STUDY ON SEROPREVALENCE OF LEPTOSPIROSIS AND ITS SEROVARS AMONG CATTLE FARMERS IN NORTHEASTERN MALAYSIA

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ABSTRACT: *A cross-sectional study was done involving 120 asymptomatic cattle farmers in six districts of Kelantan. The objectives were to determine the leptospirosis seroprevalence and to identify the predominant infecting serovars among cattle farmers. A serological test using the microscopic agglutination test (MAT) was conducted in the Institute of Medical Research with a cut-off titre for seropositivity of ≥1:100. The overall seroprevalence of leptospiral antibodies was 72.5%. The predominant serovar identified was serovar Sarawak (59.2%). A high seroprevalence of leptospiral antibodies was found among cattle farmers in Kelantan especially in Pasir Puteh district. The predominant infecting serovar was serovar Sarawak. These findings urge that more studies are required to determine the reasons for the high seroprevalence among the cattle farmers along with its transmission and pathogenicity of the local serovar Sarawak.*

Keywords: leptospirosis, seroprevalence, leptospiral antibody, microscopic agglutination test, cattle farmers

1. INTRODUCTION

Leptospirosis is considered as a disease of global public health importance as it presumed to be the most widespread re-emerging zoonotic disease in the world with potentially fatal and serious consequences for human health. It is caused by pathogenic spirochete bacteria of the genus leptospira that are transmitted directly or indirectly from animals to human [1]. It is endemic in tropical and sub-tropical countries, estimated to affect tens of millions of humans annually worldwide [2]. In high-risk groups, such as agriculture workers, the incidence may reach more than 100 per 100,000 population [3]. Various studies have shown that Malaysia is also an endemic country for leptospirosis, with an increased number of reported cases and a significant number of deaths over the past decade [4, 5, 6].

It is a zoonotic infectious disease caused by gram negative bacteria of the genus *Leptospira*. The word leptospira originated from the Greek word *leptos* meaning fine or thin and *spira* meaning coil in Latin [7]. Based on the bacteria's morphology that is thin and helically coiled. The bacteria are motile and slow growing aerobes measuring about five to 25 micrometres in length and 0.1 to 0.3 micrometres in diameter. The hooked ends of this bacterium give it a distinctive question-mark shape [8], while its thin size allows it to pass through filters that retain most other bacteria.

Leptospires are bacteria which can be either pathogenic (potential to cause disease in animals and humans) or saprophytic (free living and generally considered not to cause disease). Pathogenic leptospires are maintained in nature, in the renal tubules of certain animals. Saprophytic leptospires are found in many types of wet or humid environments ranging from surface waters and moist soil to tap water. It can be classified into more than 200 serovars with 25 serogroups based on surface antigens [3]. Leptospirosis is transmitted to humans through skin or the mucous membrane which have a contact with water, moist soil, vegetation or environmental surfaces contaminated with the urine of an infected animal.

Certain vertebrate animals especially mammalian such as cattle, buffaloes, horses, sheep, goat, pigs, dogs and rodents are natural hosts for pathogenic leptospires that house themselves in the kidneys of the hosts. These leptospires do little or no detectable harm in the host bodies and merely maintain the infection in their hosts. Hence, animals infected by leptospires are known as natural maintenance hosts. However, infection differs for humans who are not natural maintenance hosts. Pathogenic leptospires are harmful to humans and once infected, they will contract illnesses [9].

The incubation period for leptospirosis is from five to 14 days, after which the signs of infection will start to show in the patient depending on factors that include the pathogenicity of *Leptospira spp.*, virulence of the infecting bacteria, the quantity of bacteria that entered the body, and the human's immunity or susceptibility level (Bharti *et al.*, 2003; [3] . Once infected with the bacteria, the patient may present a wide range of clinical symptoms ranging from a flu-like illness to Weil's syndrome, with the case fatality rate ranging from 5% to 15%, and it is characterised by jaundice, renal failure and haemorrhage [10].

Research in Malaysia has shown that the clinical symptoms most commonly experienced by leptospirosis patients in this country are fever (98%), chills (64.2%), cough (56.9%), jaundice (44%), abdominal pain (42.9%), and hepatomegaly (40.5%) [11]. Meanwhile, research conducted in Thailand indicates that their patients' most common symptoms are fever, headache, and muscle pain in the early phase, and symptoms of meningitis, sub-conjunctival haemorrhage, jaundice, haemoptysis, hepatomegaly, diarrhoea, hypotension, and reduced urine output in the late phase [12].

From previous studies, it is learnt that leptospirosis is mainly an occupational disease. People with jobs that involve contact with the outdoor environment and animal-handling such as farmers, livestock farming, plantation workers, sewage workers, veterinarians and military personnel are those with the highest risk of leptospirosis infection [13,14]. Apart from occupational characteristics, those involved in outdoor recreational activities such as camping, and water sports such as swimming, wading, and white-water rafting in contaminated areas are also at high risk to leptospirosis infection. A recent hospital-based study reported that most

leptospirosis cases in Malaysia were among agriculture workers [15].

2. MATERIAL AND METHODS

2.1 Study Design and Population

A cross-sectional study was conducted in September 2015 involving 6 districts in Kelantan, which are northeastern state of Malaysia. Simple random sampling was applied to select 6 districts from the total of 10 districts in Kelantan. The list of all cattle farmers available in those 6 districts was required from Department of Veterinary Services Kelantan. Then, stratified random sampling was applied to the list to determine the number of farmers needed to be selected from each district.

Selected cattle farmers were approached and explained regarding our study. All the cattle farmers involved voluntarily signed the informed consent form after they were given a detailed explanation about the procedure and adequate time to decide. A member of the research team explained the informed consent individually. They were required to filled up a questionnaire and five milliliters of their blood were collected for the study. Personal information and data will not be disclosed. The questionnaires and blood samples will have serial numbers instead of names of the subjects to prevent recognition.

Reference populations of this study is the cattle farmers, where the source population are taken from multistage random sampling of cattle farms from 10 districts in Kelantan. Finally, the sampling frame are the list of cattle farmers from the 6 districts in Kelantan who fulfill the study criteria. Calculated based on a 37.5% seroprevalence of leptospirosis among animal handlers [16], 95% CI and 30% non-response rate, the estimated sample size required for the study was 120. The sampling frame consisted of cattle farmers who had been working for more than six months. Cattle farmers who are not registered to Department of Veterinary Service Kelantan are excluded. The study was granted ethical approval by the Research and Ethics Committee (Human), School of Medical Sciences, Health Campus, Universiti Sains Malaysia

2.2 Blood Samples and Serologic Tests

The consenting respondents were interviewed for socio-demographics and working practices. Venous blood samples were tested for the presence of anti-leptospiral antibodies using microscopic agglutination test (MAT) at the Institute of Medical Research (IMR) following standard methods [17]. The MAT was performed with a panel of live leptospire reference cultures obtained from the Royal Tropical Institute (World Health Organization/Food and Agriculture Organization of the United Nations Collaborating Centre for Reference and Research on Leptospirosis) in Amsterdam (Australis, Autumnalis, Bataviae, Canicola, Celledoni, Grippotyposa, Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes, Hardjoprajitno, Patoc, Tarassovi and Djasiman) and from the IMR (Melaka, Terengganu, Sarawak, Lai, Hardjobovis and Copenhagani).

Live leptospire cell suspensions representing 20 serovars were added to serially diluted serum specimens in 96 wells microtiter plates and were incubated at 30°C for 2 hours. Through dark field microscopy, agglutination was examined at a magnification of 100 times. Using the control well for comparison, agglutination was examined by observing free leptospires in each well. The MAT results were considered positive if the free leptospires approximate numbers were <50% in the control well. A titre of ≥1:100 was used as the cut-off titre for leptospirosis seropositive in this study. The level of titre indicated previous exposure to the leptospira bacteria [5].

2.3 Statistical Analysis

Data were entered and analysed using IBM Statistical Program for Social Sciences (SPSS) version 22 for Windows [18]. All continuous variables were described using means and standard deviations (SD). Frequencies and percentages were presented for categorical variables. Seroprevalence of leptospirosis was described with 95% confidence interval (CI).

3. RESULTS

Table 1: Socio-demographic characteristics of the respondents (n=120)

Variables	Frequency (%)	Mean (SD)
Age (years)		50.5 (14.94)
Gender		
Male	104 (86.7)	
Female	16 (13.3)	
Marital Status		
Married	94 (78.3)	
Single/widower	26 (21.7)	
Family Members		5.2 (2.37)
Income		
<RM1000	73(60.8)	
RM1000 to RM1999	32 (26.7)	
≥RM2000	15 (12.5)	
Education		
No formal education	5 (4.2)	
Primary school	37 (30.8)	
Secondary school	71(59.2)	
Tertiary education	7 (5.8)	
Location		
Tumpat	35 (29.2)	
Tanah Merah	10 (8.3)	
Pasir Mas	23(19.2)	
Bachok	20 (16.7)	
Pasir Puteh	20 (16.7)	
Kota Bharu	12 (10.0)	

Table 1 shows the socio-demographic characteristics of the cattle farmers. The respondents were cattle farmers whose age vary between 19 to 78 years old, with a mean age of 50.5 (SD 14.94) years old, and the majority of them were males (84.6%). Most of the respondents (65.0%) had either secondary school or tertiary education, and 37 (30.8%) had primary school educational and only 5 (4.2%) had no formal education. 15 (12.5%) of them had monthly income of RM 2000 and

above, and majority of them, 73 (60.8%) had less than RM 1000 per month.

Table 2: Serovar distribution among 87 cattle farmers determined by positive MAT (titre ≥1:100)

Serovars tested	Frequency	%
Sarawak	71	59.2
Patoc	25	20.8
Hardjobovis	8	6.7
Javanica	5	4.2
Tarrasovi	4	3.3
Grippotyphosa	3	2.5
Australis	2	1.7
Bataviae	2	1.7
Hardjoprajitno	2	1.7
Pyrogenes	2	1.7
Copenhageni	2	1.7
Pomona	1	0.8
Melaka	1	0.8
Terengganu	1	0.8
Lai	1	0.8

The distribution of serovar among 87 seropositive cases determined by the positive MAT titre ≥1:100 is shown in table 2. The predominant pathogenic serovars identified in this study was serovar Sarawak at 59.2%, followed by serovar Patoc at 20.8% and serovar Hardjobovis, Javanica and Tarrasovi at 6.7%, 4.2% and 3.3% respectively.

Table 3: Seroprevalence of leptospirosis seropositivity according to district (n=120)

District	n	MAT ≥1:100
		Frequency (%)
Pasir Puteh	20	20 (100.0)
Kota Bharu	12	10 (83.3)
Pasir Mas	23	17 (73.9)
Tumpat	35	25 (71.4)
Tanah Merah	10	7 (70.0)
Bachok	20	8 (40.0)
TOTAL	120	87 (72.5)

The overall seroprevalence of leptospirosis was 72.5% (95% CI: 0.63, 0.80). Among the respondents, cattle farmers from Pasir Puteh district showed the highest seroprevalence where all 20 of them (100.0%) was found to be seropositive. Cattle farmers from another district also noted to have a high seroprevalence ranging from 70.0% to 83.3% except for Bachok where only 40% of them was found to be seropositive to leptospirosis (Table 3).

4. DISCUSSION

This study revealed a high seroprevalence rate of 72.5% obtained from the scientific laboratory MAT, considerably higher compare with other studies in Malaysia. Using similar methods and a cut-off value of the MAT titre for comparison, Shafei *et al.* [5] reported a lower seroprevalence (24.8%) of leptospirosis among town service workers in Kelantan, which are considered another high-risk occupational group for the infection. A more recent study, which also used similar methods among oil palm plantation workers in Melaka and Johor, southern states of Malaysia showed seroprevalence rate of 28.6% [19]. Another local study conducted a long time ago among healthy paddy planters in Northeastern Malaysia using the Sensitized-erythrocyte-lysis (SEL) test also reported 24.2% seroprevalence [20].

Our finding is supported by another study among dairy cattle farm workers in India which also used MAT to determine the leptospiral seroprevalence. Seropositivity in 39 dairy cattle farm workers was reported to be 76.5% which is slightly higher to our finding. This may be due to the smaller sample size and lower titre (≥1:80) used in their study [21]. Looking at all these seroprevalence reported in other high-risk occupational group, it shows that cattle farmers with almost three times higher seroprevalence are comparably at a much higher risk for leptospiral infection.

Compared with the other high risk group mentioned before, cattle farmers are exposed not only to the environment, but they also have daily contact with their cattle and possibly other domestic and wild animals. Humans may be infected through direct contact with the carrier animal's tissue, urine, aborted fetus or blood through skin lesions, mucous membranes, or by inhalation of contaminated fluid aerosols [22]. With daily exposure, they are at higher risk to get leptospirosis as leptospira transmission occurs through direct contact with infected mammals [23] or exposure to urine-contaminated water [24, 25].

Pasir Puteh district has the highest percentage of seroprevalence where all 20 of the cattle farmers there were found to be seropositive. Almost half of them are dairy cattle's farmer, compare to other district where the cattle are mostly breed for their meats. It can be postulated that maybe the high seroprevalence among the farmers in Pasir Puteh district are due to the exposure to the cattle's urine splashes and steams during the milking process.

Cattle farmers can also be infected from their environment where leptospires are washed off from the urine-contaminated soil and are collected into the rivers and puddles. Infection occurs when cattle farmers have a contact with these contaminated waters as pathogenic leptospires can survive in moist soil and fresh water for long periods of time,

especially when the pH is slightly alkaline [21,25]. Under laboratory conditions, pathogenic Leptospiral cells survived in distilled water (pH 7.8) for 110 days, but when incubated in viscous solutions, the survival time of Leptospira increased more than three-fold (347 days) [26].

Apart from the contact with the livestock and environment, cattle farmers are also at risk to obtain wounds on their hands and other parts of their body. This circumstances are due to the nature of their job that involves lots of physical activities during handling of the cattle and risk of injury from the horn and cutting the grass by machete to fed the cattle. The higher prevalence of serovar detected in cattle farmers may also be due to their poor personal protective equipment practices. As observed during the study, only a few cattle farmers wore gloves, long sleeves and trouser while working. Some of them doesn't even wear boots while working. This practice further contributes to high exposure to the leptospire-contaminated environment. The high positivity in the seroprevalence of leptospiral antibodies among cattle farmers may also be related to rats, which are the main leptospira carrier. Rats can be found in cattle farms especially in the food storage area and the grass field.

The predominant pathogenic serovar found in our study was Sarawak (59.2%), followed by Patoc 20.8%). Similar findings are reported in another study done among oil palm plantation workers in southern states of Malaysia where the predominant serovar found was also Sarawak and Patoc with seroprevalence of 62.0% and 42.0% respectively [19]. Furthermore, a study of leptospirosis among wild animals in Sarawak, East Malaysia, reported that 72% of the seropositive samples from monkeys, rats, bats, squirrels and mongooses were also positive for the serovar Sarawak [1]. At present, the pathogenicity of the serovar Sarawak and its endemicity in Malaysia is still not well-known.

In relation to our study, there is a possibility of leptospiral transmission to occur from the cattle, if the predominant seroprevalence are of the same serovar which is serovar Sarawak. There is already one study that have been done in Southern India among dairy cattle, field rats and farm workers. Seroprevalence of 87.0 %, 51.0 % and 76.5% for cattle, rats and humans, respectively, was observed on the endemic farms. The predominant serovar in cattle and rats reported in the study were Javanica (33.2% and 50.3% respectively) followed by Autumnalis (22.1% and 14.5% respectively). Whereas, in farm workers the predominant serovar were Autumnalis (43.6%) followed by Javanica (17.9%) [27]. Local animal sources of particularly identified infecting serovar should be determined by further studies to establish the transmission pathway.

Cattle are more susceptible to leptospirosis than other domestic animals such as goats, sheep, horse, chicken and others. The incidence of leptospirosis infection in cattle can be classified into two groups. The first consists of contraction from a serovar type (e.g., Hardjo) that is carried by and well adapted to cattle. This serovar type is unaffected by regional factors or rain patterns. The second group comprises incidental infection from serovars carried by other animals in surrounding areas. These serovars are affected by surrounding environmental factors and breeding practices. The second group of leptospirosis infection commonly occurs in tropical countries [28].

In our study, serovar Hardjo types Hardjobovis and Hardjoprajitno were among the 20 serovars examined. Only small percentage which is 6.7% of our study subjects tested positive for serovars Hardjobovis and 1.7% for serovars Hardjoprajitno. This finding suggests that leptospirosis infection due to the presence of cattle is more probable in the second incidence group, in which the infecting serovars are more prevalent in tropical regions. This result also implies that the cattle was the main sources of leptospirosis infection among the cattle farmers, although an issue worth considering is the high probability that the cows became carriers by incidental infection from serovars carried by other animals.

The seroprevalence study in this high-risk occupational group of workers may reflect exposure but not necessarily to developed the leptospirosis disease. The reason is that serovar-specific antibodies are protective, and the person is considered immune to re-infection with the same serovar even if reinfection involving different serovar may still occur [29, 30].

5. CONCLUSION

The high seroprevalence indicates that cattle farmers are a high-risk group for leptospiral infection. Due to the nature of their work, they become exposed to the cattle's urine and possibly leptospire-contaminated environment through their daily work practices. Without proper personal protective equipment, the risk is very high for them to get leptospirosis disease.

Leptospira serovar Sarawak is the predominant infecting serovar detected among the seropositive cattle farmers. As our study did not include leptospirosis among cattle or any animal reservoirs, we could not conclude the pattern and interaction between humans and the animal host. We recommend further studies on local animal reservoirs, along with the surrounding environment to provide important information on predominant serovar.

Conflict of interest statement

We declare that we have no conflict of interest in the publication of this paper.

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6. REFERENCE

[1] Thayaparan, S., Robertson, I., Fairuz, A., et al., *Serological Prevalence of Leptospiral Infection in Wildlife in Sarawak Malaysia, Borneo Journal of Resource Science and Technology*, **2**(2): 79–82 (2013).

[2] Cachay, E., Vinetz, J., *A global research agenda for leptospirosis, J Postgrad Med*, **51**(3): 174-176 (2005).

[3] World Health Organization, *Human leptospirosis: guidance for diagnosis, surveillance and control, Malta*: WHO, 1-2 (2003).

[4] Ministry of Health Malaysia, *Guidelines for The Diagnosis, Management, Prevention and Control of Leptospirosis In Malaysia*, **1**: 1-4 (2011).

[5] Shafei, M., Sulong, M. R., Yaacob, N. A., et al., *Seroprevalence of Leptospirosis among Town Service Workers in Northeastern State of Malaysia, Int. J. Collab. Res. Internal Med. Public Health*, **4**(4): 395–403 (2012).

[6] Thayaparan, S., Robertson I., Fairuz A., et al., *Leptospirosis, an emerging zoonotic disease in Malaysia, Malays J Pathol*, **35**(2): 123–132 (2013).

[7] levett, P. N. & Haake, D., Leptospira species (leptospirosis). *Principles and Practice of Infectious Diseases*, **7**(3): 3059-3065 (2010).

[8] Evangelista, K. V. & Coburn, J., *Leptospira as an emerging pathogen: a review of its biology, pathogenesis and host immune response*. *Future Microbiology*, **5**(9): 1413-1425 (2010).

[9] World Health Organization, *Report of the First Meeting of the Leptospirosis Burden Epidemiology Reference Group, WHO: Geneva, Switzerland* (2010).

[10] Bharti, A. R., Nally, J. E., Ricaldi, J. N., Matthias, M. A., Diaz, M. M., Lovett, M. A., Levett, P. N., Gilman, R. H., Willig, M. R. & Gotuzzo, E., *Leptospirosis: a zoonotic disease of global importance, The Lancet infectious diseases*, **3**(12), 757-771 (2003).

[11] Riazi, M., Abdul, R. B., Fairuz, A. & Zainul, F. A., *low molecular weight lipopolysaccharide antigen preparation reactive to acute leptospirosis heterologous sera. Tropical Biomedicine*, **27**(2): 241-253 (2010).

[12] Wuthiekanun, V., Sirisukkarn, N., Daengsupa, P., Sakaraserane, P., Sangkakam, A., Chierakul, W., Smythe, L. D., Symonds, M. L., Dohnt, M. F., Slack, A. T., Day, N. P. & Peacock, S. J., *Clinical diagnosis and geographic distribution of leptospirosis, Thailand. Emerging Infectious Diseases*, **13**(1): 124-126 (2007).

[13] El Jalii, I., Bahaman, A., *A review of human leptospirosis in Malaysia, Tropical Biomedicine*, **21**(2): 113 (2004).

[14] Pappas, G., Papadimitriou, P., Siozopoulou, V., Christou, L., Akritidis, N., *The globalization of leptospirosis: worldwide incidence trends, International Journal of Infectious Diseases*, **12**(4):351-357 (2008).

[15] Rafizah, A. A. N., Aziah, B. D., Azwany, Y. N., et al., *A hospital-based study on seroprevalence of leptospirosis among febrile cases in northeastern Malaysia, Int J Infect Dis.*, **17**(6): 394–397. (2013).

[16] Sharma, S., Vijayachari, P., Sugunan, A., Natarajaseenivasan, K., Sehgal, S., *Seroprevalence of leptospirosis among high-risk population of Andaman Islands, India, The American journal of tropical medicine and hygiene*, **74**(2): 278-283 (2006).

[17] World Health Organization, *Leptospirosis: Laboratory Manual*, (2007).

[18] International Business Machines, *IBM SPSS Statistics for Windows, Version 22.0. IBM Corporation, Armonk, NY.*, (2013).

[19] Ridzuan, J. M., Aziah, B. D., Zahiruddin, W.M., *Study on Seroprevalence and Leptospiral Antibody Distribution among High-risk Planters in Malaysia, Osong Public Health and Research Perspectives*, **7**(3):168-171 (2016).

[20] Tan, D. S., *Leptospirosis in Rural Malaysia. The Medical Journal of Malaya*, **24**(4): 261–266 **(1970).**

[21] Levett, P. N., *Leptospirosis. Clin. Micro Rev*., **14**: 296–326 (2001).

[22] Hartskeerl, R., Collares-Pereira, M., Ellis, W., *Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. Clinical microbiology and infection*, **17**(4): 494-501 (2011).

[23] Kupek, E., de Sousa Santos Faversani, M. C., de Souza Philippi, J. M., *The relationship between rainfall and human leptospirosis in Florianópolis, Brazil, 1991-1996. Braz. J Infect. Dis.*, **4**:131–134 (2000).

[24] Morgan, J., Bornstein, S.L., Karpati, A. M., et al., *Outbreak of leptospirosis among triathlon participants and community residents in Springfield, Illinois, 1998. Clin. Infec. Dis*., **34**:1593–1599 (2002).

[25] Karaseva, E. V., Chernukha, Y. G., Piskunova, L. A., *Results of studying the time of survival of pathogenic Leptospira under natural conditions, J. Hyg. Epidemiology Microbiology Immunology*, **17**:339–34 (1973).

[26] Trueba, G., *Cell aggregation: a mechanism of pathogenic Leptospira to survive in fresh water, International Microbiology*, **7**: 35-40 (2004).

[27] Timoney, J. F., *Seroprevalence of Leptospira borgpetersenii serovar javanica infection among dairy cattle, rats and humans in the cauvery river valley of southern India*, **42**(3): 679-686 (2011).

[28] Lilenbaum W, Varges R, Medeiros L, Cordeiro AG, Cavalcanti A, Souza GN, Richtzenhain L, Vasconcellos SA., *Risk factors associated with leptospirosis in dairy goats under tropical conditions in Brazil. Res Vet Sci*, **84**(1): 14–17 (2008).

[29] Ganoza, C. A., Matthias, M. A., Collins-Richards, D., et al., *Determining risk for severe leptospirosis by molecular analysis of environmental surface waters for pathogenic Leptospira. PLoS Med.*, **3**(8):1329-1338 (2006).

[30] Romero, E. C., Bernardo, C. C. D. M., Yasuda, P. H., *Human leptospirosis: a twenty-nine-year serological study in São Paulo, Brazil. Rev Inst Med Trop Sao Paulo*, **45**(5): 245-248 (2003).