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CHARACTERIZATION OF LEPTOSPIRAL ISOLATES OBTAINED FROM SELECTED CATTLE FARMS IN MALAYSIA

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By

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LIST OF ABBREVIATIONS

ABTS	2, 2-Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) Diammonium Salt
BRENDA	Bacterial restriction endonuclease DNA analysis
CAAT	Cross agglutination absorption test
CFT	Complement fixation test
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
H_2O_2	Hydrogen peroxide
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IH	Institut Haiwan
JS	Johnson and Seiter
kD	Kilodalton
M	Molar
mA	Milliampere
MARDI	Malaysian Agricultural Research and Development Institute
MAT	Microscopic agglutination test
mM	Millimolar
OD	Optical density
O/N	Overnight
pH	puissance hydrogen (Hydrogen-ion concentration)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PLT	Pusat Latihan Ternakan
rpm	round per minute

SDS	Sodium dodecyl sulfate
Tric-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
UPM	Universiti Pertanian Malaysia
V	Volt
VRI	Veterinary Research Institute
v/v	volume per volume
w/v	weight per volume
µ	Micron
µg	Microgram
µl	Microlitre

Abstract of the thesis presented to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirements for the degree of Master of Science

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Chairman: Prof. Abdul Rani Bahaman

Faculty: Veterinary Medicine and Animal Science

Leptospirosis is an infectious disease of animals and man in many parts of the world. It is caused by *Leptospira* and has been classified as an important zoonotic disease. A serological survey in four selected dairy cattle farms in Malaysia revealed 36% (114/318) of the animals examined had leptospiral infection. Antibodies to serovar *hardjo* was the main (19%) serovar detected. A bacteriological survey revealed only 1.2% (3/244) of the cattle examined had leptospiral infection. Two isolates obtained have been identified as *L. hardjo* and another one as *L. pomona.*

Bacteriological study did not come across any multiple leptospiral serovar infection in the cattle farms studied. The serological prevalence of serovar *pomona* infection was very low. However, one isolate that has been identified as serovar *pomona* was isolated from cattle in Sungai Siput Farm in this study. This finding suggested that cattle in this farm might be maintaining the serovar *pomona* infection.

This study also found that isolate SS1946 grew faster than isolates H41 and F33. Growth of isolates H41 and F33 were inhibited at 13^{o}C and in presence of 8-azaguanine but isolate SS1946 was resistant to 8-azaguanine and was able to grow at 13^{o}C. Pathogenicity test in weanling hamsters (*Mesocricetus auratus*) found that isolates H41 and F33 caused death to weanling hamsters whereas isolate SS1946 did not kill the weanling hamsters. This finding suggested that the ability of leptospiral isolates to grow at 13^{o}C and in presence of 8-azaguanine and pathogenicity test using weanling hamsters can be used for differentiation of leptospiral isolates.

The enzyme-linked immunosorbant assay (ELISA) using boiled antigen gave a good agreement to the microscopic agglutination test (MAT). This finding suggested that ELISA using boiled antigen was a suitable test for screening large number of samples and useful for epidemiological study.

The leptospiral isolates were identified by MAT using known hyperimmune sera and further identification was carried out by polyacrylamide gel electrophoresis (PAGE) and bacterial restriction endonuclease DNA analysis (BRENDA). Serologically isolates H41 and F33 were identified as serovar *hardjo* whilst isolate SS1946 was identified as nonpathogenic leptospira. Analysis on their proteins by means of PAGE revealed differences between these isolates. The protein pattern of isolate H41 and F33 was similar to each other. The protein profile of these isolates was similar to the *L. hardjo* reference strain. The protein profile of isolate SS1946 was similar to the *L. pomona* reference strain.

Study of the immunogenic proteins of the three leptospiral isolates were carried out by Western blotting and immunological staining. This study found that 12, five and six immunogenic proteins from isolates H41, F33 and SS1946 respectively were detected by homologous hyperimmune sera. Two bands on isolate H41 at 35.9 and 35.2 kD were detected by the five hyperimmune sera used whilst the protein bands of isolate F33 detected by same hyperimmune sera were at 53.72 and 51.8 kD. Three protein bands of isolate SS1946 at 55.64, 51.8 and 47.96 kD were detected by all hyperimmune sera. This study showed that leptospires shared several common antigens. If a vaccine can be prepared from the immunogenic part of leptospira local isolate it may offer a solution to effective immunization against bovine leptospirosis in Malaysia.

Analysis of their DNA by restriction endonuclease analysis indicated that the DNA profile of isolates H41 and F33 were similar to each other and different to that of isolate SS1946. The DNA profile of isolates H41 and F33 was different to *L.hardjo* strain *hardjoprajitno* but similar to *L. hardjo* strain *hardjobovis* reference strain. The DNA profile of isolate SS1946 was similar to *L. pomona.*

It is concluded that the study has successfully determined the presence of leptospiral infection. Two *L. hardjo* and one *L. pomona* were isolated from cattle in the farms studied. This study has provided very useful information on the epidemiology of leptospiral infection in cattle and the approch to the control and prevention of the disease in livestock.

Abstrak tesis yang dikemukakan kepada senat Universiti Pertanian Malaysia bagi memenuhi keperluan Ijazah Master Sains

PENCIRIAN ISOLAT LEPTOSPIRA PADA LADANG TERNAKAN LEMBU TERPILIH DI MALAYSIA

Oleh: **SITI KHAIRANI BINTI BEJO**

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Pengerusi: Prof. Dr. Abdul Rani Bahaman

Fakulti: Kedoktoran Veterinar dan Sains Peternakan

Leptospirosis adalah penyakit berjangkit pada haiwan dan manusia di banyak tempat di dunia. Penyakit ini disebabkan oleh *Leptospira* dan ia dikelaskan sebagai penyakit zoonotik yang penting. Satu kajian serologi di empat ladang lembu tenusu terpilih di Malaysia telah mendapati 36% (114/318) lembu yang diperiksa menghidapi jangkitan leptospira. Antibodi kepada serovar *hardjo* adalah yang terutama (19%) dikesan. Kajian bakteriologi mendapati hanya 1.2% (3/244) daripada lembu yang diperiksa menghidapi jangkitan leptospira. Dua isolat leptospira yang dikenal pasti sebagai *L. hardjo* dan satu isolat leptospira yang dikenal pasti sebagai *L. pomona* telah diperolehi.

Kajian bakteriologi menunjukkan tiada jangkitan berganda oleh leptospira di ladang ternakan lembu yang telah dikaji. Prevalens serologi terhadap jangkitan oleh serovar *pomona* adalah sangat rendah walaubagaimanapun satu isolat serovar *pomona* telah dapat diasingkan dari lembu di Ladang Sungai Siput. Keputusan mengambarkan bahawa lembu di ladang ini berkemungkinan menyara jangkitan serovar *pomona.*

Kajian ini mendapati bahawa isolat SS1946 tumbuh lebih cepat daripada isolat H41 dan F33 . Pertumbuhan isolat H41 dan F33 terencat pada 13^{o}C dan dengan kehadiran 8-azaguanine tetapi isolat SS1946 tahan kepada kehadiran 8-azaguanine dan boleh tumbuh pada 13^{o}C. Ujian kepatogenan menggunakan hamster (*Mesocricetus auratus*) cerai susu mendapati isolat H41 dan isolat F33 menyebabkan kematian hamster manakala isolat SS1946 tidak menyebabkan kematian hamster. Keputusan ini mengambarkan bahawa kebolehan untuk tumbuh pada 13^{o}C dan dengan kehadiran 8-azaguanine serta ujian kepatogenan menggunakan hamster cerai susu boleh digunakan untuk membezakan isolat-isolat leptospira.

Assai immunoserap terikat enzim menggunakan antigen rebus memberi persetujuan yang tinggi kepada ujian pengaglutinatan mikroskopi. Keputusan ini mengambarkan bahawa assai imunoserap terikat enzim menggunakan antigen rebus adalah sesuai digunakan untuk ujian penyaringan sampel yang banyak dan berguna untuk kajian epidemiologi.

Isolat-isolat leptospira telah dikenal pasti menggunakan kaedah serologi iaitu ujian pengaglutinatan mikroskopi dan seterusnya menggunakan elektroforesis gel poliakrilamid dan analisis DNA bakteria endonuklease penyekat. Secara serologi isolat H41 dan isolat F33 adalah serovar *hardjo* dan isolat SS1946 adalah tidak patogenik. Analisa menunjukkan bahawa protin isolat H41 dan F33 adalah sama. Profail protin isolat H41 dan F33 adalah sama dengan *L. hardjo* strain rujukan. Profail protin isolat SS1946 adalah sama dengan *L. pomona* strain rujukan.

Kajian terhadap protin yang imunogenik pada isolat-isolat leptospira telah dilakukan menggunakan pemblotan Western dan pewarnaan imunologi. Kajian ini mendapati terdapat 12, lima dan enam protin imunogenik pada isolat H41, F33 dan SS1946 berturutan telah dicamkan oleh serum hiper-immun homologous.

Dua protin band isolat H41 pada 35.9 dan 35.2 kD telah dicamkan oleh semua serum hiper-immun sementara dua protin band isolat F33 telah dicamkan oleh semua serum hiper-immun pada 53.72 dan 51.8 kD. Tiga protin band isolat SS1946 telah dicamkan oleh semua serum hiper-immun pada 55.64, 51.8 dan 47.96 kD. Kajian ini mendapati leptospira berkongsi sebahagian antigen yang biasa. Jika vaksin dapat dihasilkan dari bahagian yang immunogenik pada leptospira isolat tempatan ia mungkin dapat memberi imunisasi yang efektif untuk melawan leptospirosis pada lembu di Malaysia.

Analysis DNA menggunakan enzim penyekat endonukleas *Eco*R1 menunjukkan profail DNA isolat H41 dan isolat F33 adalah sama antara satu dengan lain tetapi berbeza dengan isolat SS1946. Profail DNA isolat H41 dan isolat F33 adalah berbeza dengan *L. hardjo* strain *hardjoprajitno* strain rujukan tetapi adalah sama dengan *L. hardjo* strain *hardjobovis* strain rujukan. Profail DNA isolat SS1946 adalah sama dengan *L. pomona* strain rujukan.

Kesimpulannya, kajian ini telah berjaya memutuskan kehadiran jangkitan leptospira secara serologi dan bakteriologi. Dua *L. hardjo* dan satu *L. pomona* telah diasingkan dari lembu di ladang kajian. Kajian ini menyumbangkan maklumat berguna mengenai epidemiologi jangkitan leptospira pada lembu dan cara untuk mengawal dan mencegah penyakit ini pada ternakan.

CHAPTER 1

INTRODUCTION

Leptospirosis is an important disease which causes considerable economic loss to the cattle industry. *Leptospira interrogans* causes a persistent infection in cattle leading to abortions, stillbirth, retained placenta, weak progeny, mastitis, infertility and death (Songer *et al.*, 1983, Ellis *et al.*, 1985a, Pottos *et al.*, 1995, Abdollahpoun *et al.*, 1996) and is also a zoonotic disease (Mackintosh *et al.*, 1980, Milner *et al.*, 1980).

Evidence of leptospiral infection has been found in a wide variety of animals in Malaysia. In domestic animals, leptospirosis is often inapparent; hyperaemia, inappetence and depression are the usual signs but still it is an important economic disease since animal health and productivity are affected (Bahaman and Ibrahim, 1987). Furthermore, a majority of the human cases of leptospirosis are contracted either directly or indirectly from animals. Leptospires shed in urine of infected cattle may contaminate soil, pastures, waters, foodstuff and bedding and serve as a source of infection for other animals and human beings. Moisture, pH values of soil and environmental temperature were important factors that influenced the survival of pathogenic leptospires outside the host leading to high incidence of the leptospirosis in dairy herd workers (Gordon, 1977).

Based on immunological tests almost 200 serovars of leptospires from 25 serogroups have been identified throughout the world (Dikken, 1986). Out of this large number of serovars, 37 are currently known to be present in Malaysia (Bahaman and Ibrahim, 1987). The wet and warm environment in Malaysia allows the leptospires to survive and infect animals and humans. The majority of the serovars present in Malaysia are maintained by wildlife which act as a source of sporadic infection to domestic animals. Extensive study by Bahaman *et al.,* (1987) has established the distribution and prevalence of leptospirosis in domestic animals in Malaysia. Based on that study, cattle were shown to have the highest prevalence of leptospiral infection. Thus, the epidemiology of the leptospiral infection in cattle was selected for this study because cattle are important livestock that both directly and indirectly kept in contact with human. Furthermore, cattle are known to be an important maintenance host for serovar *hardjo* . Cattle are able to excrete leptospires in urine for a long period and readily transmit the infection to other cattle and humans (Blackmore and Hathway, 1979, Ellis *et al.,* 1981, Hathway, 1981). Pigs have been proven to be the maintenance host for serovar *pomona.* However, bacteriological survey by Bahaman and Chumponbunchorn (1993) found that cattle in Sungai Siput Farm were having persistent serovar *pomona* infection.

Many surveys make use of serological and bacteriological tests to investigate leptospiral infection. The microscopic agglutination test (MAT) is routinely used for the serodiagnosis of leptospiral infection in domestic animals (Cole *et al.,* 1973). The MAT is an effective test with high specificity for individual serovars. However, the test does have several disadvantages. It is laborious, requires maintenance of live cultures for use as antigens, is only relative serovar specific, and there is subjectivity in the reading of the test. Recently, the enzyme-linked immunosorbent assay (ELISA) has been investigated for serological diagnosis of leptospirosis in human and cattle (Thiermann and Garrett, 1983). Similarly, isolates of leptospires have been classified traditionally using serological techniques. However, these

assays are tedious and are not always reproducible (Ellis *et al.,* 1991). Beside serology, which becomes informative only after the seventh day of illness, culturing of leptospires from blood, urine or milk can be used in the diagnosis of leptospirosis.

In the last two decades, molecular methods which analyse the proteins and DNA of organisms have been successfully employed in the classification, identification and typing of bacteria. Polyacrylamide gel electrophoresis (PAGE) of proteins has been used increasingly during the past decade to get protein patterns for bacterial classification and identification. The protein patterns have been found to reflect the genome of a particular strains. A bacterial strain grown repeatedly under standard conditions produce essentially the same set of proteins. Electrophoresis of a bacterial protein sample under standard and reproducible conditions will therefore, produces a protein pattern which is characteristic for that particular strain. Proteins that were immunogenic were also investigated and used for development of effective vaccine to combat bovine leptospirosis (Le Febvre *et al.,* 1987).

Most recently, restriction endonuclease analysis has been successfully employed to type leptospires, including strains that were serologically indistinguishable (Marshall *et al.,* 1981; Robinson *et al.,* 1982; Thiermann *et al.,* 1986; Silbreck and Davies, 1989; Zuerner and Bolin, 1990; Gerritsen *et al.,* 1991). Marshall *et al.* (1981) described a means of differentiating New Zealand field isolates of *L. interrogans* serovar *hardjo* and *balcanica* by restriction endonuclease analysis of DNA and suggested that this new method had advantages over serological testing. The DNA was extracted from a culture of the organism and digested with a restriction endonuclease and the resulting fragments were separated by electerophoresis in an agarose gel. The DNA fragments in the gel were stained with ethidium bromide and examined under ultra violet light. The pattern of bands produced could be used to identify the isolate.

The objectives of the study are :

1. to determine the serological and bacteriological prevalence of leptospiral infection in cattle on selected farms in Malaysia.
2. to determine whether cattle in the Sungai Siput Farm are maintaining serovar *pomona* infection.
3. to study the protein profiles and immunogenicity of the leptospiral protein components by polyacrylamide gel electrophoresis (PAGE) and immunoblotting.
4. to study the DNA profiles of the leptospiral isolates by bacterial restriction endonuclease DNA analysis (BRENDA).

CHAPTER 2

LITERATURE REVIEW

Characterization of leptospires

Leptospires are extremely slender, spiral and flexious micro-organisms. They are actively motile and can be examined alive under dark-field microscopy. *Leptospira* are about 6-20 µm long by 0.1 µm in diameter. The hook in one or both ends is a characteristic morphological feature and the sizes of hook are variable with about 0.2-0.3 µm in overall diameter (Alexander, 1974; Turner, 1974; Blackmore and Humble, 1987).

The genus *Leptospira* consists of two species; *Leptospira interrogans* and *L. biflexa. L. interrogans* has been proposed as the pathogenic strain while *L. biflexa* as the saprophytic strain (Soltys, 1979). Members of the genus *Leptospira* are serologically heterologous (Alexander,1974). Based on the antigenic structure, the *L. interrogans* is further divided into 25 serogroups (Turner, 1974). All serovars which cross agglutinate to a high titer with one anothers' antisera are placed into a common serogroup. According to Neill *et al.*, (1986) almost 200 serovars of leptospires have been identified throughout the world. Out of this large number of serovars, 37 leptospiral serovars have been isolated in Malaysia (Bahaman and Ibrahim, 1987).

Epidemiology of leptospirosis

Investigations on wild life in various parts of the world have revealed that *Leptospira* are widely distributed. Many wild and domestic animal species have been identified as hosts of leptospiral organism (Hathaway *et al.,* 1983). However, only a few animal species are able to maintain the leptospires in their kidneys and become chronic carriers, shedding the organisms in their urine for months and perhaps, years (Leonard *et al.,* 1992).

The most important reservoirs of infection are reported to be rodents, cattle, pigs and dogs (Christmas *et al.,* 1974). *Leptospira* have also been isolated from wild birds, fish, reptiles and frogs (Michna, 1970).

Urine of healthy or diseased carriers may contaminate soil, pastures, water, foodstuffs and represents a potential source of spread (Ris and Hamel, 1978). Leptospiruria, usually evident two to three weeks after infection (Sullivan, 1974), last from a few weeks to over two years (Sullivan, 1970; Ellis and Michna, 1977; Mackintosh *et al.,* 1980; Thiermann, 1982), although it is normally intermittent (Hathaway and Little, 1983). Kidneys of carrier animals (Cargill and Davos, 1981), meat (Blackmore and Schollum, 1982) from animals slaughtered during leptospiraemia and occasionally from diseased or convalescent cattle (Michna, 1970) may play an important role in epidemiology of leptospirosis.

Each serovar is maintained in specific maintenance hosts which are characterised by the minimal clinical effects and a period of leptospiruria which is sufficient to ensure generation to generation transmission (Mackintosh *et al.,* 1980; Hathaway, 1981).

Leptospirosis in cattle is of world wide distribution. The serotypes incriminated as the causes of the disease are *icterohaemorrhagiae*, *pomona*, *grippotyphosa* and *canicola* (Jubb *et al.,* 1985). Blackmore and Humble (1987) reported that, *hardjo* is maintained in cattle and leptospiruria can persist for up to two years. In unvaccinated herds, the majority of first calf heifers will be infected and remained in the herd as a potential source of infection. Most calves will be protected from infection by maternal immunity for at least six months (Bolin et al., 1991; Goddard et al., 1991).

Leptospira interrogans serovar *pomona* and serovar *tarassovi* are maintained in pigs. Endemic infection of pigs with serovar *pomona* is more common than serovar *tarassovi* with the majority of pigs having infected kidneys at the time of slaughter. Pigs act as an important source of serovar *pomona* infection for other species of animals, especially cattle. The most common single cause of abortion in cattle is still serovar *pomona* and infected cattle remain leptospiruric for up to three months. This period of leptospiruria is however insufficient to establish endemic infections within a herd as generation to generation transmission will not occur, but it can create a temporary hazard for other animals including man (Blackmore and Humble, 1987). Acute leptospirosis in piglets caused by serovar *icterohaemorrhagiae* occurs in small outbreaks or as individual cases (Jubb *et al.,* 1985).

On a world wide basis, serovar *canicola* is the most comman serovar infecting dogs (Jubb *et al.,* 1985) and in many parts of the world dogs act as a maintenance host and as such can be a source of infection to other dogs, domestic animals and man (Bahaman and Ibrahim, 1988). The acute disease is most frequent in the age range of one to three years. Dogs which carry residual infections of serovar *canicola* in the kidneys may continue to excrete the organisms in the urine for up to three years, possibly longer (Jubb and Kennedy, 1970).

Many outbreaks of human leptospirosis resulted from indirect contact between animal and man. Generally man contracts leptospira when he intrudes into another ecosystem or when he is involved in an occupation related to animals (Mackintosh *et al.,* 1980; Milner *et al.,* 1980a). Bahaman and Ibrahim (1988) reported that leptospirosis has been closely associated with agricultural occupation and one of the most important occupation recognised to predispose is rice cultivation through contact between padi planters and infected rats.

The danger of transmission to man during handling of the kidneys of carrier animals or feeding such kidneys to dogs and cats has been emphasised by Michna (1970). In dairy countries like New Zealand, cattle are the maintenance host for serovar *hardjo* and are responsible for the large number of leptospiral cases among human population (Blackmore and Hathaway 1979; Blackmore and Humble, 1987).

Pathogenesis of leptospirosis

Leptospirosis is a contagious disease of animals and man due to infection with *Leptospira spp.* Cattle, horses, pigs, dogs and sheep are susceptible to the infection. Leptospirosis occurs in Malaysia and in many other parts of the world. Infected animals eliminate the organisms in secretions and excretions particularly in urine. The infection is principally percutaneous and the organisms spread from the point of infection into the blood stream and multiply there soon after penetrating the host's epithelium. At this time they produced no lesion but became septicaemic (Bahaman and Ibrahim, 1987).

The septicaemic phase varies widely in its manisfestation; some cases do not produce clinical illness while others cause death in one to seven days (Jubb *et al.,*1985). As the septicaemic phase subsides, the organisms localize and persist in