalah 58°C dengan 35 siklus amplifikasi. Konsentrasi dan kemurnian DNA memiliki rasio A260/A280 yang berkisar antara 1,8 hingga 2,8. **Kesimpulan:** Primer *LipL41* (500 bp) dan *IcdA* (700 bp) merupakan primer yang cocok untuk mengidentifikasi *Leptospira* patogen.

Kata kunci:
Leptospira, PCR, Primer, Kemurnian DNA, Optimisasi

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INTRODUCTION

Leptospirosis, a zoonotic disease with a worldwide distribution except in the polar region where the disease has not been reported, is now recognized as an emerging infectious disease (Lau *et al*., 2010). The diversity in specific clinical manifestations that distinguish leptospirosis adds complexity and dynamism to the disease (Moran *et al*., 2009). The genus *Leptospira* contains both pathogenic and saprophytic species belonging to the order Spirochaetales (Bharti *et al*., 2003; McBride *et al*., 2005).

Leptospirosis is frequently underdiagnosed due to nonspecific symptoms early in the disease and the difficulty of performing both culture and the reference serological test, the *Microscopic Agglutination Test* (MAT). The mortality rate in severe leptospirosis can be as high as 15% (Musso and La Scola, 2013), early diagnosis is essential for effective antibiotic treatment (Levett, 2001). Leptospirosis is cosmopolitically distributed and exists in diverse ecological habitats, including water, soil, and the tissues of various mammals, with a high prevalence in the tropics (Sethi *et al*., 2010). Humans most commonly become infected through occupational, recreational, or domestic contact with the urine of carrier animals, either directly or via contaminated water or soil. *Leptospira* are thin, helical bacteria classified into at least 12 pathogenic and 4 saprophytic species, with more than 250 pathogenic serovars (Adler and de la Peña Moctezuma, 2010).

The incidence in tropical areas is up to ten times higher, likely due to a combination of factors, including environmentalc conditions (higher temperatures, humidity, and rainfall favoring organism survival) as well as socioeconomic factors (poor sanitation, closer human contact with both rodents and domestic animals) (Hartskeerl *et al*., 2011). It contributes to an estimated 2.9 million disability-adjusted life years lost annually (Torgerson *et al*., 2015). Leptospirosis has a global distribution, with a higher incidence in the tropics and subtropics, ranging from 10 to 100 human cases per 100,000 individuals (Guerra, 2013).

Leptospirosis is reported in the South-East Asia region from time to time. Most human cases have been reported from India, Indonesia, Thailand, and Sri Lanka during the rainy season and are transmitted through rodents, with predominant species being *Leptospira* interrogans and *Leptospira* borgpetersenii (Cosson *et al*., 2014; Vijayachari *et al*., 2004). The majority of cases and deaths occur in tropical low and middle-income countries, driven by climate conditions, close human-animal contact, inadequate sewage disposal, and water treatment (Costa *et al*., 2015; Levett *et al*., 2001).

Leptospira has been virtually isolated from all mammalian species (Picardeau, 2013). Numerous mammalian species serve as natural carriers (maintenance hosts) of pathogenic *Leptospira*, including feral, farm, and pet animals (Levett, 2001). Every individual has a risk of getting infected as domestic and wild animals carry *Leptospira*. The at-risk population varies from healthcare professionals, animal caretakers, farmers, agricultural workers, fishermen, rodent catchers, water sports enthusiasts, *National Disaster Response Force* (NDRF) personnel, people who volunteer rescue operations in flood-affected areas, sanitary workers, and sewage workers. The clinical manifestations of leptospirosis range from flu-like illness to *Acute Kidney Failure* (AKF), pneumonia, jaundice, and pulmonary hemorrhages (Karpagam and Ganesh, 2020). However, many rare and uncommon clinical manifestations are being reported worldwide. Infected animals may excrete *Leptospira* intermittently or regularly for months or years, or throughout their lifetime (Picardeau, 2013).

Leptospirosis is a zoonotic disease caused by pathogenic species of the *Leptospira* genus, which can lead to severe illness and death in humans and animals (Budihal and Perwez, 2014). Molecular techniques such as PCR and sequencing have become essential tools for the accurate examination and identification of *Leptospira* species (Reis et *al*., 2013). Nonetheless, the inherent diversity within the genomes of *Leptospira* bacteria, coupled with the existence of closely related non-pathogenic species, has posed significant hurdles when it comes to the precise identification of pathogenic *Leptospira* strains. Consequently, the scientific community has diligently worked to tackle these challenges head-on. Over the years, researchers have dedicated their efforts to the development and fine-tuning advanced molecular techniques, specifically tailored to enable the specific and highly sensitive detection of various *Leptospira* species (Cosson *et al*., 2014; Vijayachari *et al*., 2004). In light of these advancements, this comprehensive primer has been meticulously crafted with the primary aim of identifying the most suitable primers for the accurate identification of pathogenic *Leptospira* strains. Additionally, it provides researchers with an extensive overview of various primers available for *Leptospira* species analysis, along with practical insights and recommendations for their optimal application.

MATERIAL AND METHOD

This study protocol had been approved by the Health Research Ethics Committee, National Institute of Health Research and Development (HERC-NIHRD) no. LB.02.01/2/KE.268/2018. This research used an observational approach to provide a comprehensive guide for researchers on optimizing molecular techniques for accurate examinations of *Leptospira* species. The research was carried out in the Proteomics Laboratory and Molecular Biology Laboratory of *Vector Control and Reservoir Disease* (IVCRD) in Salatiga, Central Java. This research was conducted from April to July 2018. It used the stored samples in the proteomics laboratory, consisting of 17 *Leptospira* cultures commonly used as positive controls, aquadest, Ssofast Eva Green Supermix, 100% ethanol, and 6 primers (Table 1). Several tools were utilized, including a

water bath, vortex, NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA), 1 µL to 10 µL micropipettes, 10 µL to 100 µL micropipettes, 100 µL to 1000 µL micropipettes, 1.5 mL eppendorf tubes, PCR tubes, and a centrifuge.

Extraction kit

DNA extraction was carried out using the DNA extraction kit (Qiagen) following the manufacturer's instructions. DNA amplification was performed in a thermal cycler using Taq DNA polymerase (Qiagen) under standard conditions. Based on the manufacturer's instructions, Qiagen could be used to extract various types of *Leptospira* from different sample sources, including whole blood, sputum, and cultures. The *Leptospira* used in this research were positive controls that had been stored. For the extraction process, we utilized alcohol, ethanol, a vortex, pipet tips, a micropipette, 1.5 mL tubes, collecting tubes, spin columns, tube trays, a water bath, and a centrifuge.

***Leptospira* detection using *Polymerase Chain Reaction* (PCR) methods**

Several primer sets corresponding to the the *LipL41*, *Adk*, *IcdA*, *seqY*, *rrs2*, and *LipL32* genes were used. The amplification process included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of amplification comprising denaturation (94°C for 30 seconds), annealing (58°C for 30 seconds), and primer extension (72°C for 1 minute) steps, with a final extension of 7 minutes at 72°C. All amplified fragments were checked on a 2% agarose gel with ethidium bromide staining.

Table 1. Composition, volume, and total concentration of each *Polymerase Chain Reaction* (PCR) reagent component

Component	Concentration	Volume (µL)
ddH_2O	-	5.5
Master mix (GoTaq™ Green)	1X	12.5
Forward primer	10 µM	1.0
Reverse primer	10 µM	1.0
DNA template	<250 ng	5.0
Total		**25.00**

***Multilocus Sequence Typing* (MLST)**

We used *Multilocus Sequence Typing* (MLST) scheme 3, which involves the analysis of six genes. The details of these six genes, namely *Adenylate Kinase (Adk), Isocitrate dehydrogenase (IcdA), outer membrane lipoprotein LipL32 (LipL32), 16S rRNA (rrs2), pre-protein translocase secY protein (secY),* and *outer membrane lipoprotein LipL41 (LipL41)*, are shown in Table 2. Many sequences of *rrs2*, *LipL32*, and *LipL41* are available in GenBank. PCR primers were designed for these genes based on GenBank Records (https://pubmlst.org/organisms/leptospira-spp/) in the conserved regions flanking the variable internal fragments of the target regions.

RESULT

In our research publication, we utilized *Leptospira* cultures commonly employed as positive controls for leptospirosis. Our primary objective was to identify the most suitable primers for the precise identification of pathogenic *Leptospira* strains. Through a comprehensive series of PCR experiments conducted under consistent amplification conditions, our investigation revealed that the primers *LipL41* and *IcdA* consistently outperformed others in the task of identifying pathogenic *Leptospira*.

Furthermore, our meticulous observations indicated that the optimal denaturation process was achieved at 95°C for 5 minutes, followed by 35 amplification cycles. Each cycle comprised denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and primer extension at 72°C for 1 minute. The process concluded with a final extension step of 7 minutes at 72°C. Subsequently, the amplified fragments underwent analysis through 2% agarose gel electrophoresis with ethidium bromide staining. Upon conducting electrophoresis on the *Leptospira* cultures using various primers, including *LipL41*, *LipL32*, *seqY*, *IcdA*, *Adk*, and *rrs2*, this research successfully optimized the primer selection, yielding the following results.

Primers optimization

LipL41 primer optimization showed that all gene targets occurred at 500 bp, as shown in Figure 1. All isolated culture *Leptospira* bacteria were obtained from the WHO Collaborating Center for Leptospirosis Identification and used as positive controls for identifying *Leptospira*. Samples 16 and 17 belonged to serovar *semaranga* and *pathok*. Therefore, they could not be captured by the target gene because *semaranga* and *pathok* are saprophytic. This indicates that *LipL41* can only capture pathogenic *Leptospira*.

Figure 2 displays the presence of *semaranga* and *pathok* strains captured by the target gene at 700 bp and 500 bp, which is not typically expected for pathogenic *Leptospira* identification. In the electrophoresis results, when using the *seqY* primer commonly used as a positive control, it is important to note that these cultures encompass both pathogenic and saprophytic strains. Significantly, *seqY* exhibits robust performance in capturing all *Leptospira* strains effectively at the 700 bp marker.

Figure 2 also shows the electrophoresis results for *LipL32*, presenting its effective capture of 16 *Leptospira* cultures but its inability to capture pathogenic *Leptospira* in sample number 17, which belongs to *djasiman*. Since we are searching for the most suitable primer to specifically capture pathogenic *Leptospira*, *semaranga* and *pathok* strains ideally should not have been captured, and pathogenic *Leptospira*, such as *djasiman*, must occur. Nevertheless, this primer proves to be useful for identifying general *Leptospira* with a DNA leader at 500 bp.

Table 2. Details of gene loci and the corresponding primer sequences used for *Multilocus Sequence Typing* (MLST) analysis

Gene	Locus	Gene size (bp)	Coordinates	PCR product size (bp)	Size of polymorphic sequence (bp)	Function	Primer sequence
Adk	LICl2852	564	3458298 - 3458861	531	430	Adenylate kinase (Adk)	**Forward** 5′GGGCTGGAAAAGGTACACAA 3′ **Reverse** 5′ ACGCAAGCTCCTTTTGAATC 3′
IcdA	LIC13244	1197	3979829 – 3981025	674	557	Isocitarate dehydrogenase (IcdA)	**Forward** 5′GGGACGAGATGACCAGGAT 3′ **Reverse** 5′ TTTTTTGAGATCCGCAGCTTT 3′
LipL41	LIC12966	1068	3603575 – 3604642	520	518	Outer membrane lipoprotein LipL41 (LipL41)	**Forward** 5′TAGGAAATTGCGCAGCTACA 3' **Reverse** 5′ GCATCGAGAGGAATTAACATCA 3′
rrs2	LIC11508	1512	1862433 – 1863944	541	542	16s ribosomal RNA (rss)	**Forward** 5′ CATGCAAGTCAAGCGGAGTA 3' **Reverse** 5′ AGTTGAGCCCGCAGTTTTC 3′
seqY	LIC12853	1383	3458869 – 3460251	549	549	Translocase preprotein secy	**Forward** 5′ ATGCCGATCATTTTTGCTTC 3′ **Reverse** 5′ CCGTCCCTTAATTTTAGACTTCTTC 3′
LipL32	LIC11352	819	1666299 – 1667117	474	474	Outer membrane lipoprotein LipL32 (LipL32)	**Forward** 5′ATCTCCGTTGCACTCTTTGC3' **Reverse** 5′ACCATCATCATCATCGTCCA 3′

Source: Ahmed *et al*. (2020)

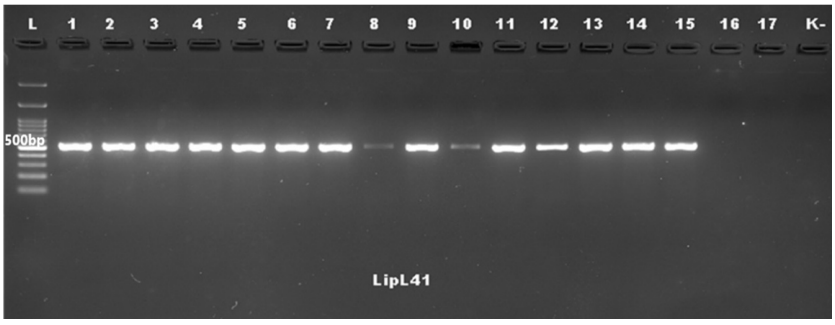

Figure 1. *Polymerase Chain Reaction* (PCR) results of culture isolate of *Leptospira* using *LipL41* primer. L= DNA leader, 1 = *canicola*, 2 = *hebdomadis*, 3 = *mini*, 4 = *robinsoni*, 5 = *pomona*, 6 = *bataviae*, 7 = *icterohaemorrhagiae*, 8 = *autumnalis*, 9 = *grippotyphosa*, 10 = *pyrogenes*, 11 = *djasiman*, 12 = *hardjo*, 13 = *rama*, 14 = *sarmin*, 15 = *manhao*, 16 = *semaranga*, 17 = *pathok*, K (-) = negative control

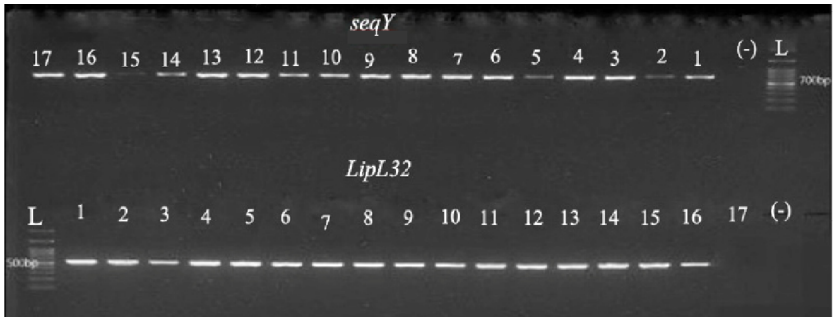

Figure 2. *Polymerase Chain Reaction* (PCR) results of culture isolate of *Leptospira* using *seqY* and *LipL32* primers. **seqY:** L= DNA leader, 1 = *djasiman*, 2 = *pyrogenes*, 3 = *sarmin*, 4 = *manhao*, 5 = *autumnalis*, 6 = *icterohaemorrhagiae*, 7 = *hardjo*, 8 = *robinsoni*, 9 = *rama*, 10 = *pomona*, 11 = *bataviae*, 12 = *hebdomadis*, 13 = *semaranga*, 14 = *grippotyphosa*, 15 = *canicola*, 16 = *pathok*, 17 = *mini*, (-) = negative control. **LipL32:** L = DNA leader, 1 = *canicola*, 2 = *sarmin*, 3 = *mini*, 4 = *robinsoni*, 5 = *pomona*, 6 = *bataviae*, 7 = *icterohaemorrhagiae*, 8 = *autumnalis*, 9 = *grippotyphosa*, 10 = *pyrogenes*, 11 = *hebdomadis*, 12 = *hardjo*, 13 = *rama*, 14 = *semaranga*, 15 = *manhao*, 16 = *pathok*, 17 = *djasiman*, (-) = negative control

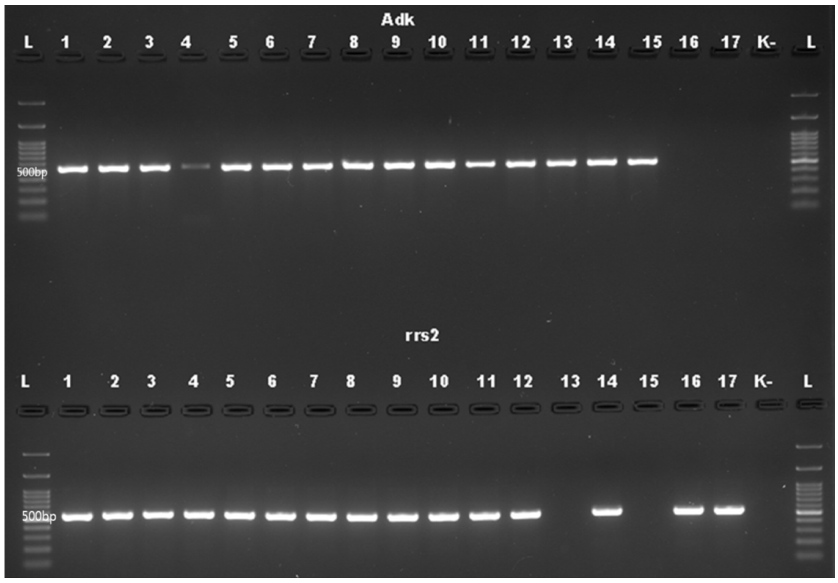

Figure 3. *Polymerase Chain Reaction* (PCR) results of culture isolate of *Leptospira* using *Adk* and *rrs2* primers. **Adk:** L = DNA leader, 1 = *canicola*, 2 = *hebdomadis*, 3 = *mini*, 4 = *robinsoni*, 5 = *manhao*, 6 = *bataviae*, 7 = *icterohaemorrhagiae*, 8 = *autumnalis*, 9 = *grippotyphosa*, 10 = *pyrogenes*, 11 = *djasiman*, 12 = *hardjo*, 13 = *semaranga*, 14 = *sarmin*, 15 = *pathok*, 16 = *rama*, 17 = *pomona*, K (-) = negative control. **rrs2:** L = DNA leader, 1= *canicola*, 2 = *hebdomadis*, 3 = *mini*, 4 = *robinsoni*, 5 = *pomona*, 6 = *bataviae*, 7 = *icterohaemorrhagiae*, 8 = *autumnalis*, 9 = *grippotyphosa*, 10 = *pyrogenes*, 11 = *djasiman*, 12 = *hardjo*, 13 = *rama*, 14 = *sarmin*, 15 = *manhao*, 16 = *semaranga*, 17 = *pathok*, K (-) = negative control

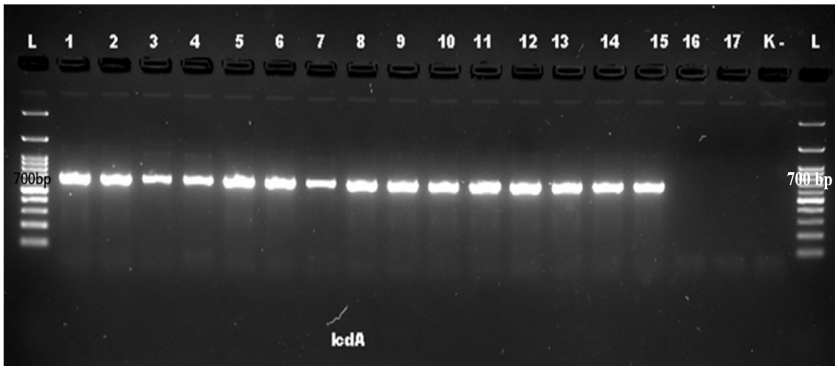

Figure 4. *Polymerase Chain Reaction* (PCR) results of culture isolate of *Leptospira* using *lcdA* primer. L = DNA leader 700 bp, 1 = *canicola*, 2 = *hebdomadis*, 3 = *mini*, 4 = *robinsoni*, 5 = *pomona*, 6 = *bataviae*, 7 = *icterohaemorrhagiae*, 8 = *autumnalis*, 9 = *grippotyphosa*, 10 = *pyrogenes*, 11 = *djasiman*, 12 = *hardjo*, 13 = *rama*, 14 = *sarmin*, 15 = *manhao*, 16 = *semaranga*, 17 = *pathok*, K (-) = negative control

In Figure 3, all bacterial cultures of pathogenic *Leptospira* are demonstrated to be captured by the gene target, resulting in a distinct band at 500 bp. However, contrary to expectations, the *rrs2* primer for *rama* and *manhao* did not produce the expected capture, while saprophytic *Leptospira* strains were detected instead. Conversely, the *Adk* primer has exhibited efficacy in capturing both saprophytic and pathogenic strains of *Leptospira* bacteria. Nonetheless, it proved inadequate in capturing the *rama* and *pomona* strains, both of which are classified as pathogenic *Leptospira* strains. Moreover, the *Adk* primer demonstrates limitations in effectively capturing the *robinsoni* strain, highlighting the need for further optimization or alternative primer design.

Primer optimization of *lcdA* showed that target genes occurred at 700 bp in Figure 4. All isolated cultures of *Leptospira* bacteria were from the WHO cc *Institute of Vector Control and Reservoir Disease* (IVRCD) Salatiga, Indonesia. Numbers 16 and 17, which belonged to serovar *semaranga* and *pathok*, could not be captured by target gene because *semaranga* and *pathok* are saprophytic.

Concentration and purity of total *Deoxyribonucleic acid* (DNA)

The DNA concentration (µg/mL) and purity (absorbance ratio at 260/280, denoted as A260/A280) were initially determined by spectrophotometry using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), where pure DNA had an A260/A280 ratios, ranging between 1.8 and 2.0 (Cheng *et al*., 2021). The concentrations and purity of the samples in this research are presented in Table 3.

Table 3. The *Deoxyribonucleic acid* (DNA) purity of the culture isolates

No.	Culture isolates	Concentration (µg/µL)	A260/A280
1	*Semaranga*	4.1	1.96
2	*Hardjo*	9.7	1.92
3	*Djasiman*	12.9	1.86
4	*Manhao*	5.9	2.17
5	*Pomona*	5.5	2
6	*Hebdomadis*	7.7	2.09
7	*Canicola*	21.8	1.64
8	*Bataviae*	13.2	1.73
9	*Pathok*	8.5	1.92
10	*Mini*	9.8	1.76
11	*Sarmin*	6.5	2.07
12	*Rama*	16.9	1.61
13	*Pyrogenes*	7.3	1.92
14	*Icterohaemorrhagiae*	5.9	2.03
15	*Grippotyphosa*	5.0	1.94
16	*Robinsoni*	4.3	1.99
17	*Autumnalis*	4.5	1.99

DISCUSSION

Leptospirosis is considered a major public health problem in many regions with endemic potential, especially following natural disasters such as floods and heavy rain. The recent study found that the six selected loci are suitable for *Multi-Locus Sequence Typing* (MLST), as they can be amplified in isolates irrespective of species. These loci are unlinked to the *L. interrogans* chromosome and exhibit a modest degree of sequence diversity (Nalam *et al*., 2010). The accurate identification and characterization of *Leptospira* species are crucial for effective disease surveillance, outbreak investigation, and treatment strategies (Sakundarno *et al*., 2014). The study by Galloway and Hoffmaster (2015) underscores the importance of continuous efforts to improve diagnostic tools for leptospirosis. The optimization of the *LipL32* PCR assay represents a significant advancement in enhancing sensitivity for detecting this potentially life-threatening disease. Further research and validation of these findings are warranted to ensure their applicability across diverse clinical and epidemiological setting (Galloway and Hoffmaster, 2015).

The optimization of primer sequences is imperative to ensure the dependable and precise identification of pathogenic *Leptospira* strains, aligning with the overarching goal of this research. Notably, *LipL41* and *IcdA* primers have demonstrated efficacy in capturing pathogenic *Leptospira* due to their selective targeting of genes associated specifically with pathogenic strains, by successfully capturing these target genes, these primers facilitate the accurate identification of pathogenic *Leptospira* bacteria, thereby contributing to the attainment of our research aim. Previous studies have corroborated the effectiveness of *LipL41* and *IcdA* primers in discerning pathogenic strains from saprophytic ones, further supporting their suitability for precise identification purposes. These findings underscore the importance of primer optimization in enhancing diagnostic accuracy and contribute to the advancement of molecular techniques for the detection and characterization of pathogenic *Leptospira* strains (Cosson *et al*., 2014; Vijayachari *et al*., 2004). However, our findings revealed limitations with the *seqY* and *LipL32* primers, as they also captured saprophytic strains, compromising their ability to precisely identify pathogenic *Leptospira*. Although commonly used in *Leptospira* research (Costa *et al*., 2015; Levett, 2001), these primers require further optimization or alternative primer design to enhance their specificity.

The utilization of *Multi-Locus Sequence Typing* (MLST) was recommended to further characterize the genetic variation within *Leptospira* species. MLST allows for the comprehensive analysis of multiple loci, then it provides a more in-depth understanding of the genetic diversity within *Leptospira* populations (Picardeau, 2013). It is important to note that sample quality plays a crucial role in obtaining accurate results. Therefore, utilizing new or well-stored samples is highly recommended to minimize the potential for contamination or degradation of genetic material (Moran *et al*., 2009).

The DNA concentration (µg/mL) and purity of the samples in this research are denoted as A260/A280, where pure DNA had A260/A280 ratios ranging between 1.8 and 2.0. If the A260/A280 ratios of *manhao*, *sarmin*, *hebdomadis*, and *icterohaemorrhagiae* exceed a ratio of 2, it may indicate prolonged waiting times during the purity test or other unknown factors affecting DNA purity. Previous studies, such as Cheng *et al*. (2021), have similarly utilized spectrophotometry to assess DNA purity in microbiological research. Their findings underscore the importance of stringent purity standards, as deviations from the optimal A260/A280 ratio range of 1.8 to 2.0 may suggest potential contamination or degradation of DNA samples. In line with our observations, elevated A260/A280 ratios beyond 2 for specific culture isolates, such as *manhao*, *sarmin*, *hebdomadis*, and *icterohaemorrhagiae*, may raise concerns regarding the integrity and quality of DNA extracted from these samples. Further investigation into the underlying factors contributing to these deviations is warranted to ensure the reliability and accuracy of subsequent molecular analyses conducted on these samples.

Our research also determined the optimal *Melting Temperature* (TM) or annealing temperature for PCR amplification to be 58°C with 35 cycles. This optimized condition ensures efficient and accurate amplification of the target genes. Therefore, the results become more robust and reliable (Slack *et al*., 2006). This research emphasizes the significance of primer optimization, sample quality, and the utilization of MLST for the accurate examination and identification of *Leptospira* species. The *LipL41* and *lcdA* primers were found to be effective in distinguishing pathogenic strains, while the *seqY*, *LipL32*, *Adk*, and *rrs2* primers require further refinement. Other researchers should consider these recommendations to enhance the reliability and specificity of their investigations into *Leptospira* species.

CONCLUSION

The findings of this research recommend using either new samples or well-preserved ones. It is worth noting that each type of sample, such as culture, blood, serum, and urine, has different sensitivities to their respective primers. The optimization of *LipL41* and *lcdA* primers effectively captured the target genes for identifying pathogenic *Leptospira*. However, the *seqY, LipL32*, and *rrs2* primers also captured saprophytic strains. Furthermore, the results obtained from the Adk primer indicate limitations in capturing certain pathogenic strains, particularly *rama* and *pamona*, alongside suboptimal performance in capturing *robinsoni*. These observations highlight the necessity for meticulous primer selection and optimization to ensure precise pathogenic *Leptospira* identification. Otherwise, the primers may prove unsuitable for accurately identifying pathogenic *Leptospira*. Moreover, it is feasible to characterize genetic variations using the MLST method by employing high-quality samples that yield positive PCR results for subsequent sequencing steps. Additionally, it is concluded that the appropriate *Melting Temperature* (TM) or annealing temperature is 58°C, coupled with 35 amplification cycles.

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REFERENCE

Adler, B., de la Peña Moctezuma, A., 2010. Leptospira and Leptospirosis. Vet Microbiol Vol. 140(3-4), Pp. 287-296.

Ahmed, A.A., Goris, M.G.A., Meijer, M.C., 2020. Development of LipL32 Real-Time PCR Combined with An Internal and Extraction Control for Pathogenic Leptospira Detection. PLoS One Vol. 15(11), Pp. e0241584.

Bharti, A.R., Nally, J.E., Ricaldi, J.N., Matthias, M.A., Diaz, M.M., Lovett, M.A., Levett, P.N., Gilman, R.H., Willig, M.R., Gotuzzo, E., Vinetz, J.M., Peru-United States Leptospirosis Consortium, 2003. Leptospirosis: A Zoonotic Disease of Global Importance. Lancet Infect Dis Vol. 3(12), Pp. 757-771.

Budihal, S.V., Perwez, K., 2014. Leptospirosis Diagnosis: Competancy of Various Laboratory Tests. J Clin Diagn Res Vol. 8(1), Pp. 199-202.

Cheng, X., Hong, X., Khayatnezhad, M., Ullah, F., 2021. Genetic Diiversity and Comparative Study of Genomic DNA Extraction Protocols in Tamarix L. Species. Caryologia Vol. 74(2), Pp. 131-139.

Cosson, J.-F., Picardeau, M., Mielcarek, M., Tatard, C., Chaval, Y., Suputtamongkol, Y., Buchy, P., Jittapalapong, S., Herbreteau, V., Morand, S., 2014. Epidemiology of Leptospira Transmitted by Rodents in Southeast Asia. PLOS Neglected Tropical Diseases Vol. 8(6), Pp. e2902.

Costa, F., Hagan, J.E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M.S., Stein, C., Abela-Ridder, B., Ko, A.I., 2015. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLoS Negl Trop Dis Vol. 9(9), Pp. e0003898.

Galloway, R.L., Hoffmaster, A.R., 2015. Optimization of LipL32 PCR Asay for Increased Sensitivity in Diagnosing Leptospirosis. Diagn Microbiol Infect Dis Vol. 82(3), Pp. 199-200.

Guerra, M.A., 2013. Leptospirosis: Public Health Perspectives. Biologicals Vol. 41(5), Pp. 295-297.

Hartskeerl, R.A., Collares-Pereira, M., Ellis, W.A., 2011. Emergence, Control and Re-emerging Leptospirosis: Dynamics of Infection in The Changing World. Clin Microbiol Infect Vol. 17(4), Pp. 494-501.

Karpagam, K.B., Ganesh, B., 2020. Leptospirosis: A Neglected Tropical Zoonotic Infection of Public Health Importance-an Updated Review. Eur J Clin Microbiol Infect Dis Vol. 39(5), Pp. 835-846.

Lau, C., Smythe, L., Weinstein, P., 2010. Leptospirosis: An Emerging Disease in Travellers. Travel Med Infect Dis Vol. 8(1), Pp. 33-39.

Levett, P.N., 2001. Leptospirosis. Clin Microbiol Rev Vol. 14(2), Pp. 296-326.

Levett, P.N., Branch, S.L., Whittington, C.U., Edwards, C.N., Paxton, H., 2001. Two Methods for Rapid Serological Diagnosis of Acute Leptospirosis. Clin Diagn Lab Immunol Vol. 8(2), Pp. 349-351.

McBride, Alan JA, Athanazio, Daniel A, Reis, Mitermayer G, Ko, Albert I, 2005. Leptospirosis : Current Opinion in Infectious Diseases. Current Opinion in Infectious Diseases Vol. 18(5), Pp. 376-386.

Moran, M., Guzman, J., Ropars, A.-L., McDonald, A., Jameson, N., Omune, B., Ryan, S., Wu, L., 2009. Neglected Disease Research and Development: How Much Are We Really Spending? PLOS Medicine Vol. 6(2), Pp. e1000030.

Musso, D., La Scola, B., 2013. Laboratory Diagnosis of Leptospirosis: A Challenge. Journal of Microbiology, Immunology and Infection Vol. 46(4), Pp. 245-252.

Nalam, K., Ahmed, A., Devi, S.M., Francalacci, P., Baig, M., Sechi, L.A., Hartskeerl, R.A., Ahmed, N., 2010. Genetic Affinities within a Large Global Collection of Pathogenic Leptospira: Implications for Strain Identification and Molecular Epidemiology. PLOS ONE Vol. 5(8), Pp. e12637.

Picardeau, M., 2013. Diagnosis and Epidemiology of Leptospirosis. Med Mal Infect Vol. 43(1), Pp. 1-9.

Reis, E.A.G., Hagan, J.E., Ribeiro, G.S., Teixeira-Carvalho, A., Martins-Filho, O.A., Montgomery, R.R., Shaw, A.C., Ko, A.I., Reis, M.G., 2013. Cytokine Response Signatures in Disease Progression and Development of Severe Clinical Outcomes for Leptospirosis. PLoS Negl Trop Dis Vol. 7(9), Pp. e2457.

Sakundarno, M., Bertolatti, D., Maycock, B., Spickett, J., Dhaliwal, S., 2014. Risk Factors for Leptospirosis Infection in Humans and Implications for Public Health Intervention in Indonesia and The Asia-Pacific Region. Asia Pac J Public Health Vol. 26(1), Pp. 15-32.

Sethi, S., Sharma, N., Kakkar, N., Taneja, J., Chatterjee, S.S., Banga, S.S., Sharma, M., 2010. Increasing Trends of Leptospirosis in Northern India: A Clinico-Epidemiological Study. PLoS Negl Trop Dis Vol. 4(1), Pp. e579.

Slack, A.T., Symonds, M.L., Dohnt, M.F., Smythe, L.D., 2006. Identification of Pathogenic Leptospira Species by Conventional or Real-Time PCR and Sequencing of The DNA Gyrase Subunit B Encoding Gene. BMC Microbiology Vol. 6(1), Pp. 95.

Torgerson, P.R., Hagan, J.E., Costa, F., Calcagno, J., Kane, M., Martinez-Silveira, M.S., Goris, M.G.A., Stein, C., Ko, A.I., Abela-Ridder, B., 2015. Global Burden of Leptospirosis: Estimated in Terms of Disability Adjusted Life Years. PLoS Negl Trop Dis Vol. 9(10), e0004122.

Vijayachari, P., Ahmed, N., Sugunan, A.P., Ghousunnissa, S., Rao, K.R., Hasnain, S.E., Sehgal, S.C., 2004. Use of Fluorescent Amplified Fragment Length Polymorphism for Molecular Epidemiology of Leptospirosis in India. J Clin Microbiol Vol. 42(8), Pp. 3575-3580.