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Isolation and phenotypic identification of Leptospire from *Rattus norvegicus* (Brown rat)

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Abstract

Leptospirosis is a bacterial zoonotic disease of worldwide importance, caused by members of pathogenic leptospire. Direct or indirect contact with the infected animal or environment results in infection. Rodents are the important reservoir of leptospire. This study was conducted to demonstrate the isolation and phenotypic identification of *Leptospira* in rodent carriers, which are central to the epidemiology of the disease. The percentage of isolation in rats in this study were 12.5. The rat isolates were confirmed with specific phenotypic tests to differentiate the pathogenic leptospire from the saprophytic leptospire. The isolates were able to grow at +28 +/- 1°C in EMJH semi-solid and liquid media, also in the presence of 5-Fluorouracil. Ellinghausen McCullough Johnson and Harris medium containing copper sulphate and 8-azaguanine inhibited the growth of these isolates at the conc. of 100 ppm and 225 µg/ml, respectively which further confirmed the pathogenic status of the isolates.

Keywords: *Leptospira* isolates, 8-azaguanine, copper sulphate, 5-Fluorouracil

Introduction

Leptospirosis is a globally emerging zoonotic disease that has a significant impact on animal and human health in countries with humid, tropical and sub-tropical climates. In India, this is exemplified by major outbreaks in Orissa (Sehgal *et al.* 2002) [13] and Mumbai (Maskey *et al.* 2006) [9]. A multitude of animal species can acquire clinical illness either from direct contact with infected animals or indirectly from the contaminated environment (Levett, 2001 and Vedhagiri *et al.* 2010) [8, 14]. Animals, especially rodents may become carriers and play a significant role in the epidemiology of leptospirosis by maintaining pathogenic *Leptospira spp.* in the renal tubules and shedding it through urine, acting as a source of environmental contamination (Faine *et al.* 1999 and Santos *et al.* 2017) [5, 12]. The average number of leptospire shed through rat urine is about 5.7×10⁶/ml (Barragan *et al.* 2017) [11]. Leptospire can be isolated by inoculating specimen on to susceptible animals like hamsters and guinea pigs. But, this approach is sparingly done due to stringent animal experimentation guidelines and cost. Culturing in bacteriological media is the most common method using blood, urine, cerebrospinal fluid, aborted foetal tissue, milk, kidney and aqueous humour. However, the slow growth rates, nutritional fastidiousness and long incubation period of some *Leptospira* isolates hamper the early diagnosis of leptospirosis. Isolation of leptospiral isolates from a particular region is the method of choice to identify circulating serovars and it can be used as representative screening antigens for epidemiological studies and research and as vaccine strains (Ezeh *et al.*, 1989) [3]. Accordingly, in this present study, attempts were made to isolate *Leptospira spp.* from the domestic rats and confirmed the pathogenic status of the isolates with phenotypic identification.

Materials and Methods

This study was conducted from November 2017 to January 2018, in and around the Zoonoses Research Laboratory. This laboratory is located in Madhavaram Milk Colony (13.15°N 80.24°E), Chennai, is a tropical zone.

The trapping of rats was conducted over for 90 days with 12 wooden rat traps (24 cm X 11 cm X 11 cm). Everyday rat traps were soaked with soapy water for 30 minutes and then washed with plain water for three times. After they were dried under sunlight and the traps with dry fish or meat baits were placed in the evening, in and around the Zoonoses Research Laboratory. The trapped live rats were immediately euthanized by using chloroform. The urine samples by direct puncture of the bladder and homogenates from kidneys were collected

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aseptically and immediately put into the EMJH medium containing 5 - Fluorouracil.

The tissue samples were triturated with two milliliters of sterile PBS and acid-washed sand by using pestle and mortar. The tissue suspension was centrifuged at 1389g for 5 min at 4°C to remove cell debris. Serial dilutions (1:10, 1:100 and 1:1000) of the supernatant of tissue suspension and urine samples were made and each dilution was inoculated in the liquid and semisolid EMJH medium containing 1% rabbit serum and 100 µg/ml 5- Fluorouracil and incubated at 28±1°C in B.O.D. incubator and examined under dark- field microscope at weekly intervals for 16 weeks before discarded as negative.

A drop of urine or kidney homogenate was placed on grease-free glass slide No.1 and coverslip was applied. The wet mount preparation was examined under the oil immersion objective (1000X) of dark- field microscope (Eclipse E600, M/s Nikon, Japan) for the detection of spirochete-like structure.

Well grown cultures (3×10⁸ leptospire/ml) of isolates along with pathogenic leptospira and saprophytic leptospira were transferred to the separate EMJH media containing 8-azaguanine, 5-Fluorouracil and copper sulphate at the concentration of 225 µg/ml, 100 µg/ml and 100ppm, respectively. On the fifth day of incubation, the first passage was done and incubated at 28 ± 1°C in a B.O.D. incubator.

Culture of the isolates containing 3×10⁸ leptospire/ml was sub-cultured in EMJH medium along with *Leptospira interrogans* serovar canicola (pathogenic leptospira), *L.biflexa* (saprophytic leptospira) and incubated at 13±1°C in B.O.D. incubator.

Results and Discussion

A total of 16 rodents were captured during the study period [Table-1]. Based on phenotypic appearance and colour, the rodents were identified as *Rattus norvegicus* (Brown rat). Out of these 16 samples, 2 urine samples showed turbidity in EMJH liquid medium after 4 weeks of incubation in the B.O.D incubator. These 2 samples were observed under dark field microscope and spiral-shaped organisms were detected. As mentioned in OIE, the low level of isolation in this study could be due to the nature of intermittent excretion of leptospire in urine by the carrier animal, failure to be present at the time of excretion. Studies on rodent isolation include 2 isolates out of 35 rat samples (5.7 %) (Natarajaseenivasan *et al.*, 2010) [10] and 18 isolates out of 140 rat urine samples (12.9 %) (Latifah *et al.*, 2017) [7]. The reason for a low percentage of trapping and isolation is the geographical location from where trapped, is a farm complex with continuous excellent rodent control operation is in place.

Out of 16 rat samples, 2 spirochete-like organisms with helical morphology and translational motility have been obtained [Figure-1]. They were thin, short, tightly coiled, motile organisms with one or both ends hooked as described earlier (Faine *et al.* 1999 and Picaudeau, 2017) [5, 11]. The detection level with darkfield microscopy is 10⁴ leptospire/ml. The morphologic appearance is typical of the genus *Leptospira*, but species diagnosis could not be made with morphologic characterization alone (Wolf, 1954) [15]. Hence the further confirmation was done with phenotypic characterization.

The isolates and pathogenic leptospira (*Leptospira canicola* serovar Canicola strain Hond Utrecht IV) were able to grow at +28 +/- 1°C in EMJH semisolid and liquid medium, also in the presence of 5-Fluorouracil [Figure-2], but the separate EMJH medium containing copper sulphate [Figure-3] and 8-

azaguanine [Figure-4] inhibited the growth of these isolates at the conc. of 100ppm and 225 µg/ml, respectively. Whereas the saprophytic leptospira (*Leptospira biflexa* serovar Patoc strain Patoc I) showed the turbidity in the tubes containing copper sulfate and 8-azaguanine separately, within 15 days of incubation. The optimum pH 7.2-7.4 for the luxuriant growth of leptospire was recorded. This result correlates with the suggestion that 8-azaguanine at 225 µg/ml could be used to differentiate pathogenic and saprophytic leptospire (Vedhagiri *et al.* 2010) [14]. There was no growth observed in EMJH liquid medium with 100ppm copper sulfate, which confirms the suggestion that pathogenic leptospire growth was inhibited at 100ppm of Copper sulphate (Zuerner *et al.* 2010) [16]. Only 1.5 µg of copper ions present in the EMJH growth medium supplement. Whereas saprophytes grow at a copper ion concentration of 500 µg in the growth medium, this can be a good screening test to differentiate saprophytes from pathogenic isolates, as it is an inexpensive reagent compared to the 8-azaguanine (Fuji and Csoka, 1960) [4]. The growth of leptospire reached maximum density at 13° C within 1 week of incubation in *Leptospira biflexa*, but no growth observed in the tubes with isolates [Figure-5]. This result is in accordance with the suggestion that saprophytic *L. biflexa* differed from pathogenic *L. interrogans* by its growth at 13° C or below (Johnson *et al.* 1967 and Bourhy *et al.* 2014) [6, 2].

Table 1: Isolation and PCR confirmation of isolates from *Rattus norvegicus*

<i>R. norvegicus</i>	no. of positive/total (% positivity)
Adults	2/12 (16.7%)
Juvenile	0/4
Total	16 (12.5%)



Fig 1: A photomicrograph of hooked leptospire in darkfield microscope (1000X)

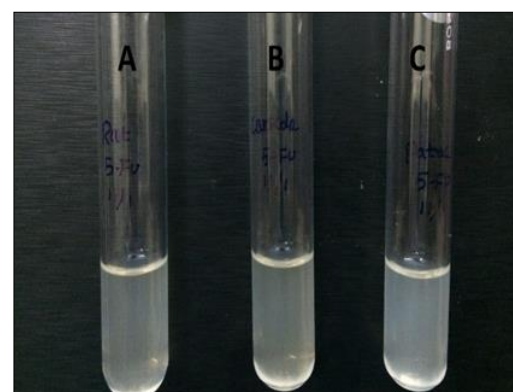


Fig 2: Sensitivity of *Leptospira* isolates to 5-Fluorouracil test

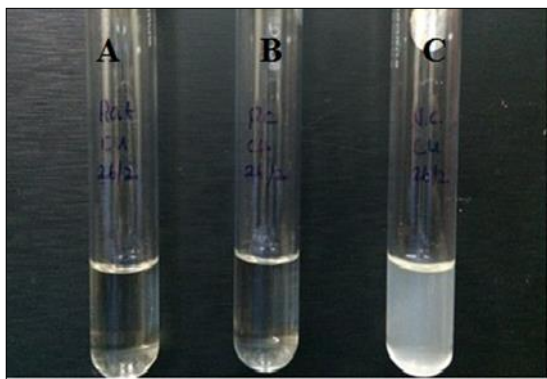


Fig 3: Sensitivity of *Leptospira* isolates to copper sulphate

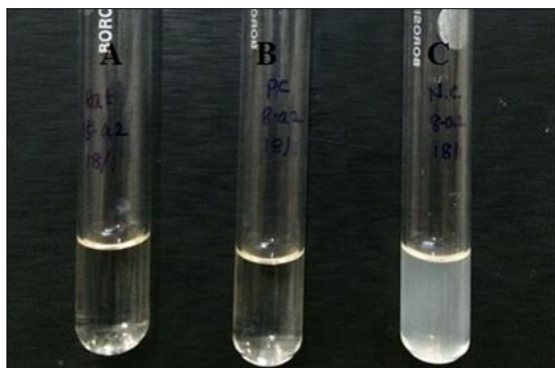
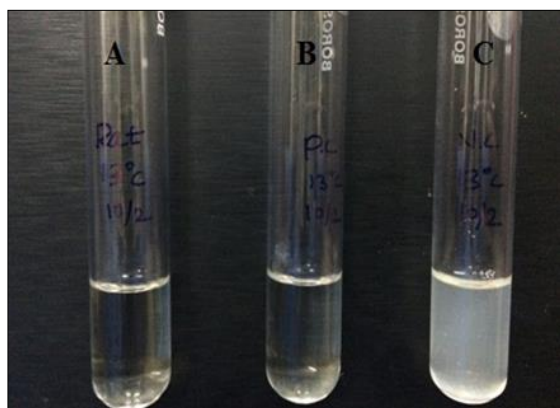


Fig 4: Sensitivity of *Leptospira* isolates to 8-azaguanine



- Sample (Rat isolate)
- Pathogenic control (*L. interrogans* serovar Canicola strain Hond Utrecht IV)
- Saprophytic control (*L. biflexa* serovar Patoc strain Patoc I)

Fig 5: Growth and viability of *Leptospira* isolates at 13°C

Conclusion

Out of 16 rats, two isolates were obtained from the rat urine samples. These two rat isolates were confirmed with specific phenotypic tests to differentiate the pathogenic leptospira from the saprophytic leptospira.

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