JTL 13 (2) DECEMBER 2024
Page: 156-165
Contents list available at Jurnal Teknologi Laboratorium
JURNAL TEKNOLOGI LABORATORIUM
Journal Homepage: www.teknolabjournal.com
ISSN 2580-0191(Online) I ISSN 2338 – 5634(Print)

Original Research

OPEN ACCESS

Optimizing DNA extraction methods for enhanced detection of pathogenic Leptospira *spp. in cultures using PCR*

Kiki Aprilia[1], Arif Mulyanto[*1], Kurnia Ritma Dhanti[1], Korri El Khobar[2], Farida Dwi Handayani[*2]

[1] Medical Laboratory Technology D4 Study Program, Faculty of Health Sciences, Universitas Muhammadiyah Purwokerto, Banyumas, Indonesia
[2] Eijkman Research Centre for Molecular Biology, National Innovation Research Agency (BRIN), Indonesia

Abstract: Leptospirosis is a worldwide zoonosis caused by *Leptospira interrogans*. Laboratory diagnosis of leptospirosis can be done through sample culture. However, microscopic observation of positive *Leptospira* spp. culture need to be supported by molecular detection to confirm the presence of pathogenic *Leptospira* spp. This study aimed to compare the effectiveness of two DNA extraction kits, from QIAGEN and Zymo Research, to extract bacterial DNA genome from positive *Leptospira* spp. culture for downstream molecular detection. This study is an analytical observational study with a cross-sectional design conducted in June-September 2023, and part of the PESTO-RITA 2023 study. Fourteen identified positive *Leptospira* spp. culture, with *Leptospira* spp. movement under dark field microscopy, was used for DNA extraction using both extraction kits. QIAGEN kit yielded higher mean DNA concentration (0.900±0.161 vs 0.790±0.167 log10 µg/mL, $p>0.05$) and better DNA purity (1.854 vs 1.632, $p<0.05$) than the Zymo Research kit. PCR results, targeting the lipL32 gene, showed that two samples extracted with QIAGEN and one sample extracted with Zymo Research were positive for pathogenic *Leptospira* spp. Our preliminary results showed that QIAGEN kit yielded better DNA concentration and purity from positive *Leptospira* spp. culture.

Keywords: DNA Concentration; DNA Extraction; DNA Purity; *Leptospira* detection; PCR.

INTRODUCTION

Leptospirosis, a zoonosis disease with a worldwide distribution, is caused by infection of pathogenic bacteria of the Spirochaete group, *Leptospira interrogans*[1]. Leptospirosis is an acute febrile infection, mostly affecting the tropical rural areas, through exposure to *Leptospira* spp. contaminated water and/or soil[2]. *Leptospira* spp. infection is transmitted by animals, especially rodents, which is its main and most widespread reservoir[3,4]. Leptospirosis has a typical clinical manifestation, making it difficult to distinguish it from other tropical diseases such as typhoid, dengue fever, malaria, and rickettsiosis. Leptospirosis can also affect multiple organs and may resulted in mortality[5–7].

Currently, there is various laboratory-based tests available to diagnose leptospirosis including IgM ELISA, Microscopic Agglutination Test (MAT), Dark Field Microscopy (DFM), Culture, Polymerase Chain Reaction (PCR), Indirect Hemagglutination Assay (IHA), and Leptodipstick Assay[9]. Among these various methods, isolation of *Leptospira* spp. through culture is a more definitive confirmation test for leptospirosis, although this method is hampered by the slow

Corresponding author.
E-mail address: fari018@brin.go.id (Farida Dwi Handayani); arifmulyanto02@gmail.com (Arif Mulyanto)
DOI: 10.29238/teknolabjournal.v13i2.480
Received 29 April 2024; Received in revised form 28 June 2024; Accepted 23 October 2024

growth characteristic of *Leptospira* spp., thus resulting in a longer culture time. In addition, the positive culture rate of *Leptospira* spp. is generally low. However, once bacterial isolates are successfully obtained, they can be very useful for further molecular genotyping and epidemiological studies[10].

Cultures of *Leptospira* spp. from the field isolates are generally prone to other bacterial contamination. In addition, its shape similarity with the other Spirochaete bacteria, makes it hard to identify the specific presence of *Leptospira* spp. in the culture. Depending on the health of its cells, *Leptospira* spp. can also change its morphological appearance to a more spherical shape instead of the typical spiral shape. Therefore, identification of *Leptospira* spp. in the culture cannot be performed based on microscopic observation alone, and need to be supported by molecular DNA testing[11].

Several studies have reported successful *Leptospira* spp. molecular detection using different source of samples. Allan *et al*. reported that *Leptospira* spp. genomic DNAs can be detected successfully from patients' plasma and serum samples, as well as urine sample, using the QIAGEN DNA extraction kit, by modifying the final elution volume into 100 µL to increase the yield DNA concentration[13]. Similarly, Goy-Thollot *et al*. also successfully extracted *Leptospira* spp. genomic DNAs from blood and urine samples of infected dogs using the same QIAGEN DNA extraction kit. They reported six positive results (out of 30 samples) for *Leptospira* spp. in the first PCR screening, and two positive results in a repeat PCR screening performed 10 months later[14]. Harran *et al*. detected the presence of *Leptospira* spp. infection by real-time PCR using genomic DNAs extracted from animal blood samples, by using a different DNA extraction kit from Zymo Research[15]. This particular DNA extraction kit has also been used to detect the presence of the *Leptospira* spp. from soil and water samples by using 16s rRNA real-time PCR[16].

Since there is no report yet on which commercial DNA extraction kit produce good quality DNA yield from cultures of pathogenic *Leptospira* spp., this study aimed to compare the quality of genomic DNAs from *Leptospira* spp. cultures extracted with two DNA extraction kits from QIAGEN and Zymo Research for *Leptospira* spp. molecular detection.

MATERIAL AND METHOD

Study design and sample collection

This is an analytical observational study with a cross-sectional design. This preliminary study is part of the Research on Genomic Characteristics and Molecular Epidemiology of PES, Leptospirosis, Rickettsiosis, and Hantavirus in East Java and Central Java (PESTO-RITA) 2023 funded by the World Health Organization (WHO) and National Innovation Research Agency (BRIN). This study was approved by the Ethics Commission for Health of the National Innovation Research Agency (No. 049/KE.03/SK/05/2023).

The study was conducted at BRIN CWS Salatiga, Indonesia from June to September 2023. The population study was all PESTO-RITA 2023 *Leptospira* spp. cultures obtained either from (1) whole blood, plasma, and urine samples from suspected Leptospirosis patients or (2) kidney samples from suspected rat reservoirs. The protocol used for *Leptospira* culture is based on the Amsterdam UMC protocol.

Sampling was done through purposive sampling. The inclusion criteria was culture with positive *Leptospira* spp. movement as identified under a dark field microscope observation. Positive *Leptospira* spp. movement was defined as morphological detection of spiral bacteria with hooks in the culture, with density around 2 x 10^8 cells/mL, and no other presence of non-spiral bacteria with hooks. The exclusion criteria was cultures with positive movement of other bacteria types including Cocci, Bacilli, Filamentous, Vibrio, and other Spirochaete species. Microscopic observations were performed twice (with second observation

performed two weeks after the first observation) by 3 different people using the 100x and 200x objective magnifications.

DNA extraction with QIAamp® DNA Mini Kit (QIAGEN)

Nucleic acid from positive *Leptospira* spp. culture was extracted using QIAamp® DNA Mini Kit (QIAGEN #51304, Hilden, Germany) according to the manufacturer's instructions. Briefly, 1 mL of media from positive *Leptospira* spp. culture, was centrifuged at 7,340 rpm for 5 minutes. The pellet was resuspended in 200 µL PBS (supplemented with 20 µL Proteinase K) and incubated at 56 °C for 1-3 hours or overnight. Following incubation, 200 µL Buffer AL was added to the mixture and homogenized by vortexing for 15 seconds. The mixture was incubated at 70 °C for 30 minutes, then centrifuged at 4,000 rpm for 1 minute. Two-hundred µL absolute ethanol were added to the mixture to precipitate the nucleic acid, continued with vortexing for 15 seconds and centrifugation at 4,000 rpm for 1 minute. The mixture was transferred into a spin column with fresh collection tube attached, then centrifuged at 8,000 rpm for 1 minute. Flow-through was discarded and fresh collection tube was attached to spin column after each centrifugation step. The spin column was washed with 500 µL of Buffer AW1 and centrifuged at 8,000 rpm for 1 minutes. The spin column was washed again with 500 µL of Buffer AW2 and centrifuged at 14,000 rpm for 3 minutes. The spin column was then transferred to a fresh 1.5 mL microtube. To elute DNA, 200 µL Buffer AE was added, followed with 1 minute incubation at room temperature and 1 minute centrifugation at 8,000 rpm. The eluted DNA was stored at -20 °C.

DNA extraction with Quick-DNA™ Miniprep Plus Kit (Zymo Research)

Nucleic acid from the positive *Leptospira* spp. culture was also extracted using Quick-DNA™ Miniprep Plus Kit (Zymo Research #D4069, California, USA) according to the manufacturer's instructions. Briefly, 1 mL of media from positive *Leptospira* spp. culture, was centrifuged at 12,000 rpm for 5 minutes. The pellet was resuspended in 200 µL PBS and mixed with 200 µL Biofluid & Cell Buffer (Red) and 20 µL proteinase K. The mixture was homogenized with vortex for 10-15 seconds and incubated at 55 °C for 30 minutes. Equal volume (420 µL) of Genomic Binding Buffer was added to the digested sample then vortexed for 10-15 seconds to homogenize. The mixture was transferred to a spin column with a fresh collection tube attached, and centrifuged at 12,000 x g for 1 minute. Flow-through was discarded and fresh collection tube was attached to spin column after each centrifugation step. The spin column was washed with 400 µL DNA Pre-Wash Buffer, then centrifuged at 12,000 x g for 1 minute. The spin column was washed two more times with 700 µL g-DNA Wash Buffer and 200 µL g-DNA Wash Buffer. The spin column was then transferred to a fresh 1.5 mL microtube. To elute DNA, 50 µL of DNA Elution Buffer was added, followed with 5 minutes incubation at room temperature and 1 minute centrifugation at maximum speed. The eluted DNA was stored at -20 °C.

Measurement of DNA concentration and purity

One µL of eluted DNA, extracted using both the QIAGEN and Zymo Research DNA extraction kits, was used to measure DNA concentration in µg/mL and purity (A260/A280) using the Thermo Scientific Nanodrop One.

PCR detection of *Leptospira* spp.

The PCR assay is performed in SimpliAmp Thermal Cycler, using *lipL32* as the gene target. The reagent mixture consisted of 12.5 µL GoTaq® Green Master Mix, 1 µL *lipL32* primers (forward: 5'–ATC TCC GTT GCA CTC TTT GC–3') and reverse: 5'–ACC ATC ATC ATC GTC CA–3'), 10 µL extracted DNA, and ddH_2O until final volume of 25 µL. The PCR cycle is as the following: pre-

denaturation at 95 °C for 5 minutes, continued with 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 second, extension at 72 °C for 1 minutes, and long extension at 72 °C for 7 minutes. The PCR products were analysed using electrophoresis in 2% agarose gel (at 90 V for 70 minutes) using the Invitrogen's SYBRSave dye. Visualization of positive band (474 bp) was performed using Bio-Rad ChemiDoc Imaging Systems.

Data analysis

Data were presented descriptively in the form of tables. T-Independent test was used to analyze the effectiveness of bacterial genomic DNA extraction of positive *Leptospira* spp. cultures using the two DNA extraction kits, QIAGEN and Zymo Research. The independent variable is the type of DNA extraction kit, while the dependent variables are the concentration and purity values of the extracted DNAs. DNA concentration and purity values from samples were presented as mean for each group and were checked for normal distribution. p-value of less than 0.05 ($p<0.05$) is considered as statistically significant by using IBM SPSS Statistic 24 software.

RESULTS AND DISCUSSION

DNA extraction from culture samples

In this study, we performed comparative analysis of DNA quality extracted from positive *Leptospira* spp. culture using two DNA extraction kit, QIAGEN and Zymo Research, as template for molecular detection. The quality of extracted genomic DNA was assessed based on two parameters, the DNA concentration and DNA purity.

Genomic DNAs were extracted from 14 positive *Leptospira* spp. cultures using both the QIAGEN kit and the Zymo Research kit. DNA extraction was performed only once with each respective kit. The mean DNA concentration of samples extracted with QIAGEN kit was 0.900±0.161 $\log_{10}$ µg/mL, while the mean DNA concentration with Zymo Research kit was 0.790±0.167 $\log_{10}$ µg/mL (Table 1). The calculated p-value for the extracted DNA concentration between the two kits was 0.642 ($p>0.05$), which indicates that there was no significant difference between the DNA concentration of samples extracted with either kit.

Efficient DNA isolation technique will produce good quality and quantity DNA yield which is pure and free from other RNA and protein contaminants[17]. Table 1 showed that samples extracted with QIAGEN kit produced higher DNA concentration (0.900±0.603 vs 0.790±0.626 $\log_{10}$ µg/mL, $p>0.05$). This result showed that despite QIAGEN producing higher DNA yield, there was no significant difference in the performance of the two DNA extraction kits in producing high concentration of extracted DNA.

The mean DNA purity of 14 samples extracted with QIAGEN kit was 1.854±0.293, while for Zymo Research kit was 1.632±0.248. The calculated p-value for the DNA purity between the two kits was 0.040 ($p<0.05$), which indicates that there is a significant difference between the DNA purity of samples extracted with QIAGEN and Zymo Research kits (Table 1).

In general, good quality DNA is defined as having a concentration above 20 µg/mL, with purity value (A260/A280) ranging from 1.8-2.0[22]. DNAs extracted with QIAGEN kit had a good average purity value compared to those extracted with Zymo Research kit (1.854±0.293 vs 1.632±0.248; $p<0.05$). The A260/280 purity ratio of less than 1.8 indicates that the DNA isolates contain protein residues, phenols, or other reagents related to the extraction protocol, while a ratio of more than 2.0 indicates the presence of RNA contamination.

Table 1. Analysis of extracted DNA concentration and purity using the two different extraction kits

Extraction Kit	Samples (n)	DNA Concentration				DNA Purity			
		Mean± SD (log_{10} µg/mL)	Median (Min–Max)	CI 95%	p-value	Mean± SD (A260/A280)	Median (Min–Max)	CI 95%	p-value
QIAGEN	14	0.900±0.161	0.879 (-0.22–1.76)	0.232 (0.368–0.587)	0.642	1.854± 0.293	1.880 (1.22–2.54)	0.103 (0.011–0.433)	0.040
Zymo Research	14	0.790±0.167	0.917 (-0.70–1.42)			1.632± 0.248	1.685 (1.06–1.93)		

Table 2 showed the complete DNA concentration and purity of the fourteen positive *Leptospira* spp. and their source samples. In general, positive *Leptospira* spp. culture from urine source sample yielded higher DNA concentration with good DNA purity compared to samples originated from blood. These observations are consistent regardless of the type of DNA extraction kits used. These results showed that both DNA extraction kits can be used for DNA extraction from positive *Leptospira* spp. culture from either blood or urine source samples. Previous study had shown that the concentration of DNAs extracted using the QIAGEN kit are stable, even after being frozen for six months[18]. We found that the DNA yield from culture originated from urine samples yielded higher average of DNA concentration. This result was in contrast with previous study showing lower DNA yield from urine samples extracted using the Zymo Research extraction kit[19].

Chemical contamination from the residue reagents from the nucleic acid extraction process can lead to an overestimation of the concentration of nucleic acids obtained[20]. On the other hand, low DNA concentrations can be caused by the low numbers of growing *Leptospira* spp. and other deposit contaminants in the culture. Sediments from the sample used in nucleic acid extraction process can cause low concentration results because these sediments can form large pellets that interfere with the DNA extraction process[21].

DNA extraction assay is a method used for DNA purification from samples using physical and/or chemical methods to separate DNA from cell membranes, proteins, and other cell components[17]. The efficiency of DNA extraction protocol is important to remove any potential inhibitors in the downstream polymerase chain reaction (PCR) analysis. Removing the inhibitors ensures that the PCR results will produce an overall representation of the existing microbial community within the sample[12]. Since culture from *Leptospira* spp. field isolates typically contain high levels of bacterial diversity, it is necessary to optimize the DNA extraction protocol so that the cells can be lysed efficiently, and good quality DNA can be obtained for downstream molecular detection.

In principle, DNA purification consists of 4 steps, lysis, binding, washing, and elution. The differences in the concentration and purity of the DNA isolates can be influenced by the type of reagents used in each extraction kit. All buffers in QIAGEN kits contain guanidine hydrochloride (GuHCl), while most of the buffers in the Zymo Research contain guanidine tyocyanate (GuSCN). GuHCl is a stronger protein denaturant and is more commonly used in DNA isolation procedures compared to GuSCN. The difference between GuSCN and GuHCl lies in their denaturing abilities, GuSCN has lower denaturing and cell homogenization capabilities compared to GuHCl. In addition, GuHCl can inactivate DNAse enzymes to produce intact DNA isolates, decrease enzyme activity found in cells and increase the solubility of hydrophobic molecules[23]. It has been reported that QIAGEN DNA extraction kit yielded better quality DNA based on several indicators such as the DNA integrity number median value, DNA fragment length in

basepairs, median coverage value, and median percentage of positions with coverage ≥ 30x[24].

Table 2. Positive *lipL32* gene PCR results from positive *Leptospira* spp. cultures

No	Sample Source	QIAGEN		Zymo Research		PCR Detection
		DNA Concentration	DNA Purity	DNA Concentration	DNA Purity	
1	Blood	0.6	1.22	2.3	1.78	Negative
2	Blood	1.1	**1.94**	0.2	1.06	Negative
3	Blood	8.3	**1.98**	9.1	1.67	Negative
4	Blood	2.6	2.54	4.0	1.57	Negative
5	Blood	2.0	2.12	0.6	1.39	Negative
6	Blood	11.7	1.75	6.6*	**1.83***	**Positive**
7	Blood	6.3	1.72	7.5	1.36	Negative
8	Urine	6.5	1.59	13.8	1.42	Negative
9	Urine	**23.9***	**1.89***	**26.5**	**1.93**	**Positive**
10	Urine	**42.5**	**1.87**	**24.0**	**1.85**	Negative
11	Urine	6.9	1.79	4.3	**1.83**	Negative
12	Urine	**57.1**	**1.92**	**20.4**	**1.85**	Negative
13	Urine	**36.1***	**1.95***	**22.0**	1.70	**Positive**
14	Urine	**20.0**	1.68	13.8	1.61	Negative

Numbers and letters in bold are to highlight (1) DNA concentration value > 20 µg/mL, (2) DNA purity value (A260/A280) from 1.8–2.0, and (3) positive PCR results. *marks the PCR-positive samples.

The variety in DNA concentration and purity in the samples may be influenced by the ability of the researcher's handling procedures during the extraction process[1]. Higher genomic DNA yields are needed for downstream molecular detection to allow for a more comprehensive reflection of the microbial community present in the sample, while low genomic DNA yields reflect an incomplete microbial community contained in the sample[12]. The yield DNA concentration from *Leptospira* spp. culture may be affected by several factors including (1) the density of *Leptospira* spp. within the culture, (2) the presence of other bacteria such as Coccus, Bacilli, Filamentous, and other Spirochaete, and (3) the presence of residual extraction reagents in the isolated DNAs. RNA contamination in the DNA isolates may cause low amplification during the PCR[1]. Other contaminants such as proteins and organic solvents will also cause interference with enzyme reactions during the PCR[20].

Molecular detection of *Leptospira* spp.

All positive *Leptospira* spp. cultures were screened by conventional PCR using *lipL32* as the target gene. PCR results showed that there were three positive *Leptospira* spp. samples out of the 14 samples, two from urine samples (no. 9 and 13) which was extracted with QIAGEN kit and one blood sample (no. 6) extracted with Zymo Research kit (Figure 1). Table 2 showed that positive PCR samples all have DNA concentration > 20 µg/mL and DNA purity between 1.80-2.00.

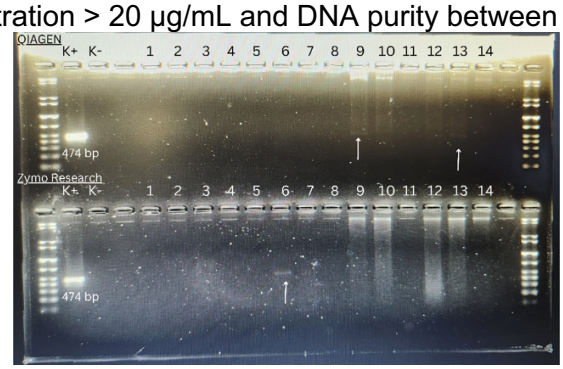

Figure 1. Conventional PCR results of positive *Leptospira* spp. cultures from 14 samples extracted with both DNA extraction kits. Samples 6, 9, and 13 were found positive by conventional *lipL32* gene PCR

DNA isolates were used as template for PCR by targeting the *lipL32* gene in *Leptospira* spp. The *lipL32* gene encodes for the lipoproteins that reside under the surface of *Leptospira*. *lipL32* as major outer membrane protein is also a virulence factor, and is only present in pathogenic species, thus making it ideal target for specific detection of pathogenic *Leptospira* spp. (*Leptospira interrogans*) [8,25]. Figure 1 showed that only three out of the 14 positive *Leptospira* spp. culture were positive for pathogenic *Leptospira* spp. This result showed that molecular detection by PCR is needed to confirm the positive microscopic identification of *Leptospira* spp. cultures to ensure true identification of pathogenic *Leptospira* spp. bacteria in the samples. Previous study had shown that molecular detection of cultured *Leptospira* spp. from biological samples is needed to confirm the presence or absence of pathogenic *Leptospira* spp.[26]. Molecular detection results will provide more definitive confirmation on the presence of *Leptospira* spp. in the culture, particularly in samples with low abundance of *Leptospira* spp., and to estimate the probability of a low estimate culture observations in epidemiologic studies with long-term follow-up.

The low molecular detection rate of positive *Leptospira* spp. culture can be associated to the presence of intermediate *Leptospira* in the culture. According to Pui *et al*., intermediate *Leptospira* is a type of *Leptospira* whose pathogenicity is not clearly known[27]. Intermediate *Leptospira* has similar characteristics to pathogenic *Leptospira* spp. One of the similar properties of intermediate *Leptospira* is possessing a *lipL32*-like protein as shown by Western and Southern blot results, but does not have a *LigA*-related gene based on the Southern blot[28,29].

The low positive rate of PCR detection may also be associated with the technical issues related to the nucleic acid amplification process. Not all of the high DNA yield (> 20 µg/mL) samples showed positive DNA band on the electrophoresis, despite having good purity value. This may be caused by the presence of contaminant RNAs[1] and/or inhibitor molecules in the samples[12,20], which may interfere with the amplification process and resulting in low amplification rate during the PCR.

This is the first study that compared the effectiveness of different DNA extraction kits for molecular detection of positive *Leptospira* spp. culture grown from human samples. Various extraction kits have been used for molecular detection of *Leptospira* spp. In previous studies, commercial kits that have been used to extract *Leptospira* DNA were from different manufacturers, including Thermo Fisher Scientific, Norgen's Urine DNA, TRIzol™ Reagent, Roche, Wizard DNA extraction kit, Promega®, and others[1,25,30]. In samples with large bacterial abundance, commercial kits are generally recommended for genomic DNA extraction, although specific type of kits may be needed based on the different origin of the sample, since different sample type may have different residual contaminant characteristics that may inhibit the downstream PCR process[19]. We have shown here that both the QIAGEN and Zymo Research DNA extraction kit is suitable for genomic DNA extraction of positive *Leptospira* spp. culture grown from both blood and urine samples. The sample numbers in this study are low, thus repeat analyses on more samples will be needed to verify the initial findings. Further, usage of a more sensitive molecular detection method such as quantitative real-time PCR might be required to improve the detection rate of pathogenic *Leptospira* spp. from positive *Leptospira* spp. culture.

CONCLUSION

Our preliminary study showed that QIAGEN kit yielded extracted genomic DNA with better concentration and purity from the 14 positive *Leptospira* spp. culture. Downstream PCR detection showed that three samples were positive for *lipL32* gene, two urine origin culture samples extracted with QIAGEN kit and one blood sample extracted with Zymo Research kit. These results showed that both

DNA extraction kits are suitable to be used for genomic DNA extraction from positive *Leptospira* spp. culture for detection of pathogenic *Leptospira* spp. by PCR. More samples are needed to confirm our preliminary results.

AUTHORS' CONTRIBUTIONS

KA: Conceptualization, Methodology, Writing - Review & Editing; AM: Review & Editing; KRD: Review & Editing; KEK: Writing – Review & Editing; FDH: Conceptualization, Methodology, Writing - Review & Editing. All authors contributed equally to every stage of the research process.

ACKNOWLEDGEMENT

The author expresses gratitude to World Health Organization (WHO) and the National Innovation Research Agency (BRIN), Indonesia for funding the research and the National Innovation Research Agency (BRIN) Co Working Space (CWS) Salatiga for facilitating this research.

FUNDING INFORMATION

This research received special grants from the World Health Organization (WHO) and the National Innovation Research Agency (BRIN), Indonesia.

DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

There is no conflict of interest.

REFERENCE

1. Handayani FD, Wijayaningsih RA, Ristiyanto, Gasem MH, Wibawa T. Comparison of DNA Extraction Methods for Molecular Identification of Pathogenic Leptospira in The Urine Samples. *Heal Sci J Indones*. 2020;11(2):77-84. doi:10.22435/hsji.v11i2.3749
2. Dittrich S, Rudgard WE, Woods KL, et al. The Utility of Blood Culture Fluid for the Molecular Diagnosis of Leptospira : A Prospective Evaluation. 2016;94(4):736-740. doi:10.4269/ajtmh.15-0674
3. Musso D, La Scola B. Laboratory diagnosis of leptospirosis: A challenge. *J Microbiol Immunol Infect*. 2013;46(4):245-252. doi:10.1016/j.jmii.2013.03.001
4. Wambi R, Worodria W, Muleme J, Aggrey S, Mugisha L. Prevalence of leptospirosis among patients attending renal and general outpatient clinics in Mulago Hospital, Kampala, Uganda. *Sci Rep*. 2022;12(1):1-7. doi:10.1038/s41598-022-12544-3
5. Dahal KP, Sharma S, Sherchand JB, Upadhyay BP, Bhatta DR. Detection of Anti- Leptospira IgM Antibody in Serum Samples of Suspected Patients Visiting National Public Health Laboratory, Teku, Kathmandu. *Int J Microbiol*. 2016;2016:1-4. doi:10.1155/2016/7286918
6. Rao M, Amran F, Aqilla N. Evaluation of a Rapid Kit for Detection of IgM against Leptospira in Human. *Can J Infect Dis Med Microbiol*. 2019;2019:10-13. doi:10.1155/2019/5763595
7. Gasem MH, Hadi U, Alisjahbana B, et al. Leptospirosis in Indonesia: Diagnostic challenges associated with atypical clinical manifestations and limited laboratory capacity. *BMC Infect Dis*. 2020;20(1):1-11. doi:10.1186/s12879-020-4903-5
8. Samrot A V, Sean TC, Bhavya KS, et al. Leptospiral Infection , Pathogenesis and Its Diagnosis — A Review. Published online 2021.

9. Budihal SV, Perwez K. Leptospirosis Diagnosis: Competancy of Various Laboratory Tests. *J Clin Diagnostic Res*. 2014;8(1):199-202. doi:10.7860/JCDR/2014/6593.3950
10. Safiee AWM, Ali MRM, Fauzi MH, et al. Leptospiral culture without 5'-fluorouracil revealed improved Leptospira isolation from febrile patients in north-eastern Malaysia. *Int J Environ Res Public Health*. 2020;17(4):1-12. doi:10.3390/ijerph17041307
11. Steinparzer R, Mair T, Unterweger C, Steinrigl A. Influence of Selective Agents (EMJH-STAFF), Sample Filtration and pH on Leptospira interrogans Serovar Icterohaemorrhagiae Cultivation and Isolation from Swine Urine. *Vet Sci*. 2021;8(90):1-14.
12. Claassen S, du Toit E, Kaba M, Moodley C, Zar HJ, Nicol MP. A comparison of the efficiency of five different commercial DNA extraction kits for extraction of DNA from faecal samples. *J Microbiol Methods*. 2013;94(2):103-110. doi:10.1016/j.mimet.2013.05.008
13. Allan KJ, Maze MJ, Galloway RL, et al. Molecular detection and typing of pathogenic leptospira in febrile patients and phylogenetic comparison with leptospira detected among animals in Tanzania. *Am J Trop Med Hyg*. 2020;103(4):1427-1434. doi:10.4269/ajtmh.19-0703
14. Goy-Thollot I, Djelouadji Z, Nennig M, Hazart G, Hugonnard M. Screening for Leptospira DNA in blood and urine from 30 apparently healthy dogs [Recherche d'ADN de leptospires pathogènes dans le sang et l'urine de 30 chiens apparemment sains]. *Rev Vet Clin*. 2018;53(3):79-86. https://www.scopus.com/inward/record.uri?eid=2-s2.0-85052979855&doi=10.1016%2Fj.anicom.2018.06.003&partnerID=40&md5=e48699dba7d30f3ec88fbb0553d4d164
15. Harran E, Abi Rizk A, Angelloz-Pessey S, et al. Molecular and Serological Identification of Pathogenic Leptospira in Local and Imported Cattle from Lebanon. *Transbound Emerg Dis*. 2023;2023:1-10. doi:10.1155/2023/3784416
16. Gorman M, Xu R, Prakoso D, Salvador LCM, Rajeev S. Leptospira enrichment culture followed by ONT metagenomic sequencing allows better detection of Leptospira presence and diversity in water and soil samples. *PLoS Negl Trop Dis*. 2022;16(10):1-14. doi:10.1371/journal.pntd.0010589
17. Gupta N. DNA Extraction and Polymerase Chain Reaction. *J Cytol*. 2019;36(2):116-117. doi:10.4103/JOC.JOC_110_18
18. Brozoski DT, Santos CF. Human DNA extraction from whole saliva that was fresh or stored for 3 , RU PRQWKV XVLQJ ¿ YH GLIIHUHQW. 2017;25(2):147-158.
19. Bali L El, Diman A, Bernard A, Roosens NHC, Keersmaecker SCJ De. Comparative Study of Seven Commercial Kits for Human DNA Extraction from Urine Samples Suitable for DNA Biomarker-Based Public Health Studies. *J Biomol Tech*. 2014;25(4):96-110. doi:10.7171/jbt.14-2504-002
20. Piskata Z, Pospisilova E, Borilova G. Comparative Study of DNA Extraction Methods from Fresh and Processed Yellowfin Tuna Muscle Tissue. *Int J Food Prop*. 2017;20(1):430-443. doi:10.1080/10942912.2017.1297953
21. Munch MM, Chambers LC, Manhart LE, et al. Optimizing bacterial DNA extraction in urine. *PLoS One*. 2019;14(9):1-13. doi:10.1371/journal.pone.0222962
22. Sophian A, Syukur A. Analysis of Purity and Concentration of Isolated DNA in Making Raw DNA of Rat Species. *Erud Indones J Food Drug Saf*. 2021;1(2):1-5. doi:10.54384/eruditio.v1i2.75
23. Amanda UD, Cartealy IC. Isolasi RNA total dari mesokarp buah kelapa sawit (Elaeis guineenssis. *PROS SEM NAS MASY BIODIV INDON*. 2015;1(2):184-188. doi:10.13057/psnmbi/m010201
24. Bonnet E, Moutet ML, Baulard C, et al. Performance comparison of three

DNA extraction kits on human whole-exome data from formalin-fixed paraffin-embedded normal and tumor samples. *PLoS One*. 2018;13(4):1-20. doi:10.1371/journal.pone.0195471
25. Podgoršek D, Ružić-Sabljić E, Logar M, et al. Evaluation of Real-Time PCR Targeting the lipL32 gene for Diagnosis of Leptospira Infection. *BMC Microbiol*. 2020;20(59):1-9. doi:10.1186/s12866-020-01744-4
26. Warnasekara J, Srimantha S, Kappagoda C, et al. Diagnostic method-based underestimation of leptospirosis in clinical and research settings; an experience from a large prospective study in a high endemic setting. *PLoS Negl Trop Dis*. 2022;16(4):1-16. doi:10.1371/journal.pntd.0010331
27. Pui CF, Bilung LM, Apun K, Su'ut L. Diversity of Leptospira spp. in Rats and Environment from Urban Areas of Sarawak, Malaysia. *J Trop Med*. 2017;2017. doi:10.1155/2017/3760674
28. Wiyata RPKT, Nugroho W. Review: Leptospirosis pada Anjing di Indonesia. *Vet Biomed Clin J*. 2021;3(2):7-22. doi:10.21776/ub.vetbioclinj.2021.003.02.2
29. Sayanthi Y, Susanna D, Lingkungan DK, Masyarakat K. Systematic Review Keanekaragaman Spesies Leptospira pada Sampel Lingkungan di Asia Diversity of Leptospira Species in The Environmental Samples in Asia: A Systematic Review. *J Kesehat Lingkung*. 2021;11(2):58-63. doi:10.47718/jkl.v10i2.1170
30. Monroy FP, Solari S, Lopez JÁ, Agudelo-Flórez P, Sánchez RGP. High diversity of leptospira species infecting bats captured in the urabá region (Antioquia-colombia). *Microorganisms*. 2021;9(9). doi:10.3390/microorganisms9091897