UNIVERSITI PUTRA MALAYSIA

EPIDEMIOLOGY OF CANINE LEPTOSPIROSIS IN KUALA LUMPUR AND SELANGOR

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By

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Chairman: Professor Dr. Abdul Rani Bahaman

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This study was conducted to determine the current state of leptospirosis in dogs in Kuala Lumpur and Selangor. The usefulness of several laboratory techniques was also evaluated for the diagnosis of leptospires and determination of leptospirosis prevalence. One hundred and sixty five serum samples were collected and examined for serological prevalence of leptospirosis. The dogs surveyed were classified into stray and pet groups. Pet dog samples were obtained from dogs which were brought to the University Veterinary Hospital at Universiti Putra Malaysia (UVH-UPM). Samples from stray dogs were obtained from Society for the Prevention of Cruelty to Animals (SPCA), and Paws Animals Welfare Society (PAWS). All serum samples were screened for leptospiral IgM and IgG antibodies, using an enzyme-linked immunosorbent assay (ELISA). Then, these serum samples were re-examined for leptospiral antibodies and serovar-specificity by the microscopic agglutination test (MAT). A serum sample was confirmed to have leptospiral infection if its MAT titre was ≥ 100, or IgM-ELISA titres of ≥ 160, or IgG-ELISA titres of more than two times of negative controls, or any combination of the above.

The study showed a high serological prevalence of leptospiral infection, particularly in the group of stray dogs. *Leptospira pomona* was found to be the most predominant serovar both in the pet and stray dogs. In previous surveys in 1955,1961, 1979 and 1986, the infection due to *L. pomona* was uncommon whilst *L. icterohaemorrhagiae* and *L. canicola* were reported to be predominant in dog populations in Malaysia. The emergence of *L. pomona* infection in dogs in Malaysia could be due to the only use of vaccines containing serovars *icterohaemorrhagiae* and *canicola.* Therefore, to prevent leptospiral infection in dogs and reduce the transmission of this disease from dogs to other animals and humans, serovar *pomona* should be included in the vaccines to be used in Malaysia.

The bacterial culture revealed no leptospires in the dogs surveyed. This could possibly be due to the fastidious nature of the organisms, stage of infection, or level of antibodies in the circulating blood. However, twenty one unknown isolates were successfully detected in blood and urine samples of the dogs surveyed by the polymerase chain reaction (PCR) and identified by low-stringency PCR technique.

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EPIDEMIOLOGI LEPTOSPIROSIS KANIN DI KUALA LUMPUR DAN SELANGOR

Oleh

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Pengerusi: Profesor Dr. Abdul Rani Bahaman

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Kajan ini dijalankan untuk menentukan tahap semasa leptospirosis dikalangan anjing di kawasan Kuala Lumpur dan Selangor. Kebaikan teknik-teknik makmal juga dinilai untuk pengenalan leptospires dan penentuan kelaziman leptospirosis. Seratus enam pulah lima contoh serum dikumpul dan diperiksa untuk kelaziman serological leptospirosis. Anjing-anjing yang dikaji dibahagikan sebagai anjing sesat dan anjing rumah. Contoh dari anjing rumah didapati daripada anjing-anjing yang dibawa ke Hospital Haiwan Universiti di Universiti Putra Malaysia (UVH-UPM). Contoh dari anjing sesat didapati daripada SPCA dan PAWS. Semasa contoh serum diselidik untuk leptospiral IgM dan IgG antibodi, dengan mengguna ELISA. Kemudian, contoh serum tersebut diperiksa semula untuk antibodi leptospiral dan serovar-specificiti melalui ujian MAT. Contoh serum disah mempunyai jangkitan leptospiral jikalau MAT titre ≥ 100, atau IgM-ELISA titre ≥ 160, atau IgG-ELISA titre melebihi dua kali ganda kawalan negatif, atau lain-lain combinasi tersebut.

Kajian ini menunjukan bahawa kelaziman serological jangkitan leptospiral adalah khasnya di kalangan anjing sesat. Telah didapati bahawa *Leptospira pomona* merupakan serovar yang terkemuka di kalangan kedua-dua anjing rumah dan anjing sesat. Didapati kajian-kajian yang pernah dijalankan dalam tahun-tahun 1955, 1961. 1979 dan 1986, jangkitan disebabkan dari *L. pomona* tidak unum manakala *L. icterohaemorrhagiae* dan *L. canicola* dilaporkan sebagai terkemuka di kalangan anjing-anjing di Malaysia. Kemunculan jangkitan *L. pomona* di kalangan anjing-anjing di Malaysia mukim disebabkan oleh penggunaan vaccine yang hanya mengandungi serovar *icterohaemorrhagiae* dan *canicola* sahaja. Oleh kerana itu, untuk mencegah jangkitan leptospiral di kalangan anjing dan untuk mengurangkan penyakit ini dari merebak daripada anjing-anjing ke binatang-binatang lain dan ke manusia, serovar *pomona* patut dimasukkan dalam senarai vaccine yang digunakan di Malaysia.

Kultur baktria menunjukkan bahawa tidak terdapat leptospires dalam kalangan anjing yang dikaji. Ini mungkin adalah kerana perangai perolah organisima peringkat jangkitan atau taraf antibodi dalam pengaliran darah. Walaubagaimanapun, dua puluh satu isolate yang tidak dikenali telah didapat dalam contoh darah dan kencing anjing-anjing yang dikaji dengan menggunakan polymerase chain reaksi (PCR) dan dikenali dengan teknik low-stringency PCR.

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µg	microgram
µl	microlitre
5-FU	5-fluorouracil
A	Adenine (only used as part of a sequence)
ABTS	2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt
bp	base pairs
C	Cytosine (only used as part of a sequence)
d	density
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetra-acetate
ELISA	enzyme-linked immunosorbent assay
G	Guanine (only used as part of a sequence)
H_2O_2	hydrogen peroxide
IgG	immunoglobulin G
IgM	immunoglobulin M
LS-PCR	low-stringency PCR
LSPs	low-stringency products
M	molar
MAT	microscopic agglutination test
mM	millimolar
MW	molecular weight

OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAWS	Paws Animal Welfare Society
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	puissance hydrogen (hydrogen-ion concentration)
rpm	round per minute
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SPCA	Society for the Prevention of Cruelty to Animals
T	Thymine (only used as part of a sequence)
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	(N,N,N′,N′ tetramethylethylenediamine)
Tric-HCl	Tris (hydroxymthyl) aminomethane hydrochloride
UPM	Universiti Putra Malaysia
UV	ultraviolet
UVH	University veterinary hospital
V	volts
v/v	volume per volume
w/v	weight per volume

CHAPTER I

INTRODUCTION

Leptospirosis, also known as haemorrhagic jaundice, mud fever, swamp fever, or rat urine disease, is an infectious bacterial disease caused by *Leptospira interrogans* (Gitton *et al.,* 1994; Pollack, 1999a). However, leptospiral infections in general are commonly referred to as Weil's disease. Weil's disease specifically refers to the type of infection that produces jaundice or a severe form (meningitis or kidney failure) of leptospirosis (James, 1997). Leptospirosis is a worldwide zoonosis, affecting farm animals, wildlife and humans (Bahaman and Ibrahim, 1988). Human leptospirosis was recognised in Europe in the 1880's but the causative organisms called leptospires were first isolated in Japan in 1914 (James, 1997).

This important zoonosis has recently been recognised as another re-emerging disease in both developing and industrialised countries (Vinetz, 1997). However, the disease occurs more commonly in tropical countries, and humans are usually infected from animal sources, where these animals excrete leptospires in urine and feces both during active illness and asymptomatic carrier stage into their environments (Bovet *et al.*, 1999). The transmission of infection is often via indirect contact with water, moist soil and food contaminated with urine of infected animals (Bahaman and Ibrahim, 1987; Bovet *et al.*, 1999). Natural reservoirs of infection are rodents and domestic animals including cattle, pigs and dogs (Soltys, 1979; Bahaman and Ibrahim, 1988; CSL Veterinary, 1999). Pathogenic members of the *Leptospira* species do not multiply in the environment, but they can survive in water

and moist soil for long periods of time if conditions are favourable for survival (Jawetz *et al.*, 1982). Thus, drinking, swimming, bathing, gardening, or handling animals may lead to human infection as leptospires can enter the body through abraded skin, mouth, or eyes (Bahaman and Ibrahim, 1987; Bovet, 1999).

Many Asian countries have the ideal environment for the maintenance and spread of leptospirosis as the combination of plantations, rice-fields, and the dense population of rodents, dogs, cats and cattle distributed throughout the regions. For example, during the rainy season, crowded streets in some countries become submerged and established a large population of rats. This provides ideal conditions for disease transmission, as do flooded rice fields (Watt, 1997). Pollack (1999a) reported that there was an outbreak in Thailand where at least 136 people died and more than 2300 others became ill in late 1999. The Bangkok Post (October 24th, 1999) also quoted the Public Health Ministry of Thailand that the toll was the highest since leptospirosis was first implicated in Thailand in 1985. The outbreak was mainly associated with heavy rains and flooding because the disease was transmitted by water contaminated with rat urine (Pollack, 1999a).

Nowadays, leptospirosis has become a common disease not only in tropical or rural areas (CSL Veterinary, 1999), but it is now increasingly recognized in deteriorating inner cities of Europe and America due to an increase in rat populations (Watt, 1997; Pollack, 1999b,c). Bahaman and Ibrahim (1988) also reported that rats were the principal natural maintenance host of leptospirosis in Malaysia. Domestic animals such as cattle, buffaloes, pigs, goats and sheep were investigated for the evidence of leptospiral infection in West Malaysia. However, dogs and cats were excluded from

the investigation at that time because they were considered as pets or small animals (Bahaman *et al*., 1987). To date, the information on leptospiral infection in Malaysian dogs and cats is still lacking. The first case of leptospirosis in domestic animals in Malaysia was a case in a dog, which was reported by Fletcher in 1928 (Bahaman *et al*., 1987). After 1928, four other investigations have been conducted on the prevalence of leptospiral infection in domestic animals in Malaysia (Bahaman *et al*., 1987). However, only a small number of dogs were investigated. Thus, results obtained were not representative of the actual prevalence of the disease.

Several serological surveys have shown that leptospirosis is widespread among dogs throughout the world (Arimitsu, 1989; Watson, 1994; Scanziani *et al*., 1995; Marshall, 1995; Weekes, 1997; Prescott, 1999). In tropical countries, like Malaysia and some other tropical regions, dogs are an important source of infection for humans. Dogs could be hazardous to humans because of their close association with people and their unsanitary habits, particularly, infants crawling on the floor or in the yard, or playing with the animals, may become infected through contact with dog urine. Cats are not as frequently infected as dogs, even though cats are still a hazard (Levett, 1999). In recent years, canine leptospirosis has been reported to be more prevalent although vaccination is widely used to prevent the disease (Gitton *et al*., 1994; Forrest *et al*., 1998). Vaccinated dogs are still at risk because the immunity is serovar specific, and the current vaccines have not included some of the recent prevalent serovars (Forrest *et al*., 1998). Thus, the potential for the bacterins to protect dogs from infection by other serovars seems limited.

Several investigations on leptospiral infection are currently based on the use of serological and bacteriological methods. Tests such as the microscopic agglutination test (MAT) and the enzyme-linked immunosorbent assay (ELISA) are commonly used for detection of leptospiral antibodies in cerebrospinal fluid (CSF) and serum (Cole *et al.*, 1973; Terpstra *et al.*, 1985). Leptospires can be demonstrated by dark-field microscopy or by isolating the organisms through culture. However, the process is very laborious and time-consuming. Cultural examination can take up to 3 months (Faine, 1982) with a low isolation rate (Bejo, 1996). Therefore, isolation by culture is primarily used for retrospective diagnosis. Leptospires can often be cultured from blood or CSF during the acute phase of infection whilst a specific antibody often cannot be detected at this stage of infection. Usually, when a specific antibody response is detected, leptospires have disappeared from the blood and bacteriuria is often intermittent (Me'rien *et al.*, 1992; Brown *et al.*, 1995). Thus, information on the stage of the infection is essential to plan and organise serological and bacteriological tests.

The polymerase chain reaction (PCR) has been used for the early diagnosis of leptospirosis and has been demonstrated to be both sensitive and rapid (Van Eys *et al.*, 1989; Me'rien *et al.*, 1992; Brown *et al.*, 1995). This is important as the infection can be detected and treated at an early stage. The specificity of the assay can be adjusted by the choice of primers (Van Eys *et al.*, 1989). The PCR has become a useful tool due to its rapid detection of small numbers of leptospires in clinical samples through its specific amplification of the leptospiral DNA. It is seen that the PCR can be used as a tool for diagnosis as well as for epidemiology studies.

In addition to the rapid detection of leptospires in clinical samples, a number of DNA-based methodologies have been applied for identification and classification of leptospiral species and serovars. Techniques such as bacterial restriction endonuclease DNA analysis (BRENDA) or restriction endonuclease analaysis (REA) (Marshall *et al.*, 1981; Robinson *et al.*, 1982) and DNA hybridisation (Millar *et al.*, 1987) may be suitable for identification, but they require large amount of purified DNA. PCR-based typing techniques such as PCR-REA, AP-PCR (arbitrarily primed - PCR), and LS-PCR (low-stringency - PCR) have become ideal for the rapid identification of leptospiral serovars because PCR-derived profiles are also less complicated and easier to compare than genomic REA profiles (Caballero *et al.*, 1994; Brown and Levett 1997).

The objectives of this study were:

1. To determine the frequency of leptospiral agglutinins in stray and pet dogs in Kuala Lumpur and Selangor,
2. To determine the bacteriological prevalence of leptospiral infection in dogs from selected areas in Malaysia,
3. To evaluate the polymerase chain reaction (PCR) technique in detection of leptospires in clinical samples and to identify the DNA profiles of leptospires detected in the dog specimens by low-stringency PCR (LS-PCR).

CHAPTER II

LITERATURE REVIEW

Classification and Nomenclature

Leptospira is a genus of the family Leptospiraceae, order Spirochaetales (Jawetz *et al*., 1982). Until recent times, the genus *Leptospira* was classified as having two species, which are *Leptospira interrogans* and *L. biflexa* (Bahaman and Ibrahim, 1987; Vinetz, 1997). *Leptospira interrogans* is the pathogenic species whereas *L. biflexa* is the saprophytic or non-pathogenic species (Bahaman and Ibrahim, 1987; Watt, 1997). More than 200 serovars from 23 serogroups have been identified for *L. interrogans* throughout the world (Wagenaar *et al*., 1994; Letocart *et al*., 1997), using conventional classification which is based on antigenic similarities (Gravekamp *et al*., 1993). Examples of these known serovars are *canicola, icterohaemorrhagiae, pomona, grippotyphosa, hardjo, australis,* and *copenhageni.* A serovar is regarded as a basic taxon at the subspecies level of leptospires (Gravekamp *et al*., 1993). Antigenically related leptospiral serovars are arranged into serogroups, that is, those serovars which cross-agglutinate to a high titre with one anothers' antisera (Bahaman and Ibrahim, 1987). These serogroups help to reduce the number of antigens used in screening unknown sera.

Now, the genus *Leptospira* is subdivided into a number of new species. This is due to a new development in molecular biology which can be used in typing (Perolat *et al*., 1993; Ralph *et al*., 1993; Letocart *et al*., 1997; Brown and Levett, 1997) and

allows grouping on the basis of DNA- relatedness (Gravekamp *et al*., 1993; Watt, 1997). This evolution of genetic classification divides the pathogenic strains into seven species (*L. interrogans, L. borgpetersenii, L. kirschneri, L. weilii, L. noguchii, L. santarosai, and L. inadai*) and four non-pathogenic species (*L. biflexa, L. meyeri, L. parva, and L. wolbachii*) (Gravekamp *et al*., 1993).

Morphology and Identification

Leptospires are tightly-coiled, thin, flexible, motile spirochetes with 5-15 μm long and 0.1 to 0.2 μm in diameter. One end of the organism is often bent and appears to be hook-like (Jawetz *et al*., 1982; Watt, 1997). The movement is mainly rotary. There is no flagellum. Electron micrographs show a thin axial filament and a delicate membrane (Soltys, 1979). The length of each cell varies during growth. Each cell grows until it doubles the length of the original cell and then it divides into two short cells by binary fission. Leptospires can be examined under dark field microscopy where they show active movement. They can also be stained by Giemsa stain or by the silver impregnation methods (Ellis and Little, 1986).

Leptospires are easily cultivated in fluid media, but pathogenic strains are fastidious in their environmental and nutritional requirements. The addition of rabbit serum, peptone and agar into a medium is usually required for routine cultivation (Soltys, 1979). However, different serovars have different minimal requirements for their isolation. Thus, culture media have to be favoured for each isolate (Bahaman and Ibrahim, 1987). Today, a number of media have been developed for the isolation and maintenance of leptospires. Apart from those enriched with rabbit serum (Soltys,