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RESEARCH ARTICLE

Detection of *Leptospira* in environmental samples of wet markets and paddy fields in Penang, Malaysia

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ABSTRACT

Leptospirosis, caused by the *Leptospira* spp., is endemic in humid subtropical and tropical climates. Several environmental settings can harbour the survival of *Leptospira;* hence, continuous surveillance of the high-risk areas is critical. This study was conducted to detect the presence of *Leptospira* in wet markets and paddy fields in Penang, Malaysia. A total of 62 soil and water samples were collected aseptically from three wet markets and two paddy fields. The samples were cultured twice on the sampling day and after two weeks of storage in the dark at ambient temperature. All environmental samples and positive cultures were subjected to deoxyribonucleic acid (DNA) extraction and polymerase chain reaction (PCR) targeting 16S rRNA, *lipL32* and *rrs* genes. 16S rRNA-positive samples were further sent for sequencing. Of 62 samples, 21 (21/62, 33.9%) tested positive via culture and PCR. Wet markets significantly had a higher detection rate of leptospires (29%) than paddy fields (4.8%). Four *Leptospira* species (*L. selangorensis, L. dzoumogneensis, L. mtsangambouensis*, and *L. meyeri*) were identified in wet markets and three (*L. meyeri*, *L. wolffii,* and *L. kmetyi*) in paddy fields. Storing the environmental samples for two weeks before isolation yielded a higher positivity (11/14) than culturing samples on the sampling day (6/14). The presence of *Leptospira* in the environment of wet markets and paddy fields in Penang although primarily intermediate and saprophyte indicates the crucial need to implement preventive measures to prevent the possibility of leptospirosis infection among the workers.

Keywords: *Leptospira*; environment; wet market; paddy field.

INTRODUCTION

Leptospirosis, caused by the pathogenic *Leptospira* species, is a common zoonotic infection worldwide (Said *et al*., 2023). Although a number of wild and domestic animals can harbour the infection, rodents remain the primary reservoir host of *Leptospira* (Ellis, 2015). Rodents usually experience a mild or asymptomatic form of infection and can shed the pathogen in their urine for a significant period of time. Once shed, *Leptospira* could persist in environmental water and moist soil for months. During this period, contaminated water and soil are the major sources of human infection, primarily through direct or indirect contact with contaminated environments. Therefore, in addition to occupations that involve direct contact with reservoir animals, such as rodent control workers, veterinarians, and butchers, occupations that might involve exposure to rodent-infested environments, including farmers, town cleaners, and garbage collectors, are also considered high-risk occupations (Samsudin *et al*., 2015).

In Malaysia, the annual incidence of leptospirosis from 2010 to 2020 ranged from 8.63 to 17.2 cases per 100,000 population (Philip & Ahmed, 2023). The tropical and humid climate has caused endemic leptospirosis in Malaysia, with occasional outbreaks associated with floods (Radi *et al*., 2018). *Leptospira* has been reported in various environmental settings in Malaysia, including the National Service Training Centers (NSTCs) in Kelantan, Terengganu (Ridzlan *et al*., 2010), and Sarawak (Pui *et al*., 2017a), wet markets and recreational lakes in Kuala Lumpur, Selangor, and Johor (Benacer *et al*., 2013a), national parks in Sarawak (Pui *et al*., 2015), recreational areas in Peninsular Malaysia (Azali *et al*., 2016; Neela *et al*., 2019; Zaki *et al*., 2020; Yap *et al*., 2021), residential areas of patients with leptospirosis in Kelantan (Ali *et al*., 2018), agricultural areas in Terengganu (Halim *et al*., 2019), Sarawak (Pui *et al*., 2017b), and livestock farms (Kamaruzaman *et al*., 2022).

Currently, the information on the presence of leptospires in wet market and paddy field environments in Malaysia is scarce yet important as these locations are favourable infestation sites for rodents. Most previous studies were conducted at recreational sites in states with higher incidences of leptospirosis, such as Kelantan, Selangor, and Sarawak. The detection of *Leptospira* in rodents infesting wet markets (Benacer *et al*., 2013b; Kamaruzaman *et al*., 2022) indicates a high possibility of its presence in the environment. Paddy fields' damp and water-logged environment creates suitable conditions for leptospiral survival. Several studies hypothesised that *Leptospira* spp. originates from the soil and is resuspended during rainfall or flood events (Bierque *et al*., 2020; Yanagihara *et al*., 2022). The existence of pathogenic and intermediate *Leptospira* spp. in

the field may threaten paddy farmers, especially when they carry out rice farming activities barefoot and without proper clothing. Furthermore, the number of studies conducted in paddy fields worldwide is limited, narrowing our knowledge about *Leptospira* in paddy field environments. Hence, this study was conducted to detect the presence of *Leptospira* spp. in wet markets and paddy fields in Penang, Malaysia, as well as to identify its species. The number of leptospirosis cases in Penang was relatively low compared to that in other states of Malaysia (Philip & Ahmed, 2023). However, many wet markets in Penang operate on a daily and weekly basis. Paddy fields in Penang are also tourist attractions. Therefore, it is crucial to monitor the presence of *Leptospira* spp. in these locations. The information gained from this study is essential for addressing the risk of leptospirosis in these sites. The effect of storing the environmental samples in the dark at ambient temperature for two weeks on the successful isolation of *Leptospira* was also investigated.

MATERIAL AND METHODS

Sample collection

The state of Penang (5.285153°N, 100.456238°E) is located in northwest Malaysia comprising mainland Seberang Perai and Penang Island. Samples were collected from three wet markets (market A, market B and market C) and one paddy field in Penang Island (paddy field A) in December 2023. Samples were also collected in one paddy field (paddy field B) in Seberang Perai in the same month. The sampling sites were selected based on convenience sampling. Five water and five soil samples were collected from each wet market, whereas eight water and eight soil samples were collected from each paddy field (Table 1). In wet markets, water samples were collected from drain water within and surrounding the markets while soil samples were collected near the drain water surrounding the markets and in waste disposal areas. For paddy fields, water samples were collected from the drain water and inside the paddy field, whereas soil samples were collected from the boundaries. Approximately 50 mL of water and 25 g of soil were collected. Water and soil samples were collected using sterile 20 mL syringes and spatulas, respectively and transferred into sterile 50 mL Falcon tubes. A 20 mL sterile syringe was used to collect each water sample at least three times to cover a larger sampling area, as *Leptospira* might be unevenly distributed in the water due to the constant flow of water. Only the outside of the syringe barrel was held to minimise potential contamination, and there was no contact with the tip and the inside of the barrel during sample collection. For soil sample collection, the spatula was thoroughly sterilised with 70% ethanol before and after sample collection and a different spatula was used for each sample. All the samples were transported to the laboratory at ambient temperature.

Sample processing

The sample processing methodology used in this study is shown in Figure 1. Each water sample was divided into 20 mL and 30 mL in separate 50 mL sterile Falcon tubes following the previous study (Narkkul *et al*., 2020). The 20 mL aliquot was used for deoxyribonucleic acid (DNA) extraction and polymerase chain reaction (PCR) as the molecular method has higher sensitivity to detect the small amount of leptospiral DNA while the 30 mL aliquot was used for isolation as culture requires high concentration of leptospires to grow in culture medium. The 20 mL aliquot was stored at -20°C until further processing for DNA extraction, while the 30 mL aliquot was stored in the dark at ambient temperature and used for two batches of culture. *Leptospira* was isolated according to a previous study (Flores *et al*., 2020), with some modifications. Approximately 3 mL of water sample from the 30 mL aliquot was filtered through a sterile membrane filter (0.22 µm) and inoculated into 5 mL of Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. For soil samples, 3 g of each soil sample was transferred to a 15 mL sterile Falcon tube and mixed homogeneously with 10 mL of sterile distilled water by inverting the tube several times. The suspensions were left to settle for one hour before 1 mL of supernatant was filtered through a sterile membrane filter (0.22 µm) and inoculated into 5 mL of EMJH medium. A previous study (Narkkul *et al*., 2020) reported that 2–4 weeks of storage of water samples in the dark at ambient temperature before culturing can improve the isolation of *Leptospira* spp. from the samples. Hence, a second batch of culture was also prepared in this study for water and soil samples using the same methods (first isolation) after storing the samples at room temperature in the dark for two weeks. All cultures were incubated at 28–30°C for three months and observed weekly under a dark-field microscope for the growth of *Leptospira.*

Molecular analysis

Culture-positive samples were subjected to DNA extraction and PCR. DNA extraction was performed on both environmental samples and positive cultures using the PrimeWay Soil DNA Extraction Kit

Table 1. Number of samples collected from each wet market and paddy field

Sampling site	Water samples	Soil samples	Total
Market A	5	5	10
Market B	5	5	10
Market C	5	5	10
Paddy field A	8	8	16
Paddy field B	8	8	16
Total	**31**	**31**	**62**

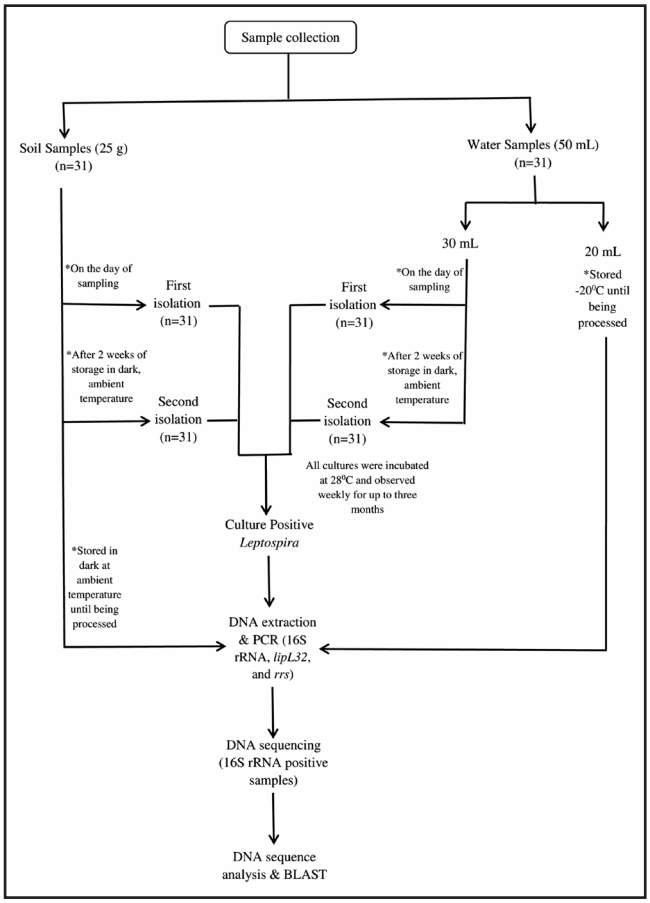

Figure 1. Flowchart of sample processing used in this study.

Table 2. Primers used in PCR

Target gene	Primer sequence 5' to 3'	Amplicon size (bp)	Reference
16S rRNA (all species)	F:GGCGGCGCGTCTTAAACATG R:TTCCCCCCATTGAGCAAGATT	331	(Cetinkaya *et al.*, 2000)
rrs (saprophyte)	F:AGAAATTTGTGCTAATACCGAATGT R:GGCGTCGCTGCTTCAGGCTTTCG	240	(Murgia *et al.*, 1997)
lipL32 (pathogenic)	F:CGCTGAAATGGGAGTTCGTATGATT R:CCAACAGATGCAACGAAAGATCCTTT	423	(Vein *et al.*, 2012)

(1st BASE) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until further processing. Three sets of primers (Table 2) were used to amplify the leptospiral genes (16S rRNA, *lipL32*, and *rrs*) in each of the extracted DNA samples (62 environmental samples and 13 positive cultures including positive and negative control). The primer set for the 16S rRNA gene is universally applicable for the detection and species identification of *Leptospira* spp. (Azali *et al*., 2016). The *lipL32* gene specifically targets pathogenic *Leptospira,* which is highly conserved among pathogenic *Leptospira*, distinguishing pathogenic and non-pathogenic strains (Ahmed *et al*., 2009; Azali *et al*., 2016; Hoke *et al*., 2008). The *rrs* gene is specifically designed to detect saprophytic *Leptospira*.

The PCR reactions were performed in a final volume of 25 µL containing 12.5 µL of DreamTagTM Green PCR Master Mix, 1 µL of 10 pmol each of the forward and reverse primers, and 10 µL of DNA samples extracted directly from the environmental samples or 5 µL of DNA samples extracted from the positive cultures. The remaining volume was filled with sterile, distilled water. For each reaction, positive controls from *Leptospira* culture positive for each target gene were used while sterile distilled water was used as a negative control.

PCR amplification was carried out using the following conditions for all three sets of primers (Pui *et al*., 2015): an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing for 30 sec at 55°C, an extension at 72°C for 1 min, and a final extension at 72°C for 5 min. A preliminary study was conducted to determine whether PCR amplification conditions were effective for all three primer sets. Because the PCR amplification conditions worked for the three sets of primers, similar PCR amplification conditions were used for all three sets of primers. PCR products were analysed by gel electrophoresis on a 1.5% agarose gel in 1× TBE buffer at 100 V for 120 min. 16S rRNA-positive cultures were sent for Sanger sequencing (Bio Basic Asia Pacific) to identify *Leptospira* species. The DNA sequence data were analysed and trimmed using MEGA 11 (Tamura *et al*., 2021) and searched for homology using BLAST in NCBI GenBank.

Statistical analysis

To determine if there are statistically significant differences in the positivity rates between wet market and paddy field and between culture and PCR, statistical analyses were conducted using SPSS version 30 and Fisher's exact test. A p-value of less than 0.05 is considered statistically significant.

RESULTS

Positivity of *Leptospira* in environmental samples in wet markets and paddy fields

A total of 62 water and soil samples were collected from three wet markets and two paddy fields in Penang. From the 62 samples, 21 (21/62=33.9%) were positive for *Leptospira* comprising culture only (n=1), PCR only (n=7) and both culture and PCR (n=13) (Table 3) ($p < 0.001$). One positive culture was heavily contaminated and attempts were made to purify the culture by filtering the culture through a sterile 0.22 µm membrane filter to a new fresh EMJH medium, however yielded no growth of *Leptospira*; therefore, DNA extraction was not performed. Water samples were positive in 22.6% (14/62), whereas soil samples were positive in 11.3% (7/62). Wet markets had a higher detection rate of leptospires (29%) than paddy fields (4.8%) (Figure 2 and Table 3) with a significant p-value ($p < 0.001$).

Influence of sample storage period on the positivity of culture

Similar to the observation in a previous study (Narkkul *et al*., 2020), storing the samples in the dark at ambient temperature for two weeks before the isolation of leptospires influenced the culture positivity. A higher number of samples were culture-positive on the second isolation. Among the 14 culture-positive samples, 57%

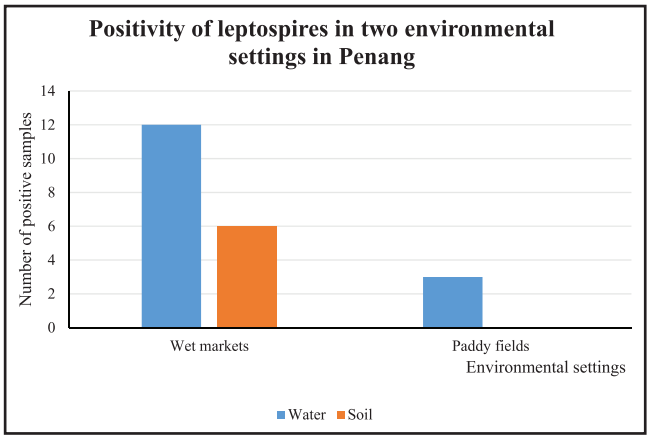

Figure 2. Number of environmental samples in wet markets and paddy fields. Wet markets showed higher positivity of leptospires compared to paddy fields.

Table 3. Samples positivity based on the type of environments and detection methods

Environments	Culture only	PCR only	Culture & PCR	Total number of positive samples	% (n=62)
Wet market	1	7	10	18	18/62 = 29%
Paddy field	–	–	3	3	3/62 = 4.8%
Total	1	7	13	21	21/62 = 33%

(8/14) were positive in the second isolation (Table 4) while only three samples were positive in the first isolation. The remaining three samples were positive in both first and second isolation (Supplementary 1: Result for wet markets and paddy fields). Overall, the second isolation (11/14) had a higher culture positivity rate than the first (6/14).

***Leptospira* species**

In this study, all samples positive for *rrs* (12 samples) and *lipL32* (two samples) genes were also positive for 16S rRNA except for two water samples which were positive for *rrs* gene only (Table 5). There were four water samples that were positive for 16S rRNA genes only. In total, only 18 samples were positive for 16S rRNA. Among 16S rRNA positive samples, only 12 samples had strong, thick bands and were sent for sequencing. The sequencing results revealed six possible *Leptospira* species circulating in the wet markets and paddy fields in Penang (Table 5). The three *Leptospira* strains isolated from paddy fields were identified as *L. meyeri*, *L. wolffii,* and *L. kmetyi*. The identified *Leptospira* strains isolated from wet markets were *L. selangorensis, L. dzoumogneensis, L. mtsangambouensis,* and *L. meyeri.*

Table 4. Positivity of leptospires based on the time of isolation

Culture	Number of positive sample (n=14)	Positivity, %
First isolation only *cultured on the day of sampling	3	21.4%
Second isolation only *cultured after two weeks of storage at dark, ambient temperature	8	57%
First and second isolation	3	21.4%
Total	14	100%

DISCUSSION

In Malaysia, leptospirosis has been commonly reported in Selangor, Kelantan, and Sarawak, whereas Penang has had fewer cases recorded from 2004 to 2021 (Philip & Ahmed, 2023). However, the figures could be underestimated because leptospirosis has symptoms similar to those of many other diseases, and therefore, there might be more unreported cases. This study is the first to report the presence of *Leptospira* and to determine the species of *Leptospira* isolates from environmental samples collected in wet markets and paddy fields in Penang, Malaysia. Additionally, the effect of the sample storage period before isolation on culture positivity was investigated to improve the recovery of leptospires from the environmental samples.

This study showed a comparatively similar positivity of *Leptospira* in wet markets (29%) and paddy fields (4.8%) to other environmental settings in Malaysia, such as livestock farms (16.9%) (Rahman *et al*., 2023), residential areas of patients with leptospirosis (42.8%) (Ali *et al*., 2018), recreational areas (33.5%) (Zaki *et al*., 2020), national parks (6.4%) (Pui *et al*., 2015), NSTCs (6.9%) (Ridzlan *et al*., 2010), and palm oil plantations (5%) (Halim *et al*., 2019). The presence of *Leptospira* in different locations in Malaysia is likely attributable to spatiotemporal factors, such as climate and season, location-based functionalities, and rodent populations. Most leptospirosis outbreaks occur in tropical and subtropical regions during the monsoon season, with the monsoon season having the highest sample positivity rate, followed by the post-monsoon and pre-monsoon seasons (Zala *et al*., 2018). In terms of location-based functionalities, the presence of *Leptospira* in places such as livestock

Table 5. PCR result and the identified *Leptospira* species based on 16S rRNA in this study

No.	Sample name	Environmental samples	Culture	Species	Group	Sequence similarity (%)
1	MAS3	–	+ (16S rRNA, *rrs*)	*L. mtsangambouensis*	Saprophytic	99.07
2	MAW1	+ (16S rRNA, *rrs*)	+ (16S rRNA, *rrs*)	*L. mtsangambouensis*	Saprophytic	99.07
3	MAW3	+ (16S rRNA)	NA	NA	NA	NA
4	MAW4	+ (16S rRNA)	NA	NA	NA	NA
5	MAW5	+ (16S rRNA)	NA	NA	NA	NA
6	MBS3	–	+ (16S rRNA, *rrs*)	NA	NA	NA
7	MBS4	–	+ (16S rRNA, *rrs*)	*L. dzoumogneensis*	Intermediate	98.80
8	MBW1	+ (16S rRNA, *rrs*)	NA	NA	NA	NA
9	MBW2	+ (16S rRNA, *rrs*)	+ (16S rRNA, *rrs*)	*L. meyeri*	Saprophytic	98.48
10	MBW4	+ (16S rRNA, *rrs*)	+ (16S rRNA, *rrs*)	*L. mtsangambouensis*	Saprophytic	98.18
11	MCS2	–	+ (16S rRNA, *rrs*)	*L. selangorensis*	Intermediate	99.09
12	MCS4	–	+ (16S rRNA, *rrs*)	*L. selangorensis*	Intermediate	99.39
13	MCS5	–	+ (16S rRNA, *rrs*)	*L. selangorensis*	Intermediate	98.78
14	MCW2	+ (16S rRNA)	NA	NA	NA	NA
15	MCW3	+ (*rrs*)	NA	NA	NA	NA
16	MCW4	+ (*rrs*)	NA	NA	NA	NA
17	MCW5	–	+ (16S rRNA, *rrs*)	*L. meyeri*	Saprophytic	98.48
18	PFAW1	–	+ (16S rRNA, *rrs*)	*L. meyeri*	Saprophytic	98.78
19	PFAW3	–	+ (16S rRNA, *lipl32*)	*L. kmetyi*	Pathogenic	97.92
20	PFAW7	–	+ (16S rRNA, *lipl32*)	*L. wolffii*	Intermediate	98.79

Note: M = market; PF = paddy field; W = water; S = soil.

farms, paddy fields, wet markets, recreational areas, and palm oil plantations can be due to occupational or leisure activities that expose people to the leptospiral habitat or *Leptospira*-contaminated urine. It is well known that rodents are the main reservoir of *Leptospira*; therefore, a higher rodent population in a particular area may suggest a higher presence of *Leptospira* and a higher risk of leptospirosis infection.

The presence of *Leptospira* in wet market environments in the present study is in accordance with previous studies (Azali *et al*., 2016; Benacer *et al*., 2013a). This study revealed that a wider species of saprophyte and intermediate *Leptospira* (*L. selangorensis, L. dzoumogneensis, L. mtsangambouensis*, and *L. meyeri*) circulating in wet market environments compared with only one pathogenic species (*L. alstonii*) identified in the previous studies (Azali *et al*., 2016; Benacer *et al*., 2013a). This study also first identified the presence of saprophytic *L. mtsangambouensis* and intermediate *L. dzoumogneensis* in Malaysia. Similar to the previous studies (Azali *et al*., 2016; Benacer *et al*., 2013a), pathogenic *Leptospira* (*L. interrogans*) was also found in the wet market in Peru (Ganoza *et al*., 2006). Several studies reported the presence of *Leptospira* spp. in rodents captured from wet markets (Benacer *et al*., 2013b; Kamaruzaman *et al*., 2022; Noh *et al*., 2024). Hence, the presence of leptospires in both rodents and environments in wet markets highlights the risk of leptospirosis among workers. In fact, several studies have reported the seroprevalence of leptospirosis among wet market workers in Malaysia (Samsudin *et al*., 2015; Rahman *et al*., 2018).

The positivity of *Leptospira* in paddy fields in the present study was similar (4.8%) to that in two previous studies conducted in Sarawak (5%) (Pui *et al*., 2017a) and Terengganu (5%) (Halim *et al*., 2019). Other countries also reported the presence of *Leptospira* in paddy fields with Indonesia (Setyaningsih *et al*., 2022) had a comparatively similar positivity (6.67%) while India (Archana *et al*., 2019; Zala *et al*., 2018; Lall *et al*., 2016) had a higher positivity (20% – 59%). This present study identified more species comprised of the three groups (saprophyte *L. meyeri*, intermediate *L. wolffii,* and pathogenic *L. kmetyi*) of *Leptospira* circulating in the paddy fields compared to only one pathogenic *Leptospira* species (*L. noguchii*) in Sarawak (Pui *et al*., 2017b). There is no information on *Leptospira* species in Terengganu. Similarly, *L. wolffii* and *L. kmetyi* were also reported in India (Lall *et al*., 2016). In addition to these two species, India also reported the presence of intermediate *L. licerasiae* and pathogenic *L. interrogans* in paddy field. The presence of pathogenic and intermediate *Leptospira* species in paddy field environments highlights the risk of leptospirosis infection to the paddy farmers. *L. kmetyi* was dominant in environmental samples collected from recreational areas in Peninsular Malaysia, indicating its role in leptospirosis outbreaks (Zaki *et al*., 2020).

The hygiene level of a particular wet market determines the presence of *Leptospira* in the rodent population. Rodents are major *Leptospira* carriers, allowing leptospires to proliferate in the renal tubules. In the present study, the wet market had a higher detection rate of *Leptospira* than the paddy fields. Although no pathogenic *Leptospira* was detected in the present study, and no study was conducted on rodents, which limits the connections with rodents, it still provides essential information on hygiene levels in these markets. This semi-closed space creates a humid environment with low aeration and protects leptospires from direct sunlight when they are shed. Meanwhile, *Leptospira's* survival in paddy fields is highly dependent on the climate. The hot climate contributes to high soil temperatures that drive out the water content in soil faster, and soil temperatures exceeding 34°C may kill leptospires in contaminated soil (Ehelepola *et al*., 2019; Yanagihara *et al*., 2022). It is interesting to observe that different *Leptospira* species were identified in wet markets and paddy fields in this study. Only *L. meyeri* was present in both types of environments. *L. meyeri* had been detected in rodents, water and soil samples in urban areas (Pui *et al*., 2017b), recreational areas (Azali *et al*., 2016) and residential areas (Benacer *et al*., 2013a), indicating that this *Leptospira* species is able to occupy a wide range of ecological niches. The remaining *Leptospira* species (*L. selangorensis*, *L. dzoumogneensis*, *L. mtsangambouensis*) identified in wet markets were newly identified *Leptospira* species (Vincent *et al*., 2019), hence, more studies are needed to determine the ecological niches that support the growth of these *Leptospira* species. *L. selangorensis* was first isolated from water collected in recreational areas (Vincent *et al*., 2019) while *L. mtsangambouensis* and *L. dzoumogneensis* were first isolated from Mayotte, an archipelago in the Indian Ocean (Vincent *et al*., 2019). *L. wolffii* and *L. kmetyi* detected in paddy field in this study were also detected in other environmental settings such as recreational areas (Zaki *et al*., 2020), forests (Neela *et al*., 2019) and residential areas (Ali *et al*., 2018) and in paddy fields in India (Lall *et al*., 2016) implying that these particular *Leptospira* species are able to adapt to the various environmental settings. According to a study conducted by Cosson and colleagues *L. interrogans* and *L. borgpetersenii* were frequently detected in humid and dry habitats, suggesting that different *Leptospira* species may have different habitat requirements (Cosson *et al*., 2014) and might explain the difference in the detected *Leptospira* species between wet markets and paddy fields. It is also noteworthy to observe that different *Leptospira* species were identified in the same type of environment in different geographical locations (Azali *et al*., 2016; Benacer *et al*., 2013a; Ganoza *et al*., 2006; Lall *et al*., 2016; Pui *et al*., 2017a). The reasons for this difference are beyond the scope of this study, however, it could be due to the differences in ecological factors such as climate, altitude and geomorphology among these areas that might influence the leptospiral diversity (Zhang *et al*., 2019).

In this study, not all samples positive for *rrs* were positive for 16S rRNA. Similarly, there were four samples positive for 16S rRNA and negative for both *rrs* and *lipL32* genes. Interestingly, the DNA of these samples were extracted directly from water. Environmental samples can vary widely in their composition, and PCR inhibitors such as humid acid, heavy metals and organic compounds might be present in the samples (Rock *et al*., 2010). The PCR inhibitors might affect the primers' efficiency in binding to the target genes. It could also be due to the low concentration of leptospiral DNA in these samples as all of these samples were culture-negative. The *rrs* gene used in this study is specifically for saprophyte *Leptospira*, however, there were four samples positive for *rrs* genes but gave intermediate (*L. dzoumogneensis* and *L. selangorensis*) species in 16S rRNA sequencing. These discrepancies might be due to the fact that the 16S rRNA gene is highly conserved among different bacterial species. While it has variable regions that can differentiate between species, these regions might not be sufficient to distinguish closely related species or strains, especially within a complex genus like *Leptospira*. There were five soil samples in which the DNA was extracted directly from the soil were PCR negative although these samples were positive by culture. This might be due to the way soil samples were processed during the extraction of DNA. Compared to the treatment of water samples, soil samples were treated more violently such as vortexing the samples due to their heterogeneous nature.

In the current study, the second batch of culture, which had two weeks of sample storage in the dark and at ambient temperature before isolation, resulted in higher positivity than the first batch of culture. This suggests that the *Leptospira* population in the initial environmental samples could be enhanced by incubating them in the dark at room temperature for a minimum of two weeks before isolation. The results of the current study were in accordance with a previous study conducted in Thailand (Narkkul *et al*., 2020), which showed an improvement in leptospiral isolation from contaminated water samples after storing the samples in the dark and at ambient temperature for 2–4 weeks before isolation in EMJH media.

The detection of *Leptospira* in the environment, particularly in paddy fields and wet markets, is vital for public health and occupational safety. Identifying pathogenic leptospires in these settings enables the early detection and prevention of leptospirosis outbreaks, which is crucial for protecting vulnerable populations, including paddy farmers, wet market workers, and visitors. Additionally, understanding the distribution and habitat preferences of different *Leptospira* species contributes to better epidemiological mapping and informs public health policies, aiding in effective control strategies.

CONCLUSION

The overall positivity for *Leptospira* was relatively high in both wet markets and paddy fields in Penang. Wet markets showed a higher detection rate of leptospires than paddy fields. This is the first study to report the presence of saprophytic *L. mtsangambouensis* and intermediate *L. dzoumogneensis* in the environment in Malaysia. To lower the risk of leptospirosis, it is crucial to implement preventive measures, including eradicating rodents, keeping the environment clean from any wastes and using protective clothing and footwear for paddy farmers. Furthermore, environmental samples should be stored in the dark at room temperature for at least two weeks before isolation.

This study has several limitations that should be addressed in future research. Increasing the sample size and expanding the sampling locations could provide a more comprehensive understanding of the presence of *Leptospira* in different environments. It is also recommended to consider sampling at different times of the year, such as during the monsoon season, to allow for a more comprehensive sampling strategy and conduct a comparative analysis across different time points. Further validation under various conditions is necessary to confirm the effects of sample storage on culture positivity. Additionally, whole-genome sequencing can be performed to identify *Leptospira* species with higher resolution and accuracy.

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Competing interests
There are no competing interests for any author.

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