MOLECULAR CHARACTERISATION OF LEPTROSPIRIAINTERROGANSISOLATED FROM ANIMAL

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Abstract: **The identification and differentiation of Leptospirainterrogansare important for epidemiological and health surveillance. The severe infection risk to organ failure and internal haemorrhage.In the present study, the animal sample(Pig, Goat, Sheep, Beef) were collected and sub cultured on lactose broth. Further they confirmed on EMJH Medium. The spirilium colony was observed under Compound microscope. The DNA was isolated and amplified with PCR using specific primers [16S RNA] and the molecular characters are confirmed by RAPD&RFLP technique, The L.Interrogans is an Multi Drug Resistant, confirmed by standard discs diffusion methods with commercial available Discs. Further the alternative remedial measure was determined by synthesised Nono particles(Zinc, Silver, and Gold).**

Key words: **Animal, 16s Nested PCR, RAPD, RFLP, Nanoparticles.**

I. INTRODUCTION

Leptospirosis is a zoonotic disease is affecting many labours i.e. Field worker, formers, meat sewage workers (Dr. Rudy A. Hartskeer 2005). Leptospirosis also is a disease of animals affecting many domestic and farm animals. 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 *Leptospirainterrogans*consist of about 300 distinct antigenic types referred to as serovars, which vary with their carrier animal species (Levett PN (2001). 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. Experts are not sure why some patients develop the severe form people who are already very ill, such as those with pneumonia, young children under five, and elderly individuals are more likely to suffer from severe Leptospirosis.**Causes of leptospirosis-** *Leptospirainterrogans*, a bacterium, may exist in raccoons, bats, sheep, dogs, mice, rats, horses; cattle, buffaloes, and pigs Contamination can persist in soil or waste for months.

People can become infected by:

- Drinking contaminated water.
- Coming into contact with contaminated water or soil if they have unhealed cuts in their skin.
- Their eyes, nose or mouth come into contact with contaminated water or soil.
- Coming into contact with the blood of an infected animal (less common).

II. 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 EMJHMedium.

III. MOLECULAR BASIS OF LEPTOSPIROSIS

Following 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

IV. MATERIALS AND METHODS

A. COLLECTION OF SAMPLE

The different animal (Pig, sheep, Beef, Goat) meat samples were collected from various area of Coimbatore city. Two strains of *Leptrospirainterrogans*(Achimia, Anthamana) were obtained from Department of Microbiology, Bharadhisan University,Tiruchirapalli , for our own confirmation.

B. ISOLATION OF ORGANISM

The organism *Leptrospirainterrogans* 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 asturbidly. They were streaked on nutrient agar plates and incubated for 24 hours at 37 ^{0}C. The spirillium colony was observed in each plate and the *Leptrospira interrogans*was confirmed physically on compound microscope at 100 X,the hock and live organism was visible. Leptrospirawas 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.

C. DNA ISOLATION

The sample was streaked on the EMJH broth. From the well grown Leptospiral *interrogans 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 agarose gel electrophoresis and the run on 50V after electrophoresis they visible on UV transilluminator. The DNA was can be stored at 20°C for later use.

D. 16S NESTED PCR AMPLIFICATION

Nested PCR assay was performed with two pairs of specific primers (Nataraja Seenivasan, Raja*etal*2011) used for primary amplification were 5′ GGCGGCGCGTCTTAAACATG 3′ 5′ GTCCGCCTACGCACCCTTTACG 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 annealingwas 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).

E. RAPD TECHNIQUE

The RAPD assay was performed with two primers B11 5′ GGCGGCGCGTCTTAAACATG 3′ 5′ GTCCGCCTACGCACCCTTTACG 3′. The reaction was performed in 50µl reaction mix contain 10x buffer, 3 mM $MgCl_2$ 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).

V. RFLP

RFLP was done in Collected *Leptospira interrogans* DNA samples. Each DNA samples was incubated with restriction enzymes EcoRI, HindIII and HaeI and 10X buffer (given in the table). The mixture was incubated for 3 hours at 37º C and run in 0.8% agarose gel for 20 minutes at 50 mV and the gel was visualized under UV- trans illuminator.

A. RFLP REACTION MIXTURE

Components	Amount
DNA	10 µl
EcoRI	2 µl
10 X assay buffer	2 µl
Distilled water	6 µl

For Collected DNA sample 2 and 3, HindIII and HaeI was added instead of EcoRI. For sample 4, all three enzymes were added. Run 0.8% agarose gel to see the digested products.

B. L.INTERROGANS IS AMULTI-DRUG RESISTANT

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), Nalidixic acid (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.

C. ALTERNATIVE REMEDIAL MEASURE

Since the isolated *Leptospira interrogans* was Multi Drug Resistance an alternative method will be used.Use of Synthesized Silver nanoparticles (plant and Microbial Source).Use of gold nanoparticles against the *Leptospira interrogans*.Use of silver nanoparticles against the *Leptospira interrogans*.Use of Plant extracts (Medicinal plants) against the *Leptospira interrogans.*

VI. RESULTS AND DISCUSSION

A. IDENTIFICATION OF LEPTROSPIRA*INTERROGANS*

Identification of Leptrospira was observed on blood agar mediumafter 24hours of incubation at 37 ˚C. The haemolysedsamples wereselected and streaked in EMJH medium plate. The grown colony was fixed in slide directly observed by microscopically. The sprillum hook individual colonies was observed is shown in the figure.

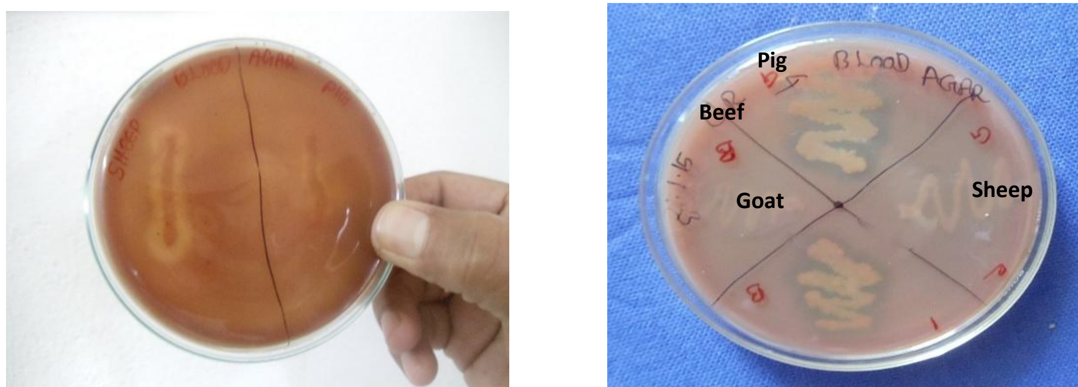

Figure 1: Observance of haemolysis on isolated samples. This shows presence of L.*interrogans in Pig, Goat, and Sheep.* L.*interrogans absent in beef.*

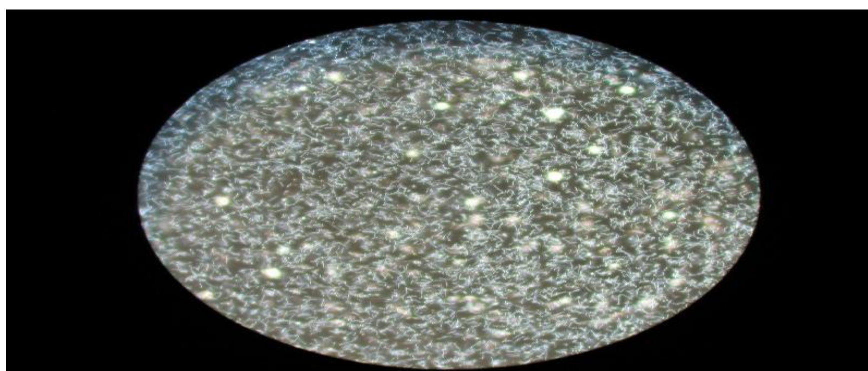

Figure 2: Observance of Spirillim and hook colony.

B. 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 theDNA visualised under UV transilluminator.

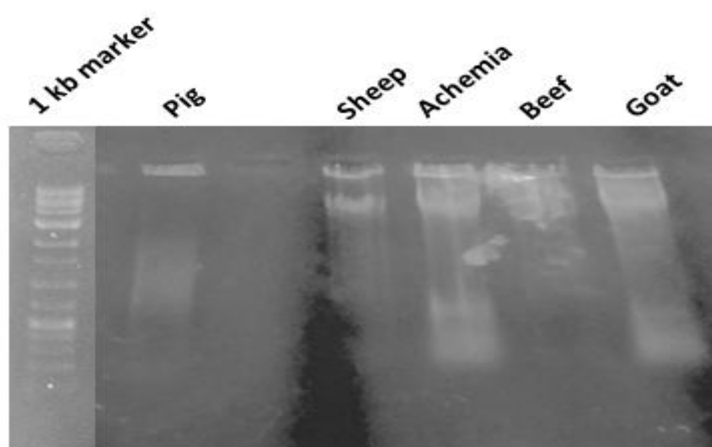

Figure 3: Isolation of DNA

C. 16S AND RAPD TECHNIQUE

The isolated Pig andGoat, sheep sample DNA was amplified 16s RNA Nested PCR with the required conditions. The amplified samples was run in 1.5% agarose gel and visualized under gel documentation is given below.

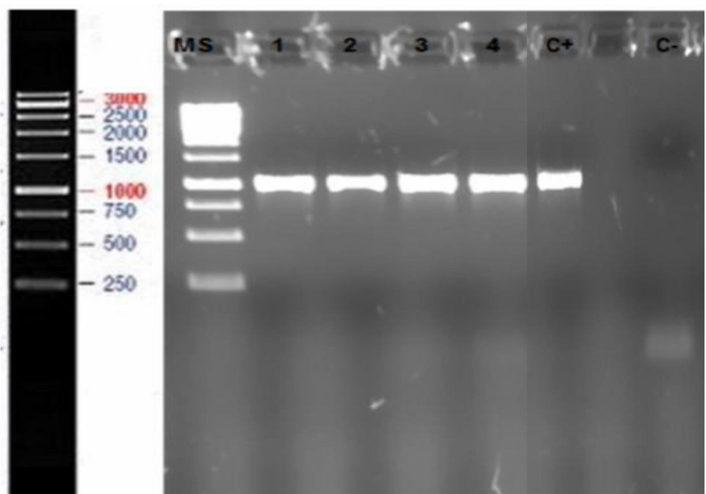

Figure 4: Nested PCR in a 1.5% agarose gel, conducted with DNA extractions, showing the positive products (1004pb) of *Leptospira* spp. MS, molecular size marker. Lines 1-4, positive extractions (Pig, Goat, sheep, achemia). Line C+, positive control. Line C-, absence of *Leptrospira*in isolated sample(Beef).

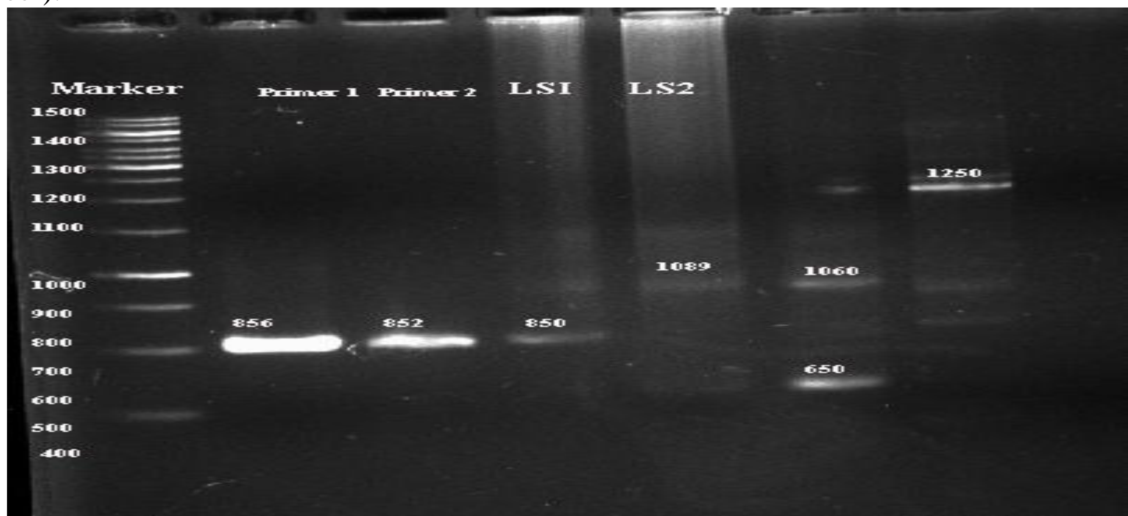

Figure 5: Random Amplified Polymorphic DNA (RAPD) technique showing positive results of isolated strains LS1-Pig, LS2- Goat, 650bp-sheep, 1250bp- Acemia.

D. RESTRICTION FRAGMENT LENGTH POLYMORPHISM –RFLP

Restriction Fragment Length Polymorphism was done in extracted DNA's. EcoRI, BamHI and HindIII enzymes were used to digest DNA. After incubation period the samples were run at 0.8% agarose gel and digested bands were observed in UV Tran illuminator.

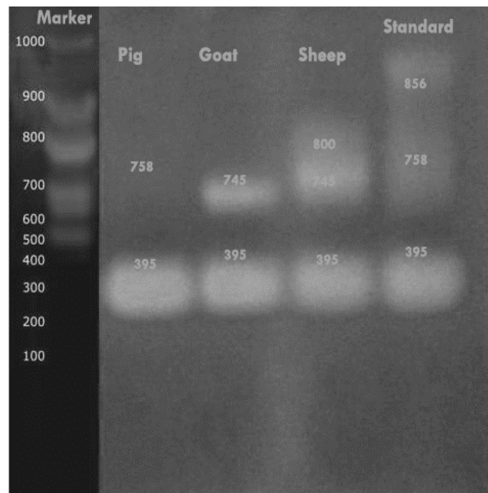

Figure 6: Restriction Fragment Length Polymorphism technique showing positive results of isolated strains LS1-Pig, LS2-Goat, LS3-sheep, LS4- Standard (Acemia)

E. CONFIRMATION OF MDR PATTERN

The isolated *Leptrospira* 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.

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

F. ALTERNATIVE REMEDIAL MEASURE

Since the isolated L. *interrogans* was Multi Drug Resistant so we used alternative method to kill the pathogenic organism. Use of synthesized Silver, Zinc, Gold Nanoparticles they are kill the organism result from zone of clearance as shown below.

Table 2: Alternative remedial measure using Synthesized Nano particles

Synthesized Nano particles	Isolated organism from different animals(Zone of Inhibition)			
	Pig	Goat	Sheep	Achemia
Zinc	2mm	4mm	6mm	6mm
Gold	2mm	Nil	Nil	Nil
Silver	5mm	2mm	3 mm	4mm
Vancomycin	6mm	6mm	5mm	11mm

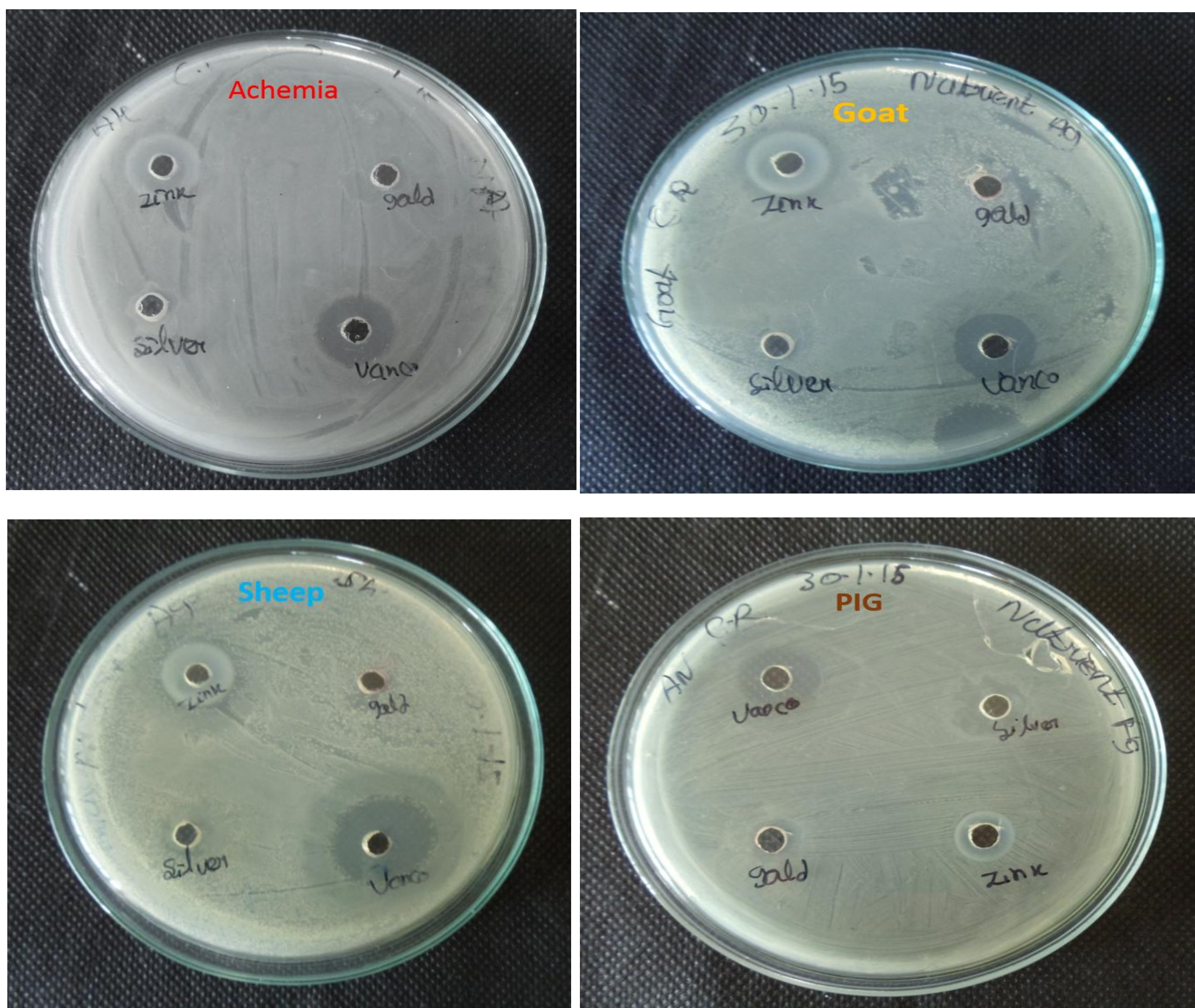

Figure 7.Alternative remedial of Achemia, Goat, Sheep, pig.

Leptospirosis is considered the most common zoonosis worldwide and is endemic in tropical environments (Katz *et al.,* 2002). 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(Radhika Krishna et al, 2008).

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

Radhika Krishna *et al*, 2008 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 non-pathogenic spp., Find RFLP for indication of genome ., 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*interrogans* among hospital case in erode.

Ramadass, *et al*, (1997) 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. Ramadass, et al, allowed the strains to DNA restriction enzyme analysis but it produce more fragments so difficult to comparison than RAPD.

In 2013, Houemenou, *et al*, Describe to asses prevalence of Leptospira in small animals. We select 90 animals kidney specimen for presence study then specimens were examined under Real Time PCR for detection of pathogenic spp.,. From kidney tissue the Leptospirsl DNA was amplified using real time PCR. To get 13.3%, 100% & 18.9% of DNA from Ratttus, Crocidura spp., and other animals respectively. Next to use Clade specific taquman PCR for further analysis. Totally we have 10 samples out of these 6 load with clade 1 and four load with in clade 2 then to analysis phylogenies. For this purpose amp icon sequence is to be used that sequence equally assigned to clade 1 have L.interrogans and L.kirschnein and L.borgpetersenii in clade 2. We conclude this small mammals is also involve major public health risk of acquire Lepto based disease.

The Pacciarini, et al., 1993, investigated 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 Identify, detectionand characterization Leptospira*interrogans*.

VII. CONCLUSION

Although no extensive prevalence study was undertaken in this region, the results of this study indicatethat *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.

In the present study the Leptospira *interrogans.*Was isolated from various animal tissues and subcultured. DNA was isolated from collected Leptospira*interrogans* species from various animal samples (Pig, Goat) was confirmed by 16S Nested PCR and also whole experiment was confirmed by 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 in the infected samples.

ACKNOWLEDGEMENT

We thankful to the management of Hindusthan College of Arts and Science, Coimbatore, for providing them necessary facilities and support to cary out this work. The authors also thankful Dr. Ragunathan,, Centre for Bioscience and Nanoscience Research Coimbatore, who giving on opportunity to do the dissertation for his valuable advice and guidance for the completion of this work.and also thank S.Komathi, Associate Professor, Department of Biotechnology, Hindusthan College of Arts and Science, Coimbatore, Tamilnadu, for providing me an opportunity do to external project. Also we thank Dr. Natarajasennivasan. , Associate Professor Department of Microbiology, for valuable help and time spending for confirmation of Leptospira.

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