

Application of polymerase chain reaction (PCR) methods in distinguishing pathogenic and saprophytic *Leptospira* from the traditional market environment in Denpasar City

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ABSTRACT

Background: Leptospirosis disease is still a health problem in Indonesia. The number of leptospirosis cases and deaths in Indonesia tends to increase. Indirect transmission of *Leptospira* bacteria can occur through poor environmental sanitation polluted by rat urine has the potential to cause leptospirosis. Traditional markets are good places for rats to breed. The purpose of this study was to detect the presence of contamination by pathogenic and saprophytic *Leptospira* bacteria in the environment around traditional markets in Denpasar City by polymerase chain reaction (PCR) method using three specific primers.

Methods: The sample is water from the market environment, taken at 19 location points from eight traditional markets in Denpasar City. After being homogenized, the sample water is filtered. Isolation of specific genes from samples by the PCR method was performed to differentiate pathogenic and saprophytic leptospira using three specific primers designed from the 16S rRNA gene. Starting from the DNA extraction stage, amplification by PCR, and detection of PCR product DNA by electrophoresis.

Results: This study found that from 19 water sampling locations, 5/19 (26.3%) point locations found specific DNA genes for pathogenic *Leptospira* bacteria. 10/19 (52.6%) point locations found specific genes for saprophytic *Leptospira* bacteria DNA, and 4/19 (21.1%) point locations found no specific genes for pathogenic or saprophytic *Leptospira* bacteria.

Conclusion: PCR examination using a combination of three primers could distinguish the presence of pathogenic *Leptospira*, saprophytic *Leptospira*, and the absence of pathogenic and saprophytic *Leptospira* simultaneously from traditional market environments in Denpasar City.

Keywords: Traditional market, *Leptospira*, PC.

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INTRODUCTION

Leptospirosis is a zoonotic disease that is widespread in various countries around the world, especially in developing countries and in countries with tropical climates. Leptospirosis is still a health problem in Indonesia and is found in various regions, including the provinces of Banten, DKI Jakarta, West Java, Central Java, Yogyakarta, East Java, Lampung, South Sumatra, Bengkulu, Riau, West Sumatra, North Sumatra, Bali, NTB, South Sulawesi, North Sulawesi, Maluku, North Kalimantan, East Kalimantan, South Kalimantan, and West Kalimantan.¹⁻³

The risk of leptospirosis disease is also related to occupations such as farming, slaughterhouse workers, veterinarians,

researchers, livestock workers, hunters, animal control, and others who are at risk of contact with soil, mud, or water contaminated with animal urine that already contains *Leptospira* bacteria. The majority of cases occurred in the productive age group, namely ages 41-50 and 21-30.^{4,5}

There has been an increase in the mortality rate due to plague in several provinces in Indonesia due to the high intensity of rain resulting in flooding.¹ In the 2009-2019, the number of morbidity and mortality cases due to leptospirosis in Indonesia tends to increase. Data for 2019 found 920 cases of leptospirosis with 122 deaths (Case Fatality Rate/CFR 13.3%) reported from 9 provinces.² Leptospirosis occurs frequently seasonally because it is

closely related to rainfall and flooding.³

Since 1907 the genus *Leptospira* has traditionally been divided into two groups based on its virulence: the saprophytic group (*Leptospira biflexa sensu lato*) and the pathogenic group (*Leptospira interrogans sensu lato*).⁶

Several microbiological examinations have been commonly performed to detect the presence of *Leptospira* bacteria both in the body and in the environment.⁷ Based on its phenotypic characteristics, such as Gram staining, colony growth, and biochemical tests, *Leptospira* bacteria cannot be differentiated at the species level.⁶ Antigenic diversity among serovars can differentiate pathogenic (*Leptospira interrogans*) and non-pathogenic or saprophytic (*Leptospira biflexa*) species.^{8,9}

Now there is a great need for fast and effective laboratory tests for the direct detection of *Leptospira* infection.¹⁰ The test currently being developed is a polymerase chain reaction (PCR) assay using specific primers that can differentiate between pathogenic and non-pathogenic *Leptospira* species.⁷

The term traditional market is defined as a gathering place for a number of sellers and buyers in the form of shops, kiosks, stalls, and tents managed by small, medium, and large traders where transactions of buying and selling of goods take place and become a place for hoarding various kinds of goods for various needs with conditions that are still not permanent.¹¹

Traditional markets with lots of stalls selling goods such as groceries can be good breeding grounds for wild rodents such as rats. Many studies show that rodents and rats are important factors in the transmission of leptospirosis to humans. The main reservoir hosts are rats, with *Leptospira* bacteria living in the kidneys and excreted through urine when urinating. Rats, as carriers of *Leptospira* bacteria in their bodies, can contaminate the environment of traditional markets, especially the water around the market, through their urine, which can contain *Leptospira* bacteria. *Leptospira* can live in fresh water, sewage, and urine for about one month. Humans during activity can be infected with *Leptospira* bacteria through contact with water, soil, mud, or plants that have been contaminated with the urine of animals carrying *Leptospira* bacteria.^{3,12}

People who are directly involved in traditional markets, including traders, buyers, market workers, and people who live around traditional markets, have the potential to be infected with *Leptospira* bacteria due to direct contact with water around traditional markets that has been contaminated with *Leptospira* bacteria, so it is necessary to detect the presence of pathogenic and saprophytic *Leptospira* in the traditional market environment using a fast and accurate PCR method. The aim of this study was to detect the presence of contamination by pathogenic and saprophytic *Leptospira* bacteria in the environment around traditional markets

in Denpasar City by PCR using three specific primers.

METHODS

This research is an exploratory study. Samples were taken at 19 point locations from eight traditional markets in Denpasar City, such as sewer water, sewage water, water reservoirs, puddles, and rivers. The location of traditional markets in the city of Denpasar is divided into the downtown Denpasar area, the South Denpasar area, and the East Denpasar area.

Samples were taken aseptically in sterile containers labeled and coded, then put into a cooler container, and immediately sent to the laboratory for processing. The water filtration technique was carried out with sterile gauze and a millipore membrane. Water samples that have been filtered using sterile gauze are followed by filtration using a 0.4 µm millipore membrane.

Specific gene detection was carried out in several stages, starting with bacterial DNA extraction, PCR amplification, and detection of the PCR product DNA by electrophoresis with a 2% agarose gel.

DNA extraction using the commercial QIAamp DNA minikit (Qiagen, Inc.). The results of the DNA extraction were then examined by the PCR method to detect specific genes that could differentiate pathogenic and saprophytic *Leptospira* using three specific primers designed from the 16S rRNA gene, namely: Lepto1(F) = 5'GTCAAACGGGTAGCAATACC3'; Lepto2(R) = 5'GTCCGCCTACACACCC TTTAC3' and Lepto3(F) = 5'AATACTGGATAGTCCCGAGAGG C3'.⁷

The conditions of the thermal cycler used were as follows: initial PCR activation step (1 cycle) 92°C for 2 minutes; 3 step cycling (35 cycles) 94°C for 2 minutes (denaturation); 54°C for 1 minute (annealing); 72°C for 1 minute (extension); and final extension at 72°C for 5 minutes. The PCR amplification results were followed by electrophoresis, and the results were viewed under shortwave UV light.

RESULTS

Electrophoresis results under shortwave UV light are presented in **Figure 1**.

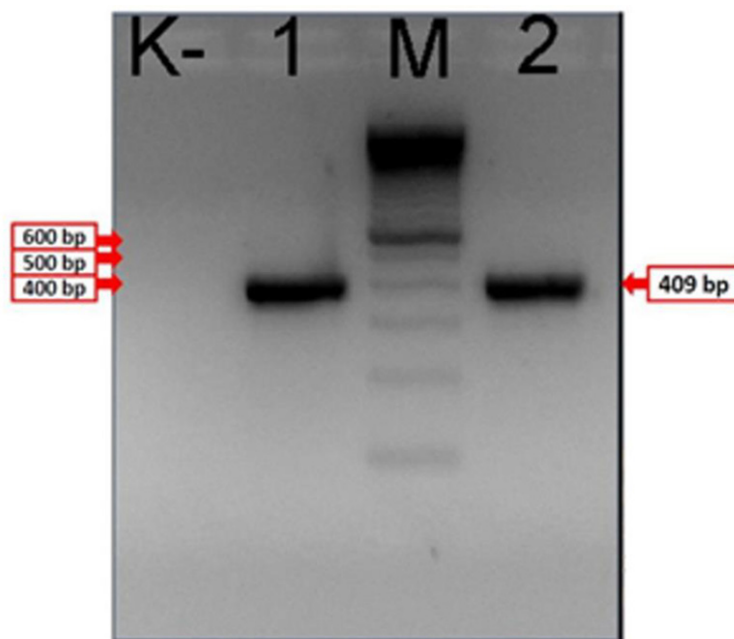


Figure 1. Electrophoresis results of PCR product control samples of pathogenic bacteria. Positive control 1, namely *Leptospira interrogans* serovar Bataviae, and positive control 2, namely *Leptospira borgpetersenii* serovar Ballum, for optimization of PCR examination, with results showing a band above 400 bp (409 bp), which appears in both positive controls. (K -)Negative Control: (1)Positive 1 control: (2) Positive 2 control: (M) : Marker

After optimizing the PCR examination on both controls, namely the positive control *Leptospira interrogans* serovar Bataviae and positive control 2, namely *Leptospira borgpetersenii* serovar Ballum were then continued to be applied to all samples with electrophoresis results under short wave UV light were presented as shown in **Figure 2** until **Figure 6**.

All the pictures show the electrophoresis results of PCR products on positive control 1 (K1), which is the bacteria *Leptospira interrogans* serovar Bataviae, and positive control 2 (K2), which is *Leptospira borgpetersenii* serovar Ballum.

After repeated PCR examinations, it appeared that there were several samples that showed positive results where there were two expected bands, namely above 400 bp (409 bp) and above 500 bp (503 bp), namely samples no. 5, 8, 10, 11, and 18.

Sample no. 5, 8, 10, 11, 18 have two bands parallel to the controls K1 and K2. It can be assumed that there are specific genes in the DNA of pathogenic *Leptospira* bacteria, so it is assumed that the five traditional market water samples contain pathogenic *Leptospira* bacteria. While samples no. 1, 3, 6, 9, 12, 13, 14, 15, 17, and 19 showed the presence of *Leptospira* saprophyte DNA. Sample no. 2, 4, 7, and 16 resulted in the absence of the two expected bands, indicating that the sample did not find pathogenic or saprophytic *Leptospira* as shown in **Table 1**.

DISCUSSION

The emergence of a band that appears on the PCR examination of the sample, that shows two parallel bands like the positive control, namely at 503 bp and 409 bp where it is suspected that it was detected in the pathogenic group, and there are samples that show one band at 503 bp where it is suspected that *Leptospira* saprophyte species were found, and there are samples that do not show both bands like the control where it is suspected that there are no pathogenic and saprophytic *Leptospira* bacteria.

The 503 bp band from the PCR results was found in the pathogenic *Leptospira* serovar Autumnalis, Bataviae, Canicola, Djasiman, Hebdomadis, Icterohaemorrhagiae, Pamona, Pyrogenes,

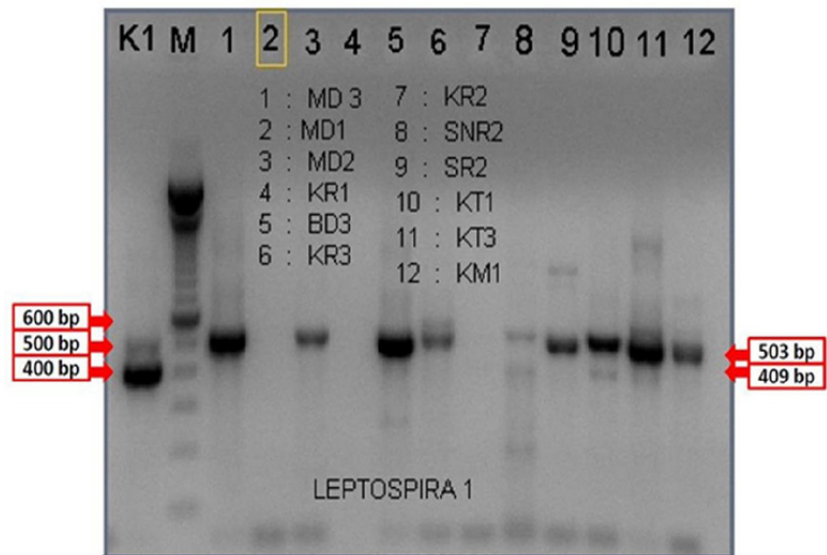


Figure 2. Electrophoresis results of PCR products from samples no.1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12. Above 400 bp (409 bp) and above 500 bp (503 bp) as seen in positive control 1 *Leptospira interrogans* serovar Bataviae. It appears that there are several samples showing the expected two bands, namely above 400 bp (409 bp) and above 500 bp (503 bp), namely samples no. 5, 8, 10, and 11. While some samples showed only 1 band above 500 bp (503 bp), namely samples no.1,3,6, and 9 which indicated the presence of *Leptospira* saprophytic DNA. (K1)Positive 1 control: (M) : Marker

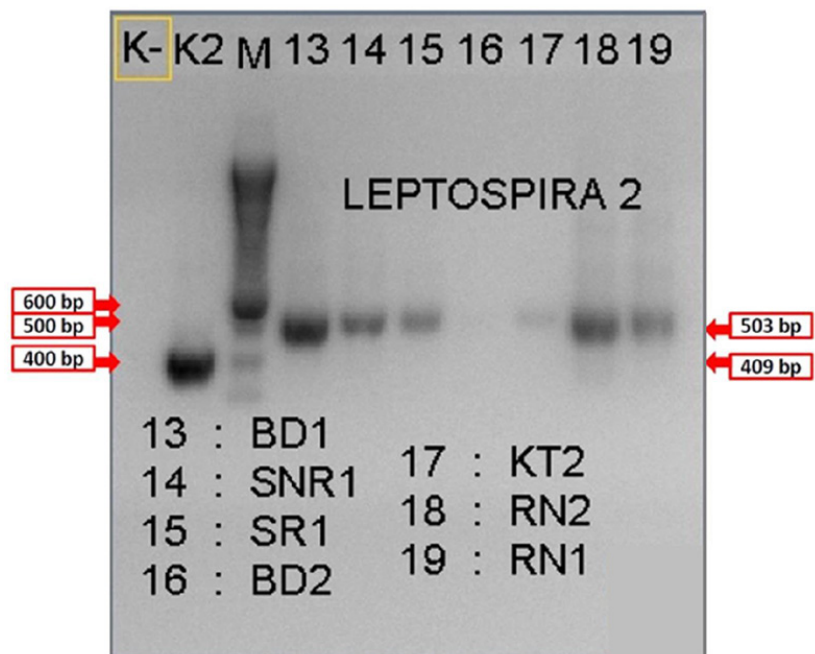


Figure 3. Electrophoresis results of PCR products from samples no.13, 14, 15, 16, 17, 18, and 19. Above 400 bp (409 bp) and above 500 bp (503 bp) as seen in positive control 2 *Leptospira borgpetersenii* serovar Ballum. Shown are several samples showing the expected presence of two bands, namely above 400 bp (409 bp) and above 500 bp (503 bp), namely sample no. 18, while some samples showed only 1 band above 500 bp (503 bp), namely samples no. 13,14,15,17, and 19 which indicated the presence of *Leptospira* saprophytic DNA. (K-)Negative Control: (K2)Positive 2 control: (M) Marker

and Sejroe. The study results showed that PCR products at 409 bp were found in pathogenic samples. PCR results are often misinterpreted as false negatives; therefore, one way that can be done is to carry out a PCR examination using a combination of three primers, namely Lepto1(F), Lepto2(R), and Lepto3(F). The results showed that using three primers simultaneously could amplify the DNA genome of 21 pathogenic *Leptospira* serovars and four saprophytic serovars. The presence of two bands at 503 bp and 409 bp was detected in the pathogenic group, whereas only one band at 503 bp was found in the saprophytic *Leptospira* species.⁷

The spectrum of leptospirosis diseases is wide and can complicate the diagnosis. Non-specific *Leptospira* laboratory tests are usually used to detect *Leptospira*.¹³ Serological tests based on the presence of *Leptospira*-specific antibodies have a weakness in that antibodies are detected within one to two weeks after the appearance of clinical symptoms, resulting in delays in treatment, especially in administering antibiotics.¹⁴ Examination of the leptospira culture method can be used for definitive diagnosis, but it also has drawbacks such as requiring technical skills, requiring time, the risk of contamination, and a high failure rate. Serological examination the microscopic agglutination test (MAT) is often used for the diagnosis of leptospirosis. This method is specific for serogroup identification but has the disadvantage that it is quite time-consuming and labor-intensive.¹⁰

Molecular assay methods such as PCR are faster, more sensitive, and more specific than other assay methods such as culture and serology.^{10,14,15} Molecular examination plays an important role in determining the presence of *Leptospira* and the diagnosis of leptospirosis.^{13,14} Molecular-based tests have often been favored for detecting the presence of leptospire in samples and can examine the genetics of *Leptospira* species. The classification of *Leptospira* species can be determined based on a comparison of the *Leptospira* genome sequences, making them more useful in epidemiological investigations of leptospirosis.^{10,16} Various studies of the *Leptospira* genome demonstrated phylogenetic relationships

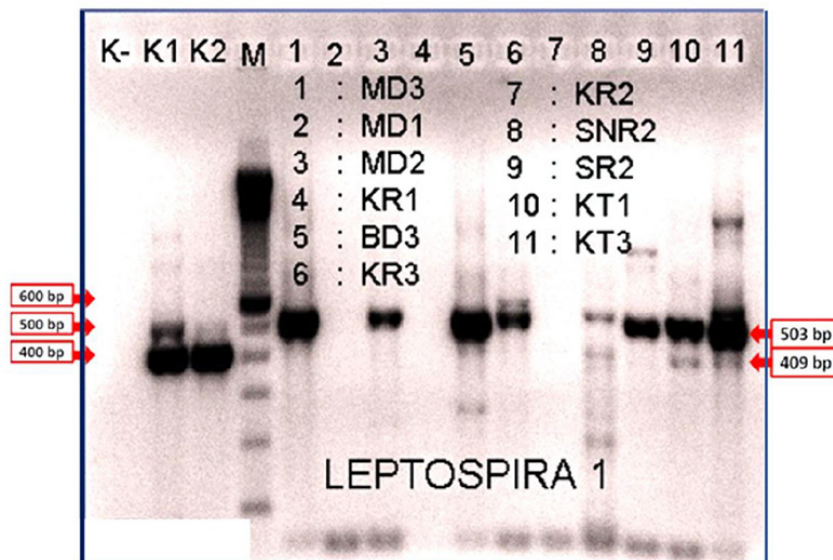


Figure 4. The results of repeated electrophoresis of PCR products from samples no.1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11. Above 400 bp (409 bp) and above 500 bp (503 bp), as seen in the positive control 1 *Leptospira interrogans* serovar Bataviae and positive control 2 *Leptospira borgpetersenii* serovar Ballum. It appears that there are several samples showing the expected two bands, namely above 400 bp (409 bp) and above 500 bp (503 bp), namely samples no. 5, 8, 10, and 11. While some samples showed the presence of only 1 band above 500 bp (503 bp), namely samples no. 3, 6, and 9, which showed the presence of *Leptospira* saprophytic DNA. (K-)Negative Control: (K1)Positive 1 control: (K2)Positive 2 control: (M)Marker



Figure 5. The results of repeated electrophoresis of PCR products from samples no.12, 13, 14, 15, 16, 17, 18, and 19. Above 400 bp (409 bp) and above 500 bp (503 bp) as seen in the positive control 1 *Leptospira interrogans* serovar Bataviae and positive control 2 *Leptospira borgpetersenii* serovar Ballum. It appears that there are several samples showing the expected two bands, namely above 400 bp (409 bp) and above 500 bp (503 bp), namely sample no. 18, while some samples showed only 1 band above 500 bp (503 bp), namely samples no. 12, 13, 14, 15, 17, and 19, which indicated the presence of *Leptospira* saprophyte DNA. (K-)Negative Control: (K1)Positive 1 control: (K2)Positive 2 control: (M)Marker

between different *Leptospira* species and demonstrated that pathogenic *Leptospira* species contain unique genes not found in non-pathogenic *Leptospira*.¹⁶

PCR examination using a combination of these three primers has been developed and used to detect and differentiate between pathogenic *Leptospira* species and saprophytic species on agarose gel, which can be applied in routine diagnosis.⁷ The PCR test method can differentiate *Leptospira* strains at the subspecies level.³

The classification of the genus *Leptospira* is quite complex. Currently, there are two classification systems in use: the traditional phenotypic classification system based on serotypes and the genotypic classification system based on DNA homology. Traditionally, *Leptospira* are grouped into two types: pathogenic species (*Leptospira interrogans*) and non-pathogenic or saprophytic species (*Leptospira biflexa*).¹⁷

Modern microbial taxonomy determination can be done based on the 16S rRNA gene, which allows identification at the species level, such as in the genus *Leptospira*.⁶ The genus *Leptospira* belongs to the Leptospiraceae family and consists of both pathogenic and nonpathogenic bacteria. Analysis of *Leptospira* bacterial DNA was able to reveal significant genetic heterogeneity in the two species, *Leptospira interrogans* and *Leptospira biflexa*, and resulted in a species reclassification based on DNA homology.¹⁷

Both pathogenic and saprophytic *Leptospira* strains can be isolated from the environment.¹⁸ *Leptospira* spp. can colonize the renal tubules of various wild mammals.³ *Leptospira* bacteria are found in the urine of infected animals and can survive for a long time in fresh water.¹⁹ *Leptospira* bacteria can survive in moist soil and fresh water for several weeks.¹⁸ Transmission to new hosts usually occurs after exposure to water contaminated with *Leptospira* bacteria.²⁰ The risk factor for the entry of bacteria into the body is the presence of a wound or injury. The presence of many rodents in the human environment increases the possibility of the environment being contaminated with urine containing *Leptospira* bacteria.⁴ Rats were identified as potential sources of infection for *Leptospira interrogans*

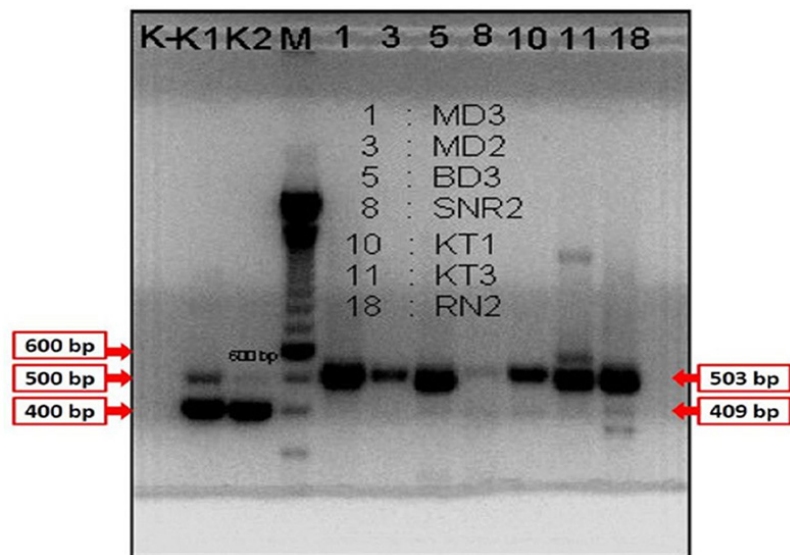


Figure 6. Repeated electrophoresis results of sample PCR products to clarify the expected presence of two bands. Above 400 bp (409 bp) and above 500 bp (503 bp), in samples suspected of having two bands parallel to the positive control 1 *Leptospira interrogans* serovar Bataviae and positive control 2 *Leptospira borgpetersenii* serovar Ballum It appears that there are several samples showing the expected two bands, namely above 400 bp (409 bp) and above 500 bp (503 bp), namely samples no. 5, 8, 10, 11, and 18. (K-)Negative Control: (K1)Positive 1 control: (K2)Positive 2 control: (M) Marker

Table 1. Results of Amplification by PCR

Results of Amplification	Sample code	Amount	Identification
One band appears	1,3,6,9,12,13,14,15,17,19	10(19)	Pathogenic <i>Leptospira</i>
Two bands appears	5, 8,10,11,18	5(19)	Saprophytic <i>Leptospira</i>
No band appears	2,4,7,16	4(19)	<i>Leptospira</i> pathogens and saprophytes were not found

serogroup Icterohaemorrhagiae and *Leptospira interrogans* serogroup Australis.³

In this study, PCR examination using a combination of three primers could distinguish the presence of pathogenic *Leptospira*, saprophytic *Leptospira*, and the absence of pathogenic and saprophytic *Leptospira* from traditional market water samples.

CONCLUSION

This study found that 5/19 (26.3%) of point locations found specific DNA genes for pathogenic *Leptospira* bacteria. 10/19 (52.6%) point locations found DNA specific genes for saprophyte *Leptospira*

bacteria, and 4/19 (21,1%) point locations in environmental water found no specific genes for pathogenic or saprophytic *Leptospira* bacteria. In this study, PCR examination using a combination of three primers could distinguish the presence of pathogenic *Leptospira*, saprophytic *Leptospira*, and the absence of pathogenic and saprophytic *Leptospira* from water samples from traditional markets in Denpasar City.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest regarding the publication of this paper.

ETHICS CONSIDERATION

Ethical clearance was obtained from the ethical committee in the Faculty of Medicine, Universitas Udayana with reference letter number :1950/UN14.2.2.VII.14/LP/2015

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AUTHOR CONTRIBUTION

The author contributes to the study from the conceptual framework, data gathering, and data analysis until reporting the study results through publication.

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