MOLECULAR DETECTION OF *LEPTOSPIRA* SPP. IN WATER FROM WET MARKETS IN KELANTAN

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CERTIFICATION

It is hereby certified that we have read this research paper entitled '**Molecular detection of *Leptospira* spp. in water from wet markets in Kelantan**' by Amirulsyafiq bin Azmir and in our opinion it is satisfactory in terms on scope, quality and presentation as partial fulfilment of the requirement for the course DVT 5436 - Research Project.

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Thank You

DEDICATIONS

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving parent, who always support my journey throughout this project.

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List of abbreviations

PCR : Polymerase Chain Reaction

LPS : Lipopolysaccharides

EMJH : Ellinghausen–McCullough–Johnson–Harris

DNA : Deoxyribonucleic acid

PBS : Phosphate Buffer Saline

GSB : Gel Sample Buffer

MAT : Microscopic Agglutination Test

ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine in partial requirement on the course DVT 5436 - Research Project

Leptospirosis is a worldwide zoonosis caused by spirochaetes from the genus Leptospira. The disease can be transmitted through direct contact with urine of a reservoir host or indirect contact through soil and water contaminated with urine from a reservoir host. A wet market is a place where fresh meat and other needs are sold. An environment with ample food increases the risk of leptospira contamination by attracting rodents. According to the findings from previous studies, wet market workers in Kelantan had a high rate of leptospirosis seroprevalence. In the present investigation, a cross-sectional study was conducted to detect presence of *Leptospira* spp. in water samples collected from wet markets in Kelantan. A total of 60 water samples were collected and polymerase chain reaction was carried out to detect the presence of leptospira DNA in water samples. As for results, all the water samples were negative for *Leptospira* spp.. In conclusion, Leptospira spp. were not detected in water samples collected from wet markets in Kelantan.

Keywords: Kelantan, Leptospira spp., Molecular detection, Water sample, Wet markets.

ABSTRAK

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

Leptospirosis ialah zoonosis di seluruh dunia yang disebabkan oleh spirochaetes daripada genus Leptospira. Penyakit ini boleh berjangkit melalui sentuhan langsung dengan air kencing perumah takungan atau sentuhan tidak langsung melalui tanah dan air yang tercemar dengan air kencing dari perumah takungan. Pasar basah ialah tempat menjual daging segar dan keperluan lain. Persekitaran yang sesuai dengan makanan yang mencukupi meningkatkan risiko jangkitan leptospirosis dengan menarik tikus. Menurut penemuan, pekerja pasar basah di Kelantan mempunyai kadar seroprevalens leptospirosis yang tinggi. Dalam penyiasatan ini, kajian keratan rentas telah dijalankan untuk mengesan kehadiran Leptospira spp. dalam sampel air. Sebanyak 60 sampel air telah dikutip dari pasar basah terpilih di Kelantan. Kaedah pengesanan molekul digunakan dalam kajian ini untuk mengesan kehadiran *Leptospira* spp, dalam air dan primer yang digunakan ialah 16sRNA. Bagi keputusan, kesemua sampel adalah negatif untuk *Leptospira* spp. Kesimpulannya, *Leptospira* spp. tidak dikesan dalam sampel air yang dikutip dari pasar basah di Kelantan.

Kata kunci : Kelantan, *Leptospira* spp., Pengesanan molekul, Pasar basah, Sampel air.

1.0 INTRODUCTION

Leptospirosis is a worldwide zoonosis caused by spirochaetes from the genus Leptospira. There are 65 known *Leptospira* spp that can categorized as pathogenic, saprophytic, and intermediate in the genus *Leptospira* (Vincent *et al*., 2019). Leptospira spp. inhabit the renal proximal tubules of reservoir animals and chronically colonize the proximal tubules of kidney tissue. Live organisms are shed intermittently in the urine, contaminating the surrounding environment (Cordonin *et al*., 2020). The lifespan of *Leptospira* spp. is determined by a variety of parameters including pH, salinity, soil moisture, and temperature (Saito *et al.,* 2013).

The annual incidence of leptospirosis is estimated to be between 0.1 and 100 per 100,000 people in tropical regions. Epidemics with an incidence of more than 100 per 100,000 are common, especially during wet seasons and floods. In certain places in Malaysia, leptospirosis is endemic. Leptospirosis incidence varies by state over time, but the trend shows a progressive rise from 1.3 to 25.9 occurrences per 100,000 people. Kelantan, a Malaysian state in the East Coast, is frequently flooded as a result of heavy rainfalls. The number of leptospirosis cases surged after the catastrophic flooding in Kelantan in 2014, with a total of 1229 cases, more than double the number of cases before the floods (Mohd-Radi *et al*, 2018).

A wet market is a place where people may buy and sell fresh meat and other needs. A good habitat with ample food has a high chance of attracting rodents, which can increase the risk of environmental contamination with leptospira, thereby increase human incidence of leptospirosis. According to a study by Rahman *et al* , wet market workers in Kelantan had a high rate (36.4%) of leptospirosis seroprevalence (Rahman *et al.,* 2018). Indirect exposure to leptospira contaminated water could be a

contributing factor for the high seroprevalence of leptospirosis among wet market laborers. Therefore, this study aims to detect and characterize leptospires in water from selected wet markets in Kelantan by a molecular detection method.

2.0 PROBLEM STATEMENT

Many rodents are found to be habitat in Kelantan's wet markets, and a recent research study indicated a significant leptospirosis seroprevalence among employees in Kelantan's wet markets (Rahman *et al*, 2018). Yet, the source of infection that contributes to a high seroprevalence of leptospirosis among wet market workers in Kelantan is unknown. Studies by Radi *et al*., (2018) and Rahman *et al*., (2020) suggested that the source of infection for leptospirosis could be from stagnated contaminated water in wet markets. Therefore, this study aims to investigate the presence of leptospira by a molecular detection method from water samples collected from the wet market in Kelantan thereby to suggest source of contamination.

3.0 RESEARCH QUESTIONS

What is the occurrence of *Leptospira* spp. presence in water from selected wet markets in Kelantan?

4.0 RESEARCH HYPOTHESIS

Leptospira spp. can be detected in water from wet markets in Kelantan.

5.0 RESEARCH OBJECTIVES

To detect *Leptospira* spp. in water collected from wet markets in Kelantan.

6.0 LITERATURE REVIEW

6.1 Morphology of *Leptospira* spp.

Leptospires are filamentous bacteria with small spirals and hook-shaped ends that are thin, flexible, and filamentous (0.1 to 0.2 m broad and 6 to 12 m long). Leptospires are motile, which means they writhe and flex while rotating around their long axis (Greene, 2012). *Leptospira interrogans sensu lato*, which included all pathogenic strains, and *Leptospira biflexa sensu lato*, which included all saprophytic strains found in the environment, were traditionally divided into two species. Based on antibody reactivity to distinct carbohydrate moieties in their outer-membrane LPS, leptospires were classified as diverse serovars within these two groupings. (Greene, 2012).

6.2 *Leptospira* spp. in environment

Saprophytic organisms are found in natural water sources and do not generally cause illness (Benacer *et al*.,2013). Pathogenic and intermediate *Leptospira* spp. inhabit in the renal proximal tubules of the reservoir animals and live organisms are intermittently shed through urine contaminating the surrounding environment. They can survive in a favorable warm humid atmosphere for several weeks and months until contact with new host. In leptospirosis endemic countries rodents are the main source for primary vector that contaminate environment and other livestock animals such as cattle, buffaloes, goats also identified as potential reservoir animals (Rahelinirina *et al*., 2019).

6.3 Detection and identification of *Leptospira* spp. from environmental samples

PCR and gel electrophoresis were used as molecular detection and identification of *Leptospira* spp. For culture and isolation, Ellinghausen McCullough Johnson Harris (EMJH) medium were utilised. For seven days up to several weeks, the inoculated medium were incubated aerobically at 30°C in dark. Then inoculated medium needs to be examined under dark field microscope to check whether there are any spirochetes (Benacer *et al.*, 2013).

7.0 MATERIAL AND METHODOLOGY

7.1 SAMPLE COLLECTION

Six wet markets in Kelantan were randomly selected for sampling in this study (Table 1). A total of 60 water samples were collected from the selected wet markets. The water samples were collected based on most preferable criteria for *Leptospira* spp., contamination and survival such as; i) stagnated water, ii) water puddles, and iii) water near the garbage sites. The water samples were collected into a sterile specimen container volume of 40 ml for each sample. The samples were kept in the at 4°C for further analysis.

Table 1: Selected wet markets for sample collection in three districts of Kelantan

Name of wet market	District of Kelantan	Number of samples
Pasar Wakaf Che Yeh	Kota Bharu	10
Pasar RTC	Kota Bharu	10
Pasar Siti Khadijah	Kota Bharu	10
Pasar Kubang Pasu	Kota Bharu	3
Pasar Taman Bendahara	Kota Bharu	7

Pasar Pasir Mas	Pasir Mas	10
Pasar Jelawat	Bachok	10
	Total samples	60

7.2 DNA EXTRACTION

Geneaid gSYNC™ DNA Extraction Kit Quick Protocol was used for rapid extraction of *Leptospira* DNA from 1 ml of water sample according to manufacturer's instructions. In the extraction procedure, 1 ml of water sample was pipetted into the 1.5 ml sterile microcentrifuge tube. The tube was centrifuged at 14,000 rpm for 5 minutes and the supernatant was discarded carefully without disturbing to the pellet at the bottom of the microcentrifuge tube. Phosphate buffered saline (PBS) 200 µl was added into the microcentrifuge tube followed by 20 µl of proteinase K. The microcentrifuge tube was incubated at 60 °C for 5 minutes. GSB 200 µl was added into the tube and the tube were shook vigorously by vortex for 30 seconds. The tube was placed in water bath incubator again for 5 minutes. The 200 µl of absolute ethanol were added into the tube and the tube was shook vigorously by vortex for 30 seconds. GS column was placed in 2 ml of collection tube and 500 µl of mixture from 1.5 ml microcentrifuge tube were transferred into the GS column. The GS column was centrifuged at 14,000 rpm for 1 minute and the flow through in the collection tube were discarded. The same collection tube was used again with the GS column, 400 µl of WI buffer was added into the GS column and was centrifuged at 14,000 rpm for 1 minute. The flow through in the collection tube were discarded, 600 µl of wash buffer was added into GS column and was centrifuged at 14,000 rpm for 1 minute. The flow through was discarded and the tube was centrifuged again for 3 minutes at 14,000 rpm. The GS column was transferred into a new sterile 1.5 ml microcentrifuge

tube and 100 µl of pre-heated elution buffer was added into the GS column. The tube was centrifuged at 14,000 rpm for 1 minute. The GS column was discarded and the microcentrifuge tube with eluted DNA was stored in freezer at -20°C until further analysis.

7.3 POLYMERASE CHAIN REACTION

Two sets of primers targeting the 16S rRNA and LipL32 genes were used to detect presence of leptospira DNA as stated in Table 2. Amplification of isolated DNA was carried out in a total of 25 µl volume which consist of 12.5 µl of PCR master mix, 2.5 µl of forward and reverse primers, and 10 µl of DNA template. Positive and negative controls were included for each experiment. PCR amplification was initiated at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 58°C for 45 seconds, DNA extension at 72°C for 30 seconds, and a final extension step at 72°C for 6 minutes with final hold at 4°C in a thermal cycler (Bio Rad).

Table 2 : Primers used for current study

Primer	Primer sequence (5'-3')	Target gene	PCR product size
16S rRNA (forward)	CATGCAAGTCAAGCGGAGTA	16S rRNA	541 bp
16S rRNA (reverse)	AGTTGAGCCCGCAGTTTTC	16S rRNA	
LipL32 (forward)	GTCGACATGAAAAAACTTTCGATTTTG	LipL32	756 bp
LipL32 (reverse)	CTGCAGTTACTTAGTCGCGTCAGAAGC	LipL32	

7.5 GEL ELECTROPHORESIS AND GEL DOCUMENTATION

The amplified products were evaluated by agarose gel electrophoresis. 2% (w/v) agarose gel was prepared by mixing 1.2 g of agarose powder in 60 ml of TBE buffer in a Schott bottle and microwaved for 2 minutes until the agarose was completely dissolved. Agarose solution was allowed to cool down and 0.1 µl of Midori Green dye was added into the solution. The agarose solution was then poured into the gel tray with well comb in place. The gel was placed at room temperature for 20 minutes until it had completely solidified.

Once solidified, the agarose gel was placed into the electrophoresis tank, which was covered with 10% (v/v) TBE buffer. 5 µl of 100 bp DNA ladder was loaded into the first lane of the gel. The PCR products were then loaded to the additional wells of the gel, which the last two wells were loaded with positive control (*L. interrogans*) and negative control (distilled water). The electrophoresis gel was then run at 100 V for 40 minutes. The DNA fragment was then visualized using GelDocTM EZ Imager which the DNA fragment was appeared as band on the gel. By using molecular weight 100 bp DNA ladder as the guide, the size of the DNA products was determined.

8.0 RESULTS

8.1 PCR 16S rRNA primer

A total of 60 water samples were collected from six wet markets in Kelantan. Amplification of the 16S rRNA of *Leptospira* spp. showed that all the samples were negative for PCR. Figure 1 shows PCR result for sample 1-24.

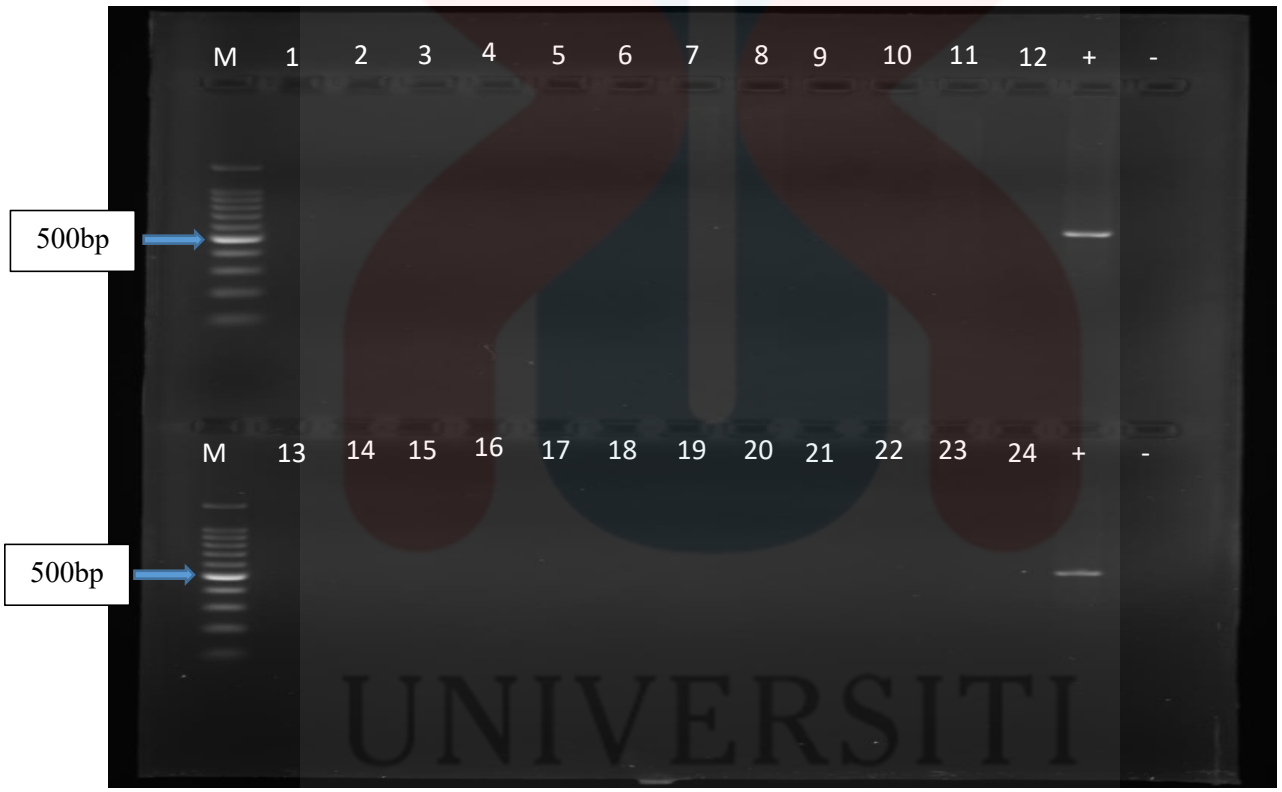

Figure 1: PCR results for samples 1-24 of *Leptospira* spp.

Lane M: 100bp DNA ladder, lane 1-10: water samples from Pasar Siti Khadijah, lane 11-13: water samples from Pasar Kubang Pasu, lane 14-23: water samples from Pasar Jelawat, lane 24: water samples from Wakaf Che Yeh, lane (+): positive control, and lane (-): negative control.

Figure 2 shows PCR result for samples 25-34, all of the samples were revealed as negative.

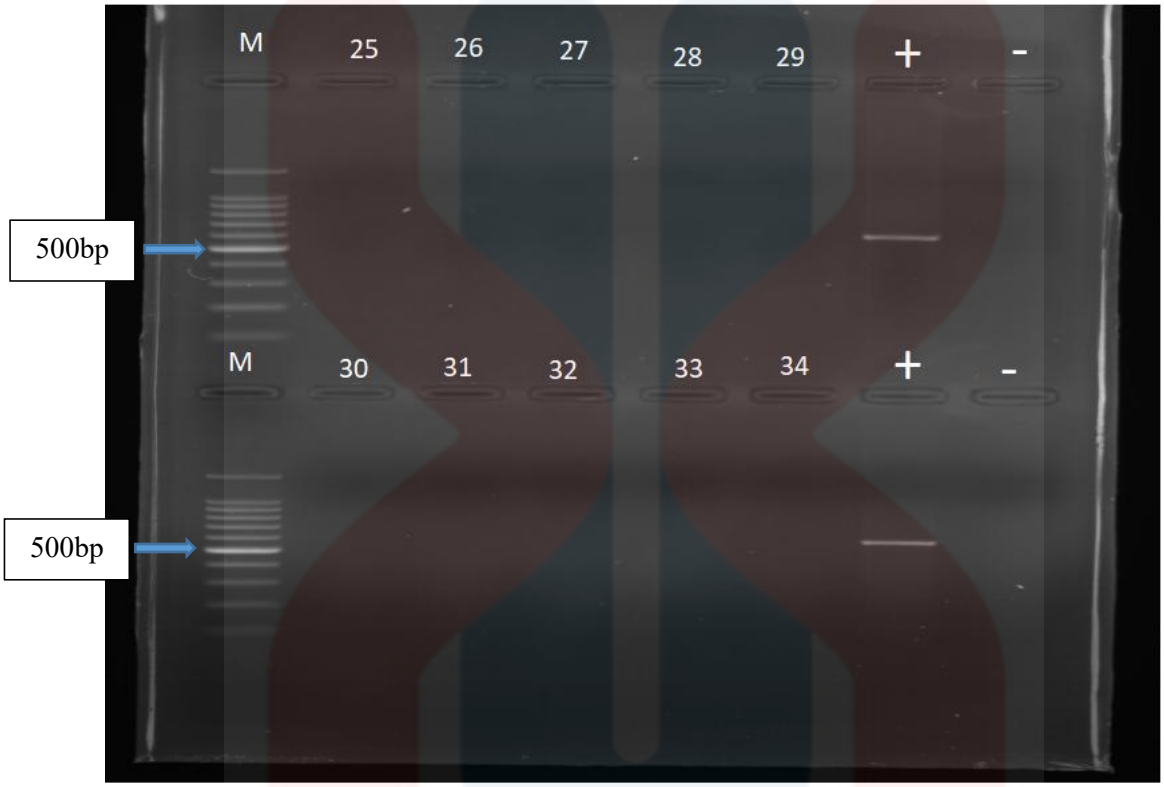

Figure 2: PCR results for samples 25-34 of *Leptospira* spp.

Lane M: 100bp DNA ladder, lane 25-33: water samples from Pasar Wakaf Che Yeh, lane 34: water samples from Pasar RTC, lane (+): positive control, and lane (-): negative control

Figure 3 shows PCR result for samples 35-58 and all the samples were gave negative for PCR.

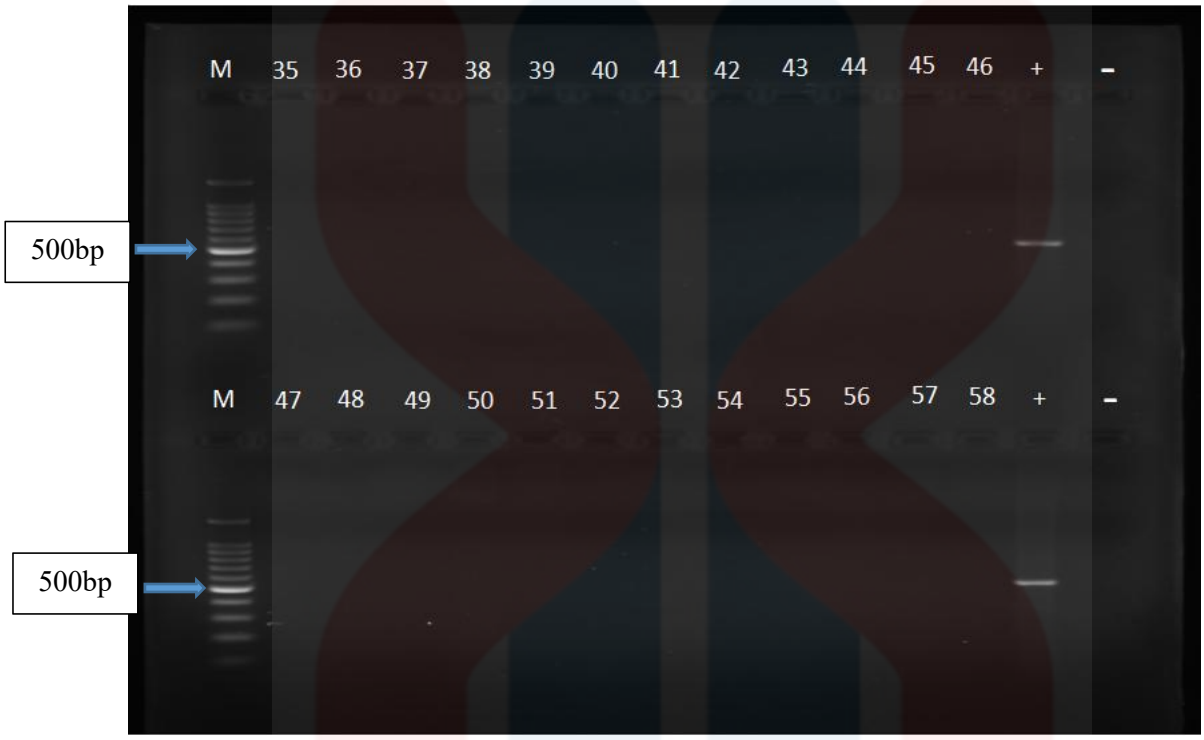

Figure 3: PCR results for samples 35-58 of *Leptospira* spp.

Lane M: 100bp DNA ladder, lane 35-43: water samples from Pasar RTC, lane 44-53: water samples from Pasar Pasir Mas, lane 54-58: water samples from Pasar Taman Bendahara, lane (+): positive control, and lane (-): negative control

Figure 4 shows PCR result for samples 59 and 60 both samples were negative.

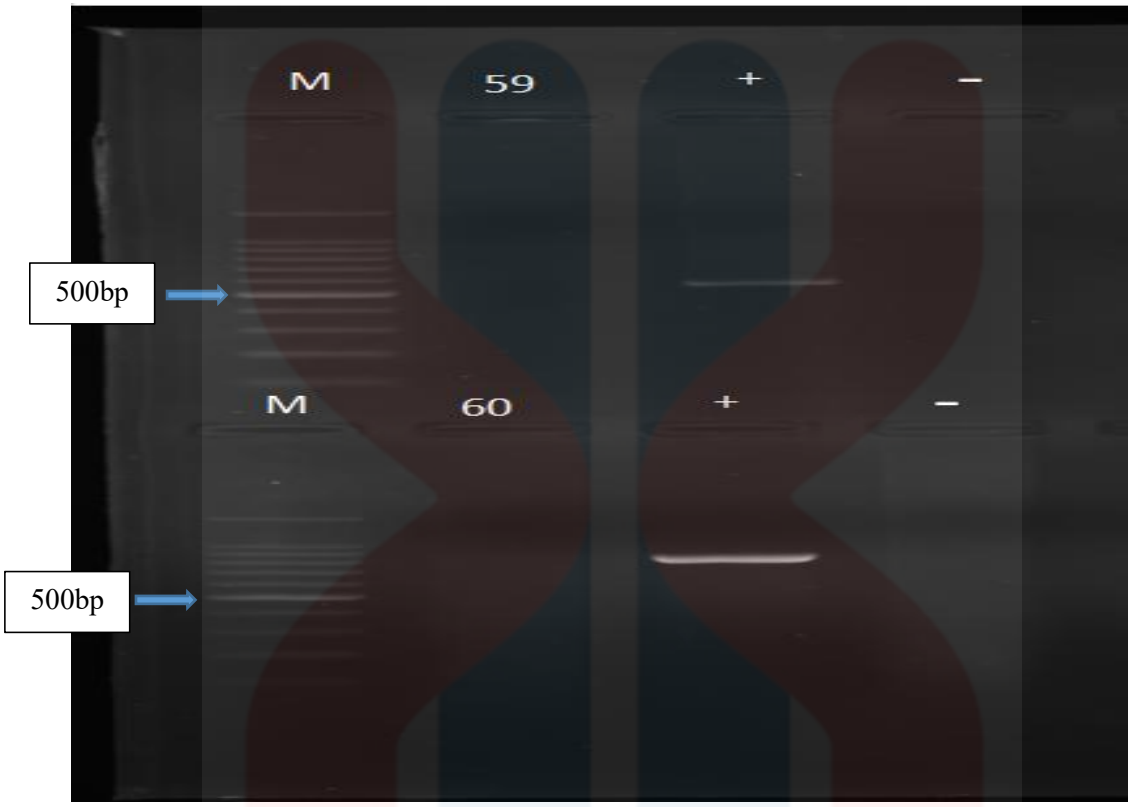

Figure 4: PCR results for samples 59-60 of *Leptospira* spp.

Lane M: 100bp DNA ladder, lane 59-60: water samples from Pasar Taman Bendahara, lane (+): positive control, and lane (-): negative control

8.2 LipL32 PCR

All 60 water samples were tested negative by using primer LipL32.

9.0 DISCUSSION

From this study, it was found that all the water samples collected from selected wet markets were negative for *Leptospira* spp. This result is quite surprising because there was a significant level of leptospirosis seroprevalence among wet market workers in Kelantan (Rahman *et al.,* 2018). Negative results of the current study may be due to several factors which can lead to false negatives and those factors are explained followingly.

The first suggestive influencing reason is leptospira free water samples collected for the current study. Leptospires are known to survive in stagnant water because this water can be contaminated with urine from a reservoir host (Haake & Levett, 2015). During the sample collection, the water sample may be free from any leptospires because it could not be contaminated with the urine of reservoir host or surrounding reservoirs may be not carriers for leptospira. This can lead to negative result of leptospires in the water samples of current study.

Samples collection at the wet markets during market closing time also can be one of the factors for negative results. The water may be already mix with detergents, because workers in the market use detergents to clean their premises at closing. Water mixed with detergent may kill the leptospira present in water and according to Levett (2001), leptospires are inhibited at low detergent concentrations. the presence of detergents in wastewater is considered to have affected leptospire survival in sewers.

Another influencing factor for negative results can be the improper sample storage after collection and during dispatch to the laboratory. During the study, the samples

were stored at 4°C for a long time. This may result DNA degradation during transportation and storage which could end up with false negative results for PCR.

The environmental temperature during sample collection time frame can be another suggestive factor. During months of sample collection, being a tropical region, there may be hot weather in Kelantan which can lead to limited survival of the organism in the collected water samples. Leptospires cannot survive in temperatures of less than 7.1°C or greater than 34°C (Ehelepola *et al*., 2019).

Detection method of *Leptospira* spp. also can be one of an influencing factors towards false negatives. This research was used molecular detection with primers targeting 16s rRNA and LipL32 gene. The used DNA extraction method, PCR protocol might not be sensitive enough to detect presence of small number of leptospira DNA in water samples. During samples collection, there are a lot of other bacteria in the water. It could be there are a few or none leptospira in the water sample. So, during DNA extraction and molecular detection, PCR the most DNA extracted is maybe other microorganisms (Wynwood et al., 2014).

Other alternative detection methods of leptospira are culture isolation and Microscopic Agglutination Test (MAT). Combination of other detection methods with current PCR might be a better solution to overcome false negatives.

10.0 CONCLUSION

In conclusion, PCR was used to examine 60 water samples. *Leptospira* spp. were found in none of the samples. The sample collection and the direct molecular detection approach are the elements that contribute to negative findings. It's possible that the water samples used in this study were contaminated with other prevalent bacteria, making DNA extraction from water more difficult. The water samples might have had a diverse population of bacteria and other contaminants, making DNA extraction and PCR reactions difficult. The failure of molecular screening might be attributed to a diverse population of bacteria and other contaminants, according to these studies. To properly discover *Leptospira* spp. in water, certain additional procedures may be required to improve on molecular screening.

11.0 RECOMMENDATION

Based on the findings from this study, the following recommendations for future studies are suggested. Instead of collection of water samples only, other environmental samples such as soil and rodents from specific environments can be consider as suggestive samples. It is recommended to avoid collecting water sample that mixed with detergents like inhibitors. The detection method of leptospira need to be expanded by including MAT and culture isolation. Other recommendation is to increase the sample size and expand the geographic distribution of sample collection thereby to reduce the chances of missing false negatives. Further proper sample dispatch and storage (-20 ^{0}C) in the laboratory is suggested to prevent DNA degradation.

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