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RESEARCH ARTICLE

Prevalence of pathogenic *Leptospira spp.* in rodents and small mammals in enzootic areas of plague in Pasuruan, Indonesia

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ABSTRACT

Leptospirosis is a globally significant yet often overlooked zoonotic infection. Several regions in Indonesia are endemic to *leptospirosis*, including Pasuruan, East Java province of Indonesia, an enzootic area of plague infection. However, other *Rodent-borne Diseases* in this area have not been reported. This study aimed to detect the presence of pathogenic *Leptospira spp.* in rats and small mammals in the enzootic plague. This was a cross-sectional study using simple random sampling. A total of 107 rats and 12 small mammals were trapped. Kidney samples of the rats and small mammals were subjected to a polymerase chain reaction (PCR) test to detect *Leptospira*, which targets the LipL32 gene. The study revealed that 7.6% (9/119) of rats in the 7 different sides (Sedaeng, Tosari, Surorowo, Petren, Pakis Bincil, Kutukan) were found to carry *Leptospira* DNA. There was a significant difference in infection rates among the sides ($p < 0.0001$). The bacteria of *Leptospira* was identified in 13.2% and 28.6% of *Rattus tanezumi* from settlement habitats and forest habitats, respectively. It was also detected in 4.4 % of *Rattus exulans* from both habitats. The findings suggest improving awareness of the spread of *Rodent-borne Diseases*, especially Leptospirosis.

1. Introduction

Leptospirosis is a neglected but essential disease globally (Tubiana *et al.,* 2013). It is a zoonotic infection caused by pathogenic *Spirochaetes* of the genus *Leptospira* (Boey *et al.,* 2019; Ningsih & Sholichah, 2018; Picardeau, 2017). Rats and mammals are the primary reservoirs maintaining of *Leptospira* in their proximal renal tubules (Boey *et al.,* 2019) and excreting the bacteria in the urine during its life cycle, which subsequently can contaminate the environment (Boey *et al.,* 2019; Joharina *et al.,* 2018; Monahan *et al.,* 2008). Small mammals do not belong to the rat's group (Ningsih & Sholichah, 2018). Rats and small mammals are strongly suspected as the host of Leptospirosis in humans, which mainly occurs due to direct contact with animals, scattered tissues, or indirect contact with soil and water with excreta such as urine or blood (Haake & Levett, 2015; Krijger *et al.,* 2019; Munoz-Zanzi *et al.,* 2020).

According to a review by Karpagam & Ganesh. (2020), Leptospirosis is a major widespread re-emerging disease. Globally, the pathogenic *Leptospira* causes 1.03 million reported severe cases and 60,000 deaths yearly (Mlowe *et al.,* 2023; Torgerson *et al.,* 2015). It is becoming a major health problem in developing and developed countries, especially in tropical and

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subtropical countries (Boey *et al.,* 2019; Mgode *et al.,* 2019; Mlowe *et al.,* 2023). High rainfall, warm air, moist/wet soils, and alkaline pH are ideal for *Leptospira* development (Samekto *et al.,* 2019).

Leptospirosis is endemic in several regions—South America, South Asia, and Southeast Asia (Philip & Ahmed, 2023). Southeast Asia has to be an endemic region for Leptospirosis with an estimated high incidence rate (Costa *et al.,* 2015; Garba & Moussa, 2021). Southeast Asia has high rainfall, frequent flooding, and hot temperatures that can impact the spread of leptospirosis, especially in agricultural-producing countries (Douchet *et al.,* 2022). Similarly, Indonesia reported a trend of increasing cases of Leptospirosis. In 2022, 1.419 leptospirosis cases were reported by ten provinces in Indonesia, including East Java. East Java is the largest contributor to leptospirosis cases, accounting for 28.3% (Ministry of Health, Republic of Indonesia, 2022). Pasuruan, a region located in East Java, has never reported cases of *Rodent-borne Diseases* other than bubonic plague. In addition, it has been one of the enzootic areas of bubonic plague in Indonesia since the outbreak in 1987 (Riyanto, 2019). Since then, Pasuruan has conducted intensive bubonic plague observations (Malikhatin & Hendrati, 2017). According to Eisen et al. (2014), bubonic plague is transmitted by lice and caused by rats, which are also potentially a major source of zoonotic agents, including leptospirosis (Parisudha *et al.,* 2020).

The incidence of leptospirosis in rats and small mammals varies depending on the geographic location (Boey *et al.,* 2019). The study in South Sulawesi showed that 9.2% of rats were positive for *Leptospira* (Ardanto *et al.,* 2018), in South Sumatra at 37.3% (Supranelfy *et al.,* 2019), and in Java at 7.7% (Khariri, 2019). Ningsih & Sholichah (2018) reported that in Purworejo, Jawa Tengah showed that 27% of *Rattus tanezumi* and 3% of *Suncus murinus* were positive for *Leptospira*.

Molecular detection of Leptospirosis is the detection of *Leptospira* DNA in samples using molecular techniques such as polymerase chain reaction (PCR) (Putro *et al.,* 2016). In particular, the detection of the LipL32 gene. Haake and Matsunaga (2010) reported that lipoprotein L32 was found only in pathogenic *Leptospira* strains and is a virulence factor. There is a lack of knowledge and information about leptospirosis and countermeasures to deal with the spread of *Leptospira* infection in rats.

To date, no reports have described the dissemination of leptospirosis in rats and small mammals in the enzootic area of plague in Pasuruan. Therefore, this study aims to detect the presence of pathogenic *Leptospira* with the LipL32 target gene in rats and small mammals in the area.

2. Materials and Methods

2.1. Study Design

This study was part of the project on the Study of Genomic Characteristics and Molecular Epidemiology of Bubonic Plague, Leptospirosis, Rickettsiosis, and Hantavirus in East Java and Central Java. The study protocol was approved by the Ethics Commission on Health Research, National Research and Innovation Agency of Indonesia (BRIN) (No 049/Ke.03/SK/05/2023.)

This cross-sectional study was conducted at the Molecular Laboratory of BRIN, Salatiga, between June and August 2023 in Pasuruan Regency, East Java, in May and June 2023. Rats and small mammals from areas were randomly selected according to the criteria of an enzootic plague area with three habitats: settlements, gardens, and forests. The population of this study consisted of all rats and small mammals in the area, consisting of 107 rats and 12 small mammals.

2.2. Rodent Trapping

The trapping of rodents was conducted using a single live trap technique with as many as 200 traps containing roasted coconut as bait. The trapping was performed for 23 days in each location of the subjects (Sedaeng, Tosari, Surorowo, Petren, Pakis Bincil, Kutukan). Traps were set at night at these locations. The traps were checked by research staff the following day. The trapped rats were placed in calico bags and taken to the field laboratory for species identification. The rats were dissected, and kidney organs were collected for molecular analysis.

2.2. Identification and Sampling

Animals were anesthetized using ketamine and xylazine in a ratio of 3 1. Identification of animals was carried out by measuring total length, tail length, hind leg length, ear length, number of nipples in females, and testicular size in male rats (length x width), as well as measuring body weight (Gunawan *et al.,* 2022).

Kidney samples were collected upon identification. The animals were placed on a tray and then subjected to dissection under anesthesia. Kidneys were collected and stored in clean tubes in an inappropriate collection protocol (Yuliadi *et al.,* 2016).

2.4. Molecular detection of *Leptospira*

Following the kit's instructions, DNA in rats and small mammals' kidneys was extracted using Genomic DNA Mini Kit Tissue Geneaid (Cat. No. GT300). The DNA template was amplified using PCR to detect pathogenic *Leptospira* with the LipL32 target gene with PRC reaction containing Go Taq® Green Master Mix (Promega, Cat. #M7122) with a formula of 12.5 µl

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Table 1. Distribution and prevalence of *Leptospira* in rats and small mammals by region

Sampling per region	N	Morphometric			*Leptospira* LipL32 Positive PCR			p-value
		Male: Female	Length (mm)	Weight (gram)	n positive	Settlement - Garden	Forest	
Rat								
Sururowo	15	5 : 10	326.3 ± 54.4	82.5 ± 44.8	5	5 / 15 (33%)	0 / 0	
Pakis-Bincil Forest	34	30 : 4	256.3 ± 26.0	42.9 ± 10.6	0	0 / 0	0 / 34 (0%)	
Sedaeng	15	5 : 10	277.7 ± 50.7	96.5 ± 5.0	0	0 / 15 (0%)	0 / 0	
Tosari	10	8 : 2	290.7 ± 44.4	78.1 ± 24.6	1	1 / 10 (10%)	0 / 0	
Kutukan Forest	17	5 : 12	292.5 ± 60.4	52.4 ± 18.0	0	0 / 0	0 / 17 (0%)	
Petren Forest	16	7 : 9	290.6 ± 51.8	64.2 ± 31.0	3	0 / 0	3 / 16 (19%)	
Total	107	60 : 47	283.2 ± 51.8	69.0 ± 31.2	9	6 / 40 (15%)	3 / 67 (4.5%)	$p<0.0001$
Small mammals								
Sururowo	4	1 : 3	216.3 ± 65.1	43 ± 1.4	0	0 / 4 (0%)	0 / 0	
Pakis-Bincil Forest	0	nd	nd	nd	0	0 / 0	0 / 0	
Sedaeng	1	0 : 1	165	60	0	0 / 1 (0%)	0 / 0	
Tosari	0	nd	nd	nd	0	0 / 0	0 / 0	
Kutukan Forest	3	1 : 2	129.3 ± 11.1	39.3 ± 1.9	0	0 / 0	0 / 3 (0%)	
Petren Forest	4	4 : 0	170.8 ± 63.1	40.0 ± 3.5	0	0 / 0	0 / 4 (0%)	
Total	12	6 : 6	175.1 ± 62.2	42.5 ± 6.6	0	0 (0 %)	0 (0%)	

*** Fisher's exact test, *nd = not determined.

master mix, 5.5 µl nuclease-free water buffer, 1 µl of each primer (forward: 5'ACTTCCGTTGCACTCTTTGC 3' and reverse: 5'ACCATCATCATCATCGTCCA 3') and 5 µl DNA template. The PCR was run using SimpliAmp Thermal Cycler (Applied Biosystems) with an initial incubation temperature of 95oC for 5 minutes, 35 cycles of amplification at 94oC for 30 seconds, 58oC for 30 seconds, and 72oC for 1 minute, and a long extension of 72oC for 7 minutes. Electrophoresis was carried out for 70 minutes with a current of 90 Volts using a 2% agarose gel containing SYBR Safe dye (Invitrogen, Cat. No. 533102). The sample was declared positive when the target gene fragment with a size of 474 bp was obtained.

2.5. Statistical Analysis

Data were obtained from the distribution of rats and small mammals per region as independent variables and the presence of the *Leptospira* positive samples as dependent variables. The data were statistically analyzed using the software of GraphPrism version 5.01 (GraphPad Software, Inc.), and significant value was determined using Fisher's exact test. A p-value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Rodent Trapping

As seen in Table 1, a total of 119 animals were obtained from three habitats (settlements, gardens, and forests) in six locations (Sedaeng, Tosari, Surorowo, Petren, Pakis Bincil, and Kutukan). Trapped animals consisted of 107 rats (90%) and 12 small mammals (10%), 66 males (55%), and 53 females (45%). Most animals were caught in forest habitats (74; 62%), with the most locations obtained in Pakis Bincil, a forest area. The morphometric analysis of animals, both for rats and small mammals, are variables that might be related to age and sex. Morphometric analysis was used to determine the species of rat based on the rat identification key.

Regarding the species, trapped rat species were identified as *Rattus tanezumi* (n=38), *Rattus tiomanicus* (n=7), *Rattus exulans* (n=45), *Niviventer fulvescens* (17), and trapped small mammals were identified as *Suncus murinus* (n=4) and *Hylomys suillus* (n=8).

3.2. Molecular Detection

The presence of *Leptospira* was detected by standard PCR. The PCR amplicons size of LipL32 fragment (474 bp) was determined on agarose gel electrophoresis (Figure. 1). From our data, out of 119 rat and small mammal kidney samples, 9 (7.6%) rats and no small mammal were positive for leptospirosis based on PCR.

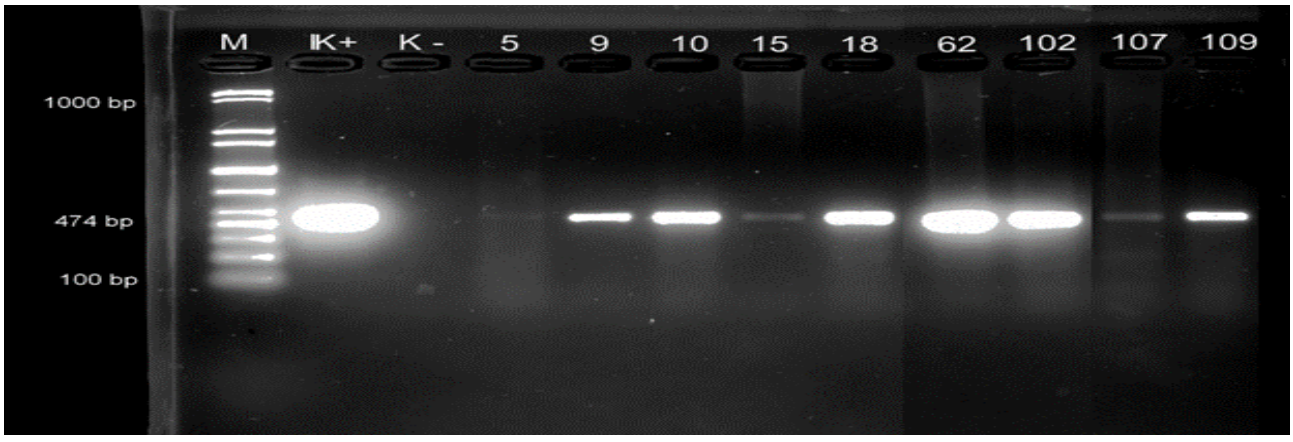

Figure 1. LipL32 gene detection results of *Leptospira* from rat kidney samples. M: Marker; K+: Control positive; K-: Control negative; 5-109: kidney sample

Table 2. Prevalence of *Leptospira* in rodents and small mammals (insectivores) by species distribution

Distribution		Leptospira Lip32 Positive PCR
Species	**N**	**n (%)**
Rat		
Rattus tanezumi	38	5 (13.2%)
Rattus tiomanicus	7	2 (28.6%)
Rattus exulans	45	2 (4.4%)
Niviventer fulvescens	17	0 (0%)
Small mammals		
Suncus murinus	4	0 (0%)
Hylomys suillus	8	0 (0%)
Total	119	9 (7.6%)

3.3. Distribution of *Leptospira*

The distribution of *Leptospira*-positive rats as reservoirs of *Leptospira* based on their habitat is presented in Table 1. The number of rats with the LipL32 gene was higher in settlement-garden habitats (6/40; 15%) compared to forest habitats (3/67; 4.5%), with a significant difference of $p < 0.0001$. It indicated *Leptospira* infected rats were near human settlements. Regarding the species, the highest prevalence of *Leptospira* was found in the rat species of *R. tiomanicus* (28.6%), followed by *R. tanezumi* (13.2%) and *R. exulans* (4.4%) (Table 2).

4. Discussion

The finding of *Leptospira* infection in rats in the enzootic area of the bubonic plague, especially the Pasuruan Regency, has not previously been reported. To our knowledge, this is the first report of leptospirosis infection in rats in the area. Pasuruan is a bubonic plague enzootic area, which is a quarantine disease and is being monitored by WHO as the outbreak area. It is still monitored for surveillance, including surveillance of rats (Malikhatin & Hendrati, 2017). In addition to being the primary source of bubonic plague, rats are reservoirs of various diseases ranging from viruses, rickettsia, protozoa, and fungi to bacteria. One source of disease in rats caused by bacteria is Leptospirosis (Ristiyanto *et al.,* 2014). Based on the bubonic plague surveillance report in the Nongkojajar area, Pasuruan Regency, the number of rats caught yearly tends to be high. The presence of these rats is very likely to cause the transmission of leptospirosis to both fellow rats and humans (Riyanto, 2019).

Leptospira detection in this study used PCR with the LipL32 target gene. Lipoprotein L32 or LipL32 is one of several targets used in the molecular analysis of *Leptospira* and is included in the outer membrane component of *Leptospira* (Satbige *et al.,* 2020). LipL32 also acts as a virulence factor found in all pathogenic species. It plays a vital role in the pathogenesis, diagnosis, and prevention of Leptospirosis (Haake & Matsunaga, 2010; Murray, 2012), which aligns with the research that Mulyani *et al.,* (2021) showed 7 out of 12 positive samples of *Leptospira* in cow urine. The LipL32 gene has high sequence conservation and the most protein in *Leptospira* (Podgoršek *et al.,* 2020)

The results of the leptospirosis examination using the PCR method with the LipL32 target gene showed that 7.6% (9/119) of the rats were positive for *Leptospira* bacteria in their kidneys (Figure 1). The positive percentage in this study was higher than that of the positive percentage in Minahasa Regency, North Sulawesi (1.9%) (Lobo *et al.,* 2020). However, this proportion is lower than that of the findings in the study by Ciwidey, West Java (23.9%) and a survey by Pati (41.2%) and Boyolali endemic areas (51.9%).

Rat species caught in the study area include *R. tanezumi*, *R. tiomanicus*, *R. exulans*, and *Niviventer fulvescens*, while *Suncus murinus* and *Hylomys suillus* are included in small mammals. Based on the results of rat capture, settlements-garden habitats are dominated by *R. tanezumi*, while *R. exulans* dominate forest habitats. According to the study of Boey et al. (2019), the two species of rats are the most commonly reported in Asian countries.

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Rattus tanezumi is the species with the highest population in the world and is most commonly found in households, especially on Java Island (Khariri, 2019; Shiels *et al.,* 2014). The results of this study showed that the prevalence of *Leptospira* in *R. tanezumi* in settlement habitats was 13.2%. Joharina et al. (2018) reported that the prevalence of *Leptospira* in *R. tanezumi* in rural environments was 18% (2/11). *Rattus tanezumi* is a commensal rat whose entire activity is carried out in the house (Khariri, 2019). These rats have the potential source of human *Leptospira* Infection, and exposure to infected rats could pose a significant public health risk (Boey *et al.,* 2019).

Our study showed a statistically significant difference between capture location and the percentage of *Leptospira* positivity in rats (Table 1). *Rattus tanezumi*, *Rattus tiomanicus*, and *Rattus exulans* live in these locations and act as the main reservoir of leptospirosis. The rats can also be a source of leptospirosis infection for rats and humans. According to Tubiana et al. (2013) rats introduced at different settlement periods contribute to the maintenance and spread of the *Icterohaemorrhagiae* serogroup. Mohd Ali et al. (2018) showed that isolated *Leptospira* spp. from settlement areas in Kelantan, Malaysia, had a high positivity rate of 42.8%.

Regarding the distribution and prevalence of *Leptospira* in rats and small mammals (Table 1), the percentage of *Leptospira* detected in rats from settlement habitats was three times higher than in forest habitats. This shows a high risk of *Leptospira* transmission from rats to humans. Densely populated settlements make it more difficult to control the cleanliness of the environment. A dirty environment creates a new habitat for rats and can increase the risk factor for leptospirosis (Sijid *et al.,* 2022). Ningsih & Sholichah (2018) reported that transmission of *Leptospira* to humans through rats increases the risk of a 7.4 times greater likelihood of leptospirosis—likewise, the results of research Katulistiwa & Lestari. (2015) showed a 10 times greater risk of developing in respondents whose houses had rats than those without. The research results by Ristiyanto et al. (2015) stated that 13.64% of *R. tanezumi* living in the house were infected with *Leptospira* bacteria, with the highest percentage caught in the kitchen area. These conditions play a role in transmission through excreted urine and have the potential to pollute the surrounding environment (Halliday *et al.,* 2013).

The percentage of *Leptospira* detection in rats from forest habitats was 4.5% and identified as *R. exulans* and *R. tiomanicus* (Table 2). According to Yuliadi et al. (2016), *R. exulans*, or field mouse, is a type of peridomestic rat that lives in agricultural land, plantations, rice fields, and even home yards, while *R. tiomanicus*, or tree rat, is a type of sylvatic rat that lives far from settlements. This discovery is evidence that there is potential for leptospirosis to occur in enzootic areas. Bubonic plague bacteria (*Yersinia pestis*) usually survive and circulate in sylvatic regions, which is called sylvatic plague. Typically, sylvatic plague occurs in forest rats.

An observation of sylvatic areas in the bubonic plague observation area shows that Petren forest, Pasuruan, is a potential area for bubonic plague. The sylvatic areas in Pasuruan are mostly highlands with temperatures ranging from 15-30oC, including Petren forest (Sinulingga *et al.,* 2022). *Yersinia pestis* bacteria live at an optimum temperature of 28oC to 30oC (Ngeleja *et al.,* 2017). The research by Sinulingga et al. (2022) in the sylvatic area showed that 27% of rats were infested with fleas in the Petren forest, with a high dominance of *Stivalus cognatus* (90%) and *Xenopsylla cheopis* (50%). These fleas act as vectors of bubonic plague (Riyanto, 2019). The species of fleas and their density per host (index fleas) have significant epidemiological value. Likewise, the results of this study on the prevalence of *Leptospira* in the Petren forest area, Pasuruan, showed 28.6% infected in the rat species *R. tiomanicus.*

Based on the findings of this study, positive rats in each habitat had the potential for infection and transmission of leptospirosis to the environment. Due to its high mobility and adaptability, rats move to find sources of needs in various environments. This movement is one way of spreading *Leptospira* bacteria (Lobo *et al.,* 2020). Human activities in forest areas are also a factor in the spread of leptospirosis. Humid and watery environments are ideal conditions for the growth and development of *Leptospira* (Khariri, 2019). Rats confirmed positive for leptospirosis can transmit leptospirosis (Pertiwi *et al.,* 2014).

5. Conclusions

This is the first report on *Leptospira* detection in rats in Pasuruan Regency, East Java. Molecular examination showed that *R. tanezumi* from settlements and forest habitats were detected to carry *Leptospira*. Likewise, *R. exulans* detected *Leptospira* in both habitats. Settlement rats had a percentage of carrying *Leptospira* three times higher than forest rats. This indicates a high risk of *Leptospira* transmission from rats to humans. Based on these results, it is essential to be aware of the spread of *Rodent-borne Diseases*, including leptospirosis, and rat control is needed.

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Conflict of interest

All authors have no conflict of interest in this article.

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