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Leptospira infection and carrier survey on rats from wet market areas in Kuala Lumpur, Malaysia

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ABSTRACT

Background & objectives: Leptospirosis is an important zoonotic infection that has caused significant mortality and morbidity worldwide. This disease is endemic in Malaysia and as a developing tropical country, leptospirosis is concerning as it threatens Malaysian public health and the country's economic sectors. However, there is limited information on leptospirosis in Malaysia, especially regarding leptospiral seroepidemiology among carriers in Malaysia. Therefore, more epidemiological information on the source of the disease and reservoir are needed for better disease control and source intervention. The objectives of this study are to gather information on *Leptospira* infection and the carrier status of rats captured from selected wet markets of Kuala Lumpur metropolitan city in Malaysia.

Methods: Live rat trappings were performed in four major wet markets in Kuala Lumpur, namely, Pudu, Chow Kit, Datuk Keramat, and Petaling Street. Animal samplings were performed for 12 months in 2017, where blood and kidney samples were collected and tested for anti-leptospiral antibodies via Microscopic Agglutination Test (MAT) and pathogenic *Leptospira* screening via Polymerase Chain Reaction (PCR) amplification of *flaB* gene.

Results: MAT showed that 34.7% (n = 50/144) of the captured rats were positive for anti-leptospiral antibody of which the most prominent serovar was Malaya followed by a local strain, IMR LEP 175. In parallel, 50 rats were also positive for pathogenic *Leptospira* DNA.

Interpretation & conclusion: This study showed that there are persistent *Leptospira* infections among rats in Kuala Lumpur wet markets and these rats are important reservoir hosts for the bacteria.

Key words Pathogenic *Leptospira*; animal leptospirosis; wet market; Malaysia; MAT; *flaB* gene

INTRODUCTION

Leptospirosis is a zoonotic disease that can infect both humans and animals. This disease is often caused by the pathogenic group of *Leptospira* species, widespread in the tropics and temperate regions, associated with rainy season and natural catastrophes such as floods or tsunamis.

Pathogenic *Leptospira* persist in the renal tubules of its carrier host or natural reservoir, which is later secreted through urine into the environment. Natural reservoirs of pathogenic *Leptospira* include many domestic and wild animals, primarily rodents. Rodents are a well-known *Leptospira* reservoir responsible for leptospirosis transmission in both urban and rural areas.

Human leptospirosis occurs through direct contact with the urine of infected carriers or through contaminated water or soil. Usually, *Leptospira* exposure in humans happen during occupational or recreational activities. In many circumstances, leptospirosis symptoms are indistinguishable from other tropical infections such as dengue. As a result, accurate diagnosis is difficult and often requires complementary laboratory investigation. Owing to these challenges, the global mortality rate of leptospirosis cases lies between 5%–15% with 350,000–500,000 reported cases of severe leptospirosis[1].

Malaysia has gazetted leptospirosis as a compulsory notifiable disease in 2010. Since then, human leptospirosis cases have gradually increased where in the first year alone, a total of 1976 cases were reported and by 2014, the number of cases quadrupled to 7806[2]. Due to the increasing and high number of human leptospirosis cases in Malaysia, it is important to establish baseline data on the prevalence of leptospirosis in its natural rodent hosts, especially in the Klang Valley where almost 30% of total Malaysians reside. Until this day, studies on detection of leptospiral antibody in Malaysian small mammals especially rodents are very limited. Data on circulating

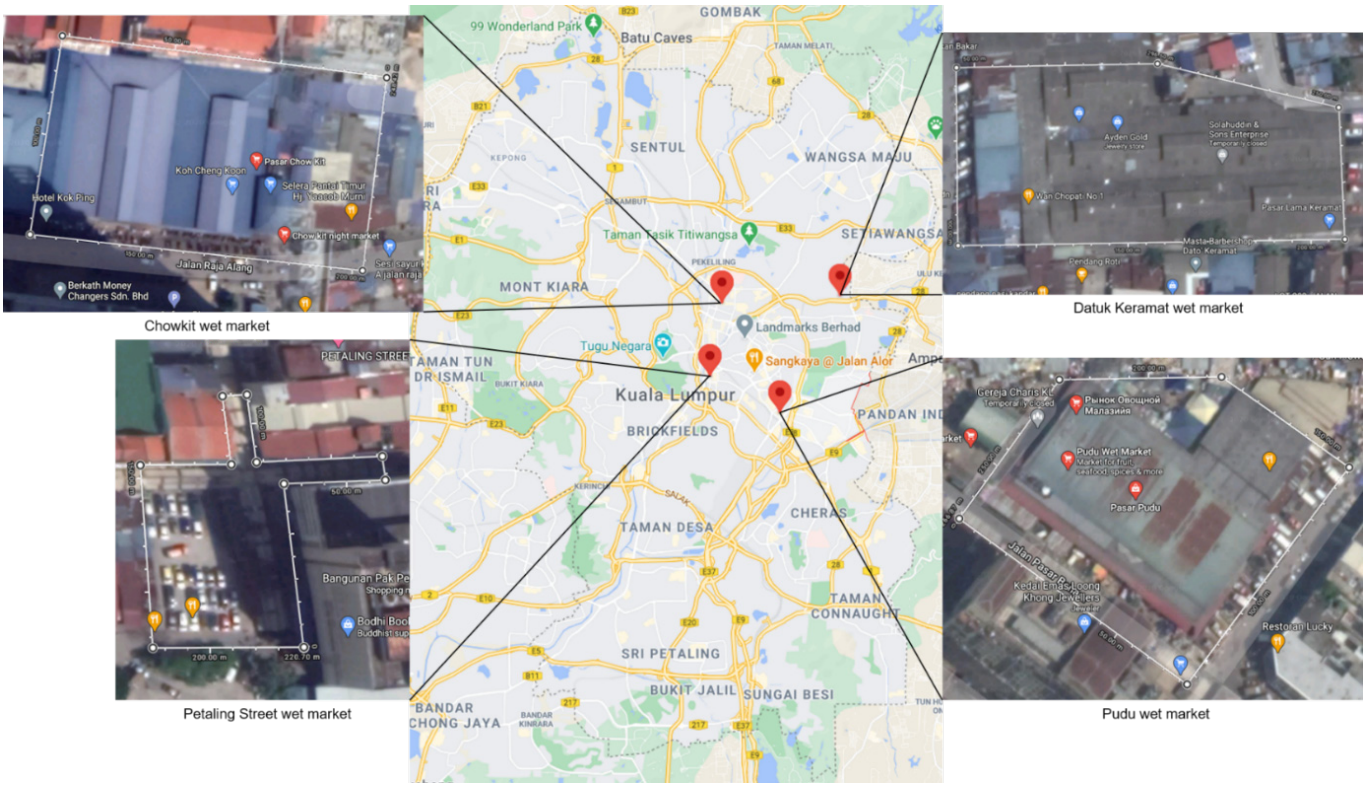

Fig. 1: Maps of selected wet market study sites in Kuala Lumpur, Malaysia (Source: Google Maps).

Leptospira serovars in rodents is important as it can be used as a reference when dealing with both human and animal leptospirosis. Hotspot in urban settings such as wet markets is an ideal place to conduct this study since this is the place where Malaysian residents have a high chance to come in contact with *Leptospira* carriers and contaminated environments.

The outcome of this study will aid in providing information on local strains detected in rats and is useful for identifying the potential origin of leptospirosis in case of future leptospirosis outbreaks in Kuala Lumpur. The findings can also be used as a reference for increasing Microscopic Agglutination Test (MAT) sensitivity by the addition of local strains in MAT panel for diagnosis or serological survey purposes.

MATERIAL & METHODS

Selection of sampling sites

Kuala Lumpur metropolitan city was selected, as it is the largest and most condensed state in Malaysia. Moreover, this city recorded the highest number of leptospirosis cases[2]. Wet markets were chosen based on their size and recommendation by the municipal authority, Kuala Lumpur City Hall (Dewan Bandaraya Kuala Lumpur, DBKL) based on which market has an abundant rodent population. Based on these criteria, four wet markets were chosen: Pudu wet market, Datuk Keramat wet market, Chow Kit wet market, and Petaling Street wet market. Figure 1 portrays the location of each wet market in Kuala Lumpur.

Animal trapping and rat identification

Random sampling was performed weekly at night in conjunction with DBKL's rodent trapping program between January and December 2017, covering both Malaysian dry season (March–September) and the wet season (October–February). Fifty wire-mesh traps with coconut and banana as bait were placed inside and around each market area. Captured rats were later euthanised with isoflurane and subsequently morphologically identified, with their species verified by a qualified expert. Any rats that died before euthanisation were excluded from the study.

Sample collection

Following euthanisation, blood and kidneys were harvested and transferred into sterile tubes. The sera were later separated by centrifugation at 10,000 rpm for 15 min, whereas the kidneys were grinded with tissue grinder into macerated tissue in Ellinghausen-McCullough-Johnson-Harris (EMJH) media. All samples were kept at -20°C until further use.

Table 1. List of *Leptospira* cultures used in MAT

Species	Serogroup	Serovar	Strain	Origin
LOCAL CULTURE				
LEP 1			Melaka	IMR Local isolate
LEP 115			Terengganu	IMR Local isolate
LEP 175			Sarawak	IMR Local isolate
LEP 22	Bataviae	Bataviae	Swart	IMR Local isolate
L. interrogans				
Hoshas		Pyrogenes	Camlo	Vietnam
L. interrogans				(IMR Local isolate)
L. interrogans	Australis	Fugis	Fudge	Malaysia
L. interrogans	Canicola	Malaya	H6	Malaysia
L. borgpetersenii	Celledoni	Whitcombi	Whitcomb	Malaysia
L. interrogans	Ictero*	Birkini	Birkin	Malaysia
L. interrogans	Pyrogenes	Biggis	Biggs	Malaysia
L. kmetyi	Tarrasovi		Bejo	Malaysia
L. interrogans	Ictero*	Lai	Langkawi	Malaysia
L. interrogans	Djasiman	Gurungi	Gurung	Malaysia
L. borgpetersenii	Hebdomadis	Worsfoldi	Worsfold	Malaysia
L. weilii	Javanica	Coxi	Cox	Malaysia
L. interrogans	Canicola	Jonsis	Jones	Malaysia
WHO CULTURE				
L. kirschneri	Gryppotyphosa	Gryppotyphosa	Mandemakers	Unknown
L. interrogans	Sejroe	Hardjo	Hardjoprajitno	Indonesia
L. biflexa	Semaranga	Patoc	Patoc I	Italy
L. interrogans	Autumnalis	Bangkinang	Bangkinang I	Indonesia
L. borgpetersenii	Sejroe	Hardjobovis	Sponselee	Netherlands
L. interrogans	Ictero*	Copenhageni	M 20	Denmark
L. interrogans	Pomona	Pomona	Pomona	Australia
L. borgpetersenii	Tarrasovi	Tarrasovi	Perepelicin	USSR

Note: Locally isolated cultures are cultures that were isolated by IMR from local samples. Ictero* - Icterohaemorrhagiae.

Microscopic Agglutination Test (MAT)

Sera were tested for the presence of anti-leptospiral antibodies via MAT according to the World Health Organization (WHO) protocol, with Malaysia Institute of Medical Research (IMR) in-house modification. Table 1 lists the serovars tested, including eight international serovars and 16 local serovars/strains. Any sample showing final titer reading of less or equal to 1:50 (1:≤50) was concluded as negative result, while any sample with final titer reading of higher or equal to 1:100 (1:≥100) was considered as positive result.

DNA extraction

DNA was extracted from the solution of macerated kidney tissue and EMJH using automated Promega Maxwell® 16 Tissue DNA Purification Kit in accordance to the manufacturers' protocols.

Polymerase Chain Reaction (PCR)

PCR procedure based on Natarajaseenivasan *et al*., 2010 were utilised for this study[3]. Reagents for this method consisted of 1x CoralLoad PCR Buffer, 1.15 mM $MgCl_2$, 200 µM dNTPs, 0.6 µM of forward *flaB* primer (5'-TCTCACCGTTCTCTAAAGTTCAAC-3'), 0.6 µM of reverse *flaB* primer (5'-CTGAATTCGGTT TCATATTTGCC-3'), 0.35U HotStar Taq® DNA Polymerase, 5 µL DNA template, and water (adjusted to 20 µL). Subsequently, PCR reactions were carried out using ABI™ GeneAmp™ PCR System 9700. The thermal cycling conditions performed were initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 60 sec, and a final elongation at 72°C for 7 min. Next, PCR reactions were analysed on 1.5% agarose gel electrophoresis at 100 volts for one hour and visualised using Bio-Rad ChemiDoc XRS+

system. Samples with an amplicon of 793 bp in size were considered positive for pathogenic *Leptospira* DNA.

Ethical statement

This study was registered and ethically approved by the Ministry of Health Malaysia NMRR-16-1000-30876 (IIR) with reference number (12)KKM/NIHSEC/P16-934 and (14)KKM/NIHSEC/P16-934.

RESULTS

Rat distribution and species identification

The total amount of rats captured from all study sites were 144, and all of them were morphologically identified and verified as *Rattus rattus*. The distribution of captured rats from each wet market are: Pudu - 44 rats, Chow Kit - 37 rats; Keramat - 32 rats; and lastly, Petaling Street - 31 rats.

Seropositivity of anti-leptospiral antibody in rats

From a total of 144 samples tested with MAT, 34.72% (50/144) showed positive result for anti-leptospiral antibody. Table 2 shows the seropositivity result of all the samples, where Pudu wet market showed the highest number of positive samples (n = 20), followed by Chow Kit (n = 11), Petaling Street (n = 10), and lastly Datuk Keramat (n = 9).

Table 2. Seropositivity of anti-leptospiral antibody in captured rats

Wet market	Seropositivity (n =144)	
	Positive, n (%)	Negative, n (%)
Pudu	20 (13.89)	24 (16.67)
Chow Kit	11 (7.64)	26 (18.06)
Petaling Street	10 (6.94)	21 (14.58)
Datuk Keramat	9 (6.25)	23 (15.97)
Total	50 (34.72)	94 (65.28)

Among the 50 MAT positive samples, six *Leptospira* serovars and 69 antibody reactions were detected. Serovar Malaya dominate the total number of antibody reaction detected (n = 26), closely followed by IMR LEP 175 (n = 23). Table 3 shows the distribution of detected serovars among all positive MAT samples. The other four detected serovars were IMR LEP 22 (Bataviae) (n = 8), Gurungi (n = 7), Hardjo type Prajitno (n = 3) and Copenhageni (n = 2). Titer reading of positive MAT, 1:100 (low antibody titer), was the most commonly recorded titer reading, whereas a high titer reading of 1:800 was least recorded. MAT also recorded several samples that displayed positive reaction to multiple serovars. Ten positive MAT samples reacted to multiple serovars and most of them reacted to serovar IMR LEP 175 and serovar Malaya, as recorded in Table 4.

Table 3. *Leptospira* serovar distribution and titer reading of positive MAT samples

Leptospira serovar	Titer				Antibody reaction (%)
	1:100	1:200	1:400	1:800	
L. interrogans Malaya	11	9	3	3	26 (37.68)
IMR LEP 175	13	9	1		23 (33.33)
L. interrogans IMR LEP 22 (Bataviae)	3	2	2	1	8 (11.59)
L. interrogans Gurungi	2	3	2		7 (10.15)
L. interrogans Hardjo type Prajitno	2	1			3 (4.35)
L. interrogans Copenhageni		1		1	2 (2.9)
Total	31	25	8	5	69

Note: Percentage of antibody reaction column is based on 69 total antibody reactions (frequency of detected serovar) out of 50 MAT positive rats.

Table 4. Positive MAT samples with multiple serovar reactions

No of antibody reaction	Serovar detected	No of sample	Titer reading
2	Malaya + Gurungi	3	Malaya 1:200 Gurungi 1:200 Malaya 1:400 Gurungi 1:400 Malaya 1:400 Gurungi 1:200
	L175 + Malaya	1	L175 1:200 Malaya 1:100
	L175 + HtP	1	L175 1:200 HtP 1:100
3	L175 + Malaya + Gurungi	3	Malaya (1:800) Gurungi (1:200) L175 (1:100) Malaya (1:800) Gurungi (1:400) L175 (1:200) Malaya (1:200) L175 (1:100) Gurungi (1:100)
	L175 + L22 + Copenhageni	1	Copenhageni (1:800) L175 (1:100) L22 (1:100)
5	L175 + L22 + HtP + Malaya + Gurungi	1	L175 (1:400) Malaya (1:200) L22 (1:100) HtP (1:100) Gurungi (1:100)

Note: L175- IMR LEP 175; HtP- Hardjo type Prajitno; L22- IMR LEP 22 (Bataviae)

Pathogenic Leptospira screening

Detection of pathogenic *Leptospira* in the kidney of captured rats yielded 50 positive samples out of a total 144 samples (34.72%). Although MAT and PCR tests shared the same result for total positive samples, the result of these two tests do not reflect the positivity of each sample in both tests, since some MAT positive samples were not PCR positive and vice versa. Table 5 shows the discrepancy result of the two tests.

DISCUSSION

Rat distribution

The finding of *Rattus rattus* as the main rat species identified in this study is in agreement with multiple past studies, which reported *R. rattus* as the most common rat species found in an urban setting[4–7]. However, this result was surprising, as it is uncommon to capture only a single species from trapping activities. This finding is possible probably due to the difference in on-site trapping locality and urban rat behaviour. Himsworth *et al*., 2014 mentioned that rat trapability can be influenced by locality of the trapping site because the density and distribution of rat population vary greatly in a short geographic distance[8]. This factor may further be influenced by urban rat behaviour, given that they are territorial in nature with small home range. Furthermore, regular rodent extermination programs by local municipal body, DBKL, and ongoing redevelopment

Table 5. Comparison of MAT and PCR positive results

Wet market	Test	
	MAT positive, n	PCR positive, n
Pudu	20	14
Chow Kit	11	12
Petaling Street	10	17
Datuk Keramat	9	7
Total	50	50

project of Chow Kit wet market at the time of trapping period may have led to the disruption of rat population in these wet markets especially on the minority species, which may have indirectly affected the number of rat species captured in this study.

Seropositivity of anti-leptospiral antibody in rats

Seropositivity of anti-leptospiral antibody in urban rodents varies widely across the globe ranging from 18.3% to 96%[4, 9–12]. This range of seropositivity is presumably to be influenced by factors such as locality, serovars used as MAT panel, and the selection of the cut-off value for MAT positive. With MAT positive cut-off value of 1:100, a high seropositivity of 34.72% was detected in this study. This finding is supported by similar findings in other studies, where seropositivity of 40% and 54% were recorded in India and locally, respectively[9, 4].

Leptospira serovar distribution and Leptospira infection

Six *Leptospira* serovars: Malaya, IMR LEP 175, IMR LEP 22 (Bataviae), Gurungi, Hardjo type Prajitno, and Copenhagen, were detected through MAT in this study. Serovar Malaya and IMR LEP 175 were the most prominent detected serovars with a total antibody reaction of 37.68% and 33.33%, respectively. This indicates that both the aforementioned serovars are endemic in the rat population of this research's study sites.

To the best of the authors' knowledge, this is the first discovery of antibody reaction against serovar Malaya in Malaysian rats although serovar Malaya was originally isolated from Malaysia. This finding is most likely because this serovar was just newly integrated into the Malaysian MAT panel by IMR, a leading Malaysian government body that is responsible for studies on *Leptospira*. The IMR creates, uses, and suggests a list of *Leptospira* serovars as MAT panel for both diagnostic and research purposes; this list is commonly followed by other laboratories in Malaysia. The IMR updated the MAT panel list in 2017, leading to the discovery of new serovars, including serovar Malaya and serovar Gurungi that were also detected in this study.

Serovar IMR LEP 175, also known as serovar Sarawak or Lepto 175 Sarawak, is one of the most prominent serovars detected in MAT conducted on Malaysian samples, as demonstrated in many local studies of both human and animal leptospirosis[13–17]. Despite its frequent mention in numerous studies, there is still limited information on this serovar about its molecular properties; there is yet any confirmation on its species. Latest information on this serovar showed that it is currently classified as an intermediate *Leptospira* strain with genetic similarity to *Leptospira wolffii* serovar Khorat strain Khorat-H2, a *Leptospira* species that has been reported to infect humans and animals in Thailand, India, and Iran[18].

In this work, MAT findings also showed the detection of both serovar Malaya and IMR LEP 175 in most of the MAT positive samples with low titer readings, 1:100, and in samples that reacted with multiple serovars. Low titer readings of 1:100 in MAT indicate the sample's past exposure to *Leptospira* and the persisting chronic infection in the animal that shed *Leptospires* through their urine[19–20]. On the contrary, the high MAT titer reading (≥ 1:400) represents recent or current *Leptospira* infection in the infected victim. Results obtained from MAT showed the presence of several *Leptospira* serovars; mainly, serovar Malaya and IMR LEP 175, which are actively circulating in rat population of Kuala Lumpur wet markets, with record of both past and recent rat infections.

Leptospira carrier status

Pathogenic *Leptospira* screening using *flaB* gene amplification produced 34.72% positive result, which means 50 out of 144 total rats in this study were renal carriers of pathogenic *Leptospira*. This result is congruent with the finding of a local study that demonstrated 31.6% positivity[21]. Compared with other pathogenic *Leptopsira* detection through PCR in a similar urban setting, positive result from kidney sample usually produced lower yield of 28.4% to 5.6%[5, 22–23]. Reasons for variation in detection rate were unclear, but it is likely influenced by factors such as study locality and the laboratory methodology used.

Comparison of positive results on MAT and PCR

This study demonstrated that MAT and PCR detection produced identical results for 50 (34.72%) positive samples. It is tempting to state that these results correlate and complement each other, but in a detailed examination, the results from each wet market do not tally for both tests. This is because the MAT result does not reflect the host carriage status[19, 24]. MAT only detects the antibody in the test subject against specific *Leptospira* serovar instead of detecting the presence of the bacteria itself in the sample. The presence of anti-leptospiral antibody indicate present or past exposure to *Leptospira*, not the carriage status. The host of a positive MAT sample may possess antibodies toward a specific serovar, but the host may not have any or carry *Leptospira* in its system. Therefore, not all positive

MAT result samples showed a positive result in the PCR test.

CONCLUSION

With evidence of multiple *Leptospira* serovars actively circulating in rat populations of Kuala Lumpur wet markets and DNA presence of *Leptospira* in the samples, it can be concluded that these wet markets are potential origins of leptospirosis in case of future leptospirosis outbreaks in human population around the aforementioned areas. Hence, there is a pressing need for precaution and prevention measures by authorities, such as regular rodent population control, better hygiene and waste management, as well as more public exposure on leptospirosis and its transmissions for outbreak prevention and improved disease risk management.

KEY MESSAGE

Rat population in Kuala Lumpur wet markets are reservoir for pathogenic *Leptospira*. There is a high probability for the study sites of this work to be the source of human leptospirosis outbreak in Kuala Lumpur, Malaysia.

Conflict of interest: None

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