DETECTION AND IDENTIFICATION OF PATHOGENIC LEPTOSPIRES IN BIO-MATERIALS

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DECLARATION

I hereby declare that the thesis, entitled **"DETECTION AND IDENTIFICATION OF PATHOGENIC LEPTOSPIRES IN BIO-MATERIALS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Introduction

1. INTRODUCTION

Leptospirosis is a zoonosis of ubiquitous distribution, infecting all types of warm-blooded vertebrates. The disease is caused by a spirochaete of the genus *Leptospira*, which are motile helical bacteria (0.1 x 6 to 12 μm) with hook shaped ends. The primary source of pathogenic leptospires is the surface of the renal tubules of carrier animals and rodent reservoirs, which readily excrete the organisms through urine. Humans, infected through direct or indirect contact with these carriers or reservoirs, are the accidental or end hosts. Though human-to-human transmission is extremely rare, congenital transplacental infection can occur (Faine, 1998).

Leptospirosis is now identified as one of the re-emerging infectious diseases. In the past, the infection had caused significant morbidity in military personel deployed in tropical areas. Now-a-days large outbreaks of leptospirosis following severe floods are of common occurrence and are not restricted to tropical regions.

Swine and cattle are the major sources of infection to human. The large volume of urine as well as the alkalinity of urine in these species favours survival of leptospires. There are no distinctive clinical signs for the disease in animals, but some are suggestive. They include elevated temperature, anorexia, depression, hemoglobinurea, icterus, anemia, abortion and agalactia and mastitis in cattle. The animals recovered from the disease act as carriers and urinary shedding of the organism occurs for variable period of time. Rodents are the natural reservoirs of infection and excrete the organism in urine throughout their lifetime.

In animals the disease has been classified into two broad categories: host adapted and non-host adapted leptospirosis. An animal infected with a host-adapted serovar of the organism is a 'maintenance' or 'reservoir' host. Exposure of

susceptible animals to non-host adapted serovars results in accidental or incidental disease, which is acute and severe unlike chronic disease in maintenance host. Each serovar is adapted to a particular maintenance host although they may cause disease in any mammalian species.

Two forms of disease are reported in humans, icteric leptospirosis with severe hepatic and renal involvement and anicteric leptospirosis, a mild flu-like syndrome. The incubation period of the disease vary from 3 to 30 days, but is usually 10 to12 days. It occurs in two phases, a septicemic phase lasting for about a week followed by an immune phase characterised by appearance of antibodies and localization of organism in immunologically sequestrated areas of body like cerebrospinal fluid (CSF), eye and kidney.

The wide spectrum of clinical signs that vary from mild to fatal forms make the diagnosis of leptospirosis difficult. The diagnostic approaches include direct microscopic demonstration of organism, isolation, serology and molecular techniques for detection of nucleic acid. Similar to any other bacterial disease current diagnosis involves detection of antigen or its components and early diagnosis is extremely important for this disease because of the multiorgan involvement and high fatality.

Dark field microscopy (DFM) for the demonstration of leptospires in clinical samples is a rapid method, but it's lower specificity and sensitivity (less than 50%), makes it highly unsatisfactory. Artifacts such as fibrin threads are referred to as "psuedospirochetes" contribute to high false positive results in DFM, which necessitates expertise in the technique. Moreover, demonstration of leptospires in clinical samples is possible only in certain stages of disease.

Serological tests are widely employed for diagnosis of leptospirosis. Among these microscopic agglutination test (MAT) is considered as the gold standard test. It is a serovar specific test and is widely used for characterization of leptospiral isolates. Though specificity and sensitivity of MAT are satisfactory, early diagnosis is not possible with this test. Moreover, it requires maintenance of large number of live cultures, which is laborious and time consuming. Contamination is also a problem especially in tropical climate. The cumbersome procedure of MAT thus reduces its preference in diagnostic labs on a routine basis.

Among serological tests enzyme linked immunosorbent assay (ELISA) can differentiate IgM and IgG antibodies in serum samples and is used for the diagnosis of recent infection. But it is a genus specific test and cannot be used for the differentiation of serovars.

Isolation of causative agent is the most confirmatory method for the diagnosis of leptospirosis. But unlike many other bacteria, the culturing of leptospires is difficult and tedious. They require special media and take about two to six weeks to grow. This delays the clinician to make a quick and prompt diagnosis. The species or the serovars of leptospires could not be differentiated based on cultural properties.

The genus *Leptospira* includes 23 serogroups and more than 250 serovars. The classification of the genus is being revised continuously and new serovars are added. A particular geographical region contains only a few serovars, but they may appear, disappear and reappear in the same region. This may be due to the fact that there is no cross protection between the serovars and the immunity is serotype specific. Vaccination is the best and the most practical approach to control the disease. So there is a need to identify prevalent serovars in an area and the tests adopted in epidemiological investigations should be suitable in this respect.

An epidemiological study, involving identification of the source of infection, isolation and identification of strains and use of such strains in vaccines to be used in an endemic area, requires single, rapid and affordable test/tests that can differentiate the pathogen in clinical samples. The search for such methods led the scientists to develop DNA based techniques such as polymerase chain reaction (PCR), restriction enzyme analysis (REA) and DNA hybridization. Now- a- days these tests alone or in combination are used for detection as well as characterization of leptospires in cultures and also in clinical samples. Among these, PCR is the most accepted one because of its sensitivity, specificity and simplicity of procedures. Restriction Enzyme Analysis of chromosomal DNA, could differentiate serovars of *Leptospira*, requires pure culture of leptospires similar to MAT. To some extend this could be circumvented by restriction enzyme analysis of PCR product. Apart from these, modifications of PCR such as arbitrarily primed PCR (AP-PCR) and low stringency PCR (LS-PCR) are found to be helpful in characterization of leptospires in clinical samples in a single reaction.

In the present study an attempt has been made to

1. Detect leptospires in clinical samples employing PCR.
2. Isolate leptospires from rodents and clinical samples.
3. Differentiate reference strains and isolates of *Leptospira* and leptospires from bio-materials by multiplex PCR, nested PCR, PCR-REA, AP-PCR and LS-PCR.

Review of Literature

2. REVIEW OF LITERATURE

2.1 HISTORY

The syndrome 'icteric leptospirosis' with renal failure in human was first reported by Weil (1886) in Germany. Goldschmidt (1887) was the first to use the term Weil's disease to denote a severe febrile illness with icterus and renal abnormalities. Stimson (1907) demonstrated finely coiled organism with hooked ends in the renal tubules of a man believed to be died of yellow fever in Neworlans for the first time. He cautiously named those organisms stained by Levaditi's silver impregnation method as *Spirochaeta interrogans* to differentiate them from other spirochetes. The etiology of leptospirosis was demonstrated independently in Germany by Uhlenhuth and Fromme (1915) and in Japan by Inada *et al.* (1916). Noguchi (1918) proposed the name *Leptospira* meaning thin spirals following detailed microscopical and cultural examinations. During 1920s and 1950s the milder forms of leptospirosis and the occupational relationships were elucidated in Japan, Indonesia and Germany (Faine, 1998).

2.2 TAXONOMY AND CLASSIFICATION

The DNA hybridization studies by Haapal *et al.* (1969) indicated a great deal of heterogeneity in leptospires and they could identify six groups based on DNA relativity among 15 serovars.

In 1962 the Leptospira subcommittee of the International Committee on Bacteriological nomenclature recommended that the genus *Leptospira* be divided into two species, *L. interrogans* representing the parasitic and *L. biflexa* the saprophytic types (Kenzy and Ringen, 1971).

The genus *Leptospira* belongs to the family *Leptospiracea* in the order *Spirochaetales*. The two *Leptospira* species are *L. interrogans,* which contains a large number of serogroups whose strains are parasitic or pathogenic for humans and animals and *L. biflexa* which contains a large number of serogroups whose strains are primarily found in fresh surface water and moist soil and are rarely isolated from humans or animals. The serovars (serotypes) of leptospires are named and antigenically related serovars are organized into serogroups (Johnson and Faine, 1984).

Yasuda *et al.* (1987) proposed a new classification of genus *Leptospira* in which the old pathogenic species *L. interrogans* and the saprophytic *L. biflexa* were subdivided in to number of new species. The genetic classification which is based on DNA homology divide leptospiral strains into four non pathogenic species *L. biflexa, L. meyeri, L. parva, L. wolbachii* and six pathogenic species *L. interrogans, L. borgpetersenii, L. weilii, L. noguchii, L. santarosai and L. inadai.* An additional genomospecies *L. kirschneri* was added later by Ramadass *et al.* (1992).

Workers at the Centers for Disease Control (CDC) recently defined 16 genomospecies of *Leptospira* that included the 11 genomospecies described previously. The genomospecies of *Leptospira* do not correspond to the previous two species *(L. interrogans* and *L. biflexa*) and indeed pathogenic and non-pathogenic serovars occur within the same species. The reclassification of leptospires on genotypic grounds is taxonomically correct and provides a strong foundation for future classifications (Levette, 2001).

The molecular classification is incompatible with the system of serogroups, which has been useful for clinicians and epidemiologists. There is a pressing need for simple molecular tools for identification of species and serovars (Levette, 2004).

2.3 PREVALENCE

Pathogenic leptospires occur naturally in a wide variety of wild and domesticated mammals throughout the world (Alexander, 1974).

The first incidence of leptospiral infection in India was reported in 1931 by Taylor and Goyle. Since then several workers have recorded the presence of *Leptospira* antibodies in the sera of man and animals in different parts of the country against at least 15 serogroups (Rajasekhar and Keshavamurthy, 1976).

In a serosurvey conducted in an agriculture based village in Tamilnadu by Natarajaseenivasan and Ratnam (1997), it was found that 61.5 per cent, 56.3 per cent, 75 per cent, 52.9 per cent and 72.5 per cent of cattle, sheep, goat, field rats and agricultural workers respectively were seropositive. Leptospirosis is an occupational disease among persons engaged in agricultural and animal husbandry activities, especially in rural environment.

Leptospirosis was a notifiable disease in Oklahoma and between 1980 and 1997 an average of 0.5 cases per year (range 0-4) have been documented (CDC, 1998).

In Brazil, leptospirosis is endemic in most of the large urban areas, where epidemic outbreaks occur after flooding caused by heavy seasonal rainfall in the summer. Since 1982, it was designated as a notifiable illness in the state of Sao Paulo (Brandao *et al.*, 1998).

Seroprevalence of *L. interrogans* serovar *djasiman* in quarantined pigs in Brazil is of great epidemiological significance, as this serovar has been referred as appearing only in human beings (Modolo *et al.*, 2000).

Leptospirosis has been documented worldwide but formal reporting systems vary widely. High-risk areas include the Caribbean islands, central and south America, South East Asia and the Pacific islands. The disease gains public attention when outbreaks occur in association with natural disasters (Hickey and Demers, 2003).

Jeyakumar *et al.* (2004) conducted microscopic and serological examination of blood samples collected from patients visiting hospitals in Chennai city and out of 150 suspected cases 70 were positive by MAT.

The prevalence rate of leptospirosis as detected by passive heamagglutination test (PHA) was 50.41 per cent in dogs, 23 per cent in cattle, 19.04 per cent in pigs, 26.19 per cent in rodents and 42.85 per cent in human beings in Thrissur, Kerala (Soman, 2004).

In a study conducted in MOSC medical college, Kolenchery, Kerala, Autumnalis was found to be the most predominating serogroup during the period January 2000 to June 2002, followed by Pyrugenes, Grippotyphosa, Bataviae, Australis, Canicola, Icterohaemorrhagiae, Hebdomadis and Lousiana (Sugathan and Varghese, 2005).

2.4. DISEASE IN ANIMALS

Leptospires have been isolated from warm-blooded animals, birds, reptiles, amphibians and arthropods. Theoretically any mammal is capable of being infected by any serovar of *L. interrogans.* However, in reality a few serovars are enzootic in a particular region which indicates that probably at a particular region a serovar is getting adapted to infect one or more animal species (Thiermann, 1984).

The majority of leptospiral infections are sub clinical and associated with foetal infections causing abortions, stillbirths and birth of weak neonates with a high death rate in cattle, sheep, horse and pigs (Radostits *et al.,* 2000).

Acute leptospirosis should be suspected in the following cases: sudden onset of agalactia (in adult milking cattle and sheep); icterus and haemoglobinuria, especially in young animals; meningitis, and nephritis and hepatitis in dogs. Chronic leptospirosis should be considered in following cases: abortions, stillbirth, birth of weak offspring (may be premature); infertility; and cases of periodic ophthalmia in horses (O.I.E., 2000).

Intraspecies and interspecies transmission within susceptible host may persist for a long time in an environment that has been contaminated with urine, especially in warm and humid climatic conditions (Machang'u *et al.,* 2004).

2.4.1 Cattle

Leptospiral infection in cattle with organisms of Hebdomadis serogroup is of mild clinical nature to the adult host. Urinary shedding is common and reproductive disorders and mastitis seems to be the salient complications. Experimental leptospiral

infections in eight pregnant cattle with organisms of Hebdomadis serogroup produced mastitis in all cows that calved, abortion in one cow and birth of premature or weak calves in two (Thiermann, 1982).

Two clinical conditions have been reported in cattle infected with serovar *hardjo*. First, a sudden onset of agalactia in dairy cattle (Ellis *et al.*, 1982) and second, abortion which occurs as a chronic sequelae to infection (Thiermann, 1984).

During an investigation of natural in utero infection of cattle by *L. interrogans* strains Ellis *et al.* (1985b) observed that majority of infected foetuses were aborted from the sixth month of gestation onwards and cows, which aborted, had not previously exhibited overt signs of agalactia. There was an association between leptospiral infections and retention of fetal membranes.

Gerristen *et al.* (1993) inoculated the eyes of cows with *L. interrogans* serovar *hardjo* subtype *hardjobovis*, because this has been reported to be the most likely route of natural infection. No clinical signs of disease were detected, but all experimentally infected cows shed leptospires in urine within four weeks after infection and all became serologically positive.

Acute clinical disease occurs more often in young calves. Cattle may develop high fever, of 104 to 107°C, depression, loss of appetite, decreased milk production and weakness. Haemoglobinurea, anemia, icterus and bloody milk are also seen (Hudson, 1996).

Infertility and milk drop syndrome occurs only in pregnant or lactating cows. Symptoms are sudden onset of fever, anorexia, immobility and agalactia. Udder will

be flabby without heat or pain and all four quarters will be equally affected (Radostits *et al.*, 2000).

2.4.2 Sheep and goats

McKeown and Ellis (1986) observed oligalactia and agalactia in ewes of various sheep farms in UK where lambs of normal birth weight were dying in the first few days of life. *L. hardjo* was isolated from blood or milk of all clinically affected ewes.

Leptospirosis in sheep and goats occurs with less frequency than in cattle and swine. The signs reported are similar to those in cattle including high fever, depression, loss of appetite, decreased milk production and weakness. Haemoglobinurea, anemia, icterus and bloody milk are also seen (Hudson, 1996).

Leptospirosis is rare in sheep and goats and most affected animals are found dead apparently from septicemia. Symptoms include fever, dyspnoea, snuffle, haemoglobinurea, and pallor of mucosa, jaundice and death in 12 hours. Abortions, oligalactia and agalactia similar to bovine milk drop syndrome have been observed in lactating ewes (Radostits *et al.*, 2000).

2.4.3 Horses

The acute phase of disease in horses following exposure to leptospires is frequently sub clinical. The infected animal may have a slight temperature rise and mild loss of appetite. Within 12 to 14 months after initial infection, the eyes of many horses show signs of uveitis, a disease commonly known as ' periodic ophthalmia' or 'moon blindness' (Hudson, 1996).

Periodic ophthalmia is a late complication of systemic leptospirosis in horses (Radostits *et al.*, 2000).

2.4.4 Pigs

Ellis *et al.* (1985a) isolated *L. bratislava* from aborted foetuses and the genital tracts of their dams and sires, and found that leptospiral abortions occurred between day 88 and 100 of gestation.

The disease in swine is largely sub clinical except for abortions, which usually occur during last two to three weeks of pregnancy. Piglets may be born weak and die shortly after birth (Hudson, 1996).

Chronic leptospirosis is the commonest form of disease in pigs and is characterized by abortions and high incidence of stillbirths (Radostits *et al.*, 2000).

2.4.5 Dogs

According to Cornwell (1994) the serotype *canicola* associated with acute interstitial nephritis and *icterohaemorrhagiae* associated with jaundice and haemorrhage are considered to be of importance in dogs. There may not be a clear-cut distinction between these diseases. The clinical signs of *L. canicola* infection include pyrexia, extreme depression, thirst, vomiting, oliguria, abdominal pain associated with swelling of kidneys, lingual and / or oral ulcers and halitosis.
L. icterohaemorrhagiae infection is associated with fever, jaundice, extreme depression, thirst, vomiting, blood stained diarrhea and petechial haemorrhage in the mucous membrane of conjunctiva.

The acute form of leptospirosis in dogs causes elevated temperature, vomiting, muscular stiffness, weakness and nephritis. In severe cases jaundice and death may occur. The signs of Central nervous system (CNS) involvement may occur with or without other clinical signs and organisms may be present in brain tissue for extended periods. Chronic leptospirosis is associated with chronic tissue degeneration. Shedding of leptospires in urine may continue for over a year (Hudson, 1996).

Per acute leptospiral infections cause massive leptospiremia, shock and death in dogs. Less severe infection causes fever, anorexia, vomiting, dehydration, increased thirst and reluctance to move. Progressive deterioration in renal functions results in oliguria and anuria. Icterus occurs in some dogs. A majority of leptospiral infections in dogs are chronic or sub clinical but the animal may have acute renal failure (Green, 2000).

The non-specific clinical signs in 31 dogs with eventual diagnosis of leptospirosis included lethargy, inappetence, dehydration, and weight loss of various severities. Other signs at presentation included vomiting, abdominal or lumbar pain, polyurea / polydypsia, tachypnea, stiff gait suggestive of arthralgia and myalgia, icterus, and lymphadenopathy. Four dogs were pyrexic, renomegaly was detected in three dogs and petechiation, oculonasal discharge, or ascites was observed in one dog each (Prescott *et al.*, 2002).

2.4.6 Rodents

Rodents are the animals most commonly infected with *Leptospira* serovars and are also the major natural reservoirs of these microorganisms (Thiermann, 1984).

Wild rats (*Rattus norvegicus*) populations in urban or rural areas are frequently infected with *L. icterohaemorrhagiae. L. ballum* is found primarily in wild mice (*Mus musculus)*, but is also present in some rat populations and has been present in commercially raised laboratory mice. Other serotypes occur with less frequency in rodent population (Hudson, 1996).

Four different species of rodents *Rattus rattus wroughtony hinton, Rattus rattus rufescens, Bandicota bengalensis* and *Bandicota indica* were identified as the natural reservoir hosts of *L. inadai* in a study conducted in Banglore (Gangadhar *et al.*, 2000).

2.5 DISEASE IN HUMANS

The infection in human being ranges from sub clinical, mild nonspecific febrile symptoms to very severe fulminating fatal infection with hepatorenal failure. Non-icteric leptospirosis is characterized by fever, headache, body pain, anorexia, nausea and conjunctival suffusion in septicaemic phase, followed by second immune phase with aseptic meningitis, uveitis and neurological lesions. In icteric leptospirosis in addition to above symptoms there will be jaundice, epistaxis, petechial haemorrhage and other complications like pneumonia, pharyngitis and iritis (Ratnam, 1994).

Majority of infections caused by leptospires are either sub clinical or of very mild severity. Small proportion of infections presents a febrile illness of sudden onset. Other symptoms include chills, headache, myalgia, abdominal pain, conjunctival suffusion and less often a skin rash (Levette, 2001).

In 90 per cent of cases, leptospirosis manifests as an acute febrile illness with biphasic course and an excellent prognosis. Non-specific signs and symptoms of leptospirosis (eg. fever, headache, nausea and vomiting) often are confused with viral illness. Ten per cent of cases will be presented as icteric leptospirosis and the signs of this form include fever, jaundice, renal failure and hemorrhage. Pulmonary, cardiac and central nervous systems are also involved frequently (Hickey and Demers, 2003).

The clinical presentation of leptospirosis varies from patient to patient. Hepatorenal failure, myocarditis, severe pulmonary haemorrhage with respiratory distress and meningitis are some of the syndromes reported commonly (Natarajaseenivasan *et al.*, 2004).

2.6 DIAGNOSIS

The use, interpretation, and value of laboratory diagnostic procedures for leptospirosis vary with the clinical history of the animal or herd, the duration of infection and the infecting serovar. Two major microbiological sequelae of leptospiral infection present particular diagnostic problems: the localization and persistence of leptospires in kidneys and in male and female reproductive tracts (O.I.E., 2000).

2.6.1 Direct microscopic demonstration.

2.6.1.1 Dark Field Microscopy (DFM)

Doherty (1966) examined urine by DFM and inferred that this method was superior to guinea pig inoculation techniques.

According to Thiermann (1982) a minimum of 1.25 X 10^4 cells/ml of urine was required to observe a single organism by DFM.

Chandrasekharan and Pankajalakshmi (1997) have described DFM after differential centrifugation of blood as a valuable technique for early diagnosis of acute leptospirosis in humans and animals when it is properly employed by experienced workers.

According to Sehgal *et al.* (2001) dark ground microscopy was the ideal technique for demonstration of leptospires in culture, but was often used in demonstrating leptospires in clinical specimens, especially blood and urine, with less than satisfactory results.

Chandrasekharan and Gomathy (2004) performed DFM for detection of leptospires and validated the results using *Leptospira* IgM antibody SERION ELISA test based on which they recommended that DFM could serve as a standard screening test for early and rapid diagnosis of leptospirosis.

2.6.1.2 Silver staining

Stimson (1907) demonstrated finely coiled organism with hooked ends in the renal tubules of a man believed to be died of yellow fever, by Levaditi's silver impregnation method and this was the first report of demonstration of leptospires in tissues.

The impression smears of kidneys and urine from rodents were stained with the silver method of Fontana and the microtome sections of kidneys with Levaditi's

technique. The kidney smear method provided a more reliable guide to the presence of infection (Pargaonker, 1957).

Gangadhar and Rajasekhar (1998a) have developed a modified silver impregnation staining for leptospires which avoids over staining, clouding of smears or development of artifacts or cracks of staining.

Sehgal *et al.* (2001) opined that though several staining techniques like silver impregnation techniques, Giemsa staining etc. have been described, their role in diagnosis was questionable.

2.6.1.3 Fluorescent Antibody Technique (FAT)

White *et al.* (1961) used FAT to detect leptospires in urine and kidneys of naturally infected dogs and suggested that compared to culturing, sterile specimens were not necessary and results might be obtained quickly for FAT.

Bolin *et al.* (1989) opined that immunofluorescence was quite sensitive when performed by experienced workers, but using generally available conjugates was not serovar specific. Immunofluorescence procedure depends on structural and antigenic integrity of the organism and these factors may be compromised in clinical specimens.

Wagenaar *et al.* (2000) reported that for routine use in their laboratory, immunofluorescence was faster, less prone to laboratory error, and easier to perform than PCR.

Immunofluorescence was used to demonstrate leptospires in kidneys from slaughtered fattening pigs, using a polyvalent antiserum to get an indication about occurrence of carriers and leptospires were detected in 69 per cent of the kidneys examined (Boqvist *et al.*, 2003).

Out of the three methods, leptospiral culture, direct immunofluorescence and polymerase chain reaction for the detection of leptospiral materials in human post-mortem samples, direct immunofluorescence was of intermediate sensitivity, confirming the presence leptospires in 11 per cent of tissue samples (Brown *et al.*, 2003).

2.6.2 Isolation

Leptospira was first isolated in India by Taylor and Goyle (1931). They isolated *L. andamana* and *L. grippotyphosa* from human patients in Andaman Islands.

Johnson and Rogers (1964) evaluated 5-Fluorouracil (5-FU) as a selective agent for growth of leptospires and found that this pyrimidine analogue was a strong bacteriostatic agent for many bacteria and concentration of 5FU as high as one milligram per milliliter had no apparent inhibitory effect on growth of leptospires. A concentration of 200-400 µg/ml was found to be useful to isolate leptospires from contaminated urine samples and to purify contaminated cultures.

Rajasekhar and Keshavamurthy (1976) cultured pieces of kidney material from 30 rodents in Fletcher's and Korthof's medium containing 10 per cent pooled inactivated rabbit serum at a temperature of 30°C, and obtained an isolate from a rat in Fletcher's medium on sixth day of incubation.

Adinarayanan and James (1980) isolated leptospires from domestic animals, rats, bandicoots, toddy cat and mongoose and was the first report of isolation from the concerned animal species in India. They have used Stuart's or Fletcher's media enriched with 10 per cent sterile haemoglobinised rabbit serum for isolation and one in ten dilutions of tissue homogenates constituted the inocula.

Ellis *et al.* (1981) cultured the kidneys of cattle, mice and badgers in an attempt to evaluate the relative importance of these species in the Epidemiology of bovine leptospirosis caused by Hebdomadis serogroup and found that catttle acted as the maintenance host for serotype *hardjo* and many of them were seronegative carriers.

Blood, urine and milk from experimentally inoculated pregnant cows were cultured in semisolid bovine serum albumin polysorbate 80 (BAP 80) medium, semisolid BAP 80 medium with 100 µg 5-FU and solid BAP 80 medium. Leptospires were cultured from early phase of mastitis only in solid medium and for urine samples semisolid medium with 5-FU was found to be useful (Thiermann, 1982).

The major advantage of bacteriologic culture is that leptospires of any serovar can be detected and subsequently identified (Bolin *et al.*, 1989).

A new culture medium in which a base medium containing sodium salts and potassium hydroxide supplemented with five per cent sheep serum was developed by Rodriguez *et al.* (1998) and was found to support the growth of leptospires. One hundred and thirty five isolates were obtained in this medium, which was devoid of conventional ingredients such as bacteriological peptone, vitamins and rabbit serum.

Gangadhar and Rajasekhar (1998b) made leptospiral isolations from 89 rodents by culturing their kidneys in EMJH semisolid medium.

Heinemann *et al.* (1999) could not isolate leptospires from any of the bovine semen samples examined, though 80 per cent of them were PCR positive..

In a study conducted by Wagenaar *et al.* (2000) PCR and culture were of equivalent sensitivity for the detection of serovar *hardjo* in urine of experimentally infected bovines. The difference in their sensitivities observed in various other studies probably reflected the rigor of culture methods that were used, variation in laboratory expertise for culturing this fastidious organisms and the variable presence of contaminating bacteria in urine samples which interfere with isolation of leptospires.

A major problem in culturing of leptospires was the contamination with other microorganisms, especially when samples were from non-sterile sources like urine, milk and aborted materials. When a total of 82 samples found positive by DFM were tested by culturing, only 50 samples yielded growth of leptospires in EMJH medium (Venkatesha and Ramadas, 2001).

Senthilkumar *et al.* (2001) opined that culture of *Leptospira* from the body fluids was the most demonstrative test but this technique is laborious and might take up to two months to get a result.

Isolation of *Leptospira* from kidneys of rats and bandicoots was attempted in Bangalore and 23 of the isolates obtained were identified as *L. inadai* by serological and PCR analysis (Gangadhar *et al.*, 2000).

Uzal *et al.* (2002) tried isolation of leptospires from aqueous humor, urine and small portion of kidney drawn with a Pasteur pipette, of normal cattle as well as of those with chronic interstitial nephritis, in EMJH media containing 0.15per cent agar (pH 7.6) at 30°C for eight weeks. Two isolates were obtained from 118 cattle with gross lesions in kidneys.

Boqvist *et al.* (2003) opined that culturing was not a suitable method for investigating presence of leptospires in epidemiological studies. Out of 32 kidneys cultured they could get only one isolate whereas 22 of them were found positive by immunofluorescence.

Of the 125 samples from different sources like human, dogs, bovines and rodents, leptospires were isolated from three rodents only (Elaiyaraja, 2003).

Harkin *et al.* (2003b) failed to isolate leptospires from dog urine that had positive results for PCR. Even a dog with acute renal failure, a MAT titer of 1: 51,200 and a strong band detected by PCR had negative culture results.

2.6.3 Serological Diagnosis

Galton *et al.* (1965) developed a micro technique for detecting *Leptospira* antibody in suspected sera using special micro plates and the reaction was observed by DFM.

Cole *et al.* (1973) developed a method for improving the original Galton micro technique for the leptospiral microscopic agglutination test.

Kawaoka *et al.* (1979) used a radioimmunoassay system utilizing a serovar specific lipopolysaccharide antigen (TM) of *Leptospira* and this was employed in elucidating the antigenic determinant group of the TM antigen.

Adler *et al.* (1980) used a solid phase enzyme linked immunosorbent assay (ELISA) to detect *Leptospira* specific IgM and IgG in sera of patients. The specificity and sensitivity of the test suggested that the ELISA anti IgM technique was a suitable method for diagnostic and epidemiological purposes.

Thiermann (1982) determined agglutinating leptospiral antibodies in serum, whey and urine by MAT and found that milk whey antibody titers closely paralleled and often exceeded serum titers.

Milner *et al.* (1985) developed an ELISA test for the detection of anti *hardjo* IgM, which was sensitive, reproducible and easy to perform.

Gerristen *et al.* (1993) studied the serologic response to *L.interrogans* serovar *hardjo* subtype hardjobovis in experimentally infected cows and the sera were ELISA positive from three to five weeks after infection, whereas the sera were MAT positive from about two weeks after infection.

Gussenhoven *et al.* (1997) studied a dipstick assay for the detection of *Leptospira* specific IgM antibodies and concluded that it was easy to perform, quick and required no electricity or special equipment.

Brandao *et al.* (1998) recommended macroscopic slide agglutination test as an alternative to MAT or even a replacement for MAT for laboratory screening during the acute phase of leptospirosis.

Levette and Whittington (1998) reported for the first time that indirect haemagglutination assay (IHA) detected both IgM and IgG antibodies. In their study IHA detected all cases of leptospirosis and the specificity was 94 per cent.

Cumberland *et al.* (1999) assessed the efficiency of IgM ELISA and MAT in the diagnosis of acute leptospirosis and IgM ELISA was found to be more sensitive and MAT more specific in diagnosing the disease.

Effler *et al.* (2000) found that the sensitivity of indirect heamagglutination assay was affected by the presumptive infecting serogroup of *Leptospira.* Since the prevalence of serovars varies with the geography, the performance of IHA should be assessed locally.

Saengjaruk *et al.* (2002) suggested that the detection of antigen in urine by the monoclonal antibody based dot ELISA had high potential for rapid, sensitive and specific diagnosis of leptospirosis at a low cost.

Harkin *et al.* (2003b) suggested that serologic results were poor for prediction of the risk of an individual dog actively shedding leptospires in urine and have poor sensitivity and poor positive predictive value.

Bajani *et al.* (2003) evaluated IgM ELISA, an IHA, an IgM dipstick assay (LDS) and an IgM dot ELISA dipstick test (DST) and suggested ELISA and DST as good choices for diagnostic testing.

According to Natarajaseenivasan *et al.* (2004l) a quantitative immunoblot analysis using acute and convalescent phase human sera for leptospiral protein

recognition was more sensitive than other serological techniques during the early stage of the disease.

Soman (2004) examined the sera samples from dogs, cattle, pigs, rodents, and human beings by MAT, PHA and ELISA. Of the three tests IgG ELISA was proved to be the most suitable test for rapid screening of population for leptospires while PHA was an effective assay for diagnosis of acute infection.

2.6.4 Molecular methods

2.6.4.1 Polymerase Chain Reaction (PCR)

Merien *et al.* (1992) developed a sensitive assay for detection of leptospires based on amplification of 16S rRNA gene and the PCR product was analysed by DNA- DNA hybridization.

Merien *et al.* (1993) detected leptospiral DNA by PCR in aqueous humor of a patient with unilateral uveitis, with genus specific primers A and B.

The primers LPI and LP2 were found to produce detectable amplification of 100 fg of leptospiral DNA. The specificity and high sensitivity of the test provided valuable tool for early diagnosis of leptospiral DNA (Kee *et al.*, 1994).

Bal *et al.* (1994) suggested that PCR analysis of urine could be more successful for early diagnosis of leptospirosis than PCR analysis of serum. It is a promising approach for early diagnosis of leptospirosis and may also be useful in studying long term urinary shedding of leptospires.

Brown *et al.* (1995) used multiplex PCR with primers G1/G2 and B64-I/B64-II for the detection of leptospires in serum and urine and found that both cuture and PCR were more often positive for sera than urine samples. They suggested that PCR could provide definitive diagnosis in the acute stage of the illness, before the antibodies were detectable.

Woo *et al.* (1997) developed a technique for the identification of pathogenic *Leptospira* Genospecies by continuous monitoring of fluorogenic hybridization probes during a rapid cycle PCR. The PCR method was rapid requiring 18 minutes, simple and flexible as DNA templates prepared by four different methods including simple boiling method could be used without adverse effects.

Romero *et al.* (1998) compared PCR, MAT and an enzyme linked immunosorbent assay for immunoglobulin M (ELISA – IgM) for detection of leptospires in samples of cerebrospinal fluid from patients with aseptic meningitis and PCR was found to be the most sensitive test.

A nested PCR was used for identification of *Leptospira* species in clinical cases of uveitis by Chu *et al.* (1998) with 80 per cent success.

In a study to compare PCR with culture isolation and serology to detect leptospires in bovine semen it was found that PCR was a method of great potential for the detection of leptospires at artificial insemination centers (Heinemann *et al.*, 1999).

Wagenaar *et al.* (2000) compared various PCR assays for detection of *L. borgpetersenii* serovar *hardjo* in bovine urine and found that the PCR assay described by Gravekamp *et al.* had the best combination of specificity (100 per cent)

and sensitivity (91 per cent). They opined that the sensitivity of DNA based diagnostic assays in urine samples varies from animal to animal and presumably there were substances in urine that inhibited recovery of DNA, inhibited the PCR reaction or inhibited binding of the DNA to a solid support.

Faber *et al.* (2000) used a nested PCR for the detection of leptospires in aqueous humor of horses and they proposed that PCR is a more reliable tool for detecting the presence of leptospires in equine recurrent uveitis.

Venkatesha and Ramadas (2001) compared molecular techniques, slot blot hybridization and PCR with routine diagnostic methods for diagnosis of leptospirosis. Both the methods were rapid and sensitive over conventional methods of isolation and culturing.

Senthilkumar *et al.* (2001) evaluated PCR for rapid diagnosis of leptospires in animals and man and study indicated that PCR assay of clinical samples was potentially useful, quick and specific diagnostic method for confirming active infection with leptospires.

Truccolo *et al.* (2001) developed a PCR assay for rapid detection and quantification of leptospires in biological samples. An ELISA microtiter plate hybridization method was developed for the quantitative determination of *Leptospira* species after PCR.

Richtzenhain *et al.* (2002) developed a multiplex PCR for the detection of *Brucella* species and *Leptospira* species DNA from aborted bovine foetus, aiming at improvement in direct diagnosis. For the detection of *Leptospira* the primers described by Merien *et al.* (1992) were used.

According to Harkin *et al.* (2003a) the PCR testing of urine had 100 per cent sensitivity and 88.3 per cent specificity. They used the primers designated as L 737 and L 1218 and all the PCR negative cases had a diagnosis other than leptospirosis or were healthy.

Harkin *et al.* (2003b) compared PCR, bacteriological culture and serologic testing in assessment of prevalence of urinary shedding of leptospirosis in dogs and recommended PCR assay to determine whether a dog is actively shedding leptospires in urine.

In a study to compare the efficiency of PCR with DFM and culture in the diagnosis of leptospirosis, PCR technique was found to be more sensitive, specific and rapid over conventional methods as it detected 41.6 per cent compared to 25.6 per cent by DFM and 2.4 percent by culture, of the samples tested (Elaiyaraja, 2003).

Brown *et al.* (2003) tried cultures, direct immune fluorescence and PCR to detect *Leptospira* organisms in human postmortem samples and among them PCR was found to be the most sensitive technique and could detect one to ten leptospires in one milliliter of sample.

DNA of 45 isolates were amplified by multiplex PCR using two sets of previously described primers G1, G2 and B64-I, B64-II and the study has indicated the utility of multiplex PCR in the rapid detection of leptospires in clinical samples (Sugathan and Varghese, 2005).

2.5.4.2 DNA Hybridization

Millar *et al.* (1987) suggested that DNA hybridization was a very sensitive technique for diagnosis of leptospirosis, allowing a result to be obtained within two days and was free from interference by urine or serum.

Bolin *et al.* (1989) compared nucleic acid hybridization, bacteriologic culture and fluorescent antibody test for the detection of *L. interrogans* serovar *hardjo* type *hardjobovis* in bovine urine and nucleic acid hybridization with radio labeled single stranded RNA probe derived from a cloned repetitive sequence was found to be the most sensitive technique. They proposed that non-radioactive label and detection systems would enhance the practicability of the use of nucleic acid probes in diagnostic laboratories.

Merien *et al.* (1992) has proposed a combined PCR-hybridization test for qualitative detection of a specific target DNA sequence of *Leptospira*. The PCR product was analysed by DNA-DNA hybridization using a 289 base pair (bp) fragment internal to the amplified product.

Merien *et al.* (1993) performed dot blot hybridization with a 289 bp digoxigenin labeled probe internal to the amplified product to confirm the results of PCR for diagnosis of leptospirosis in a patient with unilateral uveitis.

Woo *et al.* (1997) have developed an extremely rapid and specific assay for the identification of pathogenic *Leptospira* by using two species-specific fluorogenic hybridization probes, placed internal to the amplification primers for continuous monitoring of PCR products.

To improve sensitivity and specificity of PCR assays, amplified products were blotted and hybridized with probes specific for leptospiral sequences (Wagenaar *et al.*, 2000).

Venkatesha and Ramadas (2001) used a recombinant probe derived from genomic library of *australis* serovar for slot blot hybridization and could detect leptospiral DNA in 76.83 per cent of the samples tested.

Truccolo *et al.* (2001) developed a rapid and simple microtiter plate hybridization assay for the quantification of pathogenic leptospires after PCR amplification with biotinylated primers. The capture probe was covalently linked on to aminated wells of microtiter plates, using carbodiimide as a coupling agent.

Branger *et al.* (2005) used southern hybridization of genomic DNA with the integral *hap* 1 gene as a probe to show that this gene was only present in pathogenic *Leptospira* strains and confirmed the relevance of using the gene for diagnostic testing.

2.7 DIFFERENTIATION OF LEPTOSPIRES

2.7.1 Based on biological properties

A medium containing 225 µg/ml of 8-azaguanine could differentiate leptospires based on growth response. It had no effect on saprophytic leptospires (Johnson and Rogers, 1964).

Johnson and Harris (1967) tried differentiation of pathogenic and saprophytic leptospires based on the difference in biological characteristics like the minimal

growth temperature. The minimal temperature of pathogenic leptospires was between 13 to 15°C whereas that of saprophytes was between 5 to 10°C. In a medium containing 10 per cent rabbit serum all saprophytic leptospires had grown at 13°C while none of the pathogenic leptospires grew during the 30 days of incubation period.

Conventionally the differentiation between pathogenic and saprophytic leptospires is carried out by tests like pathogenicity to animals, growth response to 8-azaguanine and low temperature (13°C), conversion to spherical forms by 1M sodium chloride, lipase activity etc. which are time consuming and laborious (Noubade *et al.*, 2002).

2.7.2 Molecular methods

2.7.2.1 Polymerase Chain Reaction (PCR)

Woodward *et al.* (1991) developed a PCR test specific for *Leptospira hardjo* genotype *bovis,* based on the sequence of a repetitive element.

Gravekamp *et al.* (1993) used two sets of primers G1/G2 and B64I/B64II for the detection of seven species of pathogenic leptospires. B64I/B64II primers amplified the genome of the serovars belonging to *L. kirschneri* species.

Corney *et al.* (1993) compared random amplified polymorphic DNA (RAPD) finger printing, cross agglutination absorption and REA in identification of *Leptospira* isolates from cattle and found that RAPD finger printing was rapid and more reliable method for typing leptospires.

Perloat *et al.* (1994) have tried characterization of *Leptospira* isolates from serovar *hardjo* by ribotyping, arbitrarly primed – PCR. (AP-PCR) and mapped restriction site polymorphisms (MRSPs). AP-PCR and MRSPs in ribosomal genes proved to be quick and reliable methods for typing *Leptospira* strains and for studying intraspecific population structures.

De Caballero *et al.* (1994) found that primers proposed for the diagnosis of the pathogenic *Leptospira* species could produce complex serovar-specific patterns under low stringency PCR conditions and offered an approach to the standard identification of *Leptospira* serovar in clinical laboratories.

Brown and Levette (1997) tried three PCR based techniques *viz.*, PCR-Restriction enzyme analysis, arbitrarily primed PCR (AP-PCR) and low stringency PCR (LS-PCR) for characterization of reference strains as well as isolates of *Leptospira* and proposed AP-PCR and LS-PCR as useful methods since they are easy to perform and interpret.

Parma *et al.* (1997) used double set of primers G1/G2 and B64I/B64II for the differentiation of pathogenic from non-pathogenic leptospires in culture.

Postic *et al.* (2000) recommended that identification of new serovars should not be limited by serological procedures alone but should include at least one molecular and analytical finger printing method such as sequencing, ribotyping, Pulsed-field gel electrophoresis (PFGE), Mapped Restriction Site Polymorphism (MRSP) and AP-PCR.

Heinemann *et al.* (2000) determined the detection threshold of PCR in semen samples of bulls and evaluated the possibility of differentiation among serovars using

19 restriction endonucleases. They recommended PCR as a method of great potential for detection of leptospires in bovine A.I. centers.

The AP-PCR patterns of *Leptospira* isolates using four primers provided prominent species-specific products and these results were supported by MRSP data (Collares-Pereira *et al.*, 2000).

Barocchi *et al.* (2001) developed *Rep* 1 Primer; a single 21-mer oligo-nucleotide based on a sequence within the *Rep* 1 element and could differentiate different species and serogroups but not serovars of *Leptospira*.

Noubade *et al.* (2002) conducted PCR on pathogenic and non pathogenic leptospires from various sources like blood, urine, kidney and milk of infected animals and environmental specimens like soil and water. They used two sets of primers A/B and G1/G2 for differentiation of saprophytic and pathogenic leptospires and opined that PCR was simple, specific and rapid method for detection as well as differentiation of leptospires.

Shukla *et al.* (2002) reported the use of differentiating primers of 23S rRNA gene that amplified 500 bp DNA fragment in pathogenic serovars and 770 bp fragment in saprophytic serovars.

Ramadass *et al.* (2002) opined that AP-PCR techniques provided great potential for simple and rapid identification of leptospires at serovar level, which could be useful in molecular epidemiological studies of leptospires.

Machangu' *et al.* (2004) tested pathogenic status of two isolates of *Leptospira* by PCR with primer sets which differentiated between saprophytic and

pathogenic leptospires including G1/G2 and B64-I/B64-II. Molecular fingerprinting by PCR with IS*1533* and IS*1500* derived primers were used for characterization of the two isolates of *Leptospira.*

Branger *et al.* (2005) selected PCR primers based on *hap* 1 gene and found that specific amplification was obtained only for pathogenic strains. They opined that a PCR based on this gene would be a very useful tool for the rapid, sensitive and specific identification of pathogenic leptospires in samples for diagnosis and epidemiological survey.

2.7.2.2 Restriction Enzyme Analysis (REA)

Robinson *et al.* (1982) suggested that Bacterial Restriction Endonuclease DNA Analysis (BRENDA) would overcome many of the problems associated with serological methods of identifying serovars and allowed more precise definition of epidemiological relationships between strains and their hosts.

Marshall *et al.* (1984) described BRENDA as an ideal technique for investigating bacterial variants whose selection had come about as a result of alteration to their growth medium.

Thiermann *et al.* (1985) opined that REA was sensitive enough to differentiate among different leptospiral serovars. Their study indicated the existence of serovar *kennewicki*, which was eliminated from official serovar list because it was found to be indistinguishable from serovar *pomona* by serological methods.

All the leptospiral isolates were typed as belonging to the Australis serogroup and the strains isolated from kidney of a sow and boar have been specifically identified as serovar *bratislava* by cross agglutination absorption and REA of chromosomal DNA (Ellis *et al.*, 1985a).

Hermann *et al.* (1991) found that intact genomic DNA from *L. interrogans* strains when digested with *Not* I enzyme and submitted to PFGE could provide different restriction patterns specific to each serovar providing a reliable identification for each reference strain.

Ellis *et al.* (1991) had used REA as a taxonomic tool in the study of pig isolates belonging to Australis serogroup of *L. interrogans*.

Zuerner *et al.* (1993) demonstrated genetic heterogeneity among *hardjobovis* isolates by REA, DNA hybridization and PFGE.

Savio *et al.* (1994) differentiated *L. interrogans* serovars and *L. borg-petersenii* serovars by restriction patterns of PCR products.

Restriction digestion performed on PCR amplified rrs gene enabled the identification of pathogenic isolates at the species level (Collares-Pereira *et al.*, 2000).

Shukla *et al.* (2002) could differentiate pathogenic and saprophytic serovars of *Leptospira* by REA of 482 bp genus specific sequence of 23S rRNA gene.

2.7.2.3 DNA Hybridization

Based on DNA hybridization Hapaal *et al.* (1969) identified six DNA related groups from 15 serovars of *Leptospira* indicating a great deal of heterogeneity.

Yasuda *et al.* (1987) demonstrated by genomic DNA - DNA hybridization that the different strains of *L. interrogans* constituted not one but at least six distinct species.

According to Pucciarini *et al.* (1992) the number of repetitive elements varied in different serovars of pathogenic leptospires. They selected two probes containing repetitive sequence and were used to divide *Leptospira* species in to groups, which share genetic relatedness.

2.7.3 Serological methods

One isolate of *Leptospira* from a field rat was identified up to serovar level by Monoclonal antibody technique (Natarajaseenivasan and Ratnam, 1997).

Microscopic agglutination test remains very useful tool for epidemiological studies, identification of strains and the assessment of the probable infecting serogroup of *Leptospira* (Brandao *et al.*, 1998).

According to Levette and Whittington (1998) the definitive serological test for leptospirosis is MAT, though it requires significant expertise, the maintenance of a panel of live antigens and often requires paired serum samples, thus delaying diagnosis. Microscopic agglutination test remains useful however because of the

epidemiological information that it can provide about the leptospiral serogroup present in a population.

The serogroup and serovar of an isolate from a human patient in Andaman Islands was identified by MAT and cross agglutination absorption test (CAAT) respectively. The result of CAAT was further confirmed by monoclonal antibody technique (Sehgal *et al.*, 2000).

The use of monoclonal antibodies (MAbs) was confirmed to be generally a powerful method to identify *Leptospira* organisms at the serovar level (Collares-Pereira *et al.*, 2000).

Machang'u *et al.* (2004) classified the isolates obtained from African giant pouched rats into serovar *kenya* of serogroup *ballum* based on the results of CAAT.

In a study conducted in Thrissur, Kerala MAT was found to be the most appropriate test for epidemiological surveillance as it could identify the infecting leptospires up to the serogroup level (Soman, 2004).

Microscopic agglutination test was carried out for primary serogrouping of leptospiral isolates from human patients in MOSC medical college, Kolenchery, Kerala and were classified into 10 serogroup (Sugathan and Varghese, 2005).

2.7.4 Other techniques

Pulsed field agarose gel electrophoresis of large DNA fragments produced by rare cutting restriction enzymes offers the advantages of a simple interpretation combined with a rapid result. The pattern of each of the three serovars analysed by

using PFGE after restriction with three different endonucleases was different (Baril and Girons, 1990).

Pulsed-field agarose gel electrophoresis (PFGE) appeared to be a useful tool for the serovar identification of leptospires belonging to the serogroup Pomona and for shedding light on the problem of their classification (Ciceroni *et al.*, 2002).

Postic *et al.* (2000) conducted a study focused on different serovars of *L. meyeri* species, the classification of which has been controversial and DNA sequencing revealed large collection of heterogeneities. *Not* I restriction profiles of these serovars obtained by PFGE had confirmed the sequencing data and they recommended PFGE for characterization and differentiation of serovars of *Leptospira.*

Materials and Methods

3. MATERIALS AND METHODS

3.1 DETECTION AND DIFFERENTIATION OF LEPTOSPIRES IN BIO-MATERIALS BASED ON MOLECULAR METHODS

3.1.1 Materials

3.1.1.1 Reference strains of Leptospires

The following reference strains of leptospires representing ten different serogroups procured from National Leptospirosis Reference Centre, Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands, India and maintained in the laboratory were used for the study.

Serogroup	Serovar	Strain
1. Australis	*australis*	Ballico
2. Autumnalis	*rachmati*	Rachmati
3. Canicola	*canicola*	Hond Utrecht IV
4. Grippotyphosa	*grippotyphosa*	Moskva V
5. Icterohaemorrhagiae	*icterohaemorrhagiae*	RGA
6. Pomona	*pomona*	Pomona
7. Pyrogenes	*pyrogenes*	Salinem
8. Sejroe	*hardjo*	Hardjoprajitno
9. Tarassovi	*tarassovi*	Peripeletsin
10. Samerenga	*patoc*	Patoc 1

3.1.1.2 Bio-materials collected

3.1.1.2a Clinical samples

One hundred and seventeen samples including serum/plasma (92), urine (18), kidney tissues (5) and milk (2) were collected from suspected cases of canine/bovine/caprine/human leptospirosis. The majority of samples were collected from the cases suspected for leptospirosis presented at University

Veterinary Hospital, Mannuthy and Kokkalai. The kidney tissues collected were from the leptospirosis suspected animals autopsied at the department of Pathology. Few clinical samples were from veterinary hospitals under Animal Husbandry Department, forwarded to the department of Microbiology for diagnosis. The human samples were those referred from the medical hospitals in and around Thrissur, to the department of Microbiology for diagnosis of leptospirosis.

3.1.1.2b Bio-materials from rodent reservoirs

The kidney tissues (25) and urine (5) were collected from rats (21) and bandicoots (four) captured from the neighbouring farm premises.

The details of the samples collected are shown in table1.

Table 1. Details of the samples collected from different animal species and human.

Samples	Canines	Bovines	Caprines	Humans	Murines	Total
Serum/plasma	71	9	2	10	_	92
Urine	16	_	1	1	5	23
Kidney	4	1	_	_	25	30
Milk	_	2	_	_	_	2
Total	91	12	3	11	30	147

3.1.1.3 Phosphate Buffered Saline (PBS) stock solution (10 X)

Sodium Chloride	80 g
Potassium Chloride	2 g
Disodium Hydrogen phosphate	11.32 g

Potassium dihydrogen phosphate	2 g
Distilled water	1000 ml

The pH was adjusted to 7.4 using I N NaOH and sterilized by autoclaving at 121°C for 15 min under 15 lb pressure. The stock solution was diluted to 1 X before use.

3.1.1.4 Buffered anticoagulant

Sodium Oxalate	1.0 g
Phosphate buffered saline	100 ml

Buffered anticoagulant solution was sterilized by autoclaving at 121°C for 15 min under 15 lb pressure.

3.1.1.5 Reagents for PCR

3.1.1.5a Primers

i) Genus specific PCR

The genus specific primers A and B designed by Merien *et al.* (1992) were obtained from Alpha DNA, Canada.

A: 5'-GGC GGC TCT TAA ACA TG-3'

B: 5'-TTC CCC CCA TTG AGC AAG ATT-3'

ii) Multiplex PCR

Pathogenic species-specific primers designed by Gravekamp *et al.* (1993) were used for multiplex PCR and the sequence of these primers are given below.

G1: 5'- CTG AAT CGC TGT ATA AAA GT-3'

G2: 5'- GGA AAA CAA ATG GTC GGA AG-3'

B64-I: 5'-CTG AAT TCT CAT CTC AAC TC-3'

B64-II: 5'-GCA GAA ATC AGA TGG ACG AT-3'

The primers were custom synthesized by M/s Bangalore Genei (India).

iii) Nested PCR

Two sets of primers designed by Faber *et al.* (2000) were used for nested PCR.

Outer primer set.

5'- AGG GAA AAA TAA GCA GCG ATG TG-3'

5' -ATT CCA CTC CAT GTC AAG CC-3'

Inner primer set.

5'-GAA AAC TGC GGG CTC AAA C-3'

5'-GCT CCA CCG CTT GTG C-3'

The primers were custom synthesized by M/s Bangalore Genei (India).

iv) Arbitrarily Primed PCR (AP-PCR)

The primer designed by Brown and Levette (1997) was used.

PB 1: 5'GCG CTG GCT CAG-3'

The primer was custom synthesized by M/s Bangalore Genei (India).

v) Low stringency PCR (LS-PCR)

The primers G1/G2 designed by Gravekamp *et al.* (1993) were used for LS-PCR.

G1: 5' CTG AAT CGC TGT ATA AAA GT -3'

G2: 5' GGA AAA CAA ATG GTC GGA AG-3'

The primers were custom synthesized by M/s Bangalore Genei (India).

3.1.1.5b PCR Reaction Buffer (10 X) (Bangalore Genei, india).

This includes 500 mM Potassium chloride, 100 mM Tris- hydrochloride pH 9.0 and 15 mM Magnesium chloride

3.1.1.5c Magnesium Chloride (Bangalore Genei, india).

Magnesium Chloride with strength of 25mM

3.1.1.5d *Taq* DNA polymerase (Bangalore Genei, india).

The Taq DNA polymerase enzyme with a concentration of three units per microliter.

3.1.1.5e Deoxy ribonucleotide Triphosphates (Bangalore Genei, india).

Deoxy Nucleotide Triphosphates (dNTP mix) 10 mM (2.5 mM of each dGTP/dCTP/dATP/dTTP)

3.1.1.6 Materials for submarine agarose gel electrophoresis

3.1.1.6a Agarose

Agarose low EEO (Genei)

3.1.1.6b Tris Acetate EDTA buffer

Stock solution (50 X)(Genei)

1 X solution prepared in distilled water was used for electrophoresis.

3.1.1.6c EDTA stock solution (0.5 M) pH 8.0

EDTA disodium salt	186.1 g
Distilled water	800 ml

The pH was adjusted to 8.0 with 1 N NaOH. Distilled water was then added to make up the volume to one liter. The solution was sterilized by autoclaving at 121^0C for 15 min under 15 lb pressure.

3.1.1.6d Tris Borate EDTA Buffer (TBE) pH 8.2

a) Stock solution (10 X)

Tris base	108.0 g
Boric acid	5.0 g
EDTA (0.5 M, pH 8.0)	40 ml
Triple distilled water to make	1 liter

b) Tris Borate EDTA Buffer (TBE) 1 X

TBE stock solution	10 ml
Triple distilled water to make	100 ml

3.1.1.6e Ethidium Bromide stock solution

Ethidium bromide	10 mg
Triple distilled water	1 ml

The solution was mixed well and stored in amber coloured bottles at 4^0C.

3.1.1.6f Gel loading buffer (6 X)

Bromophenol blue	0.25 g
Xylene Xylol	0.25 g
Sucrose	40.00 g
Distilled water	100 ml

The ingredients were dissolved in distilled water, filtered and stored at 4°C.

3.1.1.6g DNA Molecular size Marker

a) pUC 18/*Sau* 3A 1-pUC 18/ *Taq* I Digest with fragments 1444,943,754,585,458,341,258,153,105,78 and 75 base pairs

b) 100 bp DNA Ladder consisting of 10 double-stranded DNA segments of 100,200,300,400,500,600,700,800,900 and 1000 base pairs

3.1.1.7 Reagents for Restriction enzyme Analysis (REA)

3.1.1.7a Restriction enzymes (RE)

Concentration and Recognition Sequence of RE were as follows:

Dde I	10 u/µl	5'...CTNAG...3'
		3'...GANTC...5' (New England Biolabs).
Hinf I	10 u/µl	5'...GANTC...5'
		3'...CTNAG...3'(IDT, USA)
Mnl I	10 u/µl	5'...CCTC (N 7)...3'
		3'...GGAG (N6)...5'(New England Biolabs).

3.1.1.7 b RE buffer (10 X)

It was supplied by the manufacturer along with RE

3.1.1.8 Materials for Polyacrylamide Gel Electrophoresis (PAGE)

3.1.1.8a Acrylamide: Bisacrylamide stock (30: 8)

Acrylamide	30.0 g
Bisacrylamide	0.8 g
Distilled water to make	100 ml

Filtered through Whatman No.1 filter paper and stored at 4°C.

3.1.2 METHODS

3.1.2.1 Standardization of molecular techniques employing the reference strains

3.1.2.1.1 Maintenance of leptospires

Stock cultures of reference strains of leptospires were maintained in Fletcher's semisolid media (3.2.5a) in BOD incubator at 28°C. The cultures were sub cultured routinely at four weeks interval. The purity of cultures was assessed by DFM and culturing on blood agar.

3.1.2.1.2 Polymerase chain reactions

3.1.2.1.2a Preparation of DNA template from cultures

A drop of culture was added to an Eppendorf tube containing 1ml of PBS (3.1.1.3). The leptospires were pelleted by centrifugation at 13,000 x g for 15 min at 4°C, washed the pellet twice with sterile PBS and then resuspended in 25 µl sterile triple glass distilled water. The samples in Eppendorf tubes were placed on a boiling water bath for 10 min and immediately kept on ice for 30 min. These were then centrifuged at 5000 x g for 10 min and the supernatant was used for PCR.

3.1.2.1.2b Reconstitution of Primers

Primers obtained in lyophilised form were reconstituted in sterile glass distilled water to a concentration of 200 pM/µl. The tubes were kept at room temperature with occasional shaking for one hour. They were then spun to pellet down the insoluble particles, and the stock solution was distributed into 10 µl aliquots and stored at - 40°C. At the time of use, the aliquots were thawed and further diluted ten fold to obtain a concentration of 20 pM/µl before using for PCR.

3.1.2.1.2c Genus specific PCR

The template DNA from reference strains was subjected to genus specific PCR using the primers A and B. Each reaction was done in a total volume of 25 µl. For five such reactions, a mastermix was prepared to contain the following reagents

Reagents	Quantity
Triple distilled water	68 µl
Magnesium Chloride (25 mM)	2.5 µl
PCR buffer (10 X)	12.5 µl
Primer A (20 pM/ µl)	5 µl
Primer B (20 pM/ µl)	5 µl
dNTPs	5 µl
Taq polymerase (3units/ µl)	2 µl

Twenty microliter of mastermix was distributed to five tubes. To twenty microliter mastermix added five microliter template. Negative control without template to monitor contamination was also set. The PCR was carried out in an automated thermal cycler (Eppendorf Mastercycler, Germany).

The programme of amplification was as follows

	First cycle	**28 cycles**	**Final cycle**
Denaturation	94°C for 3 min	94°C for 1 min	94°C for 1 min
Annealing	63°C for 1.5 min	63°C for 1.5 min	63°C for 1.5 min
Extension	72°C for 2 min	72°C for 2 min	72°C for 12 min

The amplified product was detected by Submarine Agarose gel electrophoresis (SAE) on one per cent agarose gel in TAE buffer (I X).

3.1.2.1.2d Multiplex PCR

The template DNA from reference strains was subjected to multiplex PCR using the primers G1/G2 and B64-I/B64-II. Each reaction was done in a total volume of 25 µl. For five such reactions a mastermix was prepared to contain the following reagents

Reagents	Quantity
Triple distilled water	58 µl
Magnesium Chloride (25 mM)	2.5 µl
PCR buffer (10 X)	12.5 µl
Primer G1 (20 pM/ µl)	5 µl
Primer G2 (20 pM/ µl)	5 µl
Primer B64-I (20 pM/ µl)	5 µl
Primer B64-II (20 pM/ µl)	5 µl
dNTPs	5 µl
Taq polymerase (3units/ µl)	2 µl

Five microliter of template DNA was added to 20 µl of mastermix in each tube. Negative control without template to monitor contamination was also set. Polymerase chain reaction was carried out in an automated thermal cycler (Eppendorf Mastercycler, Germany).

The programme of amplification was as follows

Denaturation	94 °C for 1.5min
Annealing	55 °C for 1min
Extension	72 °C for 2 min
Number of cycles	32

The amplified product was detected by SAE on one per cent agarose gels in TAE buffer (I X).

3.1.2.1.2e Nested PCR

The template DNA from reference strains were subjected to PCR using the outer set of primers (3.1.1.5a iii). Each reaction was done in a total volume of 25 µl. For five such reactions a mastermix was prepared to contain the following reagents.

Reagents	Quantity
Triple distilled water	68 µl
Magnesium Chloride (25 mM)	2.5 µl
PCR buffer (10 X)	12.5 µl
Forward primer (outer) (20 pM/ µl)	5 µl
Reverse primer (outer) (20 pM/ µl)	5 µl
dNTPs	5 µl
Taq polymerase (3units/ µl)	2 µl

To 20 µl of mastermix added five microliter of template. The PCR was carried out in an automated thermal cycler (Eppendorf Mastercycler, Germany).

The programme of amplification was as follows.

Denaturation	94 °C for 4 min
Denaturation	94 °C for 1 min
Annealing	55 °C for 1 min
Extension	72 °C for 1 min.
Number of cycles	30

For the nested amplification five microliter of 1/80 or 1/100 dilution of the first PCR product was used as the template. The PCR was carried out using the inner set of primers and the preparation of mastermix and the amplification programme were same as that of the first PCR.

The amplified product was detected by SAE on 1.5 per cent agarose gels in TBE buffer.

3.1.2.1.2f Arbitrarily Primed PCR

PB1 was the primer used for the AP-PCR. Each reaction was done in a total volume of 25 µl. For five such reactions a mastermix was prepared to contain the following reagents

Reagents	Quantity
Triple distilled water	68 µl
Magnesium Chloride (25 mM)	2.5 µl
PCR buffer (10 X)	12.5 µl
Primer PBI (20 pM/ µl)	10 µl
dNTPs	5 µl
Taq polymerase (3units/ µl)	2 µl

To 20 µl mastermix added five microliter template. The PCR was carried out in an automated thermal cycler (Eppendorf Mastercycler, Germany).

The programme of amplification was as follows

	First cycle	**4cycles**	**24 cycles**	**Final cycle**
Denaturation	94 °C for 7 min	94 °C for 1 min	94 °C for 1 min	94 °C for 1 min
Annealing	40 °C for 1 min	40 °C for 1 min	55 °C for 1 min	55 °C for 1 min
Extension	72 °C for 1 min	72 °C for 1 min	72 °C for 1 min	72°C for 7 min

The amplified product was detected by SAE on 1.5 per cent agarose gels in TBE buffer (1 X).

3.1.2.1.2g Low stringency PCR

The template DNA from reference strains were subjected to low stringency PCR using the primers G1/G2. Each reaction was done in a total volume of 25 µl. For five such reactions a mastermix was prepared to contain the following reagents.

Reagents	Quantity
Triple distilled water	68 µl
Magnesium Chloride (25 mM)	2.5 µl
PCR buffer (10 X)	12.5 µl
Primer G1 (20 pM/ µl)	5 µl
Primer G2 (20 pM/ µl)	5 µl
dNTPs	5 µl
Taq polymerase (3units/ µl)	2 µl

To 20 µl mastermix added five microliter of template DNA. The PCR was carried out in an automated thermal cycler (Eppendorf Mastercycler, Germany).

The programme of amplification was as follows

Initial denaturation at 94^{0}C for 5 min	**5cycles**	**24 cycles**	**Final cycle**
Denaturation	94°C for 1 min	94°C for 1 min	94°C for 1 min
Annealing	35°C for 1 min	40°C for 1 min	40°C for 1 min
Extension	72°C for 1 min	72°C for 1 min	72°C for 7 min

The amplified product was detected by SAE on 1.5 per cent agarose gels in TBE buffer.

3.1.2.1.2h Submarine Agarose gel Electrophoresis (SAE)

Submarine Agarose gel electrophoresis was performed in one per cent agarose gel in 1 X TAE buffer for the detection of amplified products of genus specific and multiplex PCR. Two hundred and fifty milligram of agarose was dissolved in 25 ml of 1 X TAE buffer by heating, to which ethidium bromide (3.1.1.6e) was added to a final concentration of 0.5 µg/ml after cooling to 50°C.

The two edges of a clean, dry, gel platform were sealed with adhesive tape and the comb was kept at proper position before pouring agarose. Once the gel was set, comb and adhesive tape were removed and the gel was then placed in buffer tank. Ten microliter of respective PCR product mixed with one microliter of 6 X gel loading buffer was loaded into the wells. Molecular size marker was

also loaded in the last well. The electrophoresis was carried out at 70 V until the bromophenol blue dye migrated more than two-third of the length of the gel.

For the detection of amplified product of nested PCR, AP-PCR and LS-PCR, electrophoresis was carried out in 1.5 per cent agarose gels. The gel was prepared by dissolving 375 mg in 25 ml TBE buffer (1 X). The rest of the procedure was as described above for one per cent agarose gels. The electrophoresis was carried out at 50 V till the Bromophenol blue dye reached more than two-third of the length of the gel.

3.1.2.1.2i Recording of results.

Gel was visualized under UV transilluminator (Hoefer, USA) and the results were documented on gel documentation system (Bio-Rad Laboratories, USA).

3.1.2.1.3 Restriction Enzyme Analysis

The PCR products of genus specific PCR and multiplex PCR were digested with restriction enzymes according to manufacturers instructions. The restriction endonuclease *Dde* I was used for the digestion of the PCR products of the primers A/B and G1/G2 whereas *Mnl* I and *Hinf* I were the enzymes used for digestion of the PCR products of the primers B64-I/B64-II. The restriction digestion of the PCR product was carried out in a total volume of 20 µl in sterile microfuge tubes.

PCR product	10 µl
RE buffer (10 X)	2 µl
RE	1 µl
Distilled Water to make	20 µl

The digestion mixture was incubated at 37°C for two hours and the reaction was stopped by heating at 65°C for 20 min.

3.1.2.1.4 Polyacrylamide Gel Electrophoresis (PAGE)

The RE digested product was separated by PAGE.

3.1.2.1.4a Preparation of eight per cent Acrylamide gel.

Acrylamide : Bisacrylamide 30:8	3.72 ml
TBE (5X)	2.8 ml
10 per cent Ammonium per sulphate	50 µl
TEMED	3 µl

Eight per cent Acrylamide was prepared by mixing the above reagents in 7.2 ml of distilled water and poured between two glass plates. Then a comb was inserted in the top and the solution was allowed to polymerise. The comb was removed after complete polymerisation. The glass plates containing Poly Acrylamide gel was transferred to the vertical slab electrophoresis system (Hoefer,USA). The wells were washed with TBE buffer (1 X) to remove unpolymerised particles and then the wells were half filled with TBE buffer (1 X). Five microliter of the digested PCR product was mixed with one microliter of gel loading buffer and carefully layered under the buffer column in the wells. Undigested PCR product and DNA molecular size marker were also loaded in separate wells. The upper and lower buffer tanks were filled with TBE (1 X) and electrophoresis was carried out at 70 V till the Bromophenol blue dye reached the bottom of the gel.

When the electrophoresis was over the slab containing the gel was dismantled, the gel was separated out and stained by placing it in 25 ml of TBE buffer (1 X) containing ethidium bromide to a final concentration of 0.5 µg/ml.

3.1.2.1.4b Recording of result.

Gel was visualized under UV transilluminator (Hoefer, USA) and the results were documented on gel documentation system (Bio-Rad Laboratories, USA).

3.1.2.2 Detection and differentiation of leptospires in bio-materials

3.1.2.2.1 Dark Field Microscopy (DFM)

3.1.2.2.1a Blood

Two milliliters of blood collected in buffered anticoagulant (3.1.1.4) was processed for DFM. The blood was centrifuged at 1000 x g for 15 min and plasma was placed on a clear grease free glass slide and applied a cover slip (18 mm square). This wet mount preparation was examined under low (10X) and high power (45X) objective of the dark field microscope. Utmost care was taken to examine as many microscopic fields as possible and a minimum of 100 high power fields were examined.

3.1.2.2.1b Urine

Five to six milliliter of urine samples collected with equal quantity of sterile PBS (3.1.1.3) was immediately centrifuged at 3000 x g for 10 min. A drop of sediment was placed on a clear grease free glass slide, applied a cover slip (18 mm square) and was examined under low (10X) and high power (45X) objective of the dark field microscope and a minimum of 100 high power fields were examined.

3.1.2.2.2 Preparation of template DNA from samples

In case of blood, one milliliter of blood was centrifuged at 1000 x g for 15 min and removed 0.5 ml of plasma. The plasma was further centrifuged at 13000 x g for 10 min at 4°C, washed the sediment twice with sterile PBS (3.1.1.3) and then resuspended in 15 µl sterile triple glass distilled water. Finally the samples in Eppendorf tubes were placed on a boiling water bath for 10 min and

immediately kept on ice for 30 min. Before setting of PCR the samples were thawed and centrifuged at 5000 x g for 10 min and the supernatant was used as template for PCR.

For urine and serum samples the procedure was similar to the blood plasma.

For processing the kidney tissues, a tiny portion of kidney cortex was triturated with one milliliter of sterile PBS. Rest of the procedure was same as for blood.

3.1.2.2.3 Detection of leptospiral DNA

The genus specific PCR using the primers A and B (3.1.1.5a i) as described for the reference strains was carried out for the detection of leptospiral DNA employing the template prepared from clinical samples and samples from rodents.

3.1.2.2.4 Differentiation of leptospires in bio-materials

Multiplex PCR, Nested PCR, AP-PCR, LS-PCR and PCR-REA standardised using the reference strains were employed for differentiation of leptospires in bio-materials.

3.2 ISOLATION OF *Leptospira*

3.2.1 Bio-materials collected

Isolation of *Leptospira* was attempted from five urine samples and twenty five kidney tissues of rodents captured from the neighbouring farm premises. In addition to this, blood samples from two dogs positive by DFM were also subjected to isolation trials.

3.2.2 Glassware

Screw capped test tubes (Borosil brand) and steristoppered test tubes (Reviera brand) were used in this study. The glassware was washed using mild soap solution, rinsed in running tap water and then in double glass-distilled water, dried and sterilized in hot air oven at 160°C for 60 min. The caps were treated similarly and sterilized by autoclaving at 121°C for 15 min under 15 lb pressure.

3.2.3 Rabbit serum

The rabbit blood was collected by cardiac puncture in to sterile syringe containing one or two drops of distilled water and was allowed to clot at room temperature. It was then transferred to 4°C, kept overnight and the serum was seperated. The serum was inactivated at 56°C for 30 min, filtered through Seitz filter and stored at – 40°C.

3.2.4 5-Fluorouracil (5-FU) solution

Hundred milligrams of 5-FU was added to five milliliter of sterile triple distilled water. To this 0.1 to 0.2 ml of 0.1N NaOH was added and heated to dissolve. The pH was adjusted to 7.4 to 7.6 using 0.1 N NaOH and the volume was made up to 10 ml with sterile triple distilled water. The solution was sterilized by filtration through a 0.2 µm membrane filter and stored at - 40°C.

3.2.5 Medium used for isolation

3.2.5a Fletcher's Semisolid Medium

The medium was prepared by autoclaving 0.25 g of the Fletcher *Leptospira* medium base (Hi-Media) in 90 ml of triple distilled water at 121°C for 15 min under 15 lb pressure, to which pooled, heat inactivated slightly haemolysed rabbit serum was added at 10 per cent level, after cooling the

medium to 55-60°C. The medium used in this study contained the following ingredients.

Ingredients	Grams/liter
Peptone	0.3
Beef extract	0.2
Sodium Chloride	0.5
Agar	1.5

The medium was dispensed in three to five milliliter quantities in screw-capped tubes , checked for sterility by incubating at 37°C for 48 h and 28°C for 7 days and was then stored at 4°C until use.

3.2.5b Fletcher's Semisolid Medium with 5-Fluorouracil (5-FU)

One milliliter of 5-FU solution (3.2.4) was added to 100 ml of Fletcher's semisolid medium to obtain a final concentration of 100 µg/ml of medium. The medium was dispensed in three to five milliliter quantities in screw-capped tubes, checked for sterility by incubating at 37°C for 48 h and 28°C for 7 days and was then stored at 4°C until use.

3.2.6 Method of isolation

Screw capped tubes containing three to five milliliters of culture medium (with 5-FU) were inoculated with one to two drops of whole blood or a drop of the plasma with aseptic precautions (Cruickshank *et al.*, 1975). The inoculum was thoroughly mixed with medium and one milliliter of medium was transferred to a second tube and from this to a third tube.

Isolation of leptospires from rodents was attempted by culturing the cortical portion of kidney and urine from the urinary bladder. Live rodents

captured were anaesthetized with chloroform and opened with aseptic precautions. The tiny portion of kidney cortex collected by a single puncture with finely drawn sterile Pasteur pipette was inoculated to culture medium with aseptic precautions, mixed properly and then inoculated into two more tubes as described for blood. All the inoculated tubes were incubated at 37°C for 24 h, followed by incubation at 28°C for two to three months. The tubes showing visual contamination were discarded. All the tubes were examined at weekly intervals by DFM and by culturing in blood agar. The contaminated tubes were discarded and those showing growth of leptospires were subcultured on to fresh media.

The urine samples from rodents were collected into sterile syringes and were cultured as for blood as described above.

3.3 DIFFERENTIATION OF ISOLATES

Five stock isolates (four from kidneys of rodents viz BT, M64, R6 and R13 and one from blood of man) maintained in the laboratory and a sixth isolate from a bandicoot obtained during the course of study were subjected to differentiation by the methods genus specific PCR (3.1.2.1.2c), multiplex PCR (3.1.2.1.2d), nested PCR (3.1.2.1.2e), AP-PCR (3.1.2.1.2f), LS-PCR (3.1.2.1.2g) and PCR-REA(3.1.2.1.3).

4. RESULTS

4.1 DETECTION AND DIFFERENTIATION OF LEPTOSPIRES IN BIO-MATERIALS BASED ON MOLECULAR METHODS

4.1.1 Standardization of molecular techniques employing the reference strains

4.1.1.1 Maintenance of reference strains

Fletcher's media supplemented with 10 per cent rabbit serum dispensed in screw-capped test tubes as well as steristoppered test tubes could support the growth of all the ten reference strains and was used for maintenance of leptospires in the laboratory at an incubation temperature of 28°C. Seven to ten day old cultures exhibited sub surface ring, which indicated the growth of leptospires. Cultures could not be maintained for more than two months in the steristoppered test tubes due to loss of moisture by evaporation. Motility was observed up to three months in cultures maintained in screw-capped test tubes. Contamination of cultures was a problem in the humid environment prevalent in the rainy season. Sub culturing in to a medium containing 5-Flourouracil (5-FU) was found to be useful in purifying the contaminated cultures.

4.1.1.2 Genus specific PCR

The template DNA prepared from the reference strains of leptospires along with a negative control were amplified by PCR with *Leptospira* genus specific primers A and B. Analysis of the electrophoresed gel revealed the presence of a 331 bp band in all the ten reference strains used in the study. The control was negative for any amplicon. Thus the genus specific PCR could amplify DNA from all the leptospires irrespective of their pathogenic status. The representative results are presented in Fig. 1.

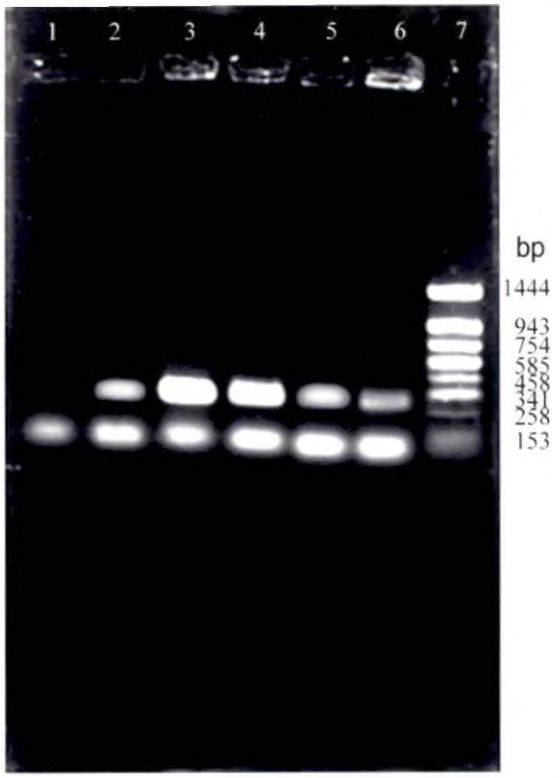

Fig. 1. Agarose gel (one per cent) electrophoresis of genus specific PCR product of representative reference strains

Lane 1: Negative control
Lane 2: *rachmati*
Lane 3: *australis*
Lane 4: *canicola*
Lane 5: *pomona*
Lane 6: *patoc*
Lane 7: pUC18/*Sau* 3A 1- pUC18/*Taq* 1 Digest

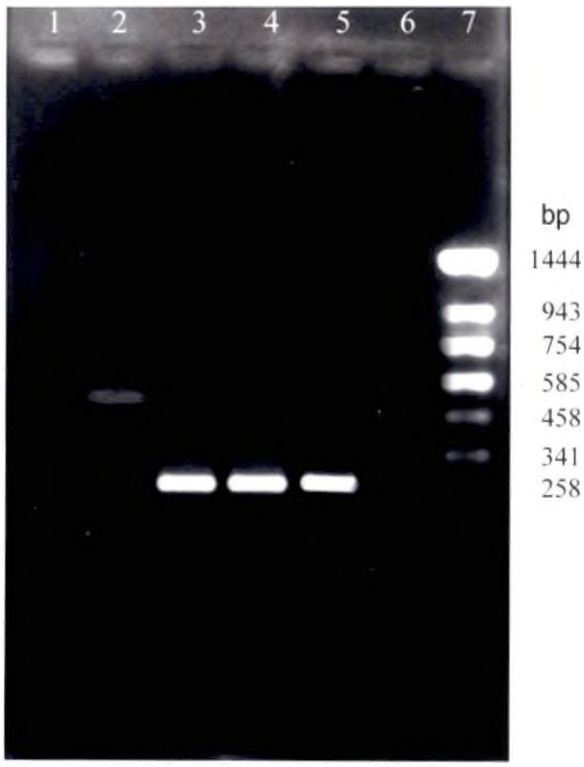

Fig. 2. Agarose gel (one per cent) electrophoresis of Multiplex PCR product of representative reference strains

Lane 1: Negative control
Lane 2: *rachmati*
Lane 3: *pyrogenes*
Lane 4: *icterohaemorrhagiae*
Lane 5: *hardjo*
Lane 6: *patoc*
Lane 7 : pUC18/*Sau* 3A 1- pUC18/*Taq* 1 Digest

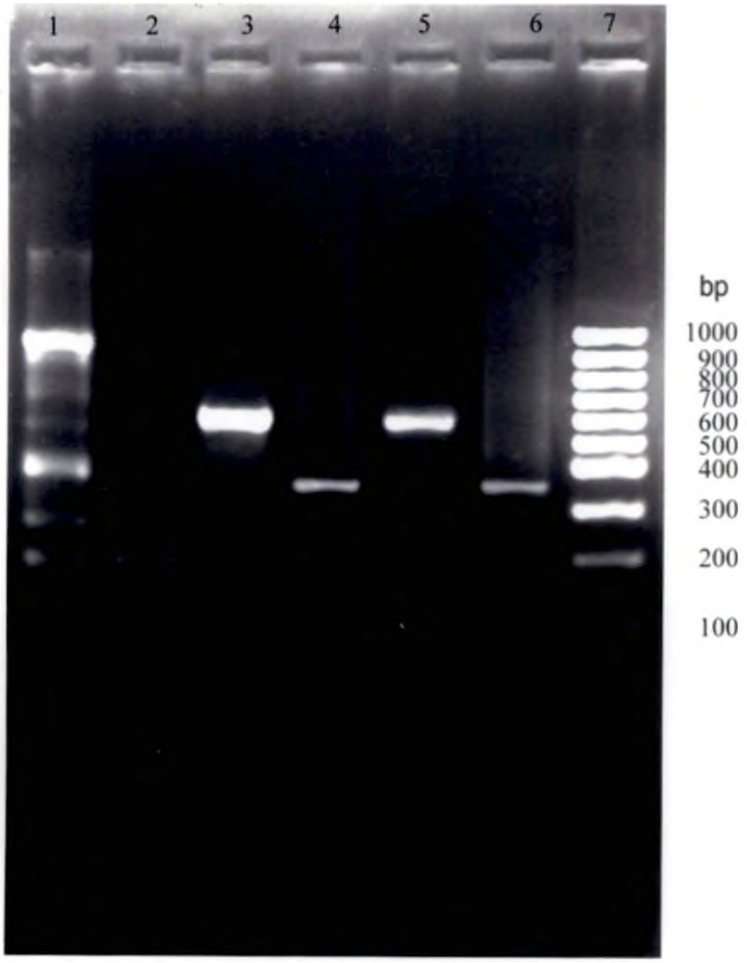

Fig. 3. Agarose gel (1.5 per cent) electrophoresis of Nested PCR products of representative reference strains

Lane 1& 2 : *rachmati*
Lane 3& 4 : *pyrogenes*
Lane 5 & 6: *hardjo*
Lane 7: 100 bp DNA ladder

4.1.1.3 Multiplex PCR

All the ten reference strains were subjected to a multiplex PCR with pathogenic species specific primers G1/G2 that can amplify DNA from six pathogenic species of *Leptospira* viz *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. inadai and L. weillii* and B64 I/B64 II that can amplify only *L. kirschneri* . Out of the ten reference serovars, the eight serovars *viz australis, canicola, grippotyphosa, hardjo, icterohaemorrhagiae, pomona, tarassovi and pyrogenes* presented 285 bp amplicon specific to the primers G1/G2, while the serovar *rachmati* presented 563 bp product specific to the primers B64 I/B64 II. The non-pathogenic serovars *patoc* did not get amplified by any of the primers. The representative results are shown in Fig. 2.

4.1.1.4 Nested PCR

An outer primer set expected to produce a 571 bp product and an internal primer set which shall produce a 370 bp product were used for nested PCR.

Nested PCR amplified DNA from all the ten reference strains including the non-pathogenic serovar *patoc*. The product of the first PCR with the outer primer set in 1/80 or 1/100 dilutions, when subjected to nested amplification with the inner primer set could present a PCR product of 370 bp as expected. All the reference serovars except *rachmati* presented PCR products of 571 bp and 370 bp. The amplification pattern of the serovar *rachmati* was different from that of others and gave multiple products for the outer primer set and a product of approximately 200 bp for the internal primers (Fig. 3).

4.1.1.5 Arbitrarily primed PCR

The primer PB1 was used for AP-PCR analysis of the reference strains. Four banding patterns were observed among the ten reference serovars (Fig. 4). The AP-PCR profiles of the serovars *australis, grippotyphosa, icterohaemorrhagiae, canicola, pomona, tarassovi* and *hardjo* were similar with amplified products of sizes ranging from 1310 to 124 bp (Profile 1). The serovars *rachmati* (bands ranging from 1437 to 124 bp - Profile 2), *pyrogenes* (bands ranging from 1011 to 124 bp - Profile 3), and *patoc* (bands from 964 to124 bp - Profile 4) had characteristic profiles.

4.1.1.6. Low stringency PCR (LS-PCR)

The primers G1/G2 were used for LS PCR. Seven different profiles were observed among the ten reference strains (Fig. 5). The specific (diagnostic) product of 285 bp was evident in all the reference strains including *patoc* and *rachmati*. The serovars *australis, pomona, hardjo* and *patoc* had same profiles (bands at 740, 410, 360, 285 and 120 bp region) (Profile 1). The banding pattern of *canicola* was close to Profile 1 but with an additional band at 140 bp regions. The profiles of *grippotyphosa* having bands at 1020, 862, 740, 490, 440, 285, 250, 150 and 120 bp regions, *rachmati* with 750,740,470, 285 and 90 bp products, *icterohaemorrhagiae* with products at 410, 360,285 and 120 bp , *tarassovi* having bands at 410, 360,285, 190 and 120 bp and *pyrogenes* with products at 410, 360, 285, 210 and 120 bp were different from each other.

4.1.1.7 Restriction enzyme analysis (REA)

The products of genus specific and multiplex PCR were subjected to REA.

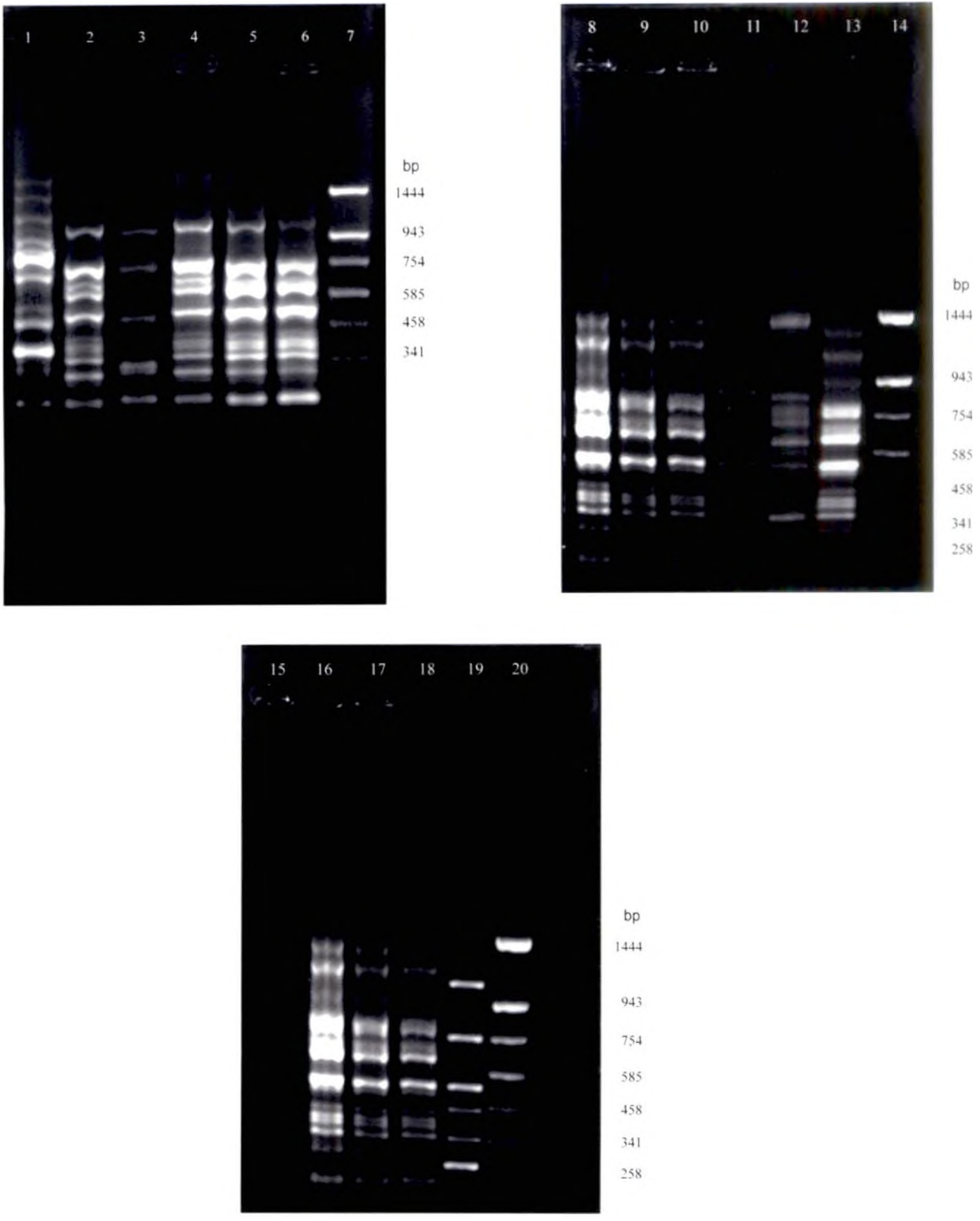

Fig. 4. Agarose gel (1.5 per cent) electrophoresis of AP-PCR products of reference strains and isolates

Lane 1: *rachmati*
Lane 2: *canicola*
Lane 3: *pyrogenes*
Lane 4: *pomona*
Lane 5: *hardjo*
Lane 6: *tarassovi*
Lane 7: pUC18/*Sau* 3A 1- pUC18/*Taq* 1 Digest
Lane 8: *australis*
Lane 9: *grippotyphosa*
Lane 10: *icterohaemorrhagiae*
Lane 11: *patoc*
Lane 12: R13
Lane 13: BT
Lane 14: pUC18/*Sau* 3A 1- pUC18/*Taq* 1 Digest
Lane 15: negative control
Lane 16: M64
Lane 17: 289
Lane 18: R6
Lane 19: B7D
Lane 20: pUC18/*Sau* 3A 1- pUC18/*Taq* 1 Digest

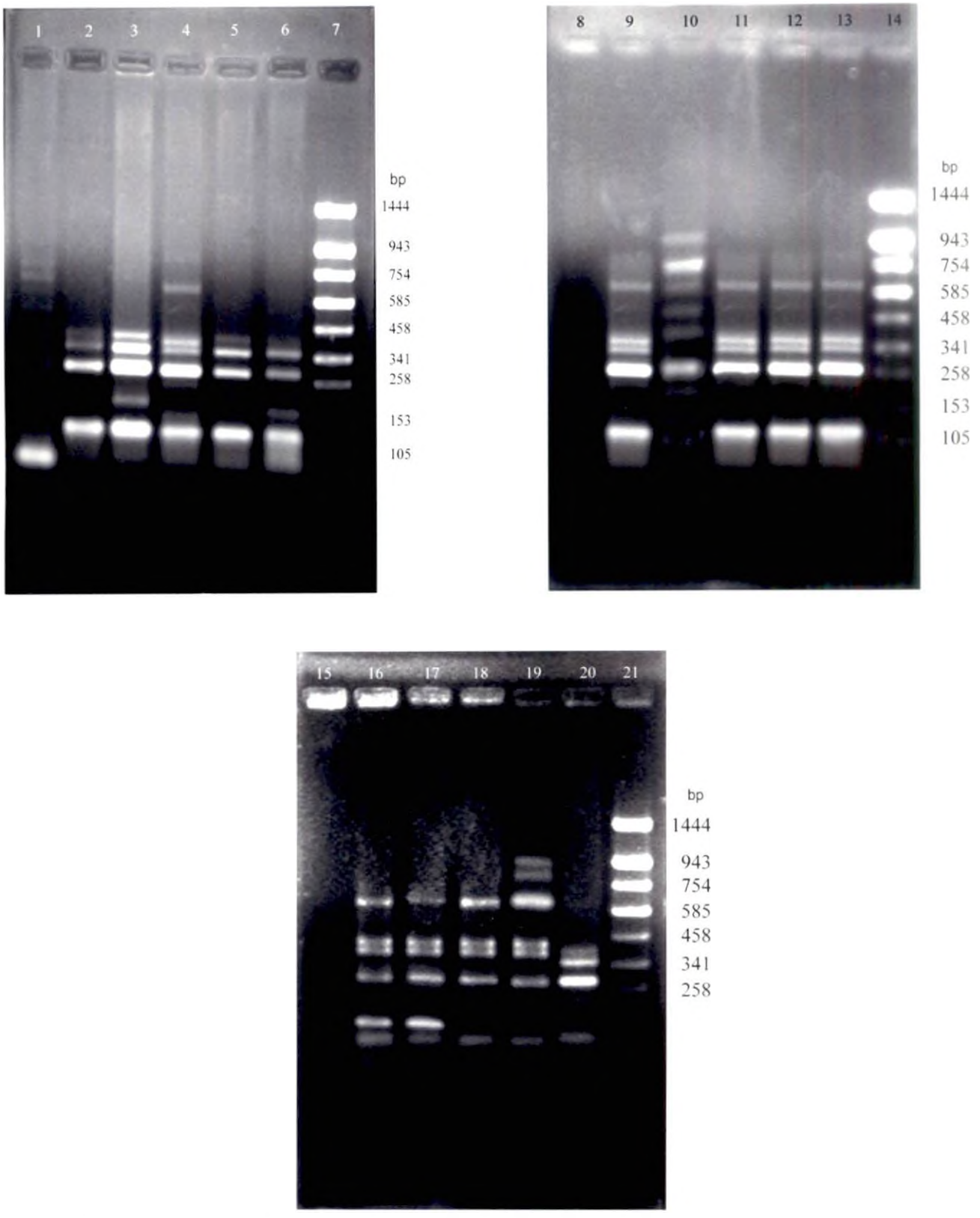

Fig.5. Agarose gel (1.5 per cent) electrophoresis of LS-PCR products of reference strains and isolates

Lane 1: *rachmati*
Lane 2: *icterohaemorrhagiae*
Lane 3: *tarassovi*
Lane 4: *canicola*
Lane 5: *pyrogenes*
Lane 6: B7D
Lane 7: pUC18/*Sau* 3A 1- pUC18/*Taq* 1 Digest
Lane 8: negative control
Lane 9: *australis*
Lane 10: *grippotyphosa*
Lane 11: *pomona*
Lane 12: *hardjo*
Lane 13: *patoc*
Lane 14: pUC18/*Sau* 3A 1- pUC18/*Taq* 1 Diges
Lane 15: negative control
Lane 16: R6
Lane 17: 289
Lane 18: M64
Lane 19: R13
Lane 20: BT
Lane 21: pUC18/*Sau* 3A 1- pUC18/*Taq* 1 Digest

The REA of genus specific PCR product of 331 bp by the enzyme *Dde* I produced three different patterns (Fig. 6). The seven serovars *viz australis, canicola, hardjo, icterohaemorrhagiae, pomona, pyrogenes* and *patoc* had a major fragment at 153 bp region. The serovars *grippotyphosa* and *tarassovi* has similar REA patterns yielding a major fragment of 210 bp. The serovar *rachmati* presented a major fragment of 245 bp on PAGE.

The REA of the 285 bp product of multiplex PCR produced three different patterns (Fig. 7). For the six serovars, *australis, canicola, icterrohaemorrhagiae, pyrogenes, hardjo* and *pomona* two bands of 120 and 85 bp (Profile 1) were observed after enzymatic digestion. The band at 85 bp region may contain two fragments of almost similar size. The serovar *grippotyphosa* had two fragments of 210 and 75 bp (Profile 2). A single fragment of 270 bp was observed for the serovar *tarassovi* (Profile 3) on digestion under the same conditions. The remaining small fragment of 15 bp might have been run out of the gel.

The 563 bp product of the multiplex PCR for the serovar *rachmati* was of low intensity. This product was digested with the enzymes *Hinf* I and *Mnl* I. Though no fragments were detected on the gel, it appeared that the 563 bp product was digested with the enzymes since the original product (563 bp) was not seen on the gel.

4.1.2 Detection and differentiation of leptospires in bio-materials

4.1.2.1 Detection of leptospiral DNA in bio-materials

4.1.2.1a Dark field microscopy (DFM)

The blood and urine samples collected from University Veterinary hospitals, Mannuthy, Kokkalai, and the blood samples collected directly from humans were subjected to DFM before processing for PCR. Out of the 68 samples examined two blood

Fig.6. Polyacrylamide gel (8 per cent) electrophoresis of *Dde* I digested genus specific PCR products showing different profiles

Lane 1: Undigested amplified product
Lane 2: *rachmati*
Lane 3: *grippotyphosa*
Lane 4: B7D
Lane 5: *canicola*
Lane 6: pUC18/*Sau* 3A 1- pUC18/*Taq*1 Digest

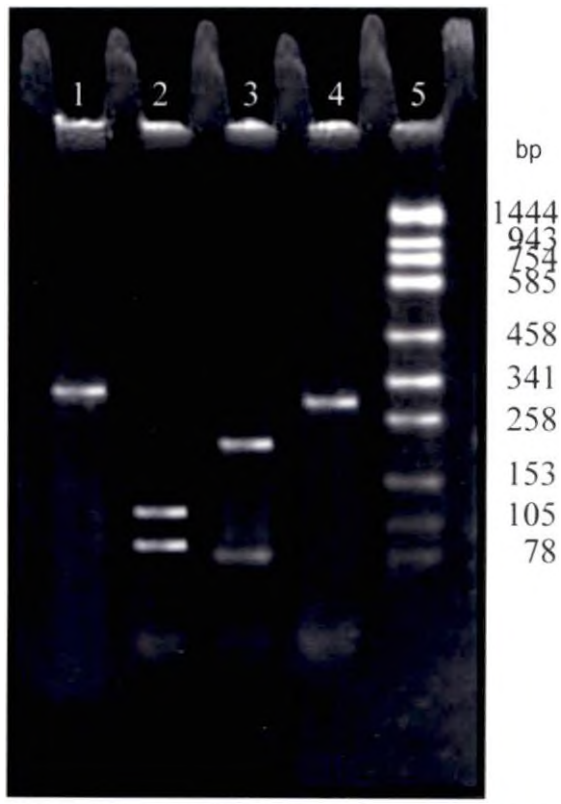

Fig.7. Polyacrylamide gel (8 per cent) electrophoresis of *Dde* I digested PCR products GI/G2 primers showing different profiles

Lane 1: Undigested amplified product
Lane 2: *australis*
Lane 3: *grippotyphosa*
Lane 4: *tarassovi*
Lane 5: pUC18/*Sau* 3A 1- pUC18/*Taq*1 Digest

samples were DFM positive and culturing was tried immediately. But later PCR and culture detected both the samples as negative for *Leptospira.* All the DFM negative samples were also negative by PCR and culture.

4.1.2.1b Polymerase chain reaction

A total of 147 samples including serum, urine, milk and kidney tissues from suspected cases of leptospirosis and kidney tissues and urine from rodents were subjected to genus specific PCR for detection of leptospiral DNA. Out of the 147 samples leptospiral DNA could be detected in nine samples. The results are shown in Fig. 8. The number of samples tested and results obtained are presented in table 2.

Among the 117 clinical samples examined amplification of leptospiral DNA was observed only in eight serum samples, four from dogs and four from cattle. All these samples were long preserved ones received from District Veterinary Centre (DVC), Kottayam and were unsuitable for culturing. Of the 30 samples from rodents the kidney tissue of one bandicoot was positive by PCR.

The differentiation of leptospires was attempted in the samples employing multiplex PCR, Nested PCR, AP-PCR and LS-PCR standardized using the reference strains.

All the samples tested positive by genus specific PCR were subjected to multiplex PCR. All the nine samples did not present any detectable amplification of DNA showing that the method was unsuitable for the differentiation of leptospires from clinical samples where the amount of DNA was low.

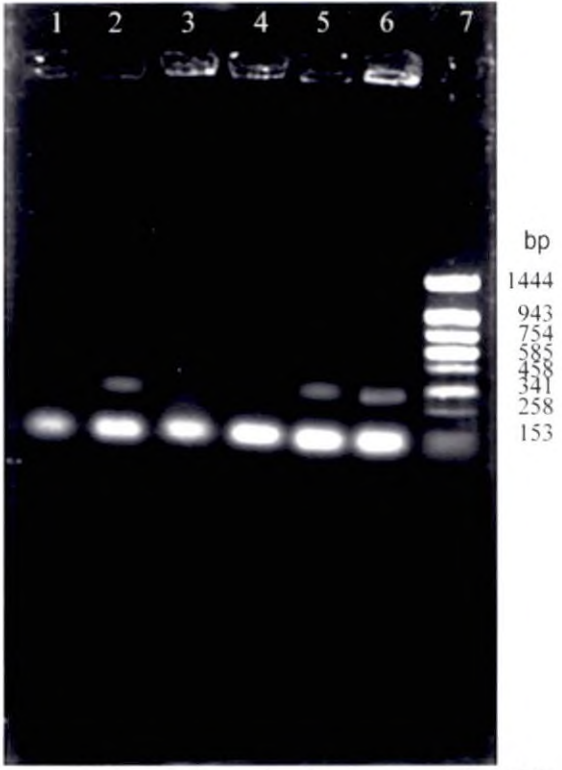

Fig. 8. Agarose gel (one per cent) electrophoresis of genus specific PCR products of representative samples

Lane 1: Negative control
Lane 2: CS-1
Lane 3: CS-4
Lane 4: CS-5
Lane 5: BK-7
Lane 6: DS-8
Lane 7: pUC18/*Sau* 3A 1- pUC18/*Taq*1 Digest

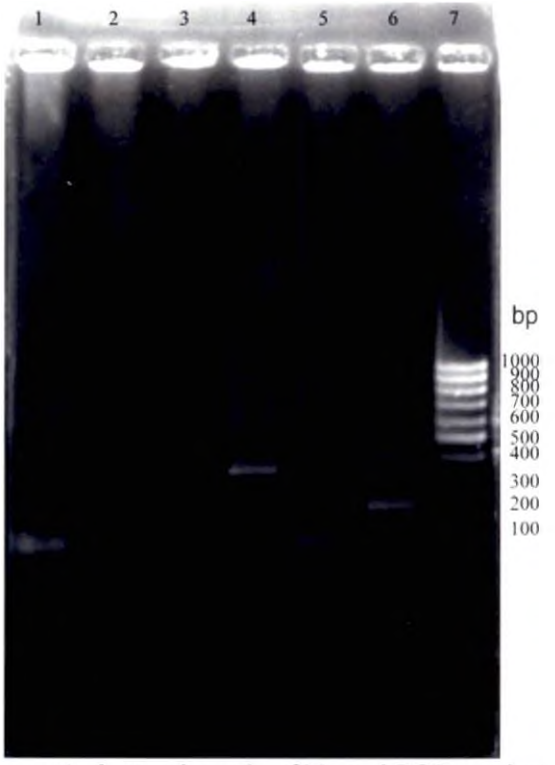

Fig. 9. Agarose gel (1.5 per cent) electrophoresis of Nested PCR products of representative samples

Lane 1& 2 : RK-5
Lane 3& 4 : CS-I
Lane 5 & 6: DS-8
Lane 7 : 100 bp DNA ladder

*CS-1, 4, 5: cattle serum 1,4, 5, BK-7: bandicoot kidney 7, DS-8: dog serum 8, RK-5: rat kidney 5

Table 2. Results of PCR on biomaterials.

	No. of Serum samples tested	No. of samples tested positive	No. of Urine samples tested	No. of samples tested positive	No. of Milk samples tested	No. of samples tested positive	No. of Kidney samples tested	No. of samples tested positive	Total no. of samples tested	Total no. of positives
Canine	71	4	16	–	–	–	4	–	91	4
Bovine	9	4	–	–	2	–	1	–	12	4
Caprine	2	–	1	–	–	–	–	–	3	–
Murine	–	–	5	–	–	–	25	1	30	1
Human	10	–	1	–	–	–	–	–	11	–
TOTAL	92	8	23	–	2	–	30	1	**147**	**9**

All the nine samples found positive by genus specific PCR were also amplified by nested PCR. For all these samples no amplification was observed for the first PCR reaction using the outer set of primers on agarose gel electrophoresis. Nested amplification using the inner primer set resulted in amplification of DNA in all the nine samples. Out of the nine samples five presented a PCR product of 370 bp whereas the remaining four had a 200 bp amplicon. Out of the five samples with 370 bp products four were serum samples from cattle and one was from the kidney of a bandicoot. The remaining four were serum samples from dogs. The representative results are shown in Fig. 9.

All the nine samples, positive by genus specific PCR and nested PCR, failed to show amplification when subjected to AP-PCR and LS-PCR as there was no detectable products on agarose gel electrophoresis.

4.2 ISOLATION

Out of the 32 samples cultured for isolation seven samples were contaminated either with bacteria or fungus. Contamination was more common in urine samples and Fletcher's media with 5-FU were used mainly to culture urine samples. The bacterial contamination was evident from 24 h of incubation whereas the fungal contaminants took five to seven days to grow.

Out of the 25 samples which were uncontaminated only one kidney sample from a bandicoot was positive for isolation of *Leptospira*. Rest of the samples was declared negative after incubation for a period of three months.

The sample which was positive for isolation was positive by genus specific PCR and motile leptospires were demonstrable by 11^{th} day of inoculation. The isolate was sub-

cultured regularly at four weeks interval and the culture could be maintained in the laboratory for further identification.

4.3 DIFFERENTIATION OF *Leptospira ISOLATES*

4.3.1 Genus specific PCR

The template DNA prepared from all six isolates of leptospires R6, R13, M64, BT, 289 and B7D were amplified by genus specific PCR with primers A and B. All the six isolates presented an amplicon of expected size of 331 bp. The results are shown in Fig. 10.

4.3.2 Multiplex PCR

All the six isolates R6, R13, M64, BT, 289 and B7D presented a 285 bp band with the primers G1 /G2 indicating that they belonged to any one of the six pathogenic species, *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. inadai* or *L. weillii* (Fig. 11).

4.3.3 Nested PCR

All the six isolates were amplified by nested PCR and PCR products of expected sizes, 571 bp and 370 bp, were obtained for all (Fig. 12).

4.3.4 Arbitrarily primed PCR

Four different banding patterns were observed among the six isolates. The AP-PCR profiles of three of the isolates 289, M64 and R6 isolated from human and bandicoots respectively were same and similar to Profile 1(1310 to 124 bp) of the reference strains.

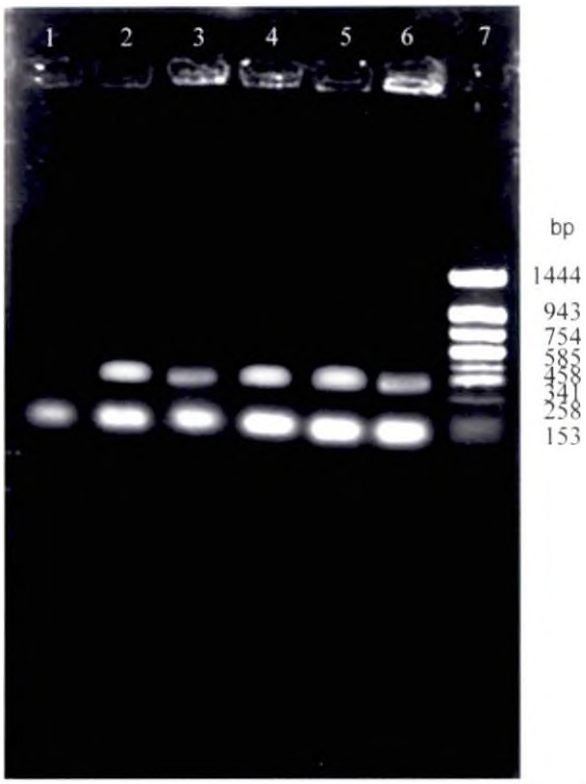

Fig. 10. Agarose gel (one per cent) electrophoresis of genus specific PCR products of *Leptospira* isolates

Lane 1: Negative control
Lane 2: R6
Lane 3: R13
Lane 4: 289
Lane 5: M64
Lane 6: BT
Lane 7: pUC18/*Sau* 3A 1- pUC18/*Taq*1 Digest

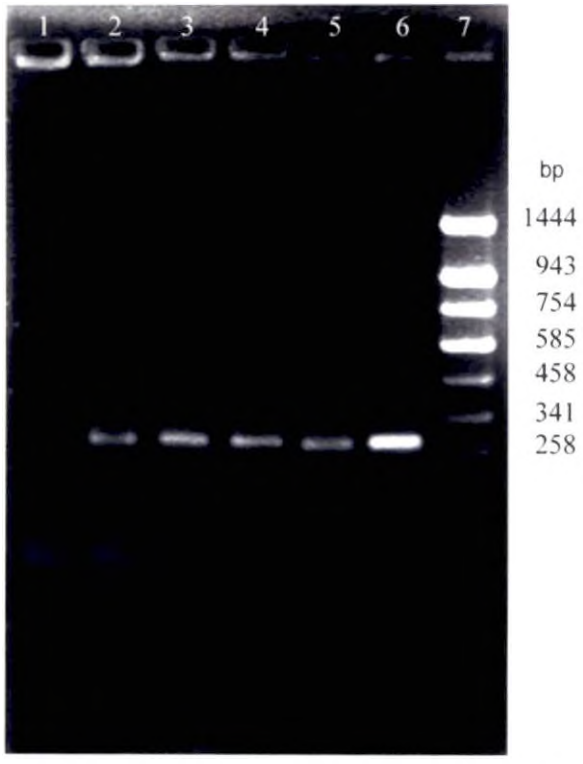

Fig. 11. Agarose gel (one per cent) electrophoresis of Multiplex PCR products of *Leptospira* isolates

Lane 1: Negative control
Lane 2: R6
Lane 3: R13
Lane 4: BT
Lane 5: 289
Lane 6: M64
Lane 7 : pUC18/*Sau* 3A 1- pUC18/*Taq*1 Digest

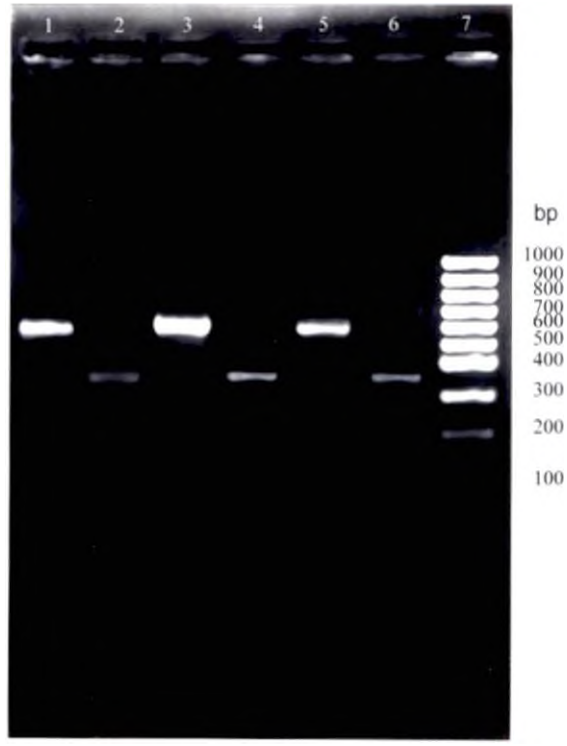

Fig. 12. Agarose gel (1.5 per cent) electrophoresis of Nested PCR products of *Leptospira* isolates

Lane 1& 2 : R6
Lane 3& 4 : R13
Lane 5 & 6 : M64
Lane 7 : 100 bp DNA ladder

The remaining three isolates (two from bandicoots and one from rat) presented distinct banding patterns unique to each of them and were different from the banding profiles presented by the reference strains. The serovar B7D presented bands from 1128 to 191 bp region (Profile 5), BT presented bands of 1252 to 124 bp (Profile 6) and R13 had products of sizes ranging from 1553 to 124 bp (Profile 7). The results are shown in Fig. 4.

4.3.5 Low stringency PCR (LS-PCR)

Five different banding patterns were observed among the six isolates (Fig. 5). The LS-PCR profile of the isolate M64 was similar to Profile 1(740, 410, 360, 285 and 120 bp) of the reference strains. The profiles of the isolates 289 and R6 were same as that of *canicola* (Profile 7) and close to Profile 1 of the reference strains with an additional band at 140 bp region. The profiles of R13 with bands at 930, 815, 740, 410, 360, 285 and 120 bp B7D having products at410, 360, 285, 210, 170 and 120 and BT with products at 385, 310, 285 and 120 bp region were different.

4.3.6 Restriction enzyme analysis (REA)

The products of genus specific and multiplex PCR were subjected to REA similar to the reference strains.

The REA of the 331 bp genus specific PCR products of all the six isolates were similar to profile 1 of the reference strains with a major fragment at 153 bp region (Fig. 6).

The REA of the 285 bp product of G1/G2 primers by the enzyme *Dde* I produced bands at 120 and 85bp for all the six isolates (Profile 1) (Fig. 7).

4.4 COMPARISON OF THE PROFILES OF AP-PCR, LS-PCR AND PCR-REA OF THE REFERENCE STRAINS AND ISOLATES

Table 3. AP-PCR profiles of the reference strains and the isolates

AP-PCR Profiles	Band size (bp)	Serovar
Profile 1	1310 - 124	*australis, canicola, grippotyphosa, hardjo, icterohaemorrhagiae, pomona, tarassovi,* R6, M64,289
Profile 2	1437 - 124	*rachmati*
Profile 3	1011 – 124	*pyrogenes*
Profile 4	964 - 124	*patoc*
Profile 5	1128 – 191	B7D
Profile 6	1252 –124	BT
Profile 7	1553 - 124	R13

Table 4. LS-PCR profiles of the reference strains and the isolates

LS-PCR Profiles	Band size (bp)	Serovar
Profile 1	740, 410, 360, 285, 120	*australis, pomona, hardjo, patoc,* M64
Profile 2	1020, 862, 740, 490, 440, 285, 250, 150, 120	*grippotyphosa*
Profile 3	750,740,470, 285,90	*rachmati*
Profile 4	410, 360,285, 120	*icterohaemorrhagiae*
Profile 5	410, 360,285, 190, 120	*tarassovi*
Profile 6	410, 360, 285, 210, 120	*pyrogenes*
Profile 7	740, 410, 360, 285, 140, 120	289, R6, *canicola*
Profile 8	930, 815, 740, 410, 360, 285,120	R 13
Profile 9	410, 360, 285, 210, 170, 120	B7D
Profile 10	385, 310, 285, 120	BT

Table 5. PCR-REA Profiles of genus specific PCR product

Enzyme: *Dde* I PCR product: 331 bp

PCR-REA Profile	Size of the major fragment (bp)	Serovar
Profile 1	153	*Patoc, icterohaemorrhagiae, australis, canicola, hardjo, pomona, pyrogenes,* R6, M64, BT, 289, R13,B7D
Profile 2	210	*grippotyphosa ,tarassovi*
Profile 3	245	*rachmati*

Table 6. PCR-REA Profiles of multiplex PCR product

Enzyme: *Dde* I PCR product: 285 bp

PCR-REA Profile	Fragment size (bp)	Serovar
Profile 1	120, 85	*icterohaemorrhagiae, australis, canicola, hardjo, pomona, pyrogenes,* B7D, R6, M64, BT, 289, R13
Profile 2	210, 75	*grippotyphosa*
Profile 3	270	*tarassovi*

Discussion

5. DISCUSSION

Leptospirosis is an acute febrile systemic disease of humans and other mammals caused by pathogenic leptospires that occur naturally in a wide variety of wild and domesticated mammals throughout the world. Rodents are the most common natural reservoirs of the infectious agent. In natural host the nesting site for leptospires is the lumen of the nephritic tubule from where they are shed in to the urine. Infections are incurred by contact with urine of carriers or indirectly by contact with streams, ponds, swamps or wet soil contaminated with urine of carriers (Alexander, 1974).

Leptospires can affect many different organ systems resulting in a wide variety of clinical presentations making the clinical recognition of leptospirosis difficult. Timely diagnosis of leptospirosis is essential because prompt, specific treatment as early in the illness as possible is important in ensuring a favourable clinical outcome (Effler *et al.*, 2000).

Rapid diagnosis of leptospirosis is important in view of an adequate early treatment. Clearly, serology does not contribute to early diagnosis of leptospirosis as antibodies become detectable on approximately the seventh day of disease. Conventional methods to detect leptospires in blood such as DFM or culturing are either unreliable or too slow to contribute to an early diagnosis. Immunofluorescence staining, immunoperoxidase staining and DNA hybridization are useful methods for detection of leptospires provided that these microorganisms are present in relatively large numbers. With the introduction of PCR rapid detection of small number of leptospires in clinical samples become practical due to specific amplification of leptospiral DNA (Gravekamp *et al.*, 1993).

The precise identification and classification of *Leptospira* species is important for epidemiological and public health surveillance, as different serovars can exhibit different host specificities and may not be associated with a particular clinical form of infection. The PCR based finger printing facilitates characterization at the inter and intra species level, and specifically the identification of pathogenic *Leptospira* isolates to the serovar level (Brown and Levette, 1997).

In the present study attempts were made to detect and differentiate leptospires by employing PCR techniques including genus specific PCR, multiplex PCR, nested PCR, AP-PCR, LS-PCR and PCR-REA. These methods were standardized by employing the standard reference serovars. The study also aimed to isolate *Leptospira* and to differentiate the isolates including those maintained in the laboratory by PCR techniques.

5.1 DETECTION OF LEPTOSPIRES

5.1.1 Dark Field Microscopy

Fresh samples obtained from clinical cases were examined by DFM before processing for PCR. Of the 68 samples examined for leptospires, only two blood samples were found positive by DFM. But both the samples were negative for isolation and identification of leptospires by PCR. All the samples found negative by DFM were also negative by PCR.

According to Chandrasekharan and Gomathy (2004) microscopic examination of tissues or body fluids was not recommended as a single diagnostic procedure since the concentration of *Leptospira* in blood may be too low and

artifacts such as fibrils and extrusions from cellular elements can be easily mistaken for *Leptospira* by the inexperienced.

Venkatesha and Ramadas (2001) subjected the clinical samples found positive on DFM to culturing and PCR. Over 60 per cent of these samples were only culture positive while 81.70 per cent of them gave positive PCR results.

In the present study, though the results of DFM did not correlate with the results of PCR and isolation trials, DFM was found to be a reliable tool for demonstration of leptospires in cultures and also to check purity of cultures.

Sehgal *et al.* (2001) opined that DFM was an ideal technique for demonstration of leptospires in cultures, but it's use as a diagnostic test for demonstration of these organisms in clinical samples is not satisfactory.

5.1.2 POLYMERASE CHAIN REACTION

The genus specific primers A and B designed by Merien *et al.* (1992) could amplify DNA from all the reference strains and isolates showing their suitability for detection of leptospires in clinical samples. These were used for PCR for detection of leptospires in samples including serum, urine, milk and kidney tissues.

Merien *et al.* (1992) designed the primers A and B based on the sequence of 16S rRNA for the detection of leptospires in body fluids.

According to Venkatesha and Ramadas (2001) the primers used for clinical diagnosis should be able to identify broad spectrum of serovars instead of a few serogroups only.

Senthilkumar *et al.* (2001) proved that PCR assay can be used not only for blood and urine, but also for other samples like CSF and milk.

Heinemann *et al.* (1999) detected leptospires in 80 per cent of bovine semen samples using the primers A and B. All these samples were negative by culture and 45 per cent of them were positive by MAT. All the PCR negative samples were negative by culture and MAT.

Out of the 147 samples from different sources, subjected to PCR nine were found positive. This included eight serum samples received from DVC, Kottayam which were long preserved ones and on which DFM and culturing methods could not be employed.

Noubade *et al.* (2002) opined that PCR has the advantage that it does not require the isolation of the organisms and detected DNA from both viable and non-viable organisms. Isolation requires viable organisms to be present in the sample, which is again influenced by various factors such as method of collection, transportation and storage.

Venkatesha and Ramadas (2001) could detect leptospires by PCR in more number of samples over culturing method and the possible reason could be that culturing needs viable organism where as PCR detects leptospiral DNA in samples.

The only one kidney tissue of the bandicoot found positive by PCR had provided one isolate of *Leptospira* by 11^{th} day of incubation. Merien *et al.* (1992) showed that PCR could surpass culturing in sensitivity of detection and was faster than culturing for isolation of leptospires (1 day versus 1 to 8 weeks).

Harkin *et al.* (2003) evaluated PCR assay on urine samples for diagnosis of leptospirosis in dogs and the sensitivity and specificity of PCR were found to be 100 and 88.3 per cent, respectively.

5.2 DIFFERENTIATION OF LEPTOSPIRES

5.2.1 Multiplex PCR

Multiplex PCR was carried out using the primers G1/G2 and B64 -I/ B64 -II described by Gravekamp *et al.* (1993). The eight reference strains and the six isolates were amplified by G1/G2 primers to give a 285 bp product and they were assumed to be belonging to any of the six species, *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. inadai* or *L. weillii.* The serovar *rachmati* was amplified by the primers B64-I/ B64-II to give a 563 bp amplicon indicating that it belonged to the species *L. kirschneri.* The non-pathogenic serovar *patoc* was not amplified by any of these primers.

Machang'u *et al.* (2004) used the primers G1/G2 and B64 -I/ B64 -II for assessing the pathogenic status of the *Leptospira* isolates from African giant pouched rats. Sugathan and Varghese (2005) reported amplification of either 285 or 563 bp fragments from all the *Leptospira* isolates subjected to amplification by G1/G2 and B64-I/B64-II, which was similar to the observation of the present study. But Parma *et al.* (1997) used the same set of primers G1/G2 and B64-I/ B64-II for the differentiation of leptospires isolated from man and animals and reported that the multiplex PCR generated either a 285 bp or 360 bp fragments depending on the pathogenic strain.

5.2.2 Nested PCR

Nested PCR was done using the two sets of primers designed by Faber *et al.* (2000), which were described to amplify a conserved region found in 16S rRNA of certain serovars of *L. interrogans* and *L. borgpetersenii.* In the present study the primers were selected expecting that it could specifically amplify serovars of these two species of *Leptospira* among those detected by G1/ G2 primers. But it amplified DNA from all the reference strains including the nonpathogenic serovar *patoc* and the isolates. The serovar *rachmati*, which belonged to *L. kirschneri* species, as evidenced by the results of multiplex PCR, presented distinct amplified products different from other reference strains. It gave multiple PCR products with the outer primer set and a product of 200 bp with the inner primers.

In case of template DNA from the clinical samples the outer primer set failed to give detectable amplification of leptospiral DNA on first PCR with the outer primers. But nested amplification with the inner primers could produce sufficient amplification of DNA that could be visualised.

Chu *et al.* (1998) identified leptospires in aqueous humor of patients with symptoms of uveitis by PCR and nested PCR was performed on all samples that were negative on the first reaction to increase the sensitivity of the test.

5.2.3 Low Stringency PCR

The primers G1/G2 described by Gravekamp *et al.* (1993) for the detection of pathogenic *Leptospira* DNA when subjected to LS-PCR could differentiate *Leptospira* serovars in the present study. Ten different banding patterns were obtained from the ten reference strains and the six isolates. Out of these 10 profiles

four patterns were almost similar differing in one or two bands. All the 10 reference serovars including *patoc* and *rachmati* and the isolates produced the specific 285 bp product under low-stringency conditions indicating its usefulness in detecting leptospires from samples. Similar observations were made by earlier workers.

De Caballero *et al.* (1994) tested the utility of LS-PCR banding patterns for the identification of leptospiral isolates by comparing with the reference strains, using the primers G1/G2 and it has been found to produce complex serovar specific patterns which maintained the specific band at 285 bp region as an internal control.

Brown and Levette (1997) used the primers G1/G2 for LS-PCR and the profiles were found to be serovar specific in some cases. They also reported that similar LS-PCR profiles were given by closely related serovars and these could usually be distinguished on the basis of two or more bands.

5.2.4 Arbitrarily Primed PCR

The primer PB1 was used for the AP-PCR analysis of the reference strains and the isolates of *Leptospira* maintained in the laboratory. Six different banding patterns were observed among the ten reference strains and the six isolates subjected to the study.

Ramadass *et al.* (2002) used the same primer PB1 for the AP-PCR analysis of leptospiral isolates and the reference serovars and the method was reported to be useful, reasonably simple and quick for typing of leptospires. In their study each of the serovar tested produced different patterns. Brown and Levette (1997) reported similar results for AP-PCR for differentiating different serovars and they observed similar patterns for closely related serovars.

Collares-Pereira *et al.* (2000) used four different primers for AP-PCR in the analysis of leptospiral strains and reported its usefulness for species identification and recommended it as a simple and rapid tool for intra-species comparison.

Perloat *et al.* (1994) found AP-PCR as a quick and reliable method for typing *Leptospira* strains for intra-species comparison.

5.2.5 Restriction enzyme analysis

The products of genus specific PCR and multiplex PCR were subjected to REA. The 331 bp product of genus specific PCR using the primers A and B resulted in three different patterns when subjected to digestion with the enzyme *Dde* I. Out of the 16 *Leptospira* cultures (ten reference strains and six isolates) the serovars *rachmati, grippotyphos* and *tarassovi* presented digestion patterns different from the common pattern of the other 13 serovars. Among these the digestion patterns of *grippotyphosa* and *tarassovi* were the same. The method could not differentiate the non-pathogenic serovar *patoc* from the pathogenic ones.

Shukla *et al.* (2002) reported that the restricton enzyme analysis with four enzymes *Apa* I, *Ban* II, *Pst* I and *Sin* I on the 482 bp genus specific region of 23S rRNA of leptospires provided clear differentiation in to pathogenic and saprophytic groups.

Savio et al. (1994) reported differentiation among majority of *Leptospira* serovars with PCR-REA using the enzymes *Hinf* I and *Dde* I.

Heinemann *et al.* (2000) conducted REA of 330 bp product of PCR using the primers A and B with the enzyme *Dde* I and obtained fragments of 80 and 250 bp for pathogenic leptospires and 10, 50, 60 and 210 bp for saprophytes.

The 285 bp products of the primers G1/G2 when subjected to REA with the enzyme *Dde* I yielded three different patterns out of the 14 *Leptospira* serovars. The serovars *grippotyphosa* and *tarassovi* presented unique digestion patterns whereas the other 12 serovars had same pattern. Thus the PCR-REA of 285 bp product of G1/G2 could differentiate *grippotyphosa* and *tarassovi*, which presented a common pattern in PCR-REA of the genus specific PCR product. The 563 product of *rachmati* with the primers B64 -I/B64 -II was of low intensity and the digestion of the products with the enzymes *Hinf* I and *Mnl* I could not yield distinguishable products.

Brown and Levette (1997) observed three polymorphic variations when the 285 bp products with primers G1 and G2 were digested with *Dde* I. The 563 bp product of multiplex PCR with the primers B64 -I/ B64 -II yielded fragments of 167 and 396 bp with *Mnl* I and 6,61, 84, 101, 121 and 187 bp with *Hinf* I.

The differentiation of leptospires in samples detected positive for leptospiral DNA could not be proceeded further and may be due to the low amount of DNA in these samples.

5.3 ISOLATION

Though bacteriologic culture procedure for leptospires is too expensive and slow for routine use, the major advantage is that leptospires of any serovar can be detected and subsequently identified (Bolin *et al.*, 1989).

Attempts were made to isolate *Leptospira* from kidney tissues and urine of rodents and also from suspected clinical samples found positive on DFM. Isolation trials were done in Fletcher's semisolid medium supplemented with 10 per cent heat inactivated haemolysed rabbit sera. Out of the 32 samples one isolate was obtained from the kidney of a bandicoot. The isolate was subsequently subcultured to the media containing 5-FU. The primary isolation was possible in the culture tube containing the tissue piece.

Rajasekhar and Kesavamurthy (1976) found Fletcher's medium superior to Korthof's medium for primary isolation of leptospires from rodents. Leptospiral growth was obtained in Fletcher's medium while the corresponding Korthof's medium tube from the same rat did not show evidence of growth of leptospires.

Johnson and Rogers (1964) found that 5-FU, an analogue of uracil, was very active in inhibiting the growth of many bacteria. The results from the growth studies indicated that leptospires were resistant to 5-FU.

Adinarayanan and James (1980) succeeded in isolating leptospires in Stuarts or Fletcher's medium with sterile 10 per cent haemoglobinised rabbit serum.

Soman (2004) tried isolation of leptospires in Fletcher's or EMJH semisolid medium with 5-FU and made primary isolation of *Leptospira* in Fletcher's semisolid medium.

The only kidney sample, which was positive for genus specific PCR could present an isolate in Fletcher's semisolid medium on 11^{th} day of incubation. All the samples, which were PCR negative, were also proved to be negative by culture.

Wagenaar *et al.* (2000) had the same observation when they compared different diagnostic techniques for detection of leptospires in urine of experimentally inoculated bovines.

In the present study out of the samples from twenty five rodents (21 rats and four bandicoots) tested for the presence of leptospires only one isolate was obtained from the kidney of a bandicoot. The samples from all the rats were negative for leptospires by PCR and culture. Among the rodents bandicoots may be playing a major role as reservoirs of *Leptospira.*

Adinarayanan and James (1980) made a study for a period of three years on leptospirosis on farm stock and wild life of Kerala. Majority of their isolations were from bandicoots and could make eight isolates out of 46 of them examined, where as only one isolation could be made out of 114 rats examined.

Ellis *et al.* (1981) could not recover leptospires from kidneys of any of the 143 mice being cultured.

The molecular techniques-genus specific PCR, multiplex PCR, nested PCR, AP-PCR, LS-PCR and PCR-REA employed in the present study were found to be useful in detection as well as differentiation of leptospires. The genus specific PCR using primers A and B for the detection of leptospires in samples could amplify DNA from all the reference strains and the isolates indicating its suitability for diagnostic purpose. The multiplex PCR using the primers G1/G2 and B64 -I/B64 -II could differentiate the non-pathogenic leptospires from the pathogenic ones. It could also differentiate *L. kirschneri* species from other six pathogenic species. Similar to genus specific PCR nested PCR could also detect all the 16 serovars of leptospires used in the study and differentiated *L. kirschneri* species from other species of

leptospires. The AP-PCR could subgroup the I6 cultures of leptospires in to seven groups. The seven reference serovars and three isolates presented same AP-PCR profile. The serovars *rachmati* and *patoc* already differentiated by multiplex PCR and the serovar *pyrogenes* presented distinct patterns. Three isolates B7D, BT and R13 had unique profiles and were not comparable to any of the reference strains. The LS-PCR could divide the 16 leptospires into 10 distinct groups, as against seven groups by AP-PCR. Out of the 16 *Leptospira* cultures, the serovars *rachmati, grippotyphosa* and *tarassovi* presented distinct digestion patterns from the common pattern of the other 13 serovars when the genus specific PCR product was subjected to digestion by the enzyme *Dde* I. The PCR-REA of 285 bp product of G1/G2 by *Dde* I could differentiate *grippotyphosa* and *tarassovi* which presented a common pattern in PCR-REA analysis of the 331 bp product of genus specific PCR by *Dde* I.

Though all the methods used in the study were capable of detecting and differentiating the leptospires, LS-PCR was found to be superior over other methods as it could detect and differentiate the serovars in one step analysis.

Summary

6. SUMMARY

Leptospirosis caused by spirochaetes of the genus *Leptospira* is considered as one of the most wide spread zoonosis worldwide. Leptospires have wide host range involving cattle, swine, dogs, rodents, wild animals and man. The disease has extreme public health importance in countries like India where man live in close association with animals. Leptospirosis is a disease of multi-organ involvement making the clinical diagnosis difficult. Rapid diagnosis of leptospirosis is extremely important for an adequate early treatment. Vaccination is the best approach to control the disease. There is no cross protection between the serovars and the immunity is serotype specific. There is a need to identify the serovars prevalent in an area to adopt control measures and the tests selected for this purpose should be simple, sensitive, specific and affordable.

In the current study an attempt has been made to detect and differentiate leptospires in the bio-materials and to differentiate *Leptospira* isolates by molecular methods. The methods employed are genus specific PCR, multiplex PCR, nested PCR, AP-PCR, LS-PCR and PCR-REA.

The molecular methods for detection and differentiation of leptospires were standardized using the reference strains. All the reference strains were amplified by genus specific PCR by the primers A and B. When subjected to multiplex PCR, out of the ten reference strains eight of the serovars were amplified by the primers G1/G2 designed to amplify DNA from six pathogenic species, whereas one of the serovar *rachmati,* was amplified by the primers B64-I/B64-II indicating its position in the species *L. kirschneri.* The non-pathogenic serovar *patoc* was not amplified by any of the above primers.

On nested PCR also the serovar *rachmati* presented an amplification pattern different from other nine strains. The nine strains of leptospires presented amplified products of 571 and 370 bp.

The AP-PCR analysis of the reference strains produced four different banding patterns. The serovars *rachmati, patoc* and *pyrogenes* presented distinct profiles whereas the other seven serovars had a common banding pattern.

The LS-PCR analysis of the reference strains could provide seven different profiles. The serovars *grippotyphosa, rachmati, icterohaemorrhagiae, tarassovi, canicola* and *pyrogenes* produced unique patterns. Most of these patterns differed in the presence or absence of one or two bands.

Restriction enzyme analysis of the genus specific and multiplex PCR products could provide differentiation among the reference serovars. The PCR-REA of the 331 bp products of genus specific PCR presented three different patterns. Seven serovars presented similar fragments upon enzymatic digestion with *Dde* I. The patterns of *grippotyphosa* and *tarassovi* were same. The serovar *rachmati* presented a unique digestion pattern.

Restriction enzyme analysis of the multiplex PCR product of 285 bp could also reveal some difference among the reference serovars when subjected to digestion with the same enzyme *Dde* I. Eight of the serovars had same digestion pattern where as *grippotyphosa* and *tarassovi* presented unique patterns.

The samples for the study included urine, blood, milk and kidney tissues from animals and man suspected for leptospirosis and the kidney and urine collected from rodents captured from neighbouring farm premises.

The blood as well as urine samples (68) collected from the clinical cases were immediately subjected to DFM before processing them for PCR. Two samples, which were found to be positive by DFM, were later confirmed as negative for *Leptospira* by PCR and isolation in culture media. DFM was found to be unsatisfactory for diagnosis due to occurrence of false positive results and was mainly employed to check purity of cultures and to detect leptospires in cultures.

One hundred and seventeen clinical samples including serum/plasma (92), urine (18), kidney tissues (five) and milk (two) were subjected to genus specific PCR using the primers A and B for the detection of leptospires. The samples from rodents, twenty five kidneys and five urine samples, were also subjected to genus specific PCR. Out of the 147 samples only 9 were positive for *Leptospira*. This included eight serum samples, four from cattle and four from dogs and one kidney sample from a bandicoot. These were also amplified by nested PCR whereas multiplex PCR, LS-PCR and AP-PCR failed to produce detectable amplification, may be due to low number of organisms in bio-materials.

Isolation was tried mainly from rodents (kidney cortex and urine) in Fletcher's semi solid medium. Out of the 32 samples one isolate was obtained from kidney of a bandicoot, which was PCR positive. All the samples, which were culture negative, were also negative by PCR.

The differentiation of leptospiral isolates was attempted using all the molecular methods standardized with reference strains. All the six isolates were found to belong to any of the six pathogenic species of leptospires by multiplex PCR, since they were amplified by the primers G1/G2. On nested PCR they presented amplification producing PCR products of expected size, 571 bp and 370 bp.

Four different AP-PCR banding patterns were observed among the six isolates. The isolates R6, M64 and 289 presented a profile similar to seven reference strains. The isolates B7D, BT and R13 presented a unique pattern, which was different from any of the four profiles of the reference strains.

The LS-PCR revealed five banding patterns among the six isolates. The isolate M64 presented a pattern similar to *australis, pomona, hardjo* and *patoc.* The profiles of 289 and R6 were similar to that of *canicola.* The isolates B7D, R13 and BT had unique profiles.

Restriction enzyme analysis of the 331 bp product of genus specific PCR produced a digestion pattern similar to seven reference strains for all the six isolates. Restriction enzyme analysis of the 285 bp product with the primers G1/G2 presented fragments of 120 and 85 bp for all the isolates similar to six reference strains.

The molecular methods were found to be capable of differentiating *Leptospira* serovars. Among the different PCR techniques used in the study LS-PCR was found to be more useful which can group the 16 leptospires in to 10 groups in one step analysis.

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DETECTION AND IDENTIFICATION OF PATHOGENIC LEPTOSPIRES IN BIO-MATERIALS

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ABSTRACT

In the present study an attempt has been made to detect and differentiate leptospires in bio-materials employing molecular techniques such as genus specific PCR, multiplex PCR, nested PCR, AP-PCR, LS-PCR and PCR-REA. Isolation trials for *Leptospira* were also made in the study and the isolates were tried to be differentiated employing the molecular methods mentioned above.

The genus specific primers A and B were used to detect leptospires in clinical samples and samples from rodents. Out of the 147 samples only nine were positive for leptospiral DNA. Out of the nine positive samples eight were serum samples (four from cattle and four from dogs) and one was kidney of a bandicoot.

Multiplex PCR, using the primers G1/G2 and B64-I/B64-II could differentiate leptospires into pathogenic and non-pathogenic ones. Among the pathogenic leptospires it could differentiate the species *L. kirschneri*, from other six pathogenic species viz *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. inadai and L. weillii.* All the six isolates were found to be belonging to any of these six species.

Nested PCR using the primers designed based on the sequence of *L. borgpetersenii* and *L. interrogans* amplified DNA from all the ten reference strains including the non-pathogenic serovar *patoc* and *rachmati* of *L. kirschneri* species. All the six isolates were amplified giving PCR products of expected sizes, 571 bp and 370 bp.

The AP-PCR could produce seven different patterns from the ten reference strains and six isolates. The profiles of three of the isolates, B7D, BT and R13 were not matching to any of the reference strains used in the study.

The LS-PCR analysis of the 16 leptospires (10 reference strains and six isolates) could provide 10 different banding patterns. The profiles of the isolate 289, R6 and *canicola* were the same. The isolate M64 produced a pattern similar to that of *australis, pomona, hardjo and patoc.* Three of the isolates R13, B7D and BT had unique patterns.

Restriction enzyme analysis of the products of genus specific and multiplex PCR could bring about differences among the serovars used in the study. The serovar rachmati presented unique digestion pattern when the 331 bp product was digested with *Dde* I. The serovars *grippotyphosa* and *tarassovi* which have similar pattern on digestion of 331 bp product with *Dde* I have shown different digestion patterns on PCR-REA of 285 bp product of the multiplex PCR.

Among the molecular methods used in the study LS-PCR was found to be more useful as it could detect and differentiate the serovars in one step analysis.