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Molecular Characterisation of *Leptospira interrogans,* Isolated from Animal Samples

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ABSTRACT

Leptospirosis is a zoonosis of worldwide distribution, endemic mainly in countries with humid subtropical or tropical climates and has epidemic potential. Leptospirosis, caused by Leptospira interrogans, is highly infectious emerging water borne zoonosis of global significance. It is an enigmatic life threatening disease, which results into high morbidity and mortality, particularly in poor resource nations. The molecular identification and differentiation Leptospirosis are important for epidemiological and health surveillance. The severe infection risk to organ failure and internal haemorrhage. In the present study, the animal samples were collected and sub cultured on lactose broth. Further they confirmed on Ellinghausen McCullough Johnsn Harris (EMJH) Medium. The spirillum colony was observed under Compound microscope. The DNA isolated and amplified with PCR using specific primers [16S RNA] and the characters are confirmed by Random amplified polymorphic DNA & Restriction Fragment length polymorphism technique. And their Multi Drug Resistant pattern was confirmed using commercial antibiotic disc.

Key words: *Leptospira interrogans, EMJH, 16S RNA Nested PCR, RAPD, RFLP.*

INTRODUCTION

Leptospirosis is a zoonotic disease affecting many labours i.e. Field worker, formers, meat, sewage workers[10]. Leptospirosis also is a disease of animals affecting many domestic and farm animals. Leptospirosis, caused by *Leptospira interrogans*, is highly infectious emerging water borne zoonosis of global significance[18]. The identification and differentiation of Leptospirosis are important for epidemiological and public health surveillance. It is most commonly transmitted from animals to humans when people come into contact with water or soil has been contaminated with animal urine then the bacterium can also enter body through the eyes or mucous membranes. In the majority of cases, infection occurred in people who either worked in or were involved in: Sewage works, Farms, and were regularly in contact with animals or infected water or soil, Sailing or canoeing.

Pathogenic *Leptospira* consist of about 300 distinct antigenic types referred to as serovars, which vary with their carrier animal species[16].

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Typically, the animals that transmit the infection to humans include rats, skunks, opossums, foxes, raccoons and other vermin. Most of the urban areas affected involve large cities in the developing world. Types of Leptospirosis, there are two main types, they are Mild leptospirosis, and severe Leptospirosis. Mild Leptospirosis - the patient experiences muscle pains, chills and possibly a headache. 90% of cases are of this type. Severe Leptospirosis - can be life-threatening. There is a risk of organ failure and internal hemorrhaging. This occurs when the bacterium infects the kidneys, liver and other major organs. People can become infected by: Drinking contaminated water, Coming into contact with contaminated water or soil if they have unhealed cuts in their skin, eyes, nose or mouth come into contact with contaminated water or soil, Coming into contact with the blood of an infected animal (less common).

Diagnosis of Leptospirosis

Leptospirosis is difficult to diagnose both in the clinic and at the laboratory. In its early stages, mild leptospirosis is hard to diagnose, because many of the symptoms are similar to flu and other common infections. Diagnostic procedures for flu are not good at identifying leptospirosis. Conventional or conformational methods for Leptospirosis such as isolation, compound microscopy, Blood agar EMJH Medium. Molecular basis of leptospirosis are the first genome sequence based molecular approaches for leptospirosis. They are, commonly used 16s RNA technique. Nested PCR, Random amplified polymorphic DNA, Restriction fragment length polymorphism.

MATERIAL AND METHODS

Collection of samples

The different animal (Pig, Sheep, Beef, Goat) meat samples were collected from various area of Coimbatore Achimia strain was procured from department of microbiology, bharathidasan University**,** for our own confirmation.

ISOLATION OF ORGANISM:

The organism *Leptrospira interrogans* was isolated from different animals (Beef, Sheep, and Pig) intestinal sample. 1g of the meat sample was mixed with 0.1 M Phosphate Buffer (pH 7.0) crushed, squashed for half hour in shaker. Then the samples were inoculated into lactose broth and the incubated at 37°C for 24hours. After incubation period microorganism growth was observed in turbidly. They were streaked on nutrient agar plates and incubated for 24 hours at 37^{o}C. The spirillium colony was observed in each plate and the *Leptrospira* was confirmed physically on compound microscope at 40 X, the hock and live organism was visible. *Leptrospira* was also confirmed on the specific medium Blood agar and Ellinghausen and Mcclulough Modified by Johnson and Harris (EMJH) Medium. Then the sample was streaked on blood agar and EMJH medium and incubated at 37°C at 24hours. After incubation the haemolysis was observed.

DNA Isolation

The sample was streaked on the EMJH broth. From the well grown Leptrospiral 2ml of culture was taken in effentof tube they centrifuge at 13000rpm for 10mins. Pellets were collected they washing twice using saline EDTA and centrifuge at 13000rpm for 10mins. Resuspend the pellets in 600µl of saline EDTA and add 50µl of lysozyme mix well and incubate at 37˚C for 30mins. Mix the suspension clearly and through by inversion the tube by several times. To that add 100µl of 10% SDS incubate the tubes at 65˚C 15min. Mix the cell suspension thoroughly. To that add Phenol: Chloroform : Isoamyl alcohol, and they are centrifuged at 12,00rpm for 10mins, collect the aqueous phase to that add 0.2 volume of sodium acetate + 5 volume of Isopropanol and centrifuge 12,00rpm for 10mins collect the pellet. To the pellet 100% Ethyl alcohol and their centrifuge and collect the pellet. To the pellet add 70% alcohol and centrifuge at 10000rpm for 5mins. To the pellet add 50µl T.E buffer. The pellet was air dried and DNA was can be loaded in agrose gel electrophoresis and the run on 50V after electrophoresis they visible on UV trans illuminator. The DNA was can be stored at 20°C for later use.

Nested PCR amplification

Nested PCR assay was performed with two pairs of specific primers[15], used for primary amplification were 5′ GGCGGCGCGTCTT AAACATG 3′ 5′ GTCCGCCTACGCACCC TTTACG 3′. The first round of reaction was performed in 50µl reaction mix contain 10x buffer, 3mM $Mgcl_2$Taqpolymerase, dNTPs and pair of primers (each 10_qm) and approximately 50gg of template DNA. The temperature profile was as follows: Initial denaturation at 95˚C for 5minutes, denaturation at 95˚C for 1minute, annealing at 60˚C for 45sec and extension at 72˚C for 1min. for 35 cycles, followed by final extension 72˚C for 7min.The second round of amplification was carried out using 1µl of the first PCR Product as template using the primers 5′CAAGTCAAGCGGAGTAGCAA 3′ 5′TAACCTGCTGCCTCCCGTA 3′.The same conditions and program of Amplification were as for the first round except annealing was set 62˚C for 45sec. Each PCR reaction was performed thrice by at least three individualsfor consistency and negative for each round PCR was maintained. The amplified products were separated on 1.2% agarose gel stained with 10 mg/ml ETBr and visualized in gel documentation system (Bio-Rad, USA).

RAPD technique

The RAPD assay was performed with two primers B11 5′ GGCGGCGCGTCTTAAA CATG 3′ 5′ GTCCGCCTACGCACCCTT TACG 3′. The reaction was performed in 50µl reaction mix contain 10x buffer, 3mM $Mgcl_2$ Taq polymerase, dNTPs and pair of primers (each 10_qm) and approximately 50gg of template DNA. The temperature profile was as follows: Initial denaturation at 95˚C for 5min, denaturation at 95˚C for 1min, annealing at 60˚C for 45sec and extension at 72˚C for 1min. for 35 cycles, followed by final extension 72˚C for 7min.The amplified products were separated on 1.2% agarose gel stained with 10 mg/ml ETBr and visualized in gel documentation system (Bio-Rad, USA).

Multi Drug Resistance (MDR) pattern

Multi-drug resistant (MDR) is defined as non-susceptibility to at least one agent in three or more antimicrobial categories. MDR bacteria are the principal cause of failure in the treatment of infectious diseases, resulting in increases in the term and magnitude of morbidity, higher rates of mortality, and a greater health cost burden. (Add author). According to Margaret Chan, Director General, World Health Organization (WHO), “Outbreaks of food-borne disease have become an especially large menace in a world bound together by huge volumes of international trade and travel. They are large in their potential in terms of geographical spread often involving multiple countries.

Confirmation test for MDR Pattern:

Antimicrobial susceptibility test (Kirby-Bauer method):

Determination of Multiple Antibiotic Resistance Index (MAR Index)

The multiple antibiotic resistance index (MAR index) was determined for each isolate by dividing the number of antibiotics against which the isolate showed resistant over the total number of antibiotics tested.

$$\text{MAR index} = \frac{\text{Number of antibiotics against which isolate showed resistance}}{\text{total no of antibiotics tested}}$$

Antimicrobial susceptibility testing was performed by the disc diffusion method using Muller Hinton agar plate (HI Media Laboratories, Mumbai, India, MV1084), according to the Clinical and Laboratory Standards Institute guidelines.

Further the multi-drug resistant (MDR) pattern was confirmed by sub culturing the isolated pathogens in Luria Bertani broth. The inoculated pathogens were incubated at 37 °C for 24 hours.

After 24 hours we prepared Muller Hinton agar plate and cotton swab the sub cultured pathogens are swabbed in the nutrient agar plate and place antibiotic disc incubated at 37˚C for 24 hours.

The antimicrobial agents tested and their corresponding concentrations were as follows: Rifampicin (2 µg/disc), Vanomycin30µg/disc, Norfloxacin (10 µg/disc), Ceftriaxone (10 µg/disc), Levofloxacin (2 µg/disc), Cephoxitin (30µg/disc), Gatifloxacin (30µg/disc), Oxacillin (30 µg/disc), Methicillin (10 µg/disc), Nalidixicacid (10 µg/disc) and Neomycin (30 µg/disc). After incubating the inoculated plates aerobically at 37 °C for 24 hours, the zone of inhibition was measured and noted down.

RESULTS AND DISCUSSION

Identification of Leptrospira

Identification of Leptrospira was observed on blood agar medium after 24hrs they observe haemolysis and the collected sample Streak at EMJH medium. The grown colony was fixed in slide directly observed by microscopically.

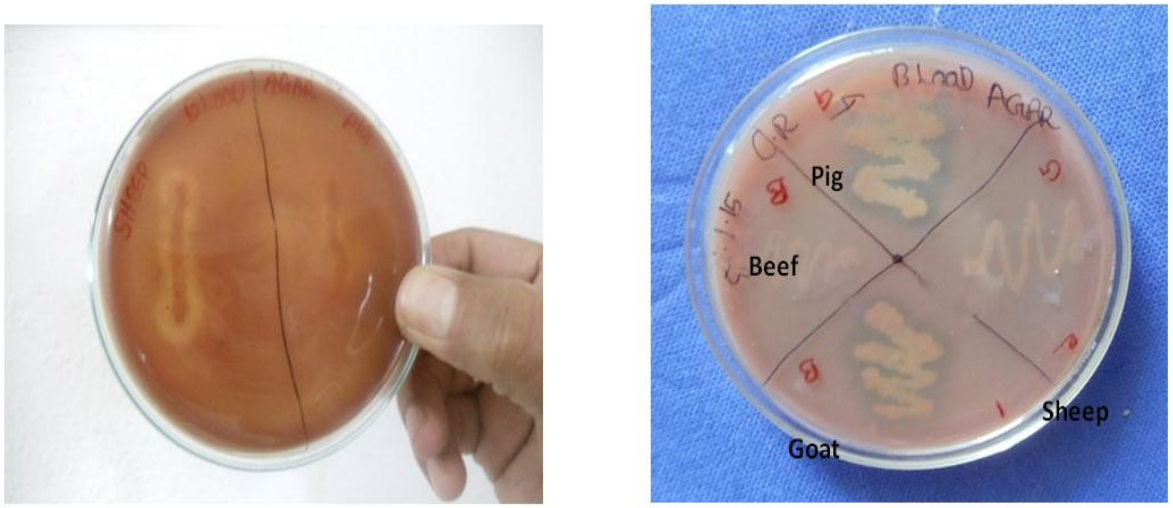

Fig. 1: Observance of haemolysis on isolated samples

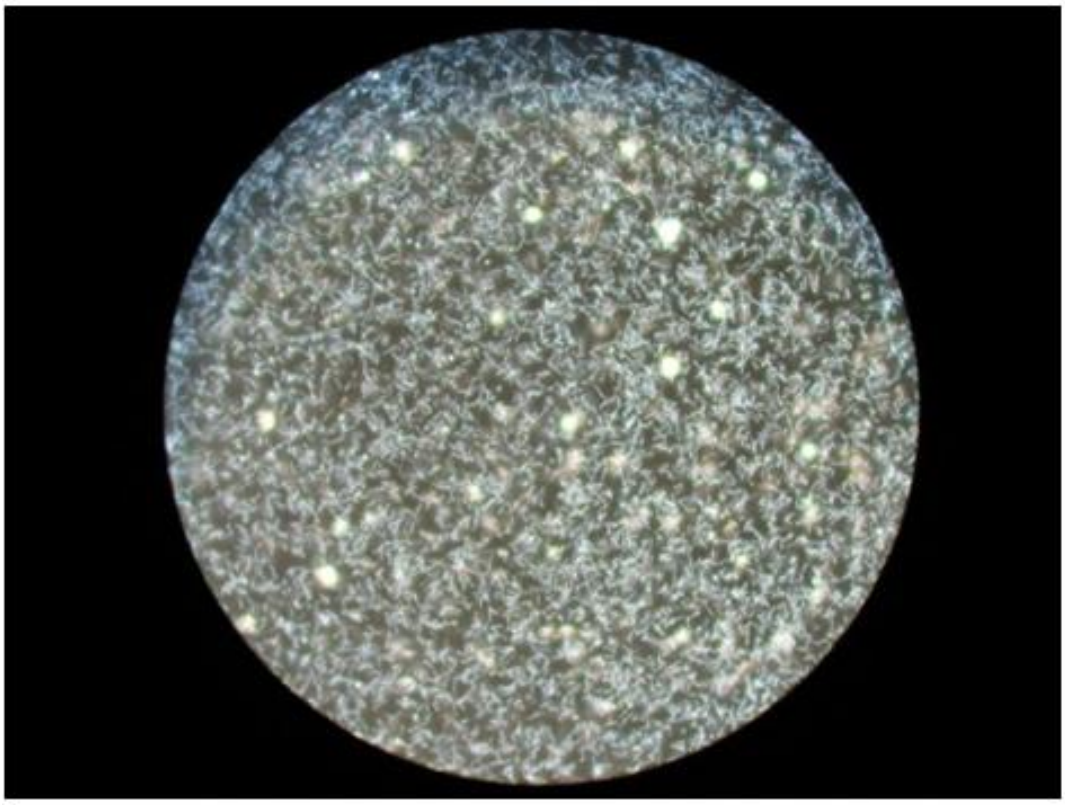

Fig. 2: Observance of spirillim colony

DNA isolation

The chromosomal DNA was isolated from Leptrospirosis using Phenol; Choloroform method. The isolated DNA were resolved in 0.8% agarose gel at 50 V and the DNA visualised under UV transilluminator.

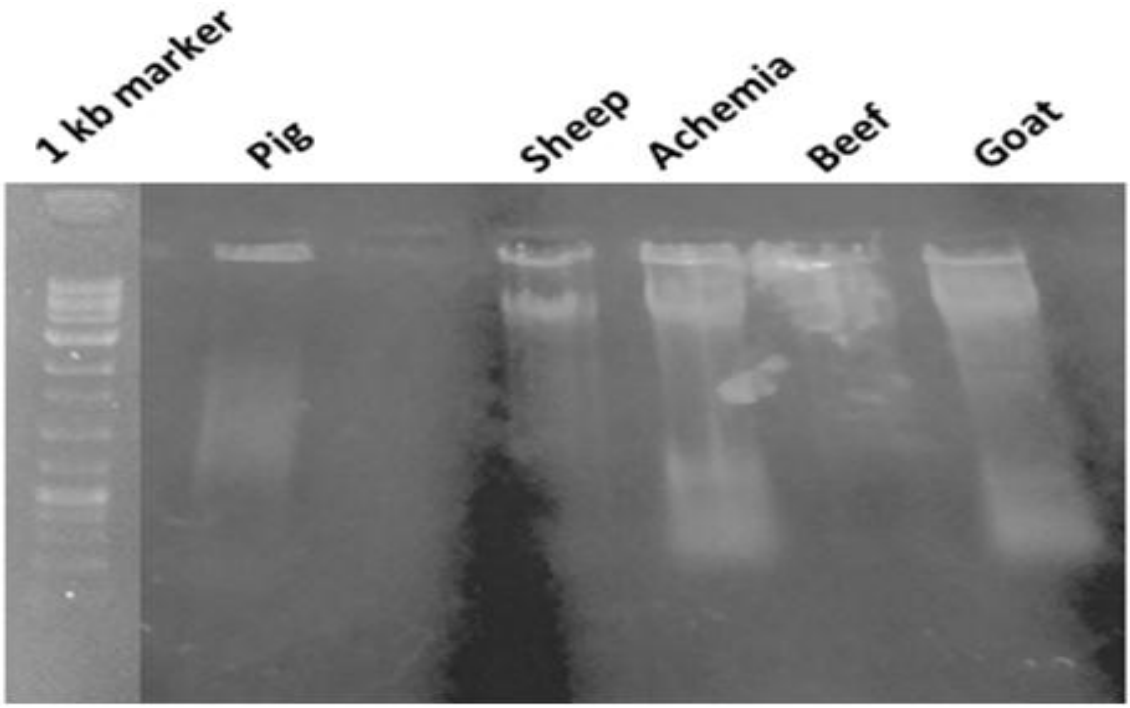

Fig. 3: Isolation of DNA

As above fig 3: L1 standard marker, LS2-Pig, LS3-Sheep, LS4-achemia, LS5-beef, LS6- Goat

16s and RAPD technique

The isolated Pig, Goat samples was amplified 16s RNA Nested PCR

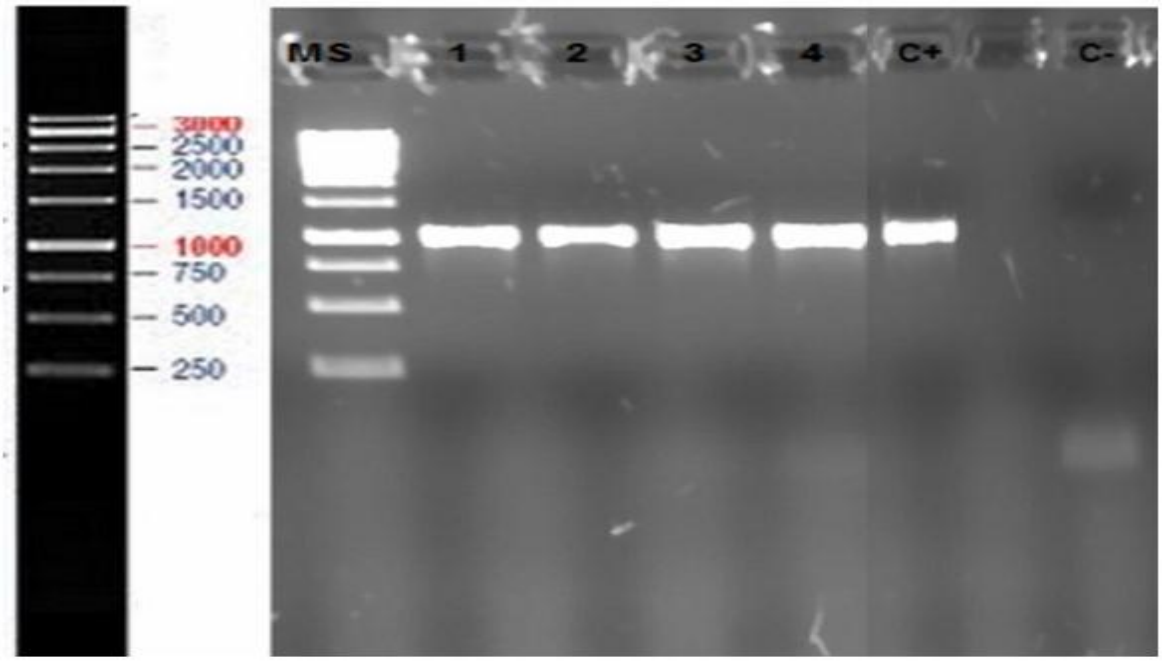

Fig. 4: Nested PCR in a 1.5% agarose gel, conducted with DNA extractions, showing the positive products (1004pb) of *Leptospira* spp. MS, molecular size marker. Lines 1-4, positive extractions (Pig, Goat, Sheep, Acemia). Line C+, positive control. Line C-, negative control (Beef).

Random Amplified Polymorphic DNA (RAPD) technique

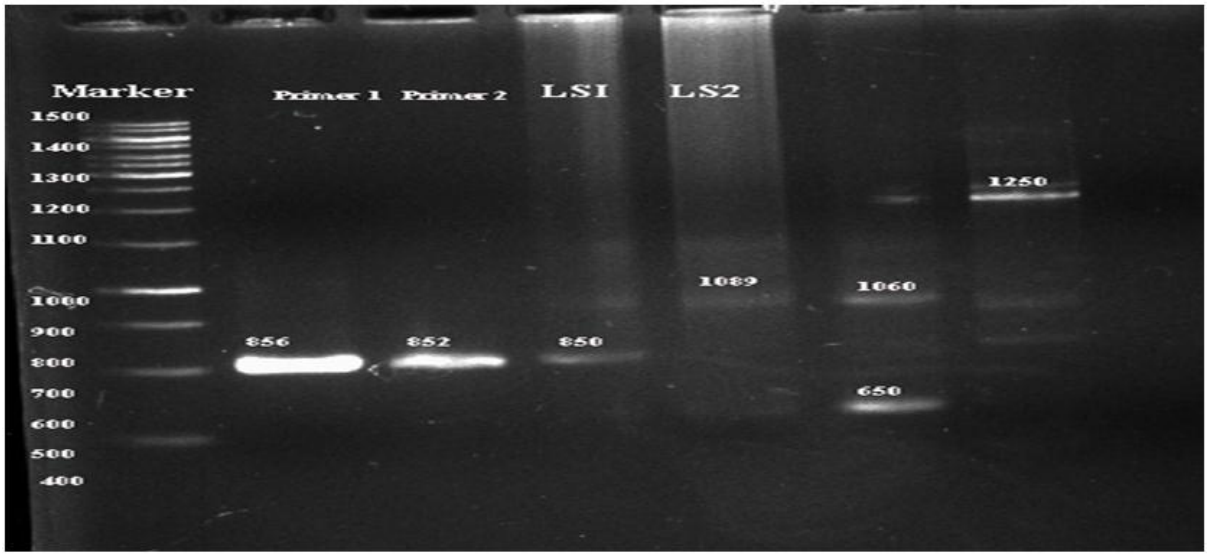

Fig. 5: Random Amplified Polymorphic DNA (RAPD) technique, technique showing positive results of isolated strains LS1-Pig, LS2- Goat, 650bp-sheep, 1250bp- Acemia

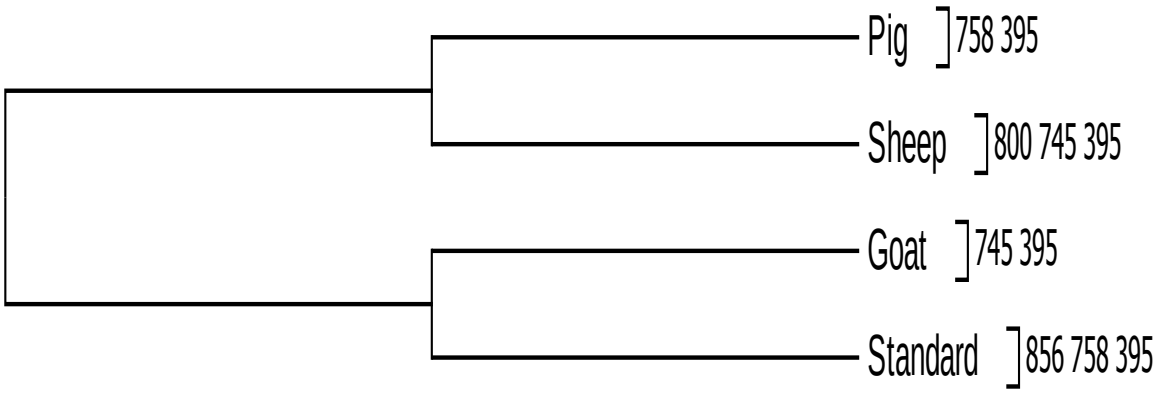

Chart 1: Phylogenetic tree analysis

Confirmation of *Leptospira*s a multi-drug resistant of strains:

The isolated *Leptospira*from collected samples is a resistance to antibiotic Discs. The MDR pattern was further confirmed by using disc diffusion method was performed to study the MDR pattern of the isolates against commercially available antibiotic discs viz., Cephoxitin (30 µg), Amikacin (30 µg), Amoxicillin (10 µg), Ceftriaxone (10 µg), Novobiocin (30 µg), Rifampicin (2 µg), Ofloxacin (2 µg), Methicillin (10 µg), Norfloxacin (10 µg), Neomycin (30 µg).

Table 1: Detection of Multi drug resistant

Antibiotic discs with concentrations	Isolated Organisms and their Zone of inhibition (in mm)			
	Pig	Goat	Sheep	Achemia (Standard Strain)
Rifampicin (2 µg)	Nil	Nil	Nil	Nil
Vanomycin (30µg)	Nil	Nil	Nil	Nil
Norfloxacin (10 µg)	Nil	Nil	Nil	Nil
Ceftriaxone (10 µg)	Nil	Nil	Nil	Nil
Levofloxacin (2 µg)	Nil	Nil	8mm	Nil
Cephoxitin (30µg)	Nil	Nil	6mm	Nil
Gatifloxacin (30µg)	Nil	1 mm	12mm	Nil
Oxacillin (30 µg)	Nil	Nil	10mm	Nil
Methicillin (10 µg)	Nil	Nil	14mm	Nil
Nalidixic acid (10 µg)	Nil	Nil	4mm	Nil

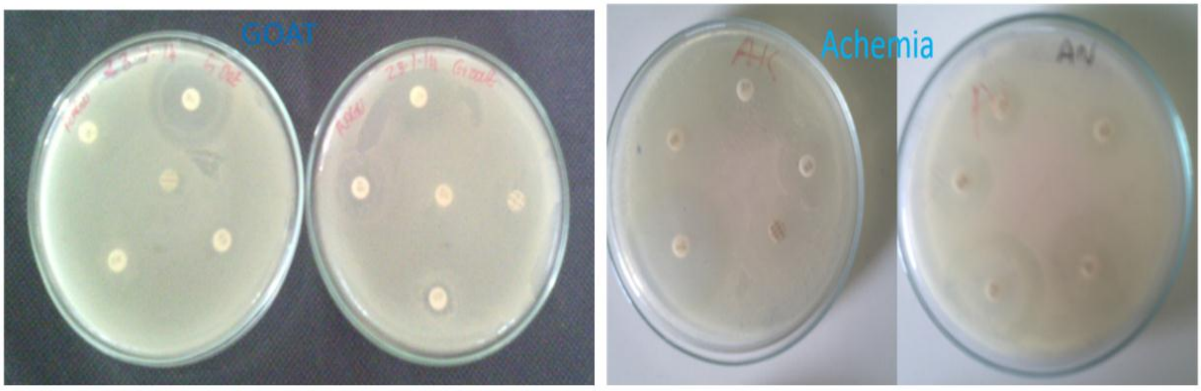

Fig. 6: Mutli drug resistant confirmation of strain I & II using commercial antibiotics

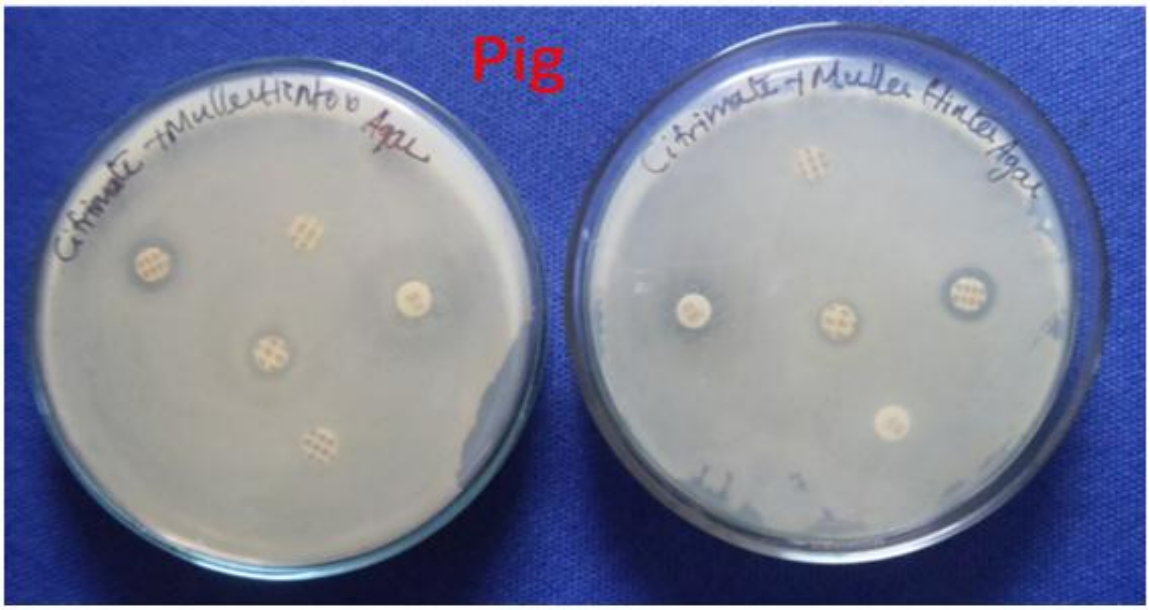

Fig. 8: Mutli drug resistant confirmation of strain III using commercial antibiotics

StrainI (Goat) showed resistivity against Rifampicin (2 µg/disc), Vanomycin30µg/disc, Norfloxacin (10 µg/disc), Ceftriaxone (10 µg/disc), Levofloxacin (2 µg/disc), Cephoxitin (30µg/disc), Gatifloxacin (30µg/disc), Oxacillin (30 µg/disc), Methicillin (10 µg/disc), Nalidixicacid (10 µg/disc) and Neomycin (30 µg/disc)) (Figure 6).

Strain II (achmia from trichi) showed resistivity againstRifampicin (2 µg/disc), Vanomycin30µg/disc, Norfloxacin (10 µg/disc), Ceftriaxone (10 µg/disc), Levofloxacin (2 µg/disc), Cephoxitin (30µg/disc), Gatifloxacin (30µg/disc), Oxacillin (30 µg/disc), Methicillin (10 µg/disc), Nalidixicacid (10 µg/disc) and Neomycin (30 µg/disc). (Figure- 6).

Strain III (Pig) showed resistivity againstRifampicin (2 µg/disc), Vanomycin30µg/disc, Norfloxacin (10 µg/disc), Ceftriaxone (10 µg/disc), Levofloxacin (2 µg/disc), Cephoxitin (30µg/disc), Gatifloxacin (30µg/disc), Oxacillin (30 µg/disc), Methicillin (10 µg/disc), Nalidixic acid (10 µg/disc) and Neomycin (30 µg/disc). (Figure- 7)

CONCLUSION

This work is always done several researchers and my work compare with those persons as follows:

Leptospirosis is considered the most common zoonosis worldwide and is endemic in tropical environments[13]. It has recently been classified as a re-emerging disease, largely because of increased recognition and recent rediscovery that it can present as a severe hemorrhagic illness, easily confused with

some viral hemorrhagic fever. Leptospirosis is emerging as an important public health problem world over and in India particularly outbreaks are frequently being reported from different regions of the country during last few years. Due to their different clinical manifestation from mild to a severe one and due to their different clinical presentation from region to region it has become very important to characterize the isolates to understand the disease deeply[21].

But the screening of Leptospirosis is very difficult to understand. This kinds of work if performed various researchers as follows.

Radhika Krishna *et al*[21]., developed new classification method for analysis of Leptospira spp., as follows. For this purpose we are isolate's eight different strains, out of this two strains from human and two strains from rat then it is subjected to flaB PCR, it has capable of pathogenic detection. The strains amplified into a 793 base pair fragment except nonpathogenic strains. That fragments digested with two different enzymes this are Hind III and Hae III. Compare this two enzymes activity Hae III effectively digest then Hind III from this comparison to detect more difference between pathogen and nonpathogenic spp. Find RFLP for indication of genome spp same are not. Finally we conclude flaB and RFLP is important tool for classification of pathogen from non-pathogenic. Human isolates also responsible for transmission of infection because of that genome has a similar sequence with Rodents.

In the year of 2004, Kalimuthusamy Natarajaseenivasan et al., studies based on the detection Leptospira in erode district. This attempt carried by isolation and serological test includes Microscopic Agglutination Test (MAT) and IgM based Enzyme linked immuno sorbent assay (ELISA). In the present study, we choose patients (10 to71 years old) with Headache body ache, fever, jaundice, and decreased urine output, mainly the patients are in agricultural field. Out of 29 patients, 26 used for diagnosis based on current Leptospiral Infection's. Further characterized by RAPD technique for check similarity between those patients. At last, we check a presence of Leptospira among hospital case in erode.

P. Ramadass, *et al*[22]., isolates 14 Leptospiral serovar Starins (serovars australis, autumnails, ballum, bataviae, canicola, grippotyphosa, hardjoprajitno, hebdomadis, iceterohaemorrhagiae, javanica, Pomona, pyrogens, panama, and tarassovi) and it characterized by RAPD using pair of primers, had unique and distinct finger print pattern. RAPD is a rapid and sensitive method for Leptospira Identification. Those person try some other bacterial DNA (Escherchia Coli, Pasteurella multocida, salmonella spp., pseudomonas spp., and Klebsiella spp.,) but show any amplification. P. Ramadass, et al, allowed the strains to DNA restriction enzyme analysis but it produce more fragments so diificult to comparison than RAPD.

M. L. Pacciarini, *et al*[19]., investicate about Leptospirosis as follows, to diaganosis Leptospirosis in such animals by to develop PCR that is amplification of specific DNA fragment. This is done in Bresscia, Italy Molecular labs. Then PCR positive samples will be rapidly detected by microtitre based assay. Again to characterized the amplified DNA product by Restriction Endonuclease analysis of PCR product and Amplified Fragment Length Polymorphisms (AFLP).

From above references, various workers should screening the Leptospirosis by using various methods like PCR, RAPD, RFLP, etc. but I use 16s Nested PCR, EMJH medium, Blood agar, RAPD, RFLP for Molecular characterization Leptospirosis. This work provide a general idea about the detection and characterization Leptospira.

In this study, I isolate *Leptospirosis interrogans* species from various animal samples (Pig, Goat) by DNA isolation and they confirmed by 16s Nested and RT PCR and also whole experiment was confirmed RAPD and RFLP using purified DNA product of collected samples. The results from this study shows that 16s Nested and RT PCR is simple and rapid method for detecting

Leptospiral serovars. Finely I conclude that two animal samples have Leptospiral Organism. The results of this study indicate that *Leptospirosis interrogans* could be the most prevalent in our region. On the other hand, leptospirosis could be a major disease causing abortions and influenza-like symptoms in our collected samples (Pig, Goat, and Sheep) as well as humans. Therefore, an animal with leptospirosis should be removed from the environment as it can be a concern in transmission of the disease to other animals and humans.

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