# MOLECULAR DETECTION OF *LEPTOSPIRA* SPP. IN SOIL FROM WET MARKET IN KELANTAN

MUHAMMAD FAIZ BIN KAMARUL ZAMAN
(D17A0020)

A RESEARCH PAPER SUBMITTED TO THE FACULTY OF VETERINARY MEDICINE, UNIVERSITI MALAYSIA KELANTAN
IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR
THE DEGREE OF
DOCTOR OF VETERINARY MEDICINE

JUNE 2022

UNIVERSITI MALAYSIA KELANTAN

## CERTIFICATION

This is to certify that we have read this research paper entitled **'Molecular Detection of *Leptospira* Spp. In Soil from Wet Market in Kelantan'** by Muhammad Faiz Bin Kamarul Zaman, and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course DVT 5436 – Research Project.

**Dr Mohammad Sabri Bin Abdul Rahman**
***DVM (UPM), MVSc(UPM)***
Lecturer,
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Supervisor)

**Dr Intan Noor Aina Binti Kamaruzaman**
***DVM (UMK), PhD (Liverpool)***
Senior Lecturer,
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Co-supervisor

## DEDICATIONS

**Special thanks for those who have given their support, guidance, advice, and aid for the completion of this project paper:**

Dr Mohammad Sabri Bin Abdul Rahman

Dr Intan Noor Aina Binti Kamaruzaman

Che Wan Salma Binti Che Wan Zalati

Public Health FYP Team (Amirulsyafiq, Fakhrul, Syazwan, Iman, Ong, and Aqilah)

Baitul Amali's Family (Kamarul Zaman, Nooraini, Fahmin, Faris, Fakhri, Fatihah, Fariha, Farhana, Natasha, Fatin, Hafizah)

DVM 5 class of 2017/2022

**Thank You**

## ACKNOWLEDGEMENT

I would like to convey my gratitude to my supervisor, Dr. Mohammad Sabri Bin Abdul Rahman, who has provided me with help, guidance, and support throughout the Final Year Project's easy and difficult phases. His mentoring made it feasible to complete this study successfully. My thanks also extend to my co-supervisor, Dr. Intan Noor Aina Binti Kamaruzaman, for providing consistent assistance and advice from the proposal stage through the drafting of the thesis, which made it simpler to comprehend.

Many thanks to Ms. Che Wan Salma for her kind assistance in ensuring that I completed my laboratory work according to time as we planned. I would want to thank all my friends especially Public Health FYP Team who were engaged in the process of doing lab work and who were willing to spend their time assisting me in finishing this research.

My last thanks go to my parents, siblings, and the rest of my family for inspiring and motivating me. Thank you for educating me either directly or indirectly about balancing study and family life. I would also want to thank them from the bottom of my heart for accompanying me on this adventure and making it possible for us to travel together.

**Contents**

**List of Table**



**List of Figure**

## ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, University Malaysia Kelantan, in partial requirement on the course DVT 5436 – Research Project

Leptospirosis is a globally significant zoonotic illness brought on by pathogenic *Leptospira* spp. Leptospira spp. have been discovered in humans, animals, and environmental materials in Malaysia. Leptospirosis can be transmitted through direct contact with the urine of a reservoir host or indirect contact through soil and water that contaminated with urine from a reservoir host. High seroprevalence of leptospirosis has been reported in wet market workers in Kelantan by previous studies. However, the source of infection is still unknown. Therefore, the aim of this research was to detect *Leptospira* spp. from the soil in wet markets in Kelantan by using polymerase chain reaction (PCR). A total of 60 samples were collected from 7 wet markets in three districts in Kelantan. After DNA extraction, PCR was performed for all samples and there was no Leptospira spp. was detected. In conclusion, pathogenic *Leptospira spp.* were not detected in soil samples collected from wet markets of Kelantan.

***Keywords**: Kelantan, Leptospira* spp., *Polymerase chain reaction, Soil, Wet market.*

## ABSTRAK

Abstrak kertas penyelidikan yang dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan, dalam keperluan separa bagi kursus DVT 5436 - Projek Penyelidikan

Leptospirosis ialah penyakit zoonosis yang ketara di seluruh dunia yang disebabkan oleh patogenik Leptospira spp. Leptospira spp. telah ditemui pada manusia, haiwan, dan bahan alam sekitar di Malaysia. Leptospirosis boleh berjangkit melalui sentuhan langsung dengan air kencing perumah takungan atau sentuhan tidak langsung melalui tanah dan air yang tercemar dengan air kencing daripada perumah takungan. Seroprevalens leptospirosis yang tinggi telah dilaporkan di kalangan pekerja pasar basah di Kelantan oleh kajian lepas. Walau bagaimanapun, punca jangkitan masih tidak diketahui. Oleh itu, tujuan penyelidikan ini adalah untuk mengesan Leptospira spp. daripada tanah di pasar basah di Kelantan dengan menggunakan tindak balas rantai polimerase (PCR). Sebanyak 60 sampel telah dikumpul dari 7 pasar basah di tiga daerah di Kelantan. Selepas pengekstrakan DNA, PCR dilakukan untuk semua sampel dan tiada Leptospira spp. telah dikesan. Kesimpulannya, patogenik *Leptospira spp.* tidak dikesan dalam sampel tanah yang dikutip dari pasar basah Kelantan.

Kata kunci: *Kelantan, Leptospira* spp., , *Pasar basah, Tanah, Tindak balas berantai polimerase.*

## 1.0 Introduction

Leptospirosis is a most common zoonotic disease that has a significant impact on public health across the world, with a high prevalence in most tropical locations, particularly where there is a significant rainfall (Haake & Levett, 2015). Kelantan is a Malaysian state on the East Coast that is frequently hit by heavy rainfall, resulting in flooding(Reuters, 2014). The number of leptospirosis cases in Kelantan increased after the floods, with a total of 1229 cases more than double the number of incidences before the floods (Mohd-Radi et al., 2018).

Leptospires can be inhabitant chronically in renal tubules and shed through urine contaminating the environment(Cordonin et al., 2020). The disease can be transmitted through direct contact with the urine of a reservoir host or indirect contact through soil and water contaminated with urine from a reservoir host (Haake & Levett, 2015). The factor of distribution of *Leptospira* spp. in the environment can be due to consistent with continuous exposure of rats as reservoirs with excretion of urine contained *Leptospira* spp. into environment(Cordonin et al., 2020).

A wet market is a place for activities that related to selling fresh meat and other consumption needed by human(Raji et al., 2017). Leptospirosis is more likely to spread in an area where rodent populations are high as large abundance of food in wet market attract rodents. Previous study reported a high seroprevalence of leptospirosis among wet market workers in Kelantan (Rahman et al., 2018a). Indirect contact via Leptospira contaminated soil can be a factor leading high seroprevalence of

leptospirosis in wet market workers. Therefore, this study aims to detect leptospires in soil from wet market in Kelantan by using a molecular detection assay.

## 2.0 Problem statement

Rahman et al., (2018) reported high seroprevalence of leptospirosis among wet market workers in Kelantan. However, the source of infection that contributed to high seroprevalence of leptospirosis among wet market workers in Kelantan is still unknown. The source of leptospirosis seroprevalence could be from *Leptospira spp.* contaminated soil or water (Azali et al., 2016; Flores et al., 2020; Pui et al., 2017). Therefore, this study aims to investigate the source of leptospirosis by a molecular detection assay from soil in wet market.

## 3.0 Research question

What is the occurrence of *Leptospira* spp. in soil from wet markets in Kelantan?

## 4.0 Research hypothesis

*Leptospira* spp. can be detect in soil at wet markets by molecular detection method.

## 5.0 Research objective

To detect *Leptospira* spp. in soil from wet markets in Kelantan by using molecular method.

## 6.0 Literature review

### 6.1 Description of *Leptospira* spp.

Leptospira belong to order of spirochaetes in family of leptospiraceae genus Leptospira. End hooks distinguish leptospires from other spirochaetes, which have a corkscrew-screw form. The FlaA and FlaB proteins, which make up the flagellar sheath and core, are found in the periplasmic region and are responsible for movement in the organism. They have a diameter of 0.1-0.2 µm and a length of 6-20 µm (Mohammed et al., 2011).

### 6.2 Availability of *Leptospira* spp. in environment

Pathogenic species of Leptospires are common aetiological agent for leptospirosis. In environment such as soil and water may contaminated with pathogenic and saprophytic species of leptospira that could not cause disease in animals or humans (Haake & Levett, 2015). Saprophytic species of *Leptospira* are most dominantly found in environment due to its fastidious nature of survival thus may be difficult to isolate and identify from soil (Azali et al., 2016; Flores et al., 2020). The environmental temperature and moisture level of soil have an impact on survivability of leptospira in soil. According to a study by Saito et al., (2013), Leptospires were highly detected on the soil that had more than 20% of moisture content (Saito et al., 2013). *Leptospira interrogans* serovar Hardjo can survive for 2 hours soil directly under the sun whereas 144 hours in soil under shaded area suggest that environmental temperature also play an important role for survival of *Leptospira* spp. in soil (Bejo et al., 2014)

## 6.3 Laboratory screening of environmental samples for Leptospira spp.

*Leptospira* species isolation process begins by culturing the soil sample in Ellinghausen McCullough Johnson Harris (EMJH) media by inoculating the sample with 0.45 µm syringe filter (Bahaman et al., 2014). Next, add combination of antimicrobials (5-fluororacil, trimethoprim, amphotericin B and Fosfomycin) to the inoculated media to enhance the selective isolation of leptospires from contaminated samples. There are number of molecular detection methods targeting several genes of leptospira from various types of samples including environmental samples. 16S rRNA and LipL32 are commonly used gene targets to detect pathogenic leptospira in environmental samples (Azali et al., 2016; Flores et al., 2020).

## 6.4 Occurrence of *Leptospira* spp in soil and water in Malaysia

Soil is the most abundant environmental component for isolation and detection of *Leptospira* species in environment compared to water (Flores et al., 2020; Miller et al., 2021). High prevalence of leptospirosis in Malaysia in human and animal was reported (Benacer et al., 2016) however information on the possible source of infection was limited. PCR was used to determine leptospira from environmental samples such as soil and water that has potential for indirect transmission to humans especially in recreational places and wet markets(Azali et al., 2016; Pui et al., 2017). According to a study by Azali et al. (2016), *Leptospira alstonii* is a pathogenic *Leptospira* species that was successfully detected by culture isolation followed by molecular detection in one of soil sample will be a factor that can cause outbreak leptospirosis will happened in a wet market (Azali et al., 2016; Miller et al., 2021).

## 7.0 Importance of the expected research finding

Malaysia as a tropical country with high seasonal rainfall, warm temperature, wet and humid climate will facilitate the prolong survival of *Leptospira* spp. in the environment. The findings of this study will assist data to identify potential sources of high prevalence of leptospirosis especially observed in Kelantan after heavy rainy season followed by flooding. Wet market has high exposure of rodents resulting possible high contamination of *Leptospira* spp. where it can affect health of both humans and animals. Thus, based on findings of this study we can suggest whether there is any environmental contamination of leptospires and relate it with future outbreaks of leptospirosis.

## 8.0 Material and methodology

### 8.1 Sample collection

A total of 60 soil samples were collected from 7 wet markets in three districts of Kelantan. Soil samples were collected from each wet market adhering specific criteria i) wet soils, ii) rain puddles, iii) garbage site, and iv) places where spoiled food has been spotted. An approximately 30 grams of covered soil with depth about 2-3 cm depth was collected per one sample and put in the 60ml sterile specimen container.

Table 1: Location for sample collection

| **Name of wet market** | **District of Kelantan** | **Number of samples** |
|---|---|---|
| Pasar Wakaf Che Yeh | Kota Bharu | 10 |
| Pasar Taman Bendahara | Kota Bharu | 7 |
| Pasar RTC Tunjong | Kota Bharu | 10 |
| Pasar Siti Khadijah | Kota Bharu | 10 |
| Pasar Kubang Pasu | Kota Bharu | 3 |
| Pasar Pasir Mas | Pasir Mas | 10 |
| Pasar Jelawat | Bachok | 10 |
| | Total of Samples | 60 |

### 8.2 Sample storage

The soil samples were stored in the chiller at 4°C until processing.

8.3 Sample processing

Sample were filtered with filter paper to isolate debris. Filter paper was folded into cone shape to fit in a glass tunnel and poured the sample into the filter paper. A filtrated liquid was transfer by using sterile 1 ml syringe into 1.5 ml microcentrifuge tube.

8.4 DNA extraction

DNA extraction was performed to isolate leptospira by using Geneaid gSYNC™ DNA Extraction Kit Quick Protocol by following manual that provided by manufacturer. 1 ml of Filtrated liquid was centrifuged for 14000 rpm for 5 minutes. Supernatant was removed carefully without disturbing the pellet. Then, 200µL of PBS and 20µL of proteinase K were added. The mixture was vortex and incubated at 60°C for 5 minutes. The mixture was mixed with 200µl of GSB buffer. Then, the mixture was shake vigorously by vortex and incubated 60°C for 5 minutes. 200µL of absolute ethanol was added and shake vigorously by vortex for 30s. The mixture was transferred into GS column with 2ml collection tube and was centrifuged 14000 rpm for 1 minute. Next, flow through content in the 2ml collection tube was discarded. 400µl of W1 buffer was added into the GS column with 2 ml of collection tube and centrifuge at 14000 rpm for 1 minute. Flow through content in the 2ml collection tube was discarded. 600µL of wash buffer was transferred into the GS column and centrifuge at 14,000 rpm for 1 minute. Then, flow through content in the 2 ml collection tube was discarded and followed by centrifuge at 14000 rpm for 3 minutes. GS column transferred into a new sterile 1.5 ml microcentrifuge tube and add 100 µl of pre-heated elution buffer was incubated at 60°C for 30 minutes. Then, centrifuged at 14000 rpm for 1 minute. The GS column was

discarded and the microcentrifuge tube with eluted DNA was stored in the freezer at -20°C until further analysis.

8.4 Polymerase chain reaction

Two sets of primers targeting virulence gene will be used for the detection of *LipL32* and 16S rRNA genes of leptospira (Table 2). A total mixture 12.5µL of 2×Master mix, 2.5µL of forward and reverse primer, and 10µL of DNA template. Amplification will be performed by using Thermal cycler (Bio-Rad Thermocycler) with initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, Primer annealing at 58°C for 45s and then, The DNA extension process at 72°C for 30 seconds. Lastly, followed by the final extension process at 72°C for 6 minutes (S. A. Rahman et al., 2021). Positive and negative controls were included for each experiment. For positive control sample that was used is *L. interrogans serovar Copenheganii*.

Table 2: Primers used for the study

| Primer | Gene | Sequence 5′–3′ | Size |
|---|---|---|---|
| 16S rRNA (forward)(Sabri et al., 2019; Sandai, 2012) | 16S rRNA | CATGCAAGTCAAGCGGAGTA | 541 bp |
| 16S rRNA (reverse)(Sabri et al., 2019; Sandai, 2012) | | AGTTGAGCCCGCAGTTTTC | |
| LipL32 (forward)(Sandai, 2012) | LipL32 | GTCGACATGAAAAAACTTTCGATTTTG | 756 bp |

| | | | |
|---|---|---|---|
| LipL32 (reverse)(Sandai, 2012) | | CTGCAGTTACTTAGTCGCGTCAGAAGC | |

8.5 Gel electrophoresis and gel documentation

0.9 g of agarose powder was measured by using weighing boat to prepare 1.5% of agarose gel. Agarose powder was mixed with 60 ml of 1x TBE buffer in a Schott bottle. The mixture was heated by using microwave for 1-3 minutes until the agarose is completely dissolved. Agarose solution was cool down to about 50 °C and add 1 ml of Midori Green dye into the agarose solution. The agarose solution was poured into the gel tray with the well comb in place. The gel was placed at room temperature for 30 minutes until it has completely solidified. Once solidified, the agarose gel was placed into the electrophoresis tank. Fill the tank with 1x TBE buffer until the gel is covered. The amplification products were analysed in Tris-borate-EDTA (TBE) buffer at 100 V for 40 minutes using electrophoresis on a 1.5% agarose gel. Gel was then pre-stained with 0.1µl of Midori Green dye. The DNA fragment was visualized by using GelDoc™ EZ Imager. The identification of amplified products was based on their band sizes of 541 bp (16S rRNA) and 756 bp (LipL32).

8.6 Statistical analysis

Data from molecular detection will be analysed by using descriptive statistical analysis with confidence interval, 95% (CIs; SPSS Statistics v.23; IBM).

## 9.0 Result

### 9.1 PCR 16S rRNA primer

PCR product for 16S rRNA is observed at 541 base pair in positive control. From figure 1-5, there were no observable band in all soil samples that collected which indicates negative for all species of Leptospira.


Figure 1: Lane M: 100 bp DNA Ladder, A1-A10: Soil samples from Pasar Siti Khadijah, D1-D2: Soil samples from Pasar Jelawat, Lane (+): Positive control, lane (-): Negative control


Figure 2: Lane M: 100 bp DNA Ladder, D3-D7: Soil samples from Pasar Taman Bendahara, F1-F7: Soil samples from Pasar Jelawat, Lane (+): Positive control, lane (-): Negative control

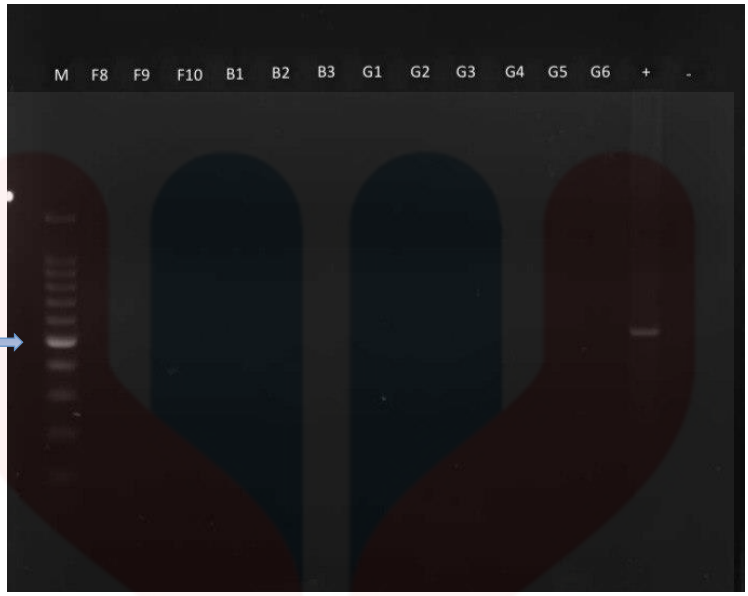


Figure 3: Lane M: 100 bp DNA Ladder, F8-F9: Soil samples from Pasar Jelawat, B1-B3: Soil samples from Pasar Kubang Pasu, G1-G6: Soil samples from Pasar Wakaf Che Yeh, Lane (+): Positive control, lane (-): Negative control

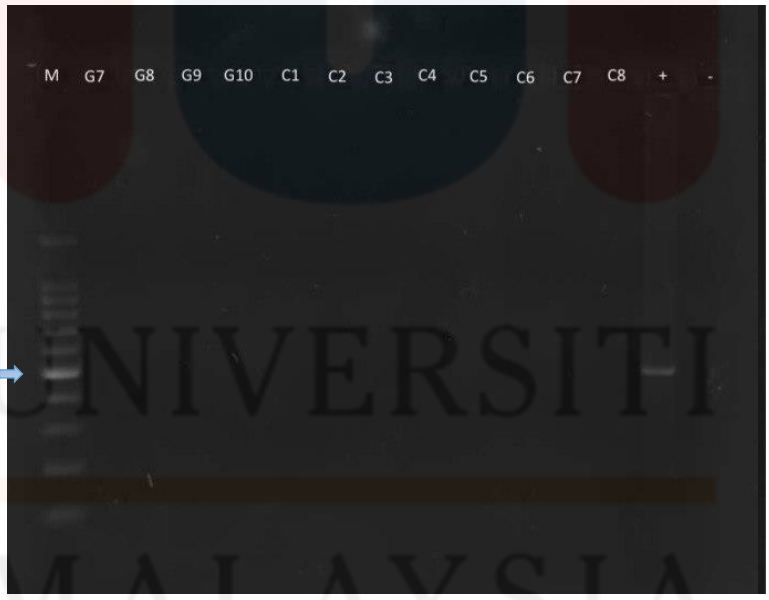


Figure 4: Lane M: 100 bp DNA Ladder, G7-G10: Soil samples from Pasar Wakaf Che Yeh, C1-C8: Soil samples from Pasar RTC, Lane (+): Positive control, lane (-): Negative control


Figure 5: Lane M: 100 bp DNA Ladder, E1-E10: Soil samples from Pasar Bandar Tasek Raja, C9-C10: Soil samples from Pasar RTC, Lane (+): Positive control, lane (-): Negative control

Table 3: PCR detection of *Leptospira* spp. using primer 16S rRNA for soil samples

| Name of wet market | Number of samples | Label | Positive sample |
|---|---|---|---|
| Pasar Siti Khadijah | 10 | A | - |
| Pasar Kubang Pasu | 3 | B | - |
| Pasar RTC Tunjong | 10 | C | - |
| Pasar Taman Bendahara | 10 | D | - |
| Pasar Pasir Mas | 7 | E | - |
| Pasar Jelawat | 10 | F | - |
| Pasar Wakaf Che Yeh | 10 | G | - |

(-) denote as negative result

## 8.2 PCR LipL32 primer

DNA product for LipL32 is observed at 756 base pair in positive control. There were no observable band in soil sample that collected which indicates negative for pathogenic leptospires. This could be indicated of all samples also resulting negative where universal primers such as 16S rRNA negative for all samples that included pathogenic leptospires.

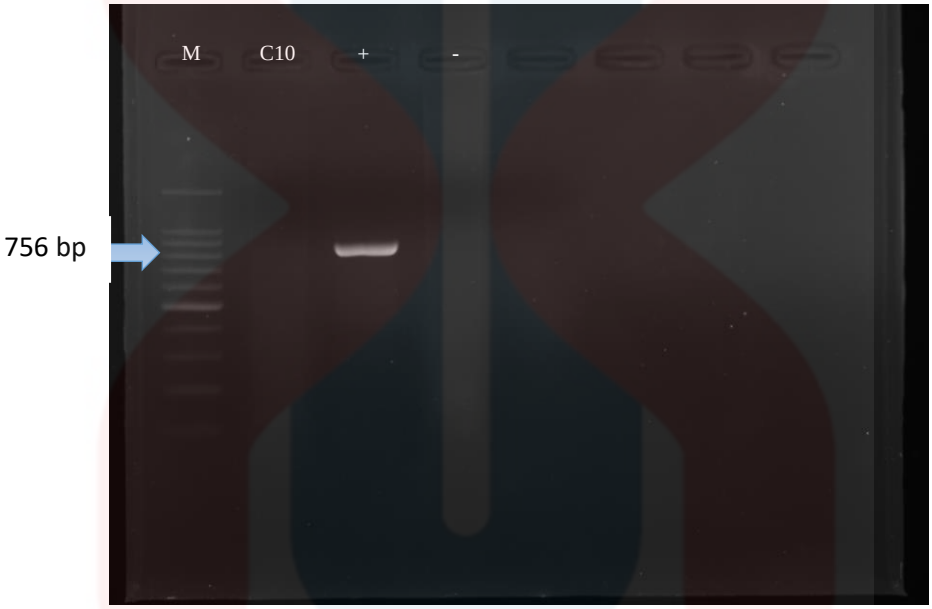


Figure 6: Lane M: 100 bp DNA Ladder, C10: Soil samples from Pasar RTC, Lane (+): Positive control, Lane (-): Negative control

## 10.0 Discussion

The polymerase chain reaction (PCR) is a DNA-based technology that has extensive used in the detection of *Leptospira spp.*(Ganoza et al., 2006). According to the findings of Zulkifli et al., (2018), supposedly the 16s rRNA primer that was designed to identify any types of Leptospira spp. did not positive for *Leptospira spp.* in the soil. The use of DNA extracted directly from uncultured soil samples is anticipated to be troublesome due to the presence of a broad pool of DNAs including both known and undiscovered microbial species, as well as plant DNAs and other cellular pollutants. Because of this, the PCR reaction's precision and sensitivity, and the primers' ability to compete against a wide range of DNA backgrounds, may be compromised. This could lead into all negative result due to multiple organism that hinder of leptospires in this study.

Additionally, the soil's moisture level may impact on survivability of leptospires. In addition, the moisture in the soil may provide leptospires with the nutrients and growth elements that they need. According to Saito et al. (2013) was found soil moisture will have more frequency to detect of *Leptospira* spp. in soil that content humidity had >20% of water content. In this research, samples were collected during rainy seasons that may humid the soil and did not collect soil directly under the sun.

Molecular detection was used for current study and did not culture and isolation were performed to any of the samples. The culture isolation might helpful during isolation of *Leptospira spp.* from environment to limit contamination of other microorganism appeared in the samples. There was an investigation of floodwater in Bangkok, Thailand, by Thaipadungpanit et al. (2013) which were successfully discovered that

certain environment samples had an amplified 16SrRNA gene that was belong to unculturable populations of *Leptospira*. Researchers observed that saprophytic leptospira is abundant in environmental samples, but pathogenic Leptospires are more typically identified in animal hosts. Because those leptospiral strains have not yet been described for their species and virulence potential, it is unknown if the non-cultivable strains of *Leptospira* that were found in above mentioned and others belong to a category that is beneficial or harmful to humans. This demonstrates the variety of the *Leptospira* population, which calls for more research, in particular into methods for the direct identification and species characterization of Leptospires in materials that do not need to culture. These uncultured leptospires might have emerged from the environment. Nevertheless, there is also a chance that they came from animals. As the environment may be contaminated by animal urine, it may temporarily harbour numerous *Leptospira* strains that may provide a health risk to people, particularly in locations frequented by humans, such as food courts, agricultural and recreational areas (Mwachui et al., 2015).

In the case failure to detect leptospires could be from incorrect transportation of samples from the wet market to the laboratory might result in the inability to identify them. During the study, there is no preservation by ice pack to preserved samples at 3°C from protein will degrade over time and decrease bacteria viability(Casanovas-Massana et al., 2018). Denaturation of protein happened when had exposure high temperature at that time can achieve 30-34°C can be a factor that led all false negative on the PCR result.

In this study also, 12 samples out of 60 samples were used syringe filter size 0.45µm to isolate Leptospira spp. and cultured on EMJH media. The result for culture still pending due to required longer period to get pure culture. All of 12 samples with filtered 0.45µm followed with PCR detection appeared negative by using 16S rRNA primers. The sensitivity and specificity of the 16S rRNA gene were both 100% ability to detect of *Leptospira spp.* on sample that had leptospires(Güven Gökmen et al., 2016). This could be either sample does not contain *Leptospira spp.* or other microorganism dominant could be hinder slow growth of leptospires

## 11.0 Conclusion

In conclusion, a total of 60 samples were tested for the presence of Leptospira spp. using direct PCR by using primers 16sRNA forward and reverse. *Leptospira* spp. has not been found in any of the soil samples that I obtained from various wet markets in Kelantan. There is possibility that the soil samples used in this investigation were contaminated by other dominant bacteria, which may have complicated DNA extraction from soil. The soil samples may have had a varied population of microorganisms and other pollutants that impeded DNA extraction and PCR reactions. These findings indicate that molecular screening failure could be due to a varied population of microorganisms and other pollutants. Some additional methods may need to improve on molecular screening to successfully detect Leptospira species in soil. However, more evaluations should be undertaken to successfully detect *Leptospira* spp. in soil by using molecular detection.

## 12.0 Recommendation

This study's results lead to the following recommendation for future research: For Samples should be process as fast as possible within two weeks must proceed with isolation, syringe filters with a diameter of 0.2-0.45 µm should be used to filter out Leptospira spp. before to inoculation on EMJH agar. Under a 20-magnification dark field microscope, the bacterial growth on EMJH medium should be examined for the presence of leptospires. The presence of leptospires was determined on motile thin helical structures with noticeable hooked ends. It is recommended by Chakraborty et al. (2011) to use sulfamethoxazole/trimethoprim (40/20 g/ml), amphotericin B (5 g/ml), and 5-fluorouracil (100 g/ml) to isolate leptospires from contaminated samples.

## References


Azali, M. A., Yean Yean, C., Harun, A., Aminuddin Baki, N. N., & Ismail, N. (2016). Molecular Characterization of Leptospira spp. in Environmental Samples from North-Eastern Malaysia Revealed a Pathogenic Strain, Leptospira alstonii. *Journal of Tropical Medicine*, *2016*. https://doi.org/10.1155/2016/2060241

Bejo, S. K., Bahaman, A. R., Saad, M. Z., & Mutalib, A. R. (n.d.). *The Survival of Leptospira interrogans Serovar Hardjo in the Malaysian Environment*. https://www.researchgate.net/publication/26591208

Benacer, D., Thong, K. L., Min, N. C., Verasahib, K. bin, Galloway, R. L., Hartskeerl, R. A., Souris, M., & Zain, S. N. M. (2016). Epidemiology of human leptospirosis in Malaysia, 2004-2012. *Acta Tropica*, *157*, 162–168. https://doi.org/10.1016/j.actatropica.2016.01.031

Casanovas-Massana, A., Pedra, G. G., Wunder, E. A., Diggle, P. J., Begon, M., & Ko, A. I. (2018). *Quantification of Leptospira interrogans Survival in Soil and Water Microcosms*. https://doi.org/10

Chakraborty, A., Miyahara, S., Villanueva, S. Y. A. M., Saito, M., Gloriani, N. G., & Yoshida, S. I. (2011). A novel combination of selective agents for isolation of Leptospira species. *Microbiology and Immunology*, *55*(7), 494–501. https://doi.org/10.1111/j.1348-0421.2011.00347.x

Cordonin, C., Turpin, M., Bringart, M., Bascands, J. L., Flores, O., Dellagi, K., Mavingui, P., Roche, M., & Tortosa, P. (2020). Pathogenic Leptospira and their animal reservoirs: testing host specificity through experimental infection. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-64172-4

Flores, B., Escobar, K., Muzquiz, J. L., Sheleby-Elías, J., Mora, B., Roque, E., Torres, D., Chávez, Á., & Jirón, W. (2020). Detection of pathogenic leptospires in water and soil in areas endemic to leptospirosis in Nicaragua. *Tropical Medicine and Infectious Disease*, *5*(3). https://doi.org/10.3390/TROPICALMED5030149

Ganoza, C. A., Matthias, M. A., Collins-Richards, D., Brouwer, K. C., Cunningham, C. B., Segura, E. R., Gilman, R. H., Gotuzzo, E., & Vinetz, J. M. (2006). Determining Risk for Severe Leptospirosis by Molecular Analysis of Environmental Surface Waters for Pathogenic Leptospira. *PLoS Medicine*, *3*(8), e308. https://doi.org/10.1371/journal.pmed.0030308

Güven Gökmen, T., Soyal, A., Kalayci, Y., Önlen, C., & Köksal, F. (2016). Comparison of 16s rrna-pcr-rflp, lipl32-PCR and ompl1-PCR methods in the diagnosis of leptospirosis. *Revista Do Instituto de Medicina Tropical de Sao Paulo*, *58*. https://doi.org/10.1590/S1678-9946201658064

Haake, D. A., & Levett, P. N. (2015). Leptospirosis in humans. *Current Topics in Microbiology and Immunology*, *387*, 65–97. https://doi.org/10.1007/978-3-662-45059-8_5

Miller, E., Barragan, V., Chiriboga, J., Weddell, C., Luna, L., Jiménez, D. J., Aleman, J., Mihaljevic, J. R., Olivas, S., Marks, J., Izurieta, R., Nieto, N., Keim, P., Trueba, G., Caporaso, J. G., & Pearson, T. (2021). Leptospira in river and soil in a highly endemic area of Ecuador. *BMC Microbiology*, *21*(1). https://doi.org/10.1186/s12866-020-02069-y

Mohammed, H., Nozha, C., Hakim, K., & Abdelaziz, F. (2011). LEPTOSPIRA: Morphology, Classification and Pathogenesis. *Journal of Bacteriology & Parasitology*, *02*(06). https://doi.org/10.4172/2155-9597.1000120

Mohd Radi, M. F., Hashim, J. H., Jaafar, M. H., Hod, R., Ahmad, N., Nawi, A. M., Baloch, G. M., Ismail, R., & Ayub, N. I. F. (2018). Leptospirosis outbreak after the 2014 major flooding event in Kelantan, Malaysia: A spatial-temporal analysis. *American Journal of Tropical Medicine and Hygiene*, *98*(5), 1281–1295. https://doi.org/10.4269/ajtmh.16-0922

Mwachui, M. A., Crump, L., Hartskeerl, R., Zinsstag, J., & Hattendorf, J. (2015). Environmental and Behavioural Determinants of Leptospirosis Transmission: A Systematic Review. *PLOS Neglected Tropical Diseases*, *9*(9), e0003843. https://doi.org/10.1371/journal.pntd.0003843

Pui, C. F., Bilung, L. M., Apun, K., & Su'ut, L. (2017). Diversity of Leptospira spp. in Rats and Environment from Urban Areas of Sarawak, Malaysia. *Journal of Tropical Medicine*, *2017*. https://doi.org/10.1155/2017/3760674

Rahman, M. H. A. A., Hairon, S. M., Hamat, R. A., Jamaluddin, T. Z. M. T., Shafei, M. N., Idris, N., Osman, M., Sukeri, S., Wahab, Z. A., Mohammad, W. M. Z. W., Idris, Z., & Daud, A. (2018a). Seroprevalence and distribution of leptospirosis serovars among wet market workers in northeastern, Malaysia: A cross sectional study. *BMC Infectious Diseases*, *18*(1). https://doi.org/10.1186/s12879-018-3470-5

Rahman, M. H. A. A., Hairon, S. M., Hamat, R. A., Jamaluddin, T. Z. M. T., Shafei, M. N., Idris, N., Osman, M., Sukeri, S., Wahab, Z. A., Mohammad, W. M. Z. W., Idris, Z., & Daud, A. (2018b). Seroprevalence and distribution of leptospirosis serovars among wet market workers in northeastern, Malaysia: A cross sectional study. *BMC Infectious Diseases*, *18*(1). https://doi.org/10.1186/s12879-018-3470-5

Rahman, S. A., Khor, K. H., Khairani-Bejo, S., Lau, S. F., Mazlan, M., Roslan, A., & Goh, S. H. (2021). Detection and characterization of Leptospira spp. in dogs diagnosed with kidney and/or liver disease in Selangor, Malaysia. *Journal of Veterinary Diagnostic Investigation*, *33*(5), 834–843. https://doi.org/10.1177/10406387211024575

Raji, M. N. A., Ab Karim, S., Ishak, F. A. C., & Arshad, M. M. (2017). Past and present practices of the Malay food heritage and culture in Malaysia. In *Journal of Ethnic Foods* (Vol. 4, Issue 4, pp. 221–231). Elsevier B.V. https://doi.org/10.1016/j.jef.2017.11.001

Rani Bahaman, A., Khairani Bejo, S., & Rahim Mutalib, A. (2014). *Detection of pathogenic Leptospira from selected environment in*. https://www.researchgate.net/publication/50377226

Reuters. (2014). *Record numbers evacuated in Malaysia's worst floods in decades By Reuters*. https://www.investing.com/news/world-news/record-numbers-evacuated-in-malaysia%E2%80%99s-worst-floods-in-decades-321473

Sabri, A. R., Khairani-Bejo, S., Zunita, Z., & Hassan. (2019). Molecular detection of Leptospira sp. in cattle and goats in Kelantan, Malaysia after a massive flood using multiplex polymerase chain reaction. In *Tropical Biomedicine* (Vol. 36, Issue 1).

Saito, M., Villanueva, S. Y. A. M., Chakraborty, A., Miyahara, S., Segawa, T., Asoh, T., Ozuru, R., Gloriani, N. G., Yanagihara, Y., & Yoshida, S. I. (2013). Comparative analysis of Leptospira strains isolated from environmental soil and water in the Philippines and Japan. *Applied and Environmental Microbiology*, *79*(2), 601–609. https://doi.org/10.1128/AEM.02728-12

Sandai, D. (2012). Rapid Diagnosis of Leptospirosis by Multiplex PCR. In *Article in Malaysian Journal of Medical Sciences*. https://www.researchgate.net/publication/236267420

Thaipadungpanit, J., Wuthiekanun, V., Chantratita, N., Yimsamran, S., Amornchai, P., Boonsilp, S., Maneeboonyang, W., Tharnpoophasiam, P., Saiprom, N., Mahakunkijcharoen, Y., Day, N. P. J., Singhasivanon, P., Peacock, S. J., & Limmathurotsakul, D. (2013). Short report: Leptospira species in floodwater during the 2011 floods in the bangkok metropolitan region, Thailand. *American Journal of Tropical Medicine and Hygiene*, *89*(4), 794–796. https://doi.org/10.4269/ajtmh.13-0124

Zulkifli, N. F., Wan, S. J., Neela, V. K., Chee, H. Y., Masri, S. N., Al-Obaidi, M. M. J., & Desa, M. N. M. (2018). Detection of leptospira species in environmental samples by amplification of 16S rRNA and rpoβ genes. *Sains Malaysiana*, *47*(8), 1795–1800. https://doi.org/10.17576/jsm-2018-4708-18