Open Veterinary Journal, (2025), Vol. 15(1): 437-445
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)

Research Article
DOI: *10.5455/OVJ.2025.v15.i1.39*

Submitted: 18/10/2024 Accepted: 19/12/2024 Published: 31/01/2025

Molecular and serological detection of *Leptospira interrogans* among wild rats in flood-prone residential areas of Indonesia

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ABSTRACT

Background: Indonesia is a tropical country with heavy rainfall, mostly in low-lying areas. Floods are one of the most common natural disasters in Indonesia, with climate change causing continuous flooding in some regions. The spread of human pathogens as a severe consequence of flooding, such as *Leptospira*, which may cause Weil's disease, is a concern for public health.

Aim: In this cross-sectional study, we compared DNA from the rat kidney to serum samples to identify pathogenic *Leptospira* using polymerase chain reaction (PCR) amplification to promote a less invasive method of collecting samples from the rat vectors.

Methods: Fifty-nine rodents inhabiting highly populated, flood-prone suburban regions were captured inside and outside houses. Following DNA extractions, we analyzed the quantity and quality of DNA concentration from the kidney and serum specimens using a nanophotometer. The *lipL32* gene was amplified to detect the pathogenic *Leptospira*.

Results: The mean value of kidney DNA was 151.67 ng/µl with an average A260/A280 value of 1.836, whereas the mean value of serum DNA was 22.08 ng/µl with an average A260/A280 value of 1.233. Twenty (33.9%) kidney DNA and 10 (16.9%) serum DNA samples showed the target DNA (*lipl32*). The multiple sequence alignment analysis revealed the *lipL32* sequences homology to *Leptospira interrogans* ser. Copenhageni.

Conclusion: Rat kidneys exhibited higher DNA amount and purity than the serum. Moreover, the PCR detection of *lipl32* revealed higher positive results in kidney DNA than serum DNA samples, with high similarity to *L. interrogans lipl32* sequences. Therefore, the kidney remains a better DNA source than serum for the molecular analysis of *Leptospira* in rats.

Keywords: *Kidney, Leptospira interrogans, lipL32, Rat, Serum.*

Introduction

Leptospirosis, or Weil's disease, is ubiquitous yet tends to occur in tropical countries with heavy rainfall (World Health Organization, 2020). The causative agent, mainly *Leptospira interrogans*, is maintained in the environment by reservoir animals. Rats are among the most common sources of human infection, as they carry the pathogen without symptoms, and both live alongside each other in the environment (Boey *et al.*, 2019). *Leptospira* survives in the renal tubules of the infected rats, which subsequently shed the pathogen in their urine (Boey *et al.*, 2019). Heavy rainfall that causes inundation in areas contaminated by the pathogen-contained urine facilitates the disease being transmitted to humans. People living in flood-prone areas face a high risk of contracting the disease because soil and floodwaters often contain *Leptospira* from animal urine, particularly from rodents, which are a common reservoir for bacteria. Some people infected by the pathogenic *Leptospira* may suffer from severe symptoms that consequently cause kidney or liver failure or meningitis that can be fatal (Brito Monteiro *et al.*, 2021).

In Indonesia, leptospirosis is one of the re-emerging bacterial zoonoses with an annual morbidity of ~39.2 per 100,000 persons in the population (World Health Organization, 2020). Data on leptospirosis in Indonesia revealed the occurrences from Java, Maluku, Sulawesi, Bali, Sumatra, and Kalimantan islands (Gasem *et al.*, 2020; World Health Organization, 2020; Sunaryo and Priyanto, 2022). About 920 cases of leptospirosis were related to 122 deaths all around Indonesia in 2019 (World Health Organization, 2020). However,

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accessible data on leptospirosis occurrence in North Sumatra is rare.

Pathogenic *Leptospira* significantly impacts kidney health and serum parameters in infected individuals (Sayyadi *et al.*, 2022). The presence of *Leptospira* in kidney tissue and serum is well-documented (Pedersen *et al.*, 2016; Sayyadi *et al.*, 2022). The choice between using the rat kidney and rat serum for DNA analysis depends on the specific objectives of a study because each sample type offers distinct advantages and limitations. However, collecting serum samples from animals allows for easier and less invasive collection than kidney tissue. As part of public health surveillance activity for *Leptospira* in the human environment, this study reported the identification of *Leptospira* in the kidney and serum of rats captured from flood-prone suburban areas of North Sumatra.

Materials and Methods

Sample collection

We collected rats from residential areas of the densely suburban population in the North Sumatra province of Indonesia from September 2022 to January 2023. Three regencies were chosen based on a preliminary survey: Asahan, Serdang Bedagai, and Batubara. We chose areas mostly affected by floods, especially after heavy rain. The sampling locations are in the residential areas of each regency. Cage traps, each with fish bait, were placed inside and outside houses. Rats were identified based on morphological traits according to the literature (Aplin *et al.*, 2003). Whole blood was collected from rats using a disposable syringe for an immediate transfer to a 1.5-ml polypropylene microtube, followed by microcentrifugation at 6,000 rpm to separate the clot. The supernatant (serum) was immediately transferred into a clean microtube and kept in a cold storage box at ~4°C. Following the rat dissection mentioned earlier, we collected the kidneys and stored them individually in a glass tube containing ethanol. Samples were collected aseptically, stored in a cold storage box, and transported as soon as possible to the laboratory for further investigation. The sample size was calculated using a formula for the single population proportion:

$$n = \frac{za^2 P(1\text{-}P)}{d^2}$$

n = sample size
za = 95% confidence level (1.96)
P = prevalence (1.5%)
d = margin of error (8%)
Thus, the minimum sample size = 25

DNA extraction and spectrophotometric analysis

The rat kidney was weighed in a microtube and then crushed aseptically using a disposable pellet pestle. After microcentrifugation at 12,000 × g for 10 minutes at room temperature, the rat serum was transferred to another 1.5 ml sterile microtube. All samples were processed individually for genomic DNA extraction according to the DNeasy Blood & Tissue kit (Qiagen, Germany) protocol. The kit was designed for the rapid purification of total DNA in a sample. The purity and yield of genomic DNA were analyzed using 1 µl of each sample and the IMPLEN NanoPhotometer N50 (Implen GmbH, Germany) at 260, 280, and 230 nm absorbances.

Detection of Leptospira by polymerase chain reaction (PCR)

Leptospira interrogans genomic DNA was obtained from the Jakarta Center for Environmental Health and Disease Control Engineering and used as a positive control. The genomic DNA (10–100 ng) was mixed with 12.5 µl KAPA2G Fast ReadyMix (Kapa Biosystems, Inc., Roche, South Africa), 0.5 µM of each forward and reverse primer, and PCR-grade water was added to reach a total volume of 25 µl for standard PCR amplification by following the kit's protocol. PCR using the Veriti thermal cycler (Applied Biosystems, Brazil) was performed with a primer set (forward: 5′-AAG CAT TAC CGC TTG TGG TG-3′ and reverse: 5′-GAA CTC CCA TTT CAG CGA TT-3′) to amplify lipL32 of pathogenic *Leptospira* (Stoddard *et al.*, 2009). The PCR condition consists of initial denaturation at 95°C for 3 minutes, 35 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 4 seconds, followed by a final extension at 72°C for 15 seconds. GelRed nucleic acid stain (EMD Millipore Corporation, USA) was added to 2% Omnipur agarose (Merck, Germany) in 1x TAE buffer (Merck, Germany) before gel agarose electrophoresis. Along with a DNA marker (Perfect DNA 100 bp Ladder, Novagen, Germany), the PCR products were loaded individually into the appropriate wells of the agarose gel in the Mini-Sub Cell GT (Bio-Rad, USA) containing 1x TAE buffer. The gel tray was then exposed to an electromotive force at 110 volts for 1 hour. The DNA bands were then visualized by exposure to UV light from a gel documentation system (Uvitec, France).

Sequencing of PCR products and data analysis

One milliliter of PCR product of each sample was subjected to DNA sequencing of the *lipL32* sequence by following the protocol of the Sanger sequencing kit using the ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA). The signal generated from the genetic analyzer was further monitored using sequencing analysis software v5.3 with KB Basecaller v1.4 (Applied Biosystems, USA) to determine the results. Descriptive statistics were done using Microsoft Office Excel 16 and GraphPad Prism 10. The McNemar test method was chosen to analyze the paired nominal data using SPSS version 23.

Ethics approval

Rodent trapping and investigation were approved by the Ethics Committee of the Universitas Sumatera Utara in agreement with the Declaration of Helsinki for animals, as registered in letter number 986/KEPK/

USU/2022. Euthanasia, blood collection, dissection, and disposition of rats were performed by trained personnel of the Indonesian Ministry of Health by following the available guidelines (American Veterinary Medical Association, 2007; Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

Results

Rat collection from flood-prone areas

A total of 59 rats were collected from the highly populated residential areas of three regencies in the North Sumatra province (Table 1), which is often affected by floods. Twenty rats were collected from Asahan Regency at around GPS coordinates of 2.982111 N 99.612722 E, 2.979190 N 99.629414 E, and 2.982695 N 99.627196 E (Fig. 1). Eleven rats from Serdang Bedagai Regency at Nagur village, Tanjungberingin subdistrict, around GPS coordinates of 3.499888 N 99.199556 E. Twenty-eight rats from Batubara Regency at around GPS coordinates of 3.217128 N 99.580925 E and 3.216558 N 99.589907 E. We morphologically identified 33 *Rattus tanezumi* and 26 *Rattus norvegicus*. *Leptospira lipL32* was detected from the kidney samples of 9 *R. tanezumi* and 11 *R. norvegicus*. *Rattus tanezumi* was the dominant species (81.8%) found in the target location at Serdang Bedagai Regency, and the dominant species (85.7%) carried pathogenic *Leptospira* in the area. However, both rat species were found equally in the target locations at Asahan and Batubara regencies.

Spectrophotometry analysis of rat kidney and serum DNA

Qualified (high-purity, high-yield) DNA samples most likely produce reliable results in many molecular biology applications. After DNA extraction, it is crucial to analyze the nucleic acid quality for further experiments. We used at least 1 µl of the sample for each UV spectrometry analysis with the low-salt DNA elution buffer as the blank. First, we measured the DNA absorption at 260 nm (A260) and analyzed the typical DNA absorbance spectrum of the samples individually. Then, we obtained the absorbance ratio at 260 and 280 nm (A260/A280) to analyze the relative purity of each DNA sample (Sambrook and Russell, 2001). As the absorption wavelength A230 may detect not only protein (Liu *et al.*, 2009) but also chemical contaminants such as salts, EDTA, and other organic compounds that were used in DNA extraction (Sambrook and Russell, 2001; Lucena-Aguilar *et al.*, 2016), we further examined the absorbance ratio at A260/A230.

DNA samples with an A260/A280 ratio of 1.8 to 2.0 and an A260/A230 ratio of 2.0 to 2.2 are considered "pure" in a UV spectrometry analysis (Sambrook an Russell, 2001; Lucena-Aguilar *et al.*, 2016). The mean value of all A260/A280 ratios from the kidney DNA samples was 1.836 (Fig. 2A). Most of the rat kidney DNA was at a relative purity with 49 of 59 (~83%) samples showing an A260/A280 ratio between a range of 1.804 and 1.972. However, only 39 (~66.1%) of the kidney DNA samples showed an A260/A230 ratio between 2.001 and 2.275 (the mean value from total samples was 2.002) (Fig. 2B). Contrary to the kidney DNA, most of the serum DNA showed low purity with the mean values of 1.233 and 1.577 for the A260/A280 and A260/A230 ratios, respectively.

UV spectrophotometric reading at A260 is suitable to calculate the nucleic acid in a sample as described in Beer–Lambert law (Sambrook and Russell, 2001), with A260 between 0.1 and 1.0 considered a reliable value (Lucena-Aguilar *et al.*, 2016). Thus, we quantified the DNA amount of each sample using this method and obtained the mean values of 151.67 and 22.08 ng/µl for the A260 readings of the kidney and serum DNA samples, respectively.

PCR detection of pathogenic Leptospira

The primers used in this study were designed following the alignment of *lipL32* sequences of several pathogenic *Leptospira* to obtain a highly consistent consensus sequence (Stoddard *et al.*, 2009). Molecular assays using the primers were guaranteed to be 100% sensitive and specific for *lipL32* detection of *Leptospira* (Stoddard *et al.*, 2009). After standard

Table 1. Characteristics of samples collected from rats.

Location	*Rattus tanezumi*				*Rattus norvegicus*				Total
	Female		Male		Female		Male		
	in	out	in	out	in	out	in	out	
Sendang Sari, Asahan	0	0	1		1	2	0	2	6
Tegal Sari, Asahan	0	0	1	2	2	1	1	0	7
Tebing Kisaran, Asahan	1	0	1	3	1	1	0	0	7
Nagur, Serdang Bedagai	2	3	3	1	1	0	1	0	11
Tanjung Tiram, Batubara	4	3	5	3	5	7	1	0	28
Total	7	6	11	9	10	11	3	2	59

"In" and "out" indicate rats collected by cage traps placed inside and outside houses, respectively.

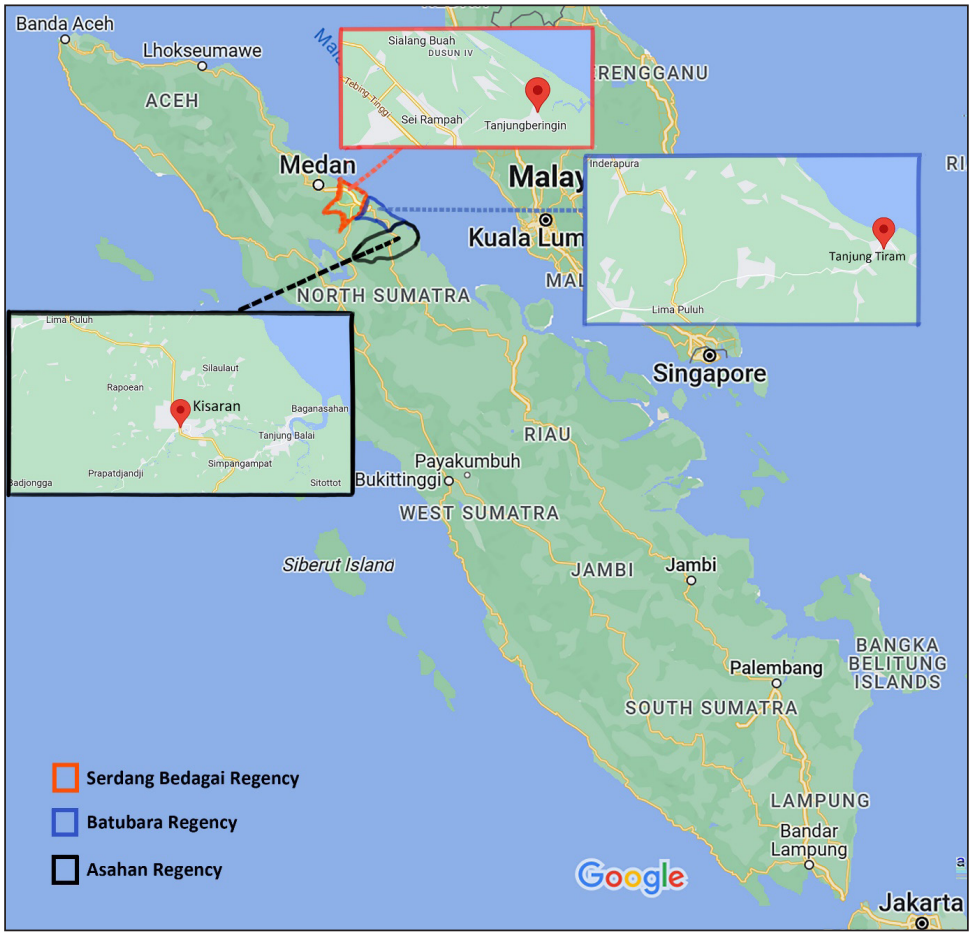

Fig. 1. Locations of rat sample collection in North Sumatra.

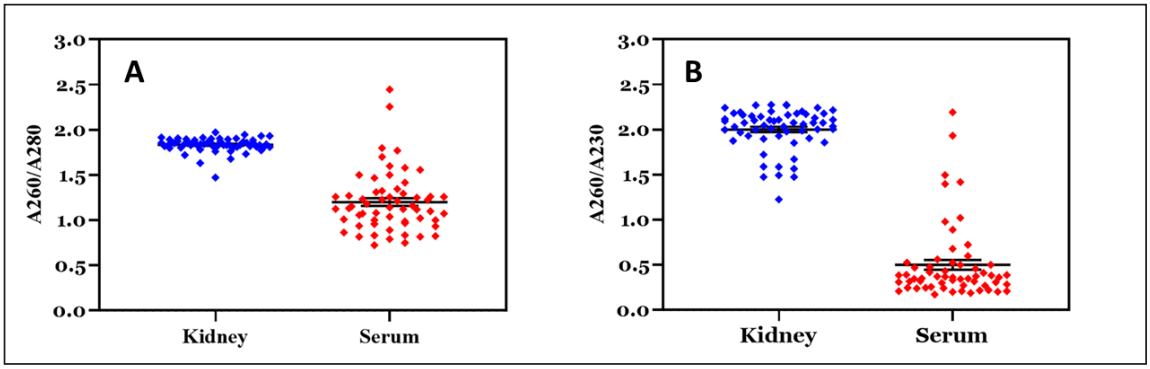

Fig. 2. Nucleic acid absorption of rat kidney and serum DNA samples by UV spectrometry analysis. A. The DNA absorbance ratio at 260 and 280 nm (A260/A280). The concentrated blue dots indicate the relative purity of kidney DNA samples with a mean value of 1.836. The scattered red dots show most of the serum DNA below the A260/A280 ratio of 1.8 (mean value = 1.223). B. The DNA absorbance ratio at A260/A230. The relative purity of serum DNA (red dots) was lower (mean value = 1.577) than that of kidney DNA (blue dots, mean value = 2.002). The black line in the plotted graphs reveals the standard error of the mean.

PCR and gel agarose electrophoresis, we detected ~242 bp fragments of *lipL32* from 33.9% (20/59) samples of rat kidney DNA. However, only 16.9% (10/59) of serum DNA samples showed similar amplicons. Figure 3A presents amplicons from the kidney DNA that were also detected from the

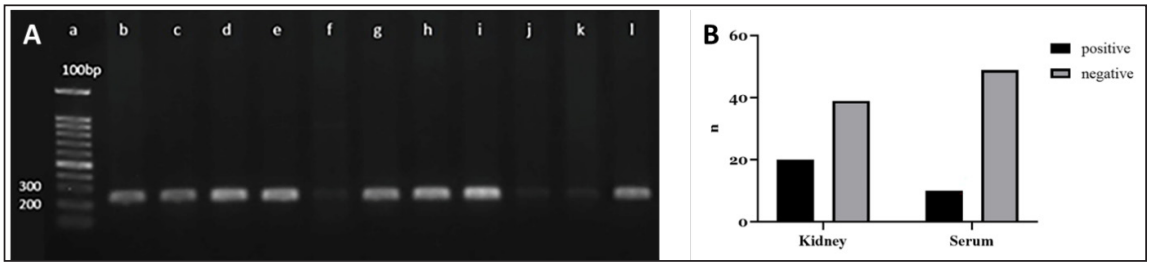

Fig. 3. *Leptospira lipL32* PCR amplification from the rat kidney and serum DNA. A. Agarose gel electrophoresis of the PCR products. The primers amplified fragments of *lipL32* sequences in the positive control DNA (lane l) and kidney DNA samples (lanes b-k), as shown by the ~242 bp bands. A 100 bp DNA ladder was used as the molecular weight marker (lane a). B. A diagram showing *lipL32* detection results from the kidney and serum DNA. Filled bars represent samples with ~242 bp bands (positive). Gray bars represent samples without the target DNA bands (negative).

serum DNA. We further analyzed the PCR results data in Figure 3B statistically to check whether the rat kidney and serum DNA samples are equally effective as a DNA source for PCR amplification of the target sequences. The McNemar test is an appropriate method to analyze the paired nominal data (Adedokun and Burgess, 2012), and the results showed a significant *p*-value = 0.021 (alpha = 0.05, confidence interval = 95%) and implied differences between the two DNA sources. Therefore, the rat kidney could be more effective as a DNA source for PCR analysis than rat serum.

***Leptospira lipL32* sequence analysis**

lipL32 is a protein-coding gene for an outer membrane surface lipoprotein of the Spirochaetales order (Paysan-Lafosse *et al.*, 2022). LipL32 is the most abundant surface protein in pathogenic *Leptospira* (Vivian *et al.*, 2009; Paysan-Lafosse *et al.*, 2022). Oligonucleotides that were constructed from a consensus sequence of pathogenic *Leptospira lipL32* provided a highly sensitive detection in the PCR assay (Stoddard *et al.*, 2009). Eight out of 10 samples of PCR products in Figure 3A were successfully sequenced by Sanger methods. We analyzed the chromatograms of the sequences individually using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA; http://www.geospiza.com). The individual sequence alignments of the eight samples using the BLAST (Altschul *et al.*, 1990) showed similar hits of homologous sequences. The first hit (97.71% identity) for sample #6 (Fig. 3A, lane h) was *L. interrogans* strain B43 *lipL32* partial CDS (GenBank *accession number* OM830322.1), whereas the uncultured *Leptospira* sp. clone C1 *lipL32* partial CDS (GenBank *accession number* MG831575.1) appeared as the first hit (100% identity) for samples #1, 2, 3, 4, 5, 7, 8 (Fig. 3A, lane b, c, d, e, g, i, k). Moreover, the BLAST identified *lipL32* partial CDS of *L. interrogans* isolate RnGZ52-2019 (GenBank *accession number* OK560918.1) as homologous sequences (100% identity) for all samples. Further, we performed multiple sequence alignment (MSA) of the sample sequences and *lipL32* partial CDS of *L. interrogans* ser. Copenhageni strain FDAARGOS_203 (GCF_002073495.2) as the reference sequences using Clustal Omega 1.2.4 (Sievers *et al.*, 2011). The matrix results indicated 51.87% identity between sample 1 and the reference sequences. Alignment between the rest of the samples (#2, 3, 4, 5, 6, 7, 8) and the reference sequences indicated 52.09% identity. The MSA result was visualized and provided in Additional File 1.

Discussion

Rodents are the major reservoir of pathogenic *Leptospira* and carry this infectious agent without any sign of illness (Boey *et al.*, 2019). Various species of rodents exist in different regions of Indonesia (Hadi *et al.*, 2021; Sunaryo and Priyanto, 2022), yet there is no accessible seroprevalence data or molecular analysis to confirm the species (Boey *et al.*, 2019; Hadi *et al.*, 2021). We identified *R. tanezumi* and *R. norvegicus* as the most prevalent carriers of pathogenic *Leptospira* in suburban residences. *Rattus norvegicus has been frequently reported as a dominant species in human habitations (*Koizumi *et al., 2022; Sun et al., 2024). Although both species* are currently equally dominant in the study area, the *R. tanezumi* population can significantly surpass *R. norvegicus* in correlation with climate change (Jing *et al.*, 2022). A population genomics approach would be suitable to analyze further the rodents' population, origin, and migration histories.

Efficient and effective nucleic acid extraction methods are crucial to obtaining a qualified DNA sample for molecular analysis. DNA extraction methods using a proteolytic enzyme (proteinase K) effectively lyse the rat kidney and serum components (Read, 2001). Moreover, proteinase K prevents DNA degradation by digesting nucleases and removing contamination in a sample (Nakajima *et al.*, 1994), providing relatively clean DNA compared to another method (Peñafiel *et al.*, 2019). The spin column with a silica matrix adsorbed DNA from the rat kidney and serum samples, allowing quick DNA purification (QIAGEN, 2020). As a result, this nucleic acid extraction method produced relatively pure DNA as shown by the rat kidney samples (Fig. 2)

and a relatively clean image of the corresponding PCR amplicons (Fig. 3A). In contrast to the kidney DNA, the overall rat serum DNA showed lower A260/A280 and A260/A230, indicating sample impurity. Contaminants such as proteins could be the reason for the serum DNA impurity.

Despite its practicability as a source for *Leptospira* DNA detection (Budihal and Perwez, 2014), the serum has lower sensitivity compared to other blood fractions (whole blood and plasma) (Stoddard *et al.*, 2009; Bourhy *et al.*, 2011). The persistence of *Leptospira* in the blood clot and the subsequent clot removal may reduce its amount in the serum (Stoddard *et al.*, 2009). A previous study demonstrated a dramatic decrease in DNA quantity referred to the lysis of whole blood cells (WBC) during storage (Huang *et al.*, 2017). Moreover, there was evidence of nucleic acid inhibition in serum specimens (Bourhy *et al.*, 2011) that presumably related to the immunoglobulin G effect in lowering DNA polymerase activity (Sidstedt *et al.*, 2018). The low sensitivity of DNA detection from serum samples in this study could be due to WBC lysis upon storage and the small concentration of *Leptospira* in serum. The latter was confirmed by measurement using a nanophotometer that showed lower serum DNA yield compared to kidney specimens. Given the fact that rats are the main carriers of *Leptospira*, there is a possibility that some rats captured in this study were in the immune phase, thus the blood-borne pathogens were eliminated by anti-*Leptospira* immunoglobulin (mainly IgM) (Vernel-Pauillac *et al.*, 2021; Rajapakse, 2022). Consequently, *lipl32* detection from rat serum DNA was significantly lower than the results shown by similar methods using the rat kidney DNA (Fig. 3B).

Leptospira lipL32 is found exclusively in the pathogenic strain and demonstrated highly conserved sequences (Haake *et al.*, 2000; Vivian *et al.*, 2009; Fernandes *et al.*, 2022; Paysan-Lafosse *et al.*, 2022). Thus, *lipL32* is likely one of the most specific and common targets for PCR detection of the pathogenic *Leptospira*. The product, LipL32, is an outer membrane protein that mediates *Leptospira* interaction with a broad range of the host's extracellular matrix proteins (Fernandes *et al.*, 2022). We detected 33.9% and 16.9% *lipL32* amplicons from the rat kidney and serum specimens, respectively. A previous study reported *lipL32 detection in environmental samples (water and soil) from the flood-prone regions of Jakarta, which implies the water contamination from the vectors' excreta, including the rodents' urine (Widiyanti et al., 2019).* The amplicons that were positive from both specimens were sequenced and analyzed for homology with the *lipL32* sequence available in the National Institutes of Health database. The amplicons' DNA sequence homology to *L. interrogans* ser. Copenhageni strain FDAARGOS_203 was confirmed by MSA with an overall 52% identity. However, the partial *lipL32* sequence of our samples hindered the analysis of a relevant protein sequence. Further investigation of the *lipL32* complete sequence of the rat DNA samples may predict a functional protein sequence to conduct a more sensitive MSA for homology analysis of the pathogenic strains.

Conclusion

The amount and purity of the rat kidney DNA were higher than the serum DNA. We detected *lipl32* in 33.9% of rat kidney DNA with high similarity to *L. interrogans lipl32* sequences, yet only 16.9% of serum DNA samples were positive for *lipl32*. Therefore, the kidney remains an ideal source for DNA analysis of *Leptospira* in rats. Despite the limitations in design and methods, this study revealed the detection of pathogenic *Leptospira* in rats from flood-prone residential areas, which will be crucial for relevant epidemiological studies in the future. Future field-based studies are required to assess the prevalence and transmission of *Leptospira* in humans, particularly in urban and rural settings, and to understand the interaction between *Leptospira* and animal reservoirs in various ecosystems for leptospirosis outbreak prediction.

Acknowledgments

We thank the head and secretary of Lembaga Penelitian Universitas Sumatera Utara. We express our gratitude to the Head of Dinas Kesehatan (Public Health Office), Mr. Harmein Harahap, MKM (Administrator of Communicable Diseases Prevention and Control of the Public Health Office) of Asahan Regency, and the team. Many thanks to the Heads of Sendang Sari, Tegal Sari, and Tebing Kisaran at Asahan Regency. We appreciate the Head of the Public Health Office and the Head of Nagur at Serdang Bedagai Regency. We extend courtesy to the Head of the Public Health Office of Batubara Regency. Our thanks as well to the Head of Tanjung Tiram Subdistrict (Sukamaju) of Batubara Regency.

Conflicts of interest

All the authors declare that they have no conflicts of interest in terms of institution, research, and funding.

Funding

The funding source of this study was the TALENTA research grant of Universitas Sumatera Utara from the USU non-PNBP fund.

Authors' contributions

EM conceptualized the research design, was involved in the fieldwork, collected and analyzed the data, wrote the original draft, and reviewed and edited the manuscript draft. DMD was involved in the research grant application and review of the original draft. SVH conceptualized the methodology and was involved in the fieldwork and data collection. AK was involved in the fieldwork and sample collection. LAS was involved in the fieldwork, sample collection, and data collection. AP and BA supervised the fieldwork and sample

collection process and verified the results. All authors have read and approved the manuscript.

Data availability

The data and material obtained during this study are available from the corresponding author upon reasonable request.

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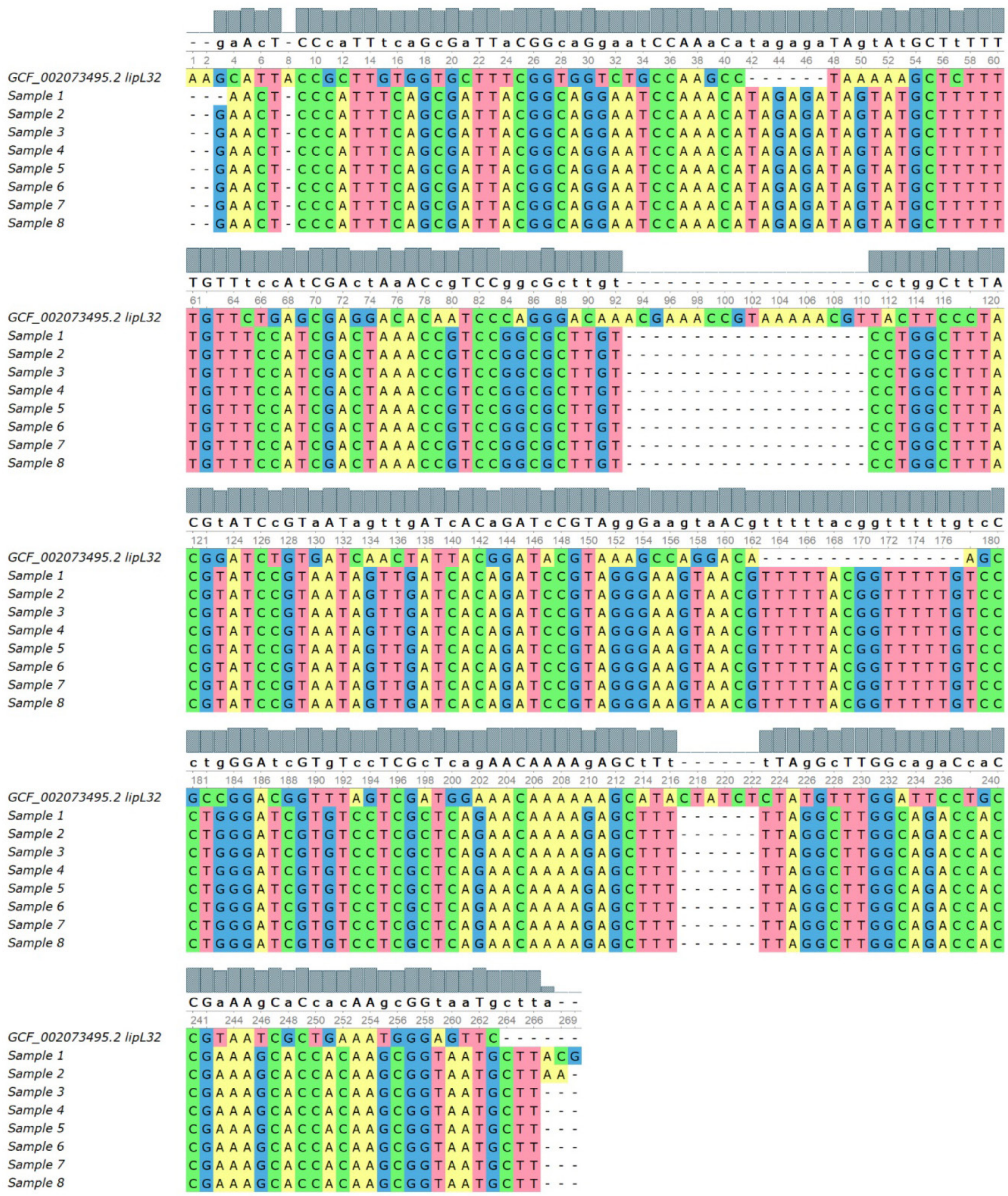
Additional File 1



Notes:

Grey bars showing consensus sequences
1st row contains the reference sequences (*lipL32* partial CDS of *L. interrogans* ser. Copenhageni strain FDAARGOS_203)
2nd to 9th rows were the query sequences (sample 1, 2, 3, 4, 5, 6, 7, and 8)

Additional File 1. Multiple sequence alignment (MSA) of the sample sequences and *lipL32* partial CDS of *L. interrogans* ser. Copenhageni (RefSeq: GCF_002073495.2).