lepto

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Detection of Pathogenic and Saprophytic *Leptospira* Bacteria Using The Polymerase Chain Reaction (PCR) Method With Three Specific Primers From Sewer Water Around Tourist Sites in Bali

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ABSTRAK
Latar belakang: Leptospirosis merupakan penyakit zoonosis yang masih menjadi masalah kesehatan di Indonesia termasuk di Bali. Bali merupakan daerah tujuan wisata yang sudah sangat terkenal di dunia, adanya kejadian penyakit infeksi menular yang menimpa wisatawan di Bali dapat memberi citra buruk pariwisata di Indonesia. Selokan pembuangan air limbah menjadi salah satu faktor risiko penularan bakteri *Leptospira* dari tikus. Suatu objek wisata dapat menjadi sumber penularan penyakit menular akibat fasilitas di tempat wisata tersebut yang tidak memenuhi standar kesehatan, seperti kondisi sanitasi yang buruk termasuk pengelolaan sampahnya. Pemeriksaan yang lebih efektif dan akurat untuk mendeteksi keberadaan bakteri *Leptospira* adalah dengan pemeriksaan *nested* PCR menggunakan primer spesifik. Tujuan dari penelitian ini adalah untuk mendeteksi keberadaan bakteri *Leptospira* patogen dan saprofit pada selokan air limbah sekitar lokasi obyek wisata di Bali menggunakan metode PCR dengan tiga primer spesifik.
Metode: Penelitian ini merupakan penelitian deskriptif exploratif secara *cross-sectional*. Sampel yang digunakan adalah air dari selokan dan sungai kecil sekitar obyek wisata di 44 titik lokasi dari 29 obyek wisata yang tersebar di beberapa kabupaten di Bali. Sampel air diambil secara aseptis kemudian dihomogenkan untuk dilakukan filtrasi. Isolasi gen spesifik sampel dilakukan dengan metode *nested* PCR menggunakan tiga primer spesifik yang dirancang dari gen 16SrRNA dengan sensitifitas dan spesifisitas yang tinggi yang dapat mengamplifikasi genom DNA dari 21 *Leptospira* serovar patogen dan empat serovar saprofit. Dimulai dari optimasi PCR, dilanjutkan pemeriksaan sampel dengan tahapan: ekstraksi DNA, amplifikasi dengan PCR dan deteksi DNA produk PCR dengan elektroforesis.
Hasil: Pada penelitian ini sebanyak 6/44 (13,6%) sampel menunjukkan satu pita pada 503 bp yang merupakan gen DNA spesifik untuk bakteri *Leptospira* saprofit dan 44/44 (100%) sampel dari 44 titik lokasi pengambilan sampel tidak ditemukan dua pita pada 503 bp dan 409 bp yang merupakan gen spesifik untuk bakteri *Leptospira* patogen.
Simpulan: Pemeriksaan PCR pada penelitian ini menggunakan kombinasi tiga primer spesifik dapat membedakan keberadaan *Leptospira* patogen, *Leptospira* saprofit, dan tidak adanya *Leptospira* patogen dan saprofit dari sampel air selokan di sekitar obyek wisata di Bali. Direkomendasikan untuk selalu menjaga kebersihan sanitasi lingkungan di sekitar tempat wisata terutama dari cemaran hewan pembawa *Leptospira*.
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Kata kunci: Selokan; Obyek wisata; *Leptospira*; PCR

ABSTRACT
Title: Detection of Pathogenic and Saprophytic Leptospira Bacteria Using The PolymeraseChainReaction (PCR) Method With Three Specific Primers From Sewer Water Around Tourist Sites in Bali

Background: *Leptospirosis is a zoonotic disease that continues to be a health concern in Indonesia, particularly Bali. Bali is a very well-known tourist destination in the world. The occurrence of infectious diseases affecting tourists in Bali can give a bad image to tourism in Indonesia. Sewers are a risk factor for the transmission of Leptospira bacteria from rats. A tourist site can become a source of transmission of infectious diseases due to facilities at the tourist site that do not meet health standards, such as poor sanitation conditions, including waste maintenance. A more effective and accurate examination to detect the presence of Leptospira bacteria is by nested PCR examination using specific primers. The aim of this research is to detect the presence of pathogenic and saprophytic Leptospira bacteria contamination in the sewers water around tourist sites in Bali using the PCR method with three specific primers.*

Method: *This research is a cross-sectional exploratory descriptive study. The samples are water from sewers and small rivers around the tourist sites at 44 locations from 29 tourist sites spread across several regencies in Bali. Sewer water samples were taken aseptically and then homogenized for filtration. The isolation of target genes from samples was performed using the nested PCR technique employing three primers specifically designed from the 16S rRNA gene. This primer set demonstrates high sensitivity and specificity, enabling the amplification of genomic DNA from 21 pathogenic Leptospira serovars as well as four saprophytic serovars. The analysis was initiated with PCR optimization and subsequently proceeded through several steps, namely DNA extraction, PCR amplification, and visualization of the amplified DNA products using electrophoresis.*

Result: *In this study, 6/44 (13.6%) samples found one band at 503bp which is specific DNA genes for saprophytic Leptospira bacteria and 44/44 (100%) samples from 44 sampling locations at tourist sites did not show two bands at 503bp and 409bp which are specific genes for pathogenic Leptospira bacteria.*

Conclusion*: The PCR analysis in this study utilized a combination of three specific primers, enabling the differentiation between pathogenic Leptospira, saprophytic Leptospira, and the absence of both types in sewer water collected from areas surrounding tourist sites in Bali. Recommendations for the community to maintain the cleanliness and sanitation of the environment around tourist attractions, especially from contamination by animals carrying Leptospira.*

Keywords*:* ***Environment; Sewers; Tourist site; Leptospira; PCR***

BACKGROUND

Cases of leptospirosis in humans have been reported in several Asian countries including Indonesia, especially during the rainy season. It is estimated that leptospirosis globally occurs from 0.1-1 per 100,000 people per year in temperate climates and 10-100 per 100,000 people per year in humid tropical areas.[1]

A study of *Leptospira* in Bali found that *Rattus norvegicus*, or sewer rats, tested positive for *Leptospira sp.* serovars *Bataviae*, *Djasiman*, and *Icterohaemorrhagiae.* The detection of *Leptospira* in sewer rats from Bali indicates the presence of pathogenic *Leptospira* that have the potential to infect humans.[2]

Incidents of leptospirosis in Bali have been reported to have occurred among foreign tourists on holiday in Bali. These tourists had a history of walking in rice paddies with bare feet and had participated in whitewater rafting on a river before becoming infected. Biomolecular testing with specific gene detection revealed that the tourists were infected with *L. weilii*.[3]

Leptospirosis remains an important zoonotic disease and a persistent public health issue in Indonesia and has been reported in numerous regions across the country.[4] The number of leptospirosis cases in Indonesia tends to increase as outbreaks have been reported in several provinces due to high rainfall intensity, which often causes flooding.[5]

Nationally, leptospirosis cases in Indonesia in 2019 were 920 cases. Although the number of cases increased, the Case Fatality Rate (CFR) decreased from 148 (CFR = 16.5%) in 2018 to 122 deaths (CFR = 13.26%) in 2019.[1]

Leptospira was exclusively categorized as *Leptospira interrogans (L. Interrogans)* and *Leptospira biflexa (L. Biflexa)*, thus separating pathogenic from non-pathogenic species. Subsequently, these two classes were subdivided into distinct serovars according to the presence of homologous antigens, comprising almost 60 serovars under *L. biflexa* and at least 225 serovars under *L. Interrogans*.[6]

The species *L. interrogans* is known to be pathogenic to humans and animals, while the species *L. biflexa* is a saphrophyte *Leptospira* that lives freely in nature and is rarely associated with human infections.*Leptospira* bacteria are transmitted to humans through direct or indirect contact with the urine of infected animals that enter the body through open skin or mucous membranes. People who are in contact with water, mud and animals are at greater risk of infection.[7]

Numerous studies have demonstrated that rats play a significant role in the transmission of leptospirosis to humans. Rats are a reservoir in the transmission of leptospirosis to humans where rats play a role in spreading *Leptospira* bacteria in nature and as a source of leptospirosis transmission to humans.[8] The reservoirs found as *Leptospira* bacteria transmitters are sewer rats (*Rattus norvegicus*), garden rats (*Rattus exulans*) and house rats (*Rattus tanezumi*). These rats are the source of infection to humans and other animals.[9]

Leptospira bacteria reside in the kidneys of rats and are expelled into the environment through their urine. Humans may become infected with *Leptospira* during daily activities through contact with water contaminated by urine from rats that carry the bacteria. Leptospira organisms are able to remain viable in freshwater, sewer water, and urine for nearly one month.[10]

Sewers or wastewater ditches are a risk factor for the transmission of *Leptospira* bacteria from rats because they can be used as a path for rats to exit or enter human residences and use its as nests. Sewers are a place that rats like to live because its have damp characteristics.[11] Open sewers pose a risk of transmission of *Leptospira* bacteria from rats through urine and rat droppings. Sewer water during the rainy season can overflow into the yard and *Leptospira* bacteria can be carried to the yard around the house.[12]

Bali is one of the provinces in Indonesia that is famous for its natural beauty and culture, making Bali a popular tourist destination for both local and foreign tourists.[13] The tourist attractions that are widely spread on the Bali island are very diverse. There are many types of tourist sites that can be visited by tourists, such as natural and cultural attractions.[14]

Tourism activities in tourist sites are dynamic. Busy tourism activities contribute to environmental cleanliness around tourist sites caused by human activities, especially those produced by domestic waste.[15] A tourist site can become a source of transmission of infectious diseases due to facilities at the tourist sites that do not meet health standards, such as poor sanitation conditions, including waste management and maintenance of waste water drainage sewers.[16]

Environments with standing water around human residence are associated with leptospirosis incidence. The physical condition of the residence, such as sanitation, especially in flood-prone areas, significanlty influences the incidence of leptospirosis.[17]

Several microbiological techniques have been commonly employed to identify the presence of *Leptospira* in both human specimens and environmental sources. A more effective and accurate examination is by nested PCR analysis using specific primers capable of differentiating between pathogenic and non-pathogenic *Leptospira* species. The primers in this study were designed based on specific gene targets within the 16S rRNA gene. Nested PCR with specific primers derived from the 16S rRNA of *Leptospira* species is often used to distinguish between pathogenic and saprophytic species. Nested PCR using a combination of specific primers can prevent misinterpretation and false negatives.[18]

The Bali provincial government has made efforts to prevent the spread of leptospirosis in Bali together with the Denpasar Health Quarantine Center (BBKK) and the Bali provincial health office involving surveillance officers, public health laboratories, community health center medical officers, and village governments, where one of the efforts is to examine the presence of pathogenic *Leptospira* in rats biomolecularly in the public health laboratory.[19]

As carriers of Leptospira, rats can play a role in contaminating the surrounding environment of tourist sites such as sewers and small rivers through the urine they excrete, which may contain *Leptospira* bacteria. Therefore, it is important to detect the presence of both pathogenic and saprophytic Leptospira in the environment surrounding tourist sites using a rapid and accurate PCR-based method.

MATERIALS DAN METHODS

This research is an exploratory study, by conducting laboratory examinations using the nested PCR method with three specific primers. The nested PCR method has high specificity because it does not produce non-specific targets and can detect the presence of a specific DNA target in a sample using a combination of primers. Nested PCR is more sensitive than conventional PCR, which uses PCR products from external primers that are re-amplified using internal primers, allowing for detection of target DNA fragments at low concentrations.[20]

The sampling locations in this study were conducted at 29 tourist sites in Bali, which are located in six districts/city in Bali, namely Denpasar City, Badung, Gianyar, Klungkung, Karangasem, and Bangli Regencies. The tourist sites used as sampling locations in this study are divided into three categories: coastal and hotel areas, rice fields and rivers, forest and mountain categories. Sample examinations were conducted at the microbiology laboratory, Faculty of Medicine, Universitas Udayana. This research was conducted between July 2023 and December 2024.

The sample for this research is water from the sewers, ditches, wastewater drainage, water reservoirs, creeks, and small rivers around the tourist sites. Sampling was conducted at 44 point locations from 29 tourist sites in Bali. The water samples were collected under aseptic conditions in sterile containers with proper labeling and coding, then kept in a cooler box and delivered directly to the laboratory for further examination. Environmental water samples were collected using sterile containers of at least 300 cc, located at suspected rat nests or areas where rats frequently pass through.The water samples were filtered using sterile gauze and a 0.4 µm millipore membrane, then circulated using a filtration pump. The resulting filtrate was collected in another container. The 0.4 µm millipore membrane filter was resuspended in 10 ml of 0.9% NaCl solution, then cut into small pieces and homogenized by vortexing. The resuspension was then aliquoted for PCR.

The detection of specific genes involved several procedures, beginning with PCR optimization, followed by bacterial DNA extraction, PCR-based amplification, and analysis of the PCR products using electrophoresis on a 2% agarose gel. Extraction of DNA was conducted using a commercial extraction kit, QIAamp DNA Mini Kit (Qiagen, Inc.) with the working procedure according to the procedure in the kit.

The extracted DNA was then processed using PCR to amplify specific genes enabling the differentiation of pathogenic and saprophytic Leptospira, with three primers designed based on the 16S rRNA gene, namely:Lepto1(F) = 5'GTCAAACGGGTAGCAATACC3', Lepto2(R) = 5'GTCCGCCTACACACCCTT TAC3' and Lepto3(F)=5'AATACTGGATAGTCCCGAGAGGC3'.[18]

The PCR amplification was performed under the following thermal cycling conditions: an initial activation step at 92°C for 2 minutes (1 cycle), followed by 35 cycles consisting of denaturation at 94°C for 2 minutes, annealing at 54°C for 1 minute, and extension at 72°C for 1 minute, with a final extension step at 72°C for 5 minutes. Following PCR amplification, the products were examined by electrophoresis and visualized using short-wave UV illumination. Optimization of PCR examination was carried out using two positive controls, namely positive control S1 using DNA gene of *L. interogans* serovar *bataviae* and positive control S2 using DNA gene of *Leptospira borgpetersenii* serovar *ballum*, which was then applied to all samples.

The data obtained in this study were analyzed based on PCR results. Positive PCR results were marked by the presence of bands corresponding to the control in the electrophoresis examination results. The number of positive results was tabulated and then analyzed descriptively and presented in narrative and table form.

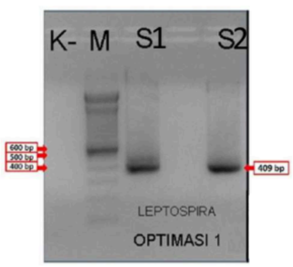
RESULTS AND DISCUSSION

Data on the number of sampling locations based on tourist site categories and the characteristics of the sewer conditions are as in the following table:

Table 1. Number of Sampling Locations Based on Tourist Site Categories and Characteristics of SewerConditions

No	Tourist site category	Number of tourist site	Number of sampling point	Characteristics of sewer condition
1	Beach and hotel area	13	19	Permanent physical condition, made of concrete, closed condition, clean, connected to residential buildings, restaurants and hotels around tourist sites
2	Rice field and river	14	22	Not made of concrete, open, dirty, and not connected to the nearby houses
3	Forest and mountains	2	3	Not made of concrete, open, dirty, and not connected to the nearby houses

The results of PCR optimization electrophoresis under shortwave UV light are presented as shown in the following image:



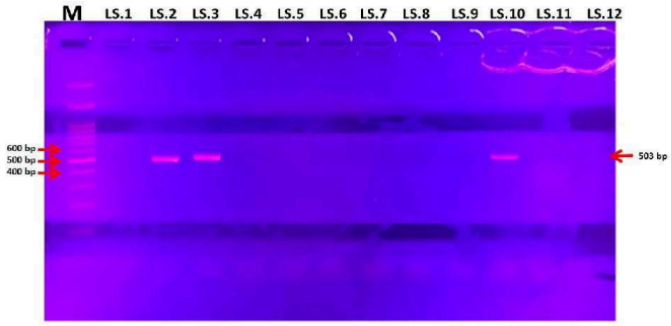
Note :
M : Marker
K(-) : Negative control
S1, S2 : Positive control

Figure 1. Figure of the electrophoresis results of PCR product optimization for the control samples of the pathogenic *Leptospira interogans* serovar *bataviae* and *Leptospira borgpetersenii* serovar *ballum* for PCR examination optimization, showing the

presence of bands above 400 bp (409 bp) which appeared in both positive controls (S) and no bands appeared in the negative control (K-).

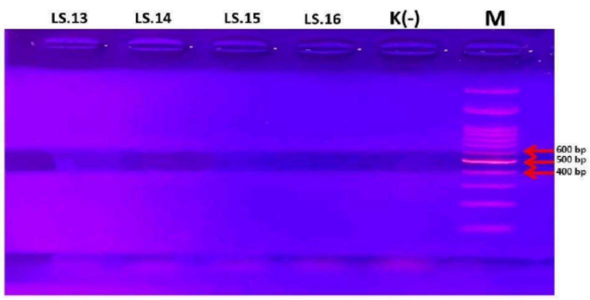
The electrophoresis results of PCR products are shown in the figure above for positive control 1 (S1), *L. interrogans* serovar *Bataviae*, and positive control 2 (S2), *Leptospira borgpetersenii* serovar *Ballum*.

The results of electrophoresis under short wave UV light on all samples are presented as in the figure below:



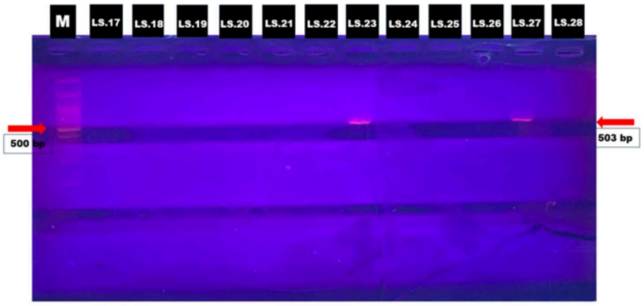
Note :
M : Marker
K(-) : Negative control
LS : Sample code

Figure 2. The result of electrophoresis from tourist sites sewer water samples show three samples showing the expected bands, which is above 500 bp (503 bp), namely samples no. LS.2, LS.3 and LS.10 which indicate the presence of saprophytic *Leptospira* DNA. While the other samples do not show any bands at all, which means there is no pathogenic or saprophytic *Leptospira* DNA.



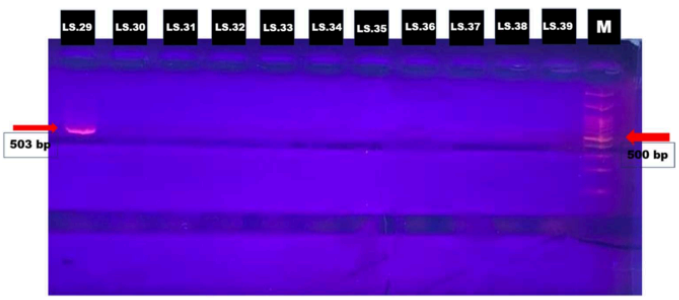
Note :
M : Marker
K(-) : Negative control
LS : Sample code

Figure 3. The result of electrophoresis from samples no. LS13, LS14, LS15 and LS16 from the tourist sites sewer water, showed no bands at all, indicating the absence of both pathogenic and saprophytic *Leptospira* DNA.



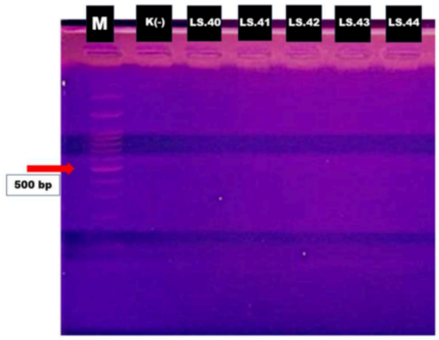
Note :
M : Marker
LS : Sample code

Figure 4. The results of electrophoresis from tourist site sewer water samples showed that there were two samples with bands above 500 bp (503 bp), namely samples LS.23 and LS.27, indicating the presence of saprophytic *Leptospira* DNA.



Note :
M : Marker
LS : Sample code

Figure 5. The result of electrophoresis from tourist site sewer water samples showed that there was only one sample that showed only one band above 500 bp (503 bp), namely sample LS.29, which showed the presence of saprophytic *Leptospira* DNA.



Note :
M : Marker
K(-) : Negative control
LS : Sample code

Figure 6. The electrophoresis results of samples no. LS40, LS41, LS42, LS43 and LS44 from tourist site sewer water samples showed no bands at all, which means there is no DNA of pathogenic or saprophytic *Leptospira*.

The following are the results of positive PCR tests as shown in Table 2:

Table2.Sample Distribution and Positive PCR Test Results

Sampel code	Tourist site category	PCR result	Interpretation
LS.2	Beach and hotel area	Positive band 503 bp	Saprophytic *Leptospira*
LS.3	Beach and hotel area	Positive band 503 bp	Saprophytic *Leptospira*
LS.10	Beach and hotel area	Positive band 503 bp	Saprophytic *Leptospira*
LS.23	Rice field and river	Positive band 503 bp	Saprophytic *Leptospira*
LS.27	Rice field and river	Positive band 503 bp	Saprophytic *Leptospira*
LS.29	Beach and hotel area	Positive band 503 bp	Saprophytic *Leptospira*

In all the figure above, the electrophoresis results of PCR products in the samples do not show any samples showing positive results in the two target bands were detected at approximately 409 bp and 503 bp. While there are six samples, namely samples LS.2, LS.3, LS.10, LS.23, LS.27 and LS.29 showing bands above 500bp (503 bp). The six samples that have bands parallel to 503 bp can be suspected of containing specific genes associated with saprophytic *Leptospira* DNA indicates that the six environmental water samples are suspected to contain saprophytic *Leptospira* bacteria.

In this study, PCR analysis using a combination of three primers was able to differentiate the presence of pathogenic *Leptospira*, saprophytic *Leptospira*, or the absence of both types in water samples collected from tourist sites. DNA detection by PCR method is a faster, more sensitive and specific method compared to several other alternative methods to detect *Leptopsira*. This method can be done either by conventional PCR or real-time PCR.[21,22]

Based on its pathogenicity, the genus *Leptospira* can conventionally be grouped into two, namely pathogenic and nonpathogenic groups. The pathogenic group comes from the *interrogans* group, while the nonpathogenic group comes from the *biflexa* saprophyte group. Epidemiological studies show that in environmental water such as sewers, rivers or lakes, *L. interrogans* and *L. biflexa* species can coexist.[23]

PCR assay using a combination of three primers has been developed and used to detect and differentiate between pathogenic and saprophytic *Leptospira* species on agarose gel which can be applied in routine diagnosis. PCR results are often misinterpreted as false negatives, therefore one way to address this is by conducting a PCR test utilizing three primer sets: Lepto1 (F), Lepto2 (R), and Lepto3 (F). The simultaneous use of three primers enables the amplification of genomic DNA from 21 pathogenic *Leptospira* serovars and four saprophytic serovars. The detection of two bands at 503 bp and 409 bp indicates the presence of pathogenic *Leptospira*, while those that only show one band at 503 bp indicate the presence of saprophytic *Leptospira* species. The presence of a 503 bp fragment in the PCR results was associated with pathogenic *Leptospira* serovars *autumnalis, bataviae, canicola, djasiman, hebdomadis, icterohaemorrhagiae, pamona, pyrogenes*, and *sejroe*.[18] The nested PCR–RFLP test using 16S rRNA is a faster and more specific leptospira test technique in distinguishing pathogenic and non-pathogenic *Leptospira spp.* in one examination.[23]

In this study, a band was found in the PCR examination, namely one band at 503bp, which is suspected to be the *Leptospira* saprophyte species, and in several samples, neither of the two bands was detected, similar to the negative control, which may indicate the presence of nonpathogenic or saprophytic *Leptospira*.

The genus *Leptospira* consists of several saprophytic and pathogenic species, both of which are able to survive and reproduce in environments that suit their needs. Infection due to pathogenic leptospira bacteria can be related to exposure to bacteria during recreation in places with less than clean environmental conditions, especially in urban environments.[24]

Examination of environmental samples requires more evaluation and attention because *Leptospira* in the environment can also be contaminated by nonpathogenic *Leptospira*, also known as saprophytes. Saprophytic *Leptospira* represented by *L. biflexa* is the most frequent environmental contaminant and often doubtful in the identification of pathogenic strains from environmental samples. *L. biflexa* is a saprophytic *Leptospira* that lives freely around the aquatic environment and is very rare as a cause of infection in humans.[6]

The presence of *Leptospira* bacteria in the sawers water indicates that there are still rats roaming around in the environment. There is a significant relationship between the physical environment around human residences, the condition of the sewers, the condition of the sewers during the rainy season, the condition of the waste collection area, and the condition of the house on the presence of *Leptospira* bacteria from rats.[12]

Several studies shows that there is a relationship between the condition of sewers and the transmission of leptospirosis cases, where sewers in poor condition have a greater risk of being contaminated with *Leptospira* bacteria compared to sewers in good condition.[25,26]

The sewers are often used as a route for rats to search for food and other activities as well as entering and exiting human residences. Rats can excrete urine that already contains *Leptospira* bacteria, thus contaminating the environment around human residences.[9]

Rat species captured in urban areas in Indonesia are predominantly *Rattus norvegicus*, followed by *Mus musculus* and *Rattus tanezumi*, with the majority being male. Leptospira bacteria have been confirmed to be abundant in male *Rattus norvegicus* rats.[27]

This study found contamination with saprophytic *Leptospira* in water samples from tourist sites near beaches, hotels, and restaurants. Beach areas with numerous hotels and restaurants are a popular tourist attraction, offering beautiful views. Hotels and restaurants are a habitat for rats because they provide abundant food supplies. Rats can travel up to 1-2 kilometers in search of food, allowing them to enter hotels and restaurants, where they can come into contact with humans.[28]

This study also found contamination with saprophytic *Leptospira* in water samples from tourist sites located in rice fields and rivers. *Leptospira* transmission is closely related to activities and locations close to rice fields and rivers. A study in Demak Regency, Central Java, showed that leptospirosis sufferers lived less than 1 kilometer from rice fields. Half of the rat population from rice field and river ecosystems was contaminated with *Leptospira*. Rats infected with *Leptospira* will excrete infectious urine along their journeys in search of food sources.[29]

Rat nests are common in beaches tourist destinations with hotels and restaurants. The presence of rats in a building increases the risk of leptospirosis by 4.51 times compared to a building without rats. The presence of rats is influenced by the physical condition of the building, such as the condition of its sewers. Shallow sewers made of non-absorbent cement make them prone to overflowing during heavy rain. Furthermore, the distance between the sewers and the building is less than 2 meters, increasing the spread of water contamination containing rat urine.[30]

Tourist sites near rivers are at greater risk of flooding, especially during rainy seasons. Rivers are ideal breeding grounds for *Leptospira*, so when rivers overflow, they are more likely to be contaminated by infectious urine excreted by rats.[29]

Leptospira can contaminate the environment through urine and feces into sewers near human residences and if flooding occurs with overflowing sewer water. During the rainy season, it is possible for sewer water to overflow so that it will affect environmental changes, namely the exposure of the surrounding environment to *Leptospira* bacteria from confirmed rats that inhabit and nest in the sewers.[12]

The absence of pathogenic *Leptospira* may be influenced by the physical condition and sanitation of the water sewers in tourist sites which are clean and well-maintained so that they do not become rat nests.

The physical condition of the sewers and small rivers around the tourist sites are mostly in good condition, where most of the sewers are closed, the water flows smoothly, is watertight, and there is a rat filter. Aspects of physical environmental conditions such as sewer conditions, garbage collection points, and waste channel conditions affect the transmission of *Leptospira* from rats. No *Leptospira* bacteria were found in several gutter points, which could be due to the smooth flow of water and high dilution of the water conditions. The good condition of the sewer can also be seen from the closed and watertight construction, the waste water flows smoothly, there is a rat filter and there is a closed absorption tank.[12]

The location of the sewers and small rivers around the tourist sites in this study are mostly far from residential areas, because the tourist sites are not located in residential areas but rather further away in the middle of rice fields, rivers, forests, mountains and beaches.The distance of overflowing and stagnant sewers from environment around the house is getting closer, thus increasing the possibility of *Leptospira* contamination.[31,32]

Another reason for the absence of *Leptospira* contamination in the tourist site's drainage could be because the sampling time for the field research was not during the rainy season. Several studies state that there is a relationship between the condition of the sewers during the rainy season and the presence of *Leptospira* bacteria. The presence of stagnant water caused by overflowing sewers due to flooding during the rainy season increases the risk factor for *Leptospira* contamination compared to areas without stagnant water.[11]

A crucial aspect in preventing the transmission of pathogens from wild animals to humans is to understand and pay attention to environmental conditions that support the survival of these animals so that the transmission of infection can be prevented.[33] *Leptospira* contamination in the sewers around tourist sites can be used as an early warning measure for the community and tourism site administrators because the presence of *Leptospira* bacteria in the water environment poses a risk of leptospirosis in humans.

The limitation of this study is did not measure parameters of environmental factors such as water pH, water temperature and other physical conditions of the water that are related to the presence of *Leptospira* bacteria in water sewers.

This research has important implications for the health of the surrounding community, tourism managers and especially the health of tourists, as well as the sustainability and preservation of the tourism site. No contamination of pathogenic *Leptospira* bacteria was found in the research samples of environmental water in tourist areas in Bali and only saprophytic *Leptospira* was found, indicating the role of nested PCR biomolecular technology with a combination of three specific primers can play a role in handling the spread of leptospirosis in tourist areas in Bali, thereby helping to increase the safety of tourist areas from infectious diseases.

CONCLUSION

In this study, 6/44 (13.6%) samples found specific DNA genes of saprophytic *Leptospira* bacteria and 44/44 (100%) samples did not find specific genes of pathogenic *Leptospira* bacteria from 44 sampling locations at tourist sites in Bali. The good and well-maintained physical condition of the sewers can affect the presence of rats in the sewers drainage, thereby affecting the occurrence of pathogenic and saprophytic *Leptospira* bacteria. The use of a combination of three specific primers can quickly and specifically differentiate the presence of pathogenic or saprophytic *Leptospira* contamination which can be used and developed in agencies that carry out the task of monitoring and controlling leptospirosis around tourist sites in Bali. This research suggestion can be continued for more detailed information by examining the DNA sequencing of *Leptospira* bacteria.

lepto

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