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Rats: Leptospirosis Reservoir in Serdang Selangor Residential Area

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Abstract: A polymerase chain reaction (PCR) technique was used for detection of the *Leptospira interrogans* gene in kidney tissue from 32 rats trapped from Serdang, Selangor Malaysia residential area in an attempt to determine a possibility rats as a reservoir of leptospirosis. Primer G1 and G2 derived from genomic DNA libraries of *Leptospira interrogans* enable the amplification of target DNA fragment from pathogenic leptospires. Positive PCR result was detected in kidney sample of one rat. Serum samples were examined for the presence of leptospiral infection by microscopic agglutination test (MAT). Serological study showed that all serum samples were negative to leptospiral antibody. The cultures of blood and kidney samples were also negative to *Leptospira*. Present study showed that PCR method was more sensitive in detection of *Leptospira* compared to culture method. The findings suggest that rats still a potential reservoir for leptospirosis and play an important role in spreading of the disease.

Key words: Rat, polymerase chain reaction (PCR), microscopic agglutination test (MAT)

Introduction

Leptospirosis is an acute generalized infectious disease caused by any of the 180-serotypes of *Leptospira interrogans*. Leptospirosis is a zoonosis of worldwide distribution, affecting a large number of wild and domestic mammals, which may behave like assymptomatic hosts. Many outbreaks of humans leptospirosis resulted from indirect contact between humans and the maintenance or carrier animals (Sanchez, 2000; Ramyasuda and Sulochana, 1998). Studies on isolates of leptospires from rats and rodents shown these mammals are the carriers of leptospires throughout the world are important reservoir of infection for domestic animals and human. The role of the rats as a source of human infection was discovered since 1971 (Ido *et al.*, 1917). *Leptospira icterohaemorrhagiae* is the most common serovar involved in rats. Bahaman *et al.* (2002) reported that *Leptospira icterohaemorrhagiae* was detected from liver, heart and blood samples obtained from patients who have been diving in one of the river in Sabah, Malaysia. They believed that the source of infection could be contaminated water with rat's urine, which contained pathogenic leptospires. In a study on 1961 examine the prevalence of leptospiral infection in rats in various localities in Malaya (to date known as Malaysia), 9% were positive for leptospiral infection (Gordon-Smith *et al.*, 1961b). In a more recent study, the serological prevalence of infection amongst 27 rats serum samples collected from Serdang, Selangor was shown to be 37% (Khairani-Bejo, 2002) and antibody to serovar *icterohaemorrhagiae* was the only antibody detected by microscopic agglutination test (MAT). Due to the importance of rats being the important reservoir of leptospires in Malaysia, this paper reports the presence of leptospires in rats in Serdang, Selangor as detected by polymerase chain reaction (PCR).

Material and Methods

Thirty-two rats were trapped from Serdang, Selangor residential area in this study. Blood samples were collected intracardiacally from the rats for isolation of leptospires and serological study. All rats were sacrificed and kidney samples were collected for leptospiral isolation and PCR. In obtaining leptospiral isolates from rats, two drops of heart blood were immediately cultured into semisolid Johnson and Seiter (JS) medium. Kidney samples collected were squashed in a 5 ml syringe and passed into universal bottle containing 10 ml of liquid JS medium. The suspension was then mixed and allowed to stand at room temperature for 10 to 15 minutes. Two drops of supernatant were inoculated into semisolid JS medium. All cultures were incubated at 30°C and examined under dark-field microscope for the presence of leptospires at 7 days interval until 12 weeks post inoculation.

Serum samples were examined for the presence of leptospiral antibodies by microscopic agglutination test (MAT). The MAT was performed using live antigen of four representative serovars usually affecting domestic animals and rats in Malaysia namely serovar *icterohaemorrhagiae, canicola, hardjo* and *pomona*. Microscopic agglutination test were carried out as described by Cole *et al.*, (1973).

In obtaining DNA from kidney samples, Wizard® Genomic DNA Purification Kit (Promega) was used in this present study. Briefly, capsule of kidney were aseptically removed. Kidney was then squeezed by forcing it through the sterile disposable 5ml syringe into a 15ml centrifuge tube containing 600μl of chilled Nucleic Lysis Solution followed by homogenized using homogenizer. Homogenized kidney solution was incubated at 65 C for 30 minutes. Subsequently 3μl of Rnase Solution was added into 1.5ml centrifuge tube containing 600μl lysate and incubated the mixture at 37°C for 15 minutes. It was cooled to room temperature for 5 minutes before 200μl Protein

Precipitation Solution was added, followed by thoroughly vortexed and cilled on ice for 5 minutes. The precipitated protein was rapidly sedimented by centrifugation. The supernatant which containing DNA was carefully transferred to a new 1.5ml microcentrifuge tube containing 600μl of room temperature isopropanol, then mix gently and centrifuged for 2 minutes at 13 000 rpm. Lastly the ethanol was removed and the white pellet of DNA was air-dry for 15 minutes. The DNA was rehydrated with 100μl DNA Rehydration Solution and incubated for 1 hour at 65 C and kept in 4°C until used.

Isolated DNA was further proceed into PCR. Forty five microliters of PCR reaction mixture containing 2 μl $MgCl_2$, 5 μl $MgCl_2$ buffer, 1μl dNTP's, 10μl primer G1 (5'CTG ATT CGC TGT ATA AAA GT 3') and primer G2 (5' GGA AAA CAA ATG GTC GGA AG 3') each, 16μl distilled water and 1μl Taq polymerase was added into 5μl rehydrated DNA in PCR tube. PCR was performed in a Thermocycle (Bio-Rad) with the conditions consisted of 35 cycles of consecutive denaturation, anneling of primers and DNA chain extension (3 minutes at 94°C, 1 minute at 55°C and 1.5 minutes at 72°C) preceded by an initial 3 minutes denaturation at 95°C and followed by a final elongation step at 72°C for 10 minutes (Khairani-Bejo, 2002).

Polymerase chain reaction products were detected by agarose gel electrophoresis. Ten microliters of amplification products were mixed with 2μl loading buffer were loaded into 2% agarose gel in TAE buffer (2M Tris-acetate, 0.05M EDTA, pH 8.3). One hundred base pair (bp) DNA ladder was loaded at the first well of the agarose gel. Positive and negative controls were also loaded into each gel. Electrophoresis was run at 70 volt for 3 hours. The gel was stained with ethidium bromide at concentration of 0.25μg/ml for 15 minutes and leptospiral DNA band was determined and analyzed using Gel Doc System (Bio-Rad).

Results and Discussion

One positive result was obtained in PCR (Fig. 1), however there were negative results for both bacteriological culture and serology (MAT). The isolating of the leptospires in laboratory media from any suspected samples either urine, blood or kidney is by itself very difficult. This is the major problem because leptospires is a fastidious organism and always fail to establish themselves with the presence of other microorganisms or contaminants. The presence of leptospires in small number inoculated into the media might also affect the growth and detectable rate. Leptospiremia only occurs during the first 7-10 days of infection (Merien *et al.*, 1992 and Levett, 2001). Thus, the chances in isolation of blood cultures are much more critical.

Microscopic agglutination test is the most widely used as serological test for the diagnosis of leptospiral infection. Four serovars namely *pomona, icterohaemorrhagiae, hardjo* and *canicola* were tested against rat serum in this study. However, all serum samples were shown negative results to leptospiral infection. This might suggested that the rats in this area were not exposed to *Leptospira* or they did expose to it since long time ago, thus the antibody was undetectable. The rat which kidney sample showed positive in PCR was also shown negative result in MAT. This finding is might due to some reasons such as localization of *Leptospira* in the convoluted tubule of reservoir host kidney may not stimulate the production of antibody. Four serovars described above were chosen depending on the importance of these serovars commonly found in rats and rodents, and also common *Leptospira* serovars, which have been demonstrated in Malaysia.

Since many years ago, PCR technology has proven its value for the direct demonstration of fastidious pathogenic microorganisms in clinical materials. Moreover, the detection of *Leptospira* by PCR was also established. The PCR is a rapid, specific and sensitive method. The used of PCR to detect leptospiral infection is important particularly at an early stage of infection, acute stage of illness before antibodies are detectable, and while other methods fail or prove unreliable. The early diagnosis of leptospirosis is very important because early treatment is necessary as it is a zoonosis that causing death if delay in treatment or unnoticed eventually. Many studies on the used of PCR to detect suspected leptospirosis were done and has been found valuable tool in the diagnosis of leptospiral infection (Gerritsen *et al.*,1991 and Brown *et al.*, 1995). The PCR is recognized as more rapid and sensitive than culturing, has proven to be specific for leptospirosis, and in contrast to serology, informative in the first week of illness (Bal *et al.*, 1994). This study showed the practical value of PCR for diagnosis of leptospiral infection while other methods were failed to detect it. Furthermore, only one day time was required for getting result by PCR method which involved DNA extraction, running PCR processes and gel electrophoresis. This is a faster diagnostic method compared to culture and MAT that require days to weeks for the growth of organisms. Thus, the results showed that PCR is more sensitive than culture and MAT methods.

The number of rats infected with leptospires was extremely low in this study, which is only one of thirty-two (3.1%). The low leptospiral infection rate may also due to the epidemiology of leptospirosis has been modified by changes in animal husbandary, climate and human behavior. Rats and rodents have been recognized as maintenance host and carrier of leptospires since 1917 and in Malaysia (Fletcher, 1982). A study done by Gordon-Smith *et al.* (1961a) showed five serovars: *canicola, australis, javanica, ballum* and *pomona* have been frequently isolated from rats in Malaysia. Although, serovars *icterohaemorrhagiae* and *habdomadis* did isolated from the rats in Malaysia (Wisseman *et al.*, 1955 and Gordon-Smith *et al* .,1961a). Different strains of rats were studied on their

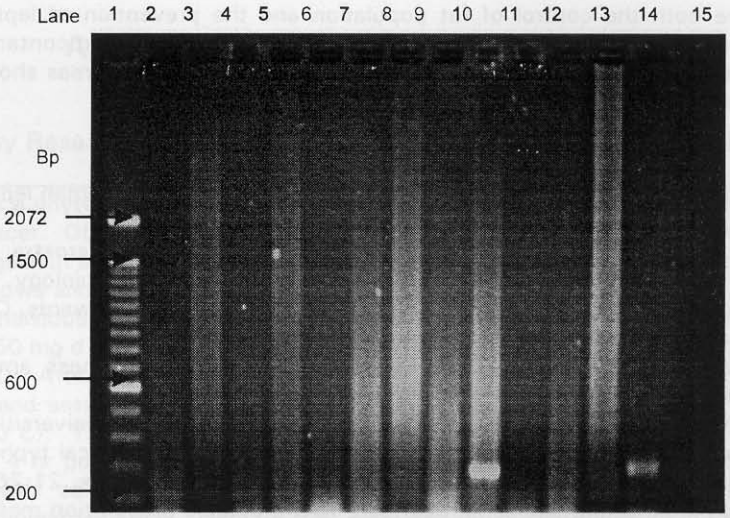

Fig. 1: Detection of leptospiral DNA in rat kidney by PCR. Gel electrophoresis of PCR products obtained from kidney sample from rats in Serdang Selangor area. Lanes: 1, 100 base pair (bp) molecular marker; 2-13, Negative PCR results; 10, positive sample generated band at 285 base pair; 14, positive control;15, negative control. An arrow indicated the DNA fragment of 285 base pairs that was amplified

leptospiral infection from different sources (Gordon-Smith *et al.,* 1961a). They found that the majority (15/23) of the isolates from the Icterohaemorrhagiae serogroup were obtained from house rats, *Rattus norvegicus*, whilst most (37/42) of the strains from the Javanica serogroup were isolated from *Rattus argentiventer* found in rice fields. A study by Chang, 1996 on the prevalence of leptospiral infection in rats shown a moderate to high (55%), compared to the present study that was very low. However, high infection rates may be depend on their study environment where samples were collected, which might be an endemic area, suitable conditions for leptospires to survive, many available maintenance hosts and direct contact between leptospiral infected livestock, wildlife and rats. The prevalence of leptospiral infection rate in dogs was 42.1% (Wisseman *et al.,* 1955), 17.6% (Gordon-Smith *et al.,* 1961a) and 56.6% (Joseph, 1979). Recently a study done by Phumoona *et al.* (2000) showed 36% of dogs (44/165) were found serological positive with the serovar *pomona* and *icterohaemorrhagiae* constitute majority of the infections. In 1979, Shophet reported that leptospiral infections are not commonly seen in cats in New Zealand. While in Malaysia, Gordon-Smith *et al.* (1961a) revealed that 10.3% of cats were infected with serovar *pomona*. In 1998, the study of seroprevalence of infected cats was 37.9% (Norhaliza, 1998).

One study showed that leptospirosis is endemic and widespread in Malaysia (Ungku Omar, 1967). Surveys in human have also shown a high prevalence of antibodies to leptospires throughout Malaysia and the highest distribution was found among laborers working in rubber estates and those dealing with sewage, drainage, forestry, town cleaning and anti-malaria work. It has been reported that rats appeared to be the main natural maintenance host of leptospires in this country (Gordon-Smith *et al.,* 1961a). Apart of rats, the evidence of leptospiral infection has also been found in a wide variety of animals in Malaysia. Domestic animals now constitute the major source of infection for man. The serotypes commonly involved are *pomona* and *tarrasovi* carried by pigs and cattle and *hardjo* carried by cattle (Sullivan, 1974; Khairani-Bejo and Bahaman, 2000). Household pets such as dogs and birds might constitute another potential source of infection for man and animals (Chang, 1996; Phumoona *et al.,* 2000). These animals may harbour leptospires for quite long periods and so may act as important maintenance host. Transmission of infection is usually through contaminated water although direct urinary contaminated water although direct urinary contamination may occur in certain circumstances. The recent case of leptospirosis where occurred in Sabah Malaysia, 23 of 304 participants were infected due to their activities which involved the contacting of natural environment. (Centers for Disease Control and Prevention, 2000).

Due to the importance of certain leptospiral serovars that constitute major source of infection, thus four of these serovars namely *pomona, hardjo, icterohaemorrhagiae* and *canicola* were chosen in this study. Although there is no positive result from the selected tested, does not mean that these leptospiral serovars are not present or may be other serovars are constitute in this area because there is one positive sample detected by PCR. Present study shown that the infection rate in rats in this area is not significantly high, but this also does not mean that human and animal infection can be reduced or only little precautions taken against leptospirosis. This is extremely important in towns, where human infections were probably from the house rat, which although having a low rate, is numerous and in close contact with man (Gordon-Smith *et al.,* 1961a). Thus, the prevention of human

leptospirosis should involve both the control of rat population and the prevention of leptospiral infection in household pets in residential areas. Improvement in living condition has lessened man contact with rats. Further investigation on prevalence of leptospiral infection in rats in housing or residential areas should be carry- out to know the status of leptospirosis in Malaysia.

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