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The Survival of *Leptospira interrogans* Serovar *Hardjo* in the Malaysian Environment

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Abstract: The survival time of *Leptospira interrogans* serovar *hardjo* in water, soil and cattle urine in Malaysia were evaluated. The survival time of serovar *hardjo* in the six types of water samples varied considerably. The longest survival time recorded was for 264 hours (11 days) in river water with between pH 6.7 and pH 7.3 placed in shaded area. The shortest survival time was recorded in seawater pH 6.5 to pH 6.8, with dissolved solid salt content between 3.78% and 3.85%. The leptospires were killed almost immediately after inoculation into seawater. The survival time of serovar *hardjo* in the three types of soil with three different water content, kept under either direct sun or shaded area were variable. The minimum survival time was 2 hours and maximum was 144 hours (6 days). Serovar *hardjo* survived in undiluted urine for only 2 hours under direct sun and 6 hours under shaded area. The survival time of serovar *hardjo* was longer in urine diluted with distilled water compared to undiluted urine. In diluted urine under direct sun, serovar *hardjo* can survive for up to 48 hours (2 days), under shaded area for up to 72 hours (3 days) and in refrigerator (4°C) for 984 hours (41 days). Under experimental conditions serovar *hardjo* survived in water, soil and urine remained infective up to 1 to 7 days post inoculation. These findings suggested that survived organisms would provide a source of infection for human and animals exposed to the contaminated environment.

Key words: Leptospira, survival time, water, soil, urine

Introduction

Environmental factors have frequently been shown to influence the development of leptospirosis in animals and human beings. In addition to direct contact, leptospirosis may be transmitted by exposure to infective materials in the environment that permit survival of leptospires (Ellis, 1986). The prevalence of leptospiral infection depends on environmental factors which allow survival of leptospires outside the host and the susceptibility of man and animals to pathogenic leptospires. Water and soil contaminated with urine from infected animals are of primary importance in the transmission of the disease (Caldas, 1979). Several environmental factors affect the survival time of the pathogenic leptospires. The most important is the pH of the urine, the soil and the water in which the urine is excreted and the local temperature. Heavy rainfall, natural water bodies and the abundance of different animal species favor the transmission of the leptospires to other animals and man (Szyfres, 1976).According to Malaysian Meteorological Service (MMS), the characteristic features of the climate of Malaysia are uniform temperature, high humidity and heavy rainfall throughout the year (MMS, 1999). The day temperature is between 27°C to 32°C and night temperature is between 21°C to 24°C. The mean monthly relative humidity is between 70% to 90% varying from place to place and from month to month. The distribution of rain in the country is according to season. The incidence of leptospirosis in cattle is higher in warm and wet climatic conditions where leptospires have a better opportunity to survive. Duration of survival of pathogenic leptospires outside the host requires a warm, moist environment and pH of soil and water that is near neutral (Michna, 1970). High rainfall, warm and wet climatic condition make leptospirosis endemic in Malaysia. Thus, twenty-nine serovars of pathogenic leptospires have been isolated from natural waters and wet soils in Malaysia (Alexander *et al.*, 1975). Leptospirosis caused by serovar *hardjo* is an important zoonotic disease worldwide (Sehgal *et al.*, 1999) and it is endemic in cattle population in Malaysia (Bahaman *et al.*, 1987). However, information on the survival of serovar *hardjo* in environment under Malaysia field condition was scanty. Thus, the objectives of this present study were to determine the survival time of serovar *hardjo* in cattle urine, water and soil in Malaysia and to determine the infectivity of survival serovar *hardjo* in urine, water and soil to experimental animals.

Materials and Methods

Local isolate of serovar *hardjo* obtained from cattle was used in the present study. The organisms were grown for seven days at 30°C in liquid JS medium. The number of viable organisms per ml of culture was obtained by counting the number of organism present in a drop of inoculum of known volume using the dark-field microscopy. An inoculum containing approximately $1x10^5$ leptospires/ml was prepared. Six types of water namely rainwater,

pond water, tap water, seawater, river water and drain water were collected from four different locality in Peninsular Malaysia. Three types of soil namely clay, sand and loam were also collected from various parts of Peninsular Malaysia. Urine samples were collected from four female cattle that were free from leptospires were given furosemide (Lasix®; Hoechst Animal Health) at approximately 0.7 mg/kg body weight by intramuscular injection prior to urine collection. These water, soil and urine samples were used in the study of survival time of serovar *hardjo* outside the host. The pH values of each water, soil and urine samples were measured using pH meter (CRISON, microph 2002). Four water samples each were placed in different conditions including shaded area (27°C) and direct sun (32°C). One hundred (100) ml of water samples were placed in conical flasks and were inoculated with serovar *hardjo* culture at the concentration of $1x10^5$ leptospires per ml. The waters were sampled at 2, 4, 8, 12 and 24 hours, and daily until 15 days, and weekly up to 60 day p.i. Two kilograms (kg) of the soils were completely dried by heating at 40°C for three days in hot air oven to destroy any free-living leptospires present. Small plastic cups measuring 70 mm x 70 mm x 20 mm and each containing 10g of dried soil were placed in a plastic tray. Sterile distilled water was added in to four trays each to give final concentrations of 23, 29 and 33 per cent of the total soil weight respectively. These soil-water ratios were maintained throughout the experiment by frequent replacement of the small water loss. The trays were sealed in plastic bags to prevent water loss and were left to stabilize for several days in the environment before each tray was inoculated with 1×10^5 leptospires. The small plastic cups were withdrawn from each of the plastic tray at 2, 4, 8, 12 and 24 hours and daily until the 15 day, and weekly until the 60 day p. i. The soil samples were then placed in sterile beaker containing 20 ml of sterile distilled water. The beaker was agitated to suspend the soil particles and the suspension was left to stand for 2 hours. The supernatant was collected for culture.Urine samples were prepared into two concentrations namely the undiluted and diluted urine samples. The diluted urine sample was prepared in distilled water to make 1: 10 dilution. The urine samples were then placed in the three different environmental conditions namely shaded area (27°C), direct sun (32°C) and refrigerated (4°C). One hundred (100) ml of the undiluted and diluted urine were placed in conical flasks and were inoculated with serovar *hardjo* culture. Inoculated urine samples with serovar *hardjo* were sampled at 2, 4, 8, 12 and 24 hours, daily until the 15 days and weekly until the 60 days p.i.

Weanling hamster was choused as experimental animal in determine the infectivity of survival serovar *hardjo* in urine, water and soil. Weanling hamsters, obtained from a leptospire-free colony, were inoculated intraperitoneally with 0.5 ml of serovar *hardjo* inoculated water, soil and urine samples collected at days 0, 7, 14, 21 and 28 p.i. The inoculated hamsters were observed for the present of clinical signs related to leptospiral infection for 35 days. Samples of kidneys were cultured either at death or on the days 35 p.i. Blood samples were collected from hamsters at days 0, 7, 14, 21, 28 and 35 p.i. The antibody against serovar *hardjo* was detected by ELISA following the method described by Adler *et al.,* (1982).

Results and Discussion

The conditions of shaded and direct sun areas used in this experiment represent the average natural condition in a typical Malaysian cattle farm and the number of leptospires in the inoculum was approximately the number of leptospires released by a cow. The experiment demonstrated the survival times of serovar *hardjo* in six types of water that were frequently in contact with human and cattle population namely rain water, pond water, river water, sea water, pipe water and drain water. Serovar *hardjo* when inoculated into rainwater, pond water, river water, pipe water and drain water could survive between 6 and 264 hours (11 days) under shaded area (Table 1). Environmental temperature and pH values were important factors that influence the survival time of leptospires in water. The longest survival time of serovar *hardjo* was recorded in river water pH between 6.7 and 7.3 placed in shaded area. These findings showed that, river could be a source of leptospiral infection. In this study, serovar *hardjo* was recorded to survive in river water for 264 hours (11 days). These findings, however, revealed that the survival time of serovar *hardjo* in river water was shorter than the study conducted by Hellstrom and Marshall (1978) who reported that leptospires could survive in river water for up to 94 days. This results is important as many activities like military operation, jungle tracking, picnicking and fishing are related to rivers. The survival time of serovar *hardjo* in river water placed under direct sun was shorter compared to water placed under shaded area. Environmental factors in the jungle such as canopy of trees, temperature of around 27°C and unpolluted water may increase the ability of leptospires to survive longer in the environment.

In drain water, serovar *hardjo* survived for 192 hours when placed under shaded area and survived for only 6 hours when placed under direct sun. The pH of drain water was between 6.7 and 7.5 but the survival time of serovar *hardjo* was shorter in drain compared to river water. This probably due to the polluted drain water compared to river water. Leptospires need fresh water to remain viable in the environment. In the polluted drain water, leptospires were often overgrown by other contaminants. Minzat and Tomescu (1975), however, reported that the average

Table 1: The survival time of serovar *hardjo* in different types of water under direct sun (32°C) and shaded are (27°C)

Types of water	pH of water	Survial time of serovar *hardjo* (hours)	
		Direct sun	Shaded area
Pipe	6.8	6	72-120
Pond	6.7-7.2	6	72-96
Rain	6.6-6.9	2-6	6
Sea	6.5-6.8	0	0
River	6.7-7.2	6	264
Drain	6.7-7.5	6	192

Table 2: The survival time of serovar *hardjo* in different types of soil under direct sun (32°C) and shaded area (27°C)

Type of soils	pH of soils	Survival time of serovar hardjo (hours) in different water contents (%)					
		Direct sun			Shaded area		
		23%	29%	33%	23%	29%	33%
Loam	5.5-5.6	2	2-4	2-4	2-4	12-24	120-144
Clay	6.5-6.7	2	2-4	2-4	2-4	6-10	96-144
Sand	5.5-6.0	2	2	2-4	2-4	8-10	48-96

Table 3: The Survival time of servovar hardjo in diluted and undiluted urine under direct sun (32°C), shaded area (27°C) and refrigerator (4°C)

Conditions	Survival time of serovar hardjo (hours) in cattle urine	
	Diluted urine	Undiluted urine
Direct sun (32°C)	48	0 - 2
Shaded area (27°C)	72	2 - 6
4°C	48-984	2 - 48

period of survival of pathogenic leptospires in sewage effluent was between 24 and 48 hours with a maximum of 96 hours.

The survival time of serovar *hardjo* in pond water was between 72 and 96 hours under shaded areas and 6 hours under direct sun. The pH of water was recorded between 6.7 and 7.2. The ability of serovar *hardjo* to survive in pond water is an important factor as pond water is normally used as water source in cattle farms. Pond water can be contaminated with serovar *hardjo* when infected cattle urinate into the pond or the surrounding area. It then becomes a source for leptospiral infection to cattle, other animals and humans that come in contact with the contaminated pond. Alexander *et al.* (1964) reported that leptospires could survive in pools and stagnant water for a few weeks whilst Ryn and Lin (1966) reported that leptospires only survived for one week in these types of water. However, the survival time of serovar *hardjo* in pond water was much shorter in this study. This could be due to the differences in the environmental temperature between temperate and tropical countries. Direct sun with high environmental temperature (32°C) can easily kill leptospires in the environment.

The present study demonstrates that leptospires can survive between 72 and 120 hours in chlorinated pipe water. Michna (1970) reported that leptospires remained viable for up to 30 days in sterile pipe water. Although the ability of serovar *hardjo* to survive in pipe water was shorter in this study compared to the study by Michna (1970), it still gave a point to consider that serovar *hardjo* can survive even in chlorinated pipe water. Pipe water was a source of water for the majority of the people in this country. Pipe water is considered as clean and safe water to consume as it is treated with chlorine to kill pathogenic bacteria. People who use pipe water are suppose to be of less risk to leptospiral infection, since leptospires can only survive for up to 120 hours. Urine of infected cattle or other carrier animals such as rats can contaminate pipe water in the water tanks. This can become a source of leptospiral infection to human and animals. Reports on outbreaks of human leptospirosis as a result of bathing in water contaminated with urine of carrier farm animals have been reported (Jackson *et al.*, 1993). It is suggested that all water containers be covered to avoid from contamination.

The survival time of serovar *hardjo* in rainwater with pH 6.8 was no longer than 6 hours under shaded area and direct sun. The finding was different to the finding of Krischner and Maguire (1957). Krischner and Maguire (1957) reported that serovar *pomona* from cattle urine diluted with rain water survived for up to three weeks. This difference might be due to the different serovar or environmental condition under which the experiments were carried out. Previous workers regarded environmental conditions are important in the survival of leptospires in the environment (Smith and Turner, 1961; Twigg *et al.,* 1969). Many clinical outbreaks and natural leptospiral infection in cattle have been reported in environmental conditions favorable to the survival of the leptospires such as high rainfall and stagnant rainwater (Sullivan and Callan, 1970).

This study has shown that leptospires died almost immediately in seawater. Michna (1970) reported that survival time of leptospires in seawater containing 3.45 per cent salinity was for 18 to 24 hours. Although a case of leptospirosis in sea lion in California was reported by McIhatten *et al.* (1971), the source of infection was not clearly determined. Schuffer (1934) tested the survival of leptospires in water from various source in the Netherlands and from the North Sea on the Dutch Coast. It was shown that leptospires could survived for 10 days in lake water with low salinity (40 mg of chlorine per liter or less) whereas in water from the North Sea with 13 000 to 17 000 mg chlorine, the survival time was less than 1 day. Chang *et al.* (1948) recorded that in seawater with a total salt content of 22000 mg per liter, leptospires could survive between 18 and 20 hours. The use of sea water for washing fish was considered by Hampson (1946) as a likely explanation of the low incidence of leptospirosis among fish workers in Grimsby, England as compared to the high rate in Aberdeen, Scotland where fresh water was used (Davidson *et al.,* 1934). The finding from this study indicated that sea recreation is safe to people since leptospires were not able to survive in sea water under Malaysia conditions.

Leptospires can survive in water under Malaysian field condition. Leptospires survive longer under shaded area than under direct sun. The high temperature and the strong ultraviolet ray under direct sun apparently killed the serovar *hardjo* rapidly. This finding was similar to the finding of Polanen (1941), who reported that leptospires were readily destroyed by ultraviolet light. The thermal death point of leptospires was carefully studied by Chang *et al.* (1948). They found that, when suspended in water, leptospires were killed on exposure to a temperature at 45°C for 30 minutes; at 50°C for 10 minutes; at 60°C for 10 seconds and at 70°C the organisms could not survive for more than 10 seconds. Leptospires are sensitive to acids and are seldom found in water of pH less than 6.8. However, in this study serovar *hardjo* was still viable at pH 6.6, but for not more than 6 hours. Sardjito and Zuelzer (1929) found abundant leptospira in alkaline waters in Sumatera, but were absent from the more acidic waters in Java. In the Andaman Island, Taylor and Goyle (1931) recorded that leptospires were frequently present in waters of pH 6.9 or more, but were absent from those of pH 6.6 or less. Okazaki and Ringen (1957) noted that the survival time of leptospires in solutions of different acidities varied with the temperature at which they were maintained. It was suggested that the duration of leptospires survived in water was also depending on the environmental temperature. The experiment demonstrated that environmental temperature and water content of soils played important factors influencing the survival time of serovar *hardjo* in three types of soil including sand, clay and loam. Serovar *hardjo* can survive in the soil samples placed under direct sun as short as 2 hours (Table 2). In contras the organism can survive up to 144 hours p.i. in loam and clay soils with 33% water content kept in shaded area. The pH of the soils is thought by most observers to be significant in the survival of leptospires, which prefer an alkaline environment (Martin *et al.,* 1967 and Kingscote, 1970). Although some workers found organism to be maintained under acidic conditions (Gordon-Smith and Turner, 1961; Twigg *et al.,* 1969), the present study showed that leptospires could survive up to 144 hours in soils with pH between 5.5 and 6.7. Moisture, pH of soil and temperature are critical for the survival of leptospires outside the host. The survival time of serovar *hardjo* in soil was shorter compared to the findings by Hellstrom and Marshall (1978) who carried out similar study in New Zealand. They reported that leptospires could survive for up to 42 days in wet soils under winter field conditions.

Karaseva *et al.* (1973) reported that the duration of leptospires surviving on soil surface is in direct proportion to its moisture. They found that leptospires survived only for a few hours in dry matter. The shortest survival times was recorded where the moisture of the soil was low (9.5-16.5%), with little shade and pH of 5.5. The soil used in the present experiment was acidic, with pH as low as 5.5, same as pH of soil used by Karaseva *et al.* (1973). In moist soils with 40-60% water contents and pH of 6.9-7.4, leptospires were preserved for 4 to 7 days. Smith and Self (1955) who made careful experiments on the survivality of serovar *australis* found that this serovar survived in soil for 43 days, whilst Kirschner and Maguire (1957) reported that serovar *pomona* survived in the soil for 21 days. The effect of moisture content of soil was studied by Okazaki and Rigen (1957). In dry soil, no leptospires could be detected after half hour by direct microscopy examination or by culture after 2.5 hours. In damps soil, they could neither be seen after 3 days nor cultured after 5 days. Under supersaturated conditions, they were still visible at 193 days and could be cultured for up to 183 days.

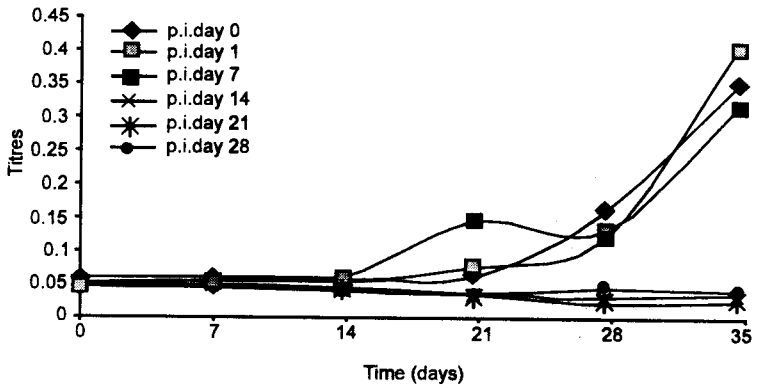

Fig. 1: Antibody titers of serovar hardjo in hamsters inoculated with water samples detected by ELISA

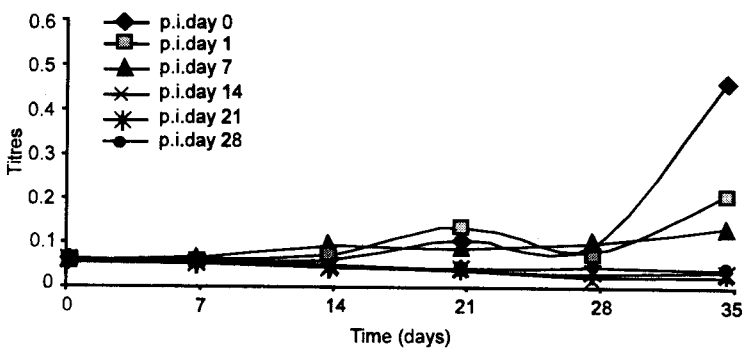

Fig. 2: Antibody titres of serovar *hardjo* in hamsters inoculated with soil samples detected by ELISA

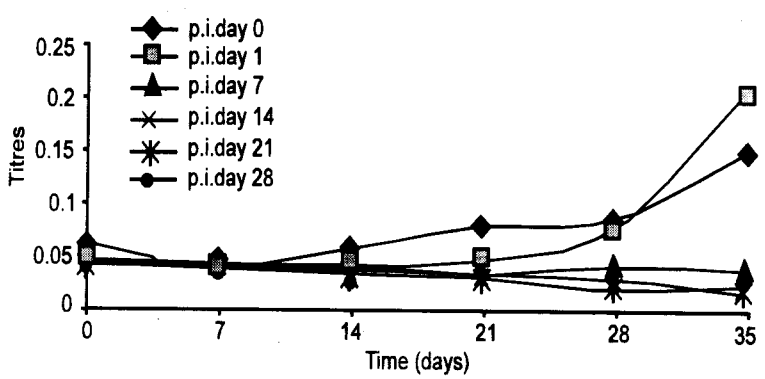

Fig. 3: Antibody titers of serovar hardjo in hamsters inoculated with urine samples detected by ELISA

The survival time of serovar *hardjo* in undiluted cattle urine was up to 48 hours when urine samples were kept at 4°C. However, the survival time of serovar *hardjo* in undiluted urine was reduced when kept in direct sun and shaded area with temperature 32°C and 27°C respectively (Table 3). The urine pH value of cattle used in this present experiment was 8. The pH value was similar with the urine samples used by Leonard *et al.* (1992b) who

reported that the urine pH value of normal adult cattle was in the region of 7 to 8. They reported that leptospires could survive many hours in bovine urine. In this study leptospires survived for not more than 2 hours in undiluted urine placed under direct sun and for up to 6 hours in shaded area. The environmental temperature where the samples were kept affected the survival time of leptospires in undiluted urine. There was no significant change of urine pH throughout the study.

This present study showed that serovar *hardjo* can survived longer for up to 48 hours in diluted urine placed in direct sun when compared to undiluted urine. The ability of serovar *hardjo* to survive in cool temperature (4°C) is a practical important on the isolation of leptospires from urine samples. It is recommended that urine samples should be kept under low temperature prior to isolation of the samples in the laboratory. The ability of serovar *hardjo* to survive in diluted urine is also a practical important in isolation of the organism. Furthermore diluted urine containing serovar *hardjo* present in the environment might a risk to man and animals to be infected with the organism. Leonard *et al.* (1992) reported that serovar *hardjo* survived for up to 18 hours in urine of cattle at pH 7.3.

Under experimental conditions serovar *hardjo* survived and remained infective for 1 to 7 days p.i. (Figs. 1, 2 and 3). The infectivity study showed that serovar *hardjo* could survive in water, soil and urine samples and did not cause death to the inoculated hamsters. The ability of leptospires to survive in the environment will facilitate transmission of this disease from one animal to another. It was suggested that serovar *hardjo* is able to survive in water, soil and urine under Malaysian field conditions and will provided a source of infection for human and animals exposed to the contaminated environment.

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