172109

COMPARISON OF POLYMERASE CHAIN REACTION WITH CONVENTIONAL METHODS FOR THE DIAGNOSIS OF LEPTOSPIROSIS

ELAIYARAJA. M.

Thesis submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University, Thrissur

2003

Department of Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR - 680651
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DECLARATION

I hereby declare that the thesis, entitled **"COMPARISON OF POLYMERASE CHAIN REACTION WITH CONVENTIONAL METHODS FOR THE DIAGNOSIS OF LEPTOSPIROSIS"** 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.

Mannuthy,
23.09.03

ELAIYARAJA. M.

CERTIFICATE

Certified that the thesis entitled **"COMPARISON OF POLYMERASE CHAIN REACTION WITH CONVENTIONAL METHODS FOR THE DIAGNOSIS OF LEPTOSPIROSIS"** is a record of research work done independently by **Shri. Elaiyaraja. M.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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We, the undersigned members of the Advisory Committee of **Shri. Elaiyaraja, M.,** a candidate for the degree of Master of Veterinary Science in Microbiology, agree that the thesis entitled **"COMPARISON OF POLYMERASE CHAIN REACTION WITH CONVENTIONAL METHODS FOR THE DIAGNOSIS OF LEPTOSPIROSIS"** may be submitted by Shri. Elaiyaraja, M., in partial fulfilment of the requirement for the degree.

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Elaiyaraja, M

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Introduction

1. INTRODUCTION

Leptospirosis is a zoonotic disease of ubiquitous occurrence, caused by infection with pathogenic spirochaetes of the genus leptospira, which affects a wide range of hosts such as domestic animals, humans, wild animals and rodents. The natural foci of infection have been presumed as domestic animals and rodents, which contribute for wide spread of the infection in man. In livestock industry the disease leads to major production losses as it causes abortion, still birth, infertility, decreased milk production and death.

The incidence of the disease is significantly higher in tropical countries than in temperate regions. Leptospirosis is mainly an occupational disease and the agricultural workers show the most frequent occurrence of infection. The clinical symptoms of leptospirosis in man range from mild influenza-like symptoms to fatal jaundice and death, while in animals they vary from inapparent infection to acute septicaemia, anaemia, abortion or fatal jaundice.

The disease has gained extreme public health importance, especially in countries like India, because of huge livestock, rodent and wildlife populations, poor sanitary conditions, poor animal management and close association between man and animals, providing a congenial environment for the spread of the disease. The occurrence of large outbreaks of leptospirosis is usually observed following severe floods. Recently in India, an outbreak has been reported during the post cyclone investigation in Orrisa (WHO, 2000).

Many places in South India are known to be endemic for leptospirosis. These include Chennai and Madurai in the state of Tamil Nadu, Kolencherry and Kochi in the state of Kerala and some areas in the state of Karnataka.

Leptospirosis has been traditionally diagnosed by demonstration of leptospires in blood by dark field microscopy (DFM), isolation of leptospires in culture and retrospectively by the detection of specific antibodies by agglutination

Review of Literature

2. REVIEW OF LITERATURE

The syndrome Leptospirosis, with icterus and renal failure, was first reported over 100 years ago by Weil (1886). The term Weil's disease was first used by Goldsmidt (1887) to denote a severe febrile illness with jaundice and renal abnormalities. Leptospirosis is presumed to be the most wide spread zoonosis in the world (Turner, 1970), which affects wild and domestic animals and humans (Alexander, 1974).

Leptospiroris is a general term that denotes all infections of man and animals by spirochaetes of the genus Leptospira. The basic taxon of the leptospires is the serovar. The genus *Leptospira* was divided into two species, *Leptospira interrogans* comprising all pathogenic strains, and *L. biflexa*, containing the saprophytic strains (Johnson and Faine 1984) and this has been replaced by genotypic classification comprising a number of genospecies including all serovars of both *L. interrogans* and *L. biflexa* (Yasuda *et al.*, 1987).

2.1 INCIDENCE

Weil's disease was reported from many parts of the world (Jaeger, 1892, Chowdry, 1903). Inada *et al.* (1916) were the first to demonstrate these organisms in the liver tissue of a guineapig which had been infected with blood from a patient suffering from Weil's disease.

Stuart (1946) showed that forty per cent of the Glasgow house dogs have been infected with *L. canicola.* There are also several reports of leptospirosis among domestic and wild animals from various parts of the world (Keast *et al.* 1963; Michna, 1967; Alexander, 1974; Watson *et al.* 1976).

The incidence of the disease is significantly higher in warm climate countries than in temperate regions, mainly due to longer survival of leptospires in the warm and humid environmental conditions (Ratnam, 1994).

Leptospirosis is now identified as one of the emerging infectious diseases exemplified by recent large outbreaks in Southeast Asia, the United States (CDC, 1998), India (WHO, 2000) and most recently leptospirosis has been reported among athletes from several countries, who were participated in Eco Challenge Sabah 2000 competition in Malaysia (CDC, 2000).

2.2 PREVALENCE IN INDIA

Ayyar (1932) reported the incidence of canine leptospirosis in India, while studying an outbreak of leptospiral jaundice among dogs in Madras, which was the first record of leptospirosis in India.

Pargaonker (1957) detected leptospiral infection in 10.6 per cent of the rats from the city of Hyderabad. The organisms were demonstrated by direct smear examination stained with Fontana's method or sections stained with Lavaditi's techniques.

Adinarayanan *et al.* (1960) during their investigation, found that leptospirosis due to serotypes *L. sejroe, L. medanensis and L. sarekoebing* was responsible for a disease condition among cattle population in Uttar Pradesh.

The presence of leptospiral agglutinins in sheep and goats was reported earlier (Pargaonker1964; Tripathy 1977).

Adinarayanan and James (1980) in their three year study isolated 28 strains of leptospira, 14 from pig, eight from bandicoots, one each from aborted foetus of a sheep, a goat, a rat and a mongoose and one from a piggery effluent. Serogroups identified were Autumnalis, Hebdomadis, Javanica and Tarassovi. These were the first reported isolations from animals in India.

Srivastava *et al.* (1983) examined 965 sera samples from animals and man and reported 69 (7.2 per cent) samples were positive for leptospiral antibodies. He found leptospiral antibodies in 10.1 per cent of cattle sera, 9.7 per cent of horse sera, 6.0 per cent of sheep sera, 6.0 per cent of goat sera and 5.8 per cent buffalo sera.

Manickavel *et al.* (1991) were the first to report the incidence of leptospirosis among sheep in Tamil Nadu.They investigated an outbreak of jaundice, abortions and death among sheep flocks, in Tirunelveli District, and observed high agglutination titres on serological investigation against *L. interrogans* serovar *pomona* in sera of affected animals.

Natarajaseenivasan and Ratnam (1997) conducted a serosurvey in an agricultural village of south India and reported seroprevalence of 61.5 per cent, 56.3 per cent, 75 per cent, 52.9 per cent and 72.5 per cent among cattle, sheep, goat, field rats and in agricultural workers respectively.

Mrunalini and Ramasastry (2000) reported the seroprevalence of leptospirosis among cattle, buffaloe and sheep in organized farms in Andhra Pradesh .

Bharadwaj *et al.* (2002) reported an outbreak of leptospirosis during the rainy season in Mumbai, India. A seropositivity of 43.7 per cent by microagglutination test and 46.1 per cent by IgM enzyme-linked immunosorbent assay, were detected.

Natarajaseenivasan *et al.* (2002) reported a seroprevalence of 68.3 per cent, 52.9 per cent, 50.0 per cent, 66.6 per cent and 52.1 per cent among rice mill workers, cattle, dogs, cats and rats respectively for leptospirosis in Salem District, South India.

2.3 DISEASE IN ANIMALS

2.3.1 Disease in Cattle

Leptospirosis in cattle produces wide variety of disease manifestations including fever, inappetance, and flaccid udder, loss of milk production, abortion, haemoglobinuria, haemolytic anaemia, death and rarely icterus (Kenzy and Ringen, 1983).

Thiermann (1984) repoted clinical signs like pyrexia, anorexia, acute haemolytic anaemia, haemoglobinuria, jaundice, decreased milk production and red colouration of milk in adult cattle infected with leptospirosis.

2.3.2 Disease in Dogs

Ayyar (1932) had described the symptoms of leptospirosis as sudden off-feed, sanguineous discharge from the nostrils, conjunctivitis, passing of faeces with blood and death in Madras hounds. Icterus in mouth, lips, haemorrhages in the lips and under the subcutis in the region of sternum, were also recorded in few cases.

Kenzy *and* Ringen (1983) reported that in dogs the type of disease produced by each serotype was different. Serotype *canicola* produced uremia but very little or no jaundice, whereas serotype *icterohaemorrhagiae* produced the typical jaundice of Weil's disease.

Venkataraman and Nedunchelliyan (1990) reported a case of Stuttgart disease in a dog with a history of anorexia, vomiting and dysentery. Symptoms such as dullness, depression, dehydration, temperature of 39.8°C, ulcers in oral cavity and scanty urine were observed.

Brown *et al.* (1996) observed clinical signs like lethargy, anorexia, vomiting and abdominal pain in dogs infected with leptospires.

2.3.3 Disease in Swine

Ferguson and Power (1956) observed fever, joint stiffness, inappetance and abortion in sows experimentally infected with leptospires.

Infections in pigs were usually subclinical (or) asymptomatic, and caused abortion in late pregnancy. Occasionally metritis, icterus, anaemia, fever and meningoencephalitis (Cole, 1990) were also observed in pigs.

2.4 DISEASE IN HUMAN BEINGS

Clinical presentation of leptospirosis is biphasic with the acute or septicemic phase lasting about a week, followed by the immune response characterized by antibody production and excretion of leptospires in urine (Turner, 1967).

Faine (1982) indicated that Leptospirosis was often misdiagnosed as influenza, hepatic disease, or fever of unknown origin, as it caused a variety of clinical manifestations. The disease varied from subclinical infection to a severe illness with multi- organ involvement.

Majority of infections caused by leptospires are either subclinical or of very mild severity. Small proportion of infections, present a febrile illness of sudden onset. Other symptoms include chills, head-ache, myalgia, abdominal pain, conjunctival suffusion and less often a skin rash (Levett, 2001).

2.5 CARRIER STATUS

The role of rat as a source of human infection was discovered in 1917 (Ido *et al.*, 1917). Rodents have been shown to be the main carriers of pathogenic leptospires throughout the world and represent hazard for infection in humans and domestic animals (Twigg *et al.*, 1969).

Carrier state may be transient in accidental host, but in maintenance hosts leptospires may be present for years. Cattle, dogs, horses and pigs act as important reservoirs for man and they act as maintenance hosts as well as amplification hosts for rodent maintained leptospires (Penn and Pritchard, 1990).

2.6 DIAGNOSTIC METHODS

2.6.1 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.

Dark field microscopic examination of body fluids such as blood, urine, CSF and dialysate fluid have been used, but was both insensitive and lacking specificity. Approximately 10^4 leptospires/ml are necessary for one cell per field to be visible by dark field microscopy. Artifacts such as fibrin or protein threads, which may show Brownian motion may lead to misinterpretation in DFM of blood (Turner, 1970).

Presence of intact leptospires is necessary to get a positive result in DFM, which are often confused with proteinaceous filaments known as pseudo-leptospires (Rahman and Macis, 1979).

Hagan and Bruner (1988) reported the diagnosis of leptospiral infection by dark field microscopic examination of body or tissue fluids, especially, when the organisms were present in large numbers.

Chandrasekaran and Pankajalakshmi (1997) demonstrated leptospires with DFM, by differential centrifugation of blood and concluded that DFM was useful in the early diagnosis of leptospirosis.

Chandrasekaran *et al.* (1998) used DFM to show the etiology of leptospira in panuveitis cases and demonstrated leptospires in 82 per cent of the cases, of which 75 per cent were detected by low speed centrifugation and 7 per cent by high speed centrifugation.

Vijayachari *et al.* (2001) evaluated the diagnostic accuracy of dark ground microscopy (DGM) by comparing it with tests like culture, microscopic

agglutination test (MAT), IgM- ELISA and Lepto-Dipstick and concluded that DGM was an unreliable test as it showed low specificity and low sensitivity.

2.6.1.2 Fluorescent Antibody Test (FAT)

Hodges and Ekdahl (1973) used fluorescent antibody technique for the serological differentiation of leptospiral serotypes in cultures and in bovine urine.

Ellis *et al.* (1982) demonstrated leptospires by immunofluorescence in specimens like liver, lung, kidney, and cotyledon of aborted foetus. Direct fluorescent antibody technique, using incident-light microscopy, was found to be more successful in demonstrating foetal *hardjo* infection.

Bolin *et al.* (1989) demonstrated leptospires in urine samples by fluorescent antibody staining with fluorescein-labelled rabbit anti-*hardjo* serum. The FAT was more rapid than bacteriologic culture and FAT also depended on the structural and antigenic integrity of the organism and these factors might be compromised in clinical specimens.

Appassakij *et al.* (1995) tested sera from 175 patients with clinically suspected leptospirosis by the immunofluorescent antibody assay and compared with microscopic agglutination method.

2.6.1.3 Immunoperoxidase Test

Terpstra *et al.* (1983) demonstrated leptospires in blood and urine by immunoperoxidase staining method.

Ellis *et al.* (1983) described the development and testing of a peroxidase-labelled antibody method for the detection of leptospires in tissues and smears of infected fluids, that could detect leptospires in formalin fixed paraffin embedded kidney sections and found good correlation with cultural results.

Immunoperoxidase test was evaluated for the diagnosis of leptospirosis by demonstration of leptospires in blood and urine of experimentally infected bull calves (Koothan *et al.*, 1987).

2.6.2 Antigen Detection

When several methods were tested to detect leptospires in samples, radioimmunoassay (RIA) was the most sensitive method, followed by ELISA. Countercurrent immunoelectrophoresis and staphylococcal coagglutination were the least sensitive (Adler *et al.*, 1982).

Enzymatic radioimmunoassay (ERIA) was more sensitive than DFM, but less sensitive than culture when applied to porcine urine. Enzymatic radioimmunoassay, unlike the other two methods, was suitable for urine samples which had been stored frozen for several months (Chappel *et al.*, 1984).

Monoclonal antibodies (Mabs), specific to all members of the genus *Leptospira* and also specific to the pathogenic serovars, were produced for the detection of antigen in urine by the monoclonal antibody-based dot-ELISA..This test had high potential for rapid, sensitive and specific diagnosis of leptospirosis at low cost (Saengjaruk *et al.*, 2002).

2.6.3 Isolation of Leptospires

Murphy *et al.* (1958) attempted to recover leptospires from urine of 31 dogs that were serological reactors, by intraperitoneal inoculation of hamsters. Blood sample from the heart of hamsters collected on 4, 7, 11 and 28 day post inoculation were cultured on Fletcher's semisolid medium. *Leptospira pomona* was isolated from one dog, while attempts in other 30 dogs were unsuccessful.

Addition of two per cent pooled rabbit serum to semi-solid commercial EMJH medium with EMJH enrichment and 0.5 mg of 5-fluorouracil per ml was found to enhance the growth rate and success of isolation of *Leptospira interrogans* serovar *hardjo* from bovine urine (Te Brugge and Nicolette Louw, 1985).

Venugopal *et al.* (1990) isolated leptospires from a human case by inoculation of heparinized blood into different culture media (Korthof's,

Fletcher's and EMJH media) and by animal inoculation (Guinea pigs and hamsters). The isolate was identified as serogroup autumnalis by comparative microscopic agglutination test.

Venkataraman *et al.* (1994) demonstrated the presence of leptospires in 13 urine samples from dogs which were all seroreactors, only one isolation could be made by direct inoculation of the urine sample in EMJH semisolid medium.

Natarajaseenivasan and Ratnam (1997) isolated serovar *javanica* of serogroup Javanica from one field rat out of the attempts made in 34 field rats, urine samples of 30 normal cattle and 10 healthy human beings. Semisolid EMJH medium enriched with 2 per cent rabbit serum and containing 100µg per ml of 5-fluorourail as selective agent were used for the isolation.

Gangadhar and Rajashekar (1998) isolated leptospires from different species of rodents trapped from several human dwellings and animal habitats, by culturing their kidneys in EMJH semisolid medium. Out of 500 rodents examined leptospires were isolated only from 89 of them.

Natarajaseenivasan and Ratnam (2000) successfully isolated 12 leptospira isolates from various sources like, dead albino mice, dead Wistar rats, field and house rats, sheep and ailing human urine samples. Isolates from mice, Wistar rats, house rat, sheep and field rats were typed as serovar *javanica* of serogroup Javanica and 2 human isolates were identified as serovar *autumnalis* and *canicola*. EMJH semisolid medium with 2 per cent rabbit serum, 0.1 per cent sodium pyruvate and 100 µg per ml of 5-flurouracil as selective agent were used for isolation.

Petersen *et al.* (2001) reported the first isolation of *Leptospira fainei* serovar *hurstbride* from human patients and established the involvement of the new species *L. fainei* in human disease. Species was determined by serology, polymerase chain reaction and sequencing of 16S rDNA.

2.6.4 Serological Diagnosis

2.6.4.1 Microscopic Agglutination Test (MAT)

Microscopic agglutination test for the serodiagnosis of leptospirosis was first developed by Schuffner and Mochtar (1927).

Galton *et al.* (1965) developed a microtechnique for detecting leptospira antibodies in suspected sera using special microplates. The reactions were observed by DFM. The major disadvantage with this technique was that only agglutinated leptospires were visualized.

Although MAT remains as the reference test for the serological diagnosis of leptospirosis, it lacks sensitivity, consistency and could not differentiate titres of infection from vaccinal titres and could not identify most chronic carriers of leptospires as well (Thiermann, 1984).

Hathaway *et al.* (1986) reported that the MAT was the best test of choice when used as a screening test in investigating the possibility of *L. hardjo* infection in herds of cattle. At least 30 animals (or 10 per cent of large groups) should be bled and animals of various ages should be included.

The reference method for the serological diagnosis of leptospirosis is MAT, in which patient sera are reacted with live antigen suspensions of leptospiral serovars. After incubation, the serum antigen mixtures are examined microscopically for agglutination, and the titres are determined (Levett, 2001).

2.6.4.2 Macroscopic Agglutination Test

Galton *et al.* (1958) described a macroscopic agglutination test for the rapid screening of sera from humans and animals. Twelve serovars combined into four pools was used as antigen and the test was found to be as sensitive as the microscopic test.

Srivastava (1990) studied the efficacy of the slide agglutination test(SAT) for detecting leptospirosis in various animal species and man. He opined that

SAT alone might not be useful for detecting leptospirosis in cattle and sheep, as it poorly correlated with MAT. He concluded that low MAT titres and a strong SAT reaction in animals could be suggestive of a recent infection.

Brandao *et al.* (1998) evaluated a commercially available SAT by comparing it with an IgM-ELISA and the MAT for the diagnosis of human leptospirosis. Both the SAT and IgM-ELISA had a sensitivity and specificity of 99 per cent. The SAT and ELISA were convenient methods for rapid and early screening for leptospirosis. Slide agglutination test was inexpensive, easy and quick than ELISA, and could be used in the conventional laboratories.

2.6.4.3 Passive Haemagglutination Test

Chang *et al.* (1957) were the first to isolate an erythrocyte sensitizing substance that could be used for passive haemagglutination test for detecting leptospiral antibodies.

Sulzer and Jones (1973) described indirect haemagglutination test for the diagnosis of leptospirosis. Soluble antigen from serotype *patoc* was used to sensitize the sheep erythrocytes, which were then fixed with glutaraldehyde. They concluded that this test was more reliable than the conventional microscopic agglutination test and that the sensitized fixed cells could be stored for at least a year.

Srivastava *et al.* (1985) standardized an indirect haemagglutination (IHA) test for the detection of antibodies against leptospira organisms in different animal species. Indirect haemagglutination was found to be quite simple and sensitive.

Levett and Whittington (1998) evaluated a commercially available indirect haemagglutination assay (IHA) with multiple serum specimens from 107 patients for leptospirosis. Indirect haemagglutination detected both IgM and IgG classes of antibodies in human sera, the sensitivity and specificity of IHA for the detection of acute leptospirosis were 100 and 94 per cent respectively.

Investigation of serum specimens from 27 dogs showed concordance between IHA and an IgM ELISA. They concluded that the performance of IHA was simple, and required no specialized equipment.

Effler *et al.* (2000) evaluated indirect haemagglutination assay for diagnosis of acute leptospirosis in Hawaii. The sensitivity of the IHA was found to be low, particularly early in the course of illness, limiting its usefulness for diagnosing acute infection.

2.6.4.4 Latex Agglutination Test (LAT)

The latex agglutination test for the detection of leptospirosis had been described (Kelen and Labzoffsky, 1960).

A rapid semi-quantitative latex agglutination test (LAT) for the detection of leptospiral antibodies in serum samples of man and animals was standardized (Ramadass *et al.*, 1999). The efficacy of the LAT was compared with the plate assay. The rapidity, simplicity and economics of the LAT were found to fulfill the requirements of a screening test, for leptospiral antibodies.

Smits *et al.* (2000) evaluated a newly developed latex agglutination assay for the detection of genus-specific leptospiral antibodies in human sera. Stabilized dyed latex particles coated with a broadly reactive leptospiral antigen was utilized. The assay is easy to perform and the reagents have a long shelf-life, even at tropical temperatures; these factors together make the assay suitable as a rapid screening test for leptospirosis.

2.6.4.5 *Enzyme Linked Immunosobent Assay (ELISA)*

Adler *et al.* (1980) used ELISA for the detection of leptospira-specific IgM and IgG in the sera of patients infected with leptospires. The specificity and sensitivity of the test suggested that IgM-ELISA technique was a suitable method for detecting leptospiral antibodies in human sera for diagnosis and epidemiologic purposes.

Cousins *et al.* (1985) studied the leptospira-specific IgM and IgG antibody response in sera collected from cattle inoculated with live leptospires. Enzyme linked immunosorbent assay measuring IgM antibody was assessed as a serological technique to detect recent infection and they suggested that the IgM-ELISA might be useful as an aid to diagnose field outbreaks of leptospirosis in cattle.

Terpstra *et al.* (1985) used ELISA to detect specific IgM and IgG antibodies in sera from humans with current or past leptospirosis. The IgG titres were clearly higher with the homologous antigen in the past infections. The ELISA test, by the detection of characteristic patterns of IgM and IgG in the acute phase of the disease, makes an important contribution to the rapid diagnosis of human leptospirosis.

Mendoza and Prescott (1992) studied the axial filament ELISA (AF-ELISA) assay and whole cell ELISA for serodiagnosis of leptospirosis in swine. A sensitivity of 97.1 per cent and specificity of 93.1 per cent were observed for AF-ELISA in comparison to the microscopic agglutination test. The AF-ELISA was found to be a sensitive and specific test for the detection of antibodies against *L. interrogans* on a species rather than serovar level.

Gussenhoven *et al.* (1997) developed an easy to perform dipstick method for the detection of leptospira specific IgM antibodies in human serum samples and observed a high degree of concordance between the results of the dipstick assay and IgM-ELISA.

Winslow *et al.* (1997) assessed the ability of the commercially available Pan Bio leptospira IgM ELISA to diagnose early or recent leptospira infection by testing sera with known MAT titres. This test was sufficiently sensitive as an initial screen for leptospiral infections, followed by confirmation of positive results by MAT.

Sehgal *et al.* (1999) evaluated the lepto dipstick assay for detecting IgM antibodies and its adaptability in situations existing in developing and tropical countries. The lepto dipstick had a sensitivity of 78.7 per cent, and specificity of 88.3 per cent. This test was found to be valuable as a rapid screening test for the diagnosis of leptospirosis.

Smits *et al.* (1999) performed a multicentre evaluation of a dipstick assay for the detection of leptospira-specific immunoglobulin M (IgM) antibodies. The sensitivity of the dipstick assay increased from 60.1 per cent for acute phase serum samples to 87.4 per cent for convalescent phase samples. The specificities for these two groups of samples were 94.1 and 92.7 per cent respectively. The results were concordant with the results of ELISA for the detection of specific IgM antibodies.

Srivastava and Tiwari (1999) reported an IgG based Dot-ELISA for the diagnosis of leptospirosis in goats and found that Dot-ELISA could be a better replacement for MAT.

Matsuo *et al.* (2000) utilized exocellular mannan from *Rhodotorula glutinins* as an immuno reactive antigen in serological diagnosis of leptospirosis and found it to be useful as an antigen in ELISA as it specifically cross reacted with the anti-leptospiral antibodies.

The diagnostic utility of recombinant antigens in ELISA for serodiagnosis of leptospirosis was evaluated by Flannery *et al.*, 2001. The sensitivity of recombinant Lip 32 IgG- ELISA was found to be 56 and 94 per cent in acute and convalescent phases respectively. They concluded that recombinant Lip L32 could be a useful antigen for the serodiagnosis of leptospirosis.

Levett *et al.* (2001) evaluated the immunoglobulin M (IgM) dipstick assay and the indirect hemagglutination assay (IHA) for serological diagnosis of leptospirosis and compared the results with IgM- ELISA and MAT. The

sensitivity and specificity of the IgM-dipstick assay were found to be 98 and 90.6 per cent respectively.

Relative superiority of IgM + IgG combined conjugate ELISA for the detection of leptospiral antibodies was studied and indicated that this test could be well utilized in seroprevalence studies. (Natarajaseenivasan and Ratnam, 2001).

2.6.5 Molecular Diagnosis

2.6.5.1 *Primers*

Several primer pairs for PCR detection of leptospires have been described. Primers were designed from repetitive elements (Woodward *et al.*, 1991), or specific genes, most frequently from rRNA genes (Merien *et al.*, 1992; Gravekamp *et al.*, 1993; Kee *et al.*, 1994).

The primers developed by Merien *et al.*, and Gravekamp *et al.*, have been evaluated extensively by testing clinical samples (Merien *et al.*, 1995; Brown *et al.*, 1995).

2.6.5.2 *Polymerase Chain Reaction*

Van Eys *et al.* (1989) first developed PCR for the detection of leptospires in urine samples of infected cattle. Urine samples were investigated using PCR assay, culture isolation, dot and quick blot hybridization. This comparative study suggested that amplification by PCR might be a valuable method for the detection of leptospires in cattle urine.

Polymerase chain reaction specific for *Leptospira hardjo* genotype *bovis* was developed (Woodward *et al.*, 1991). In preliminary screening experiment 24 *Leptospira hardjo bovis* isolates, could be amplified.

Merien *et al.* (1992) developed a sensitive assay for the detection of leptospires, based on the amplification of the leptospira *rrs* (16S) gene by PCR,

and suggested that PCR assay could be used on biological samples such as CSF, urine, or blood as a diagnostic tool for cases of suspected leptospirosis.

Zhang and Dai (1992) developed a sensitive assay for diagnosis of leptospirosis, using PCR. Eight different serovars of *Leptopira interrogans* were detected, but not the DNAs from *L. biflexa*, Leptonema, virus and human.

Two sets of primers G1 and G2, derived from genomic DNA libraries of leptospiral serovar *icterohaemorrhagiae* (strain RGA), and B641 and B65II derived from *bim* (strain 1501), enabled the amplification of DNA from leptospiral reference strains belonging to the pathogenic leptospiral species. The practical value of PCR for early diagnosis of leptospirosis was shown, and the results were compared with those of culturing and concluded that PCR was more sensitive (Gravekamp *et al.*, 1993).

Merien *et al.* (1993) detected leptospiral DNA by PCR in aqueous humor of a patient with unilateral uveitis. They amplified a 331 bp fragment by PCR and confirmed the results by dot blot hybridization with a 289 bp digoxigenin-labeled probe internal to the amplified DNA fragment.

Bal *et al.* (1994) tested urine samples from patients at different stages of Leptospirosis, to determine whether the use of PCR for detection of leptospires in urine could be a valuable alternative to culturing. Leptospires were detected in approximately 90 per cent of urine samples, and they concluded that the detection of leptospires in urine with PCR was a promising approach for early diagnosis.

Kee *et al.* (1994) designed a pair of primers (LP1 and LP2) from a highly conserved EcoR1 fragment of *Leptospira interrogans* isolated in Korea. A 274 bp could be detected by PCR amplification of target DNA from cultured *L. interrogans* and also in DNA samples purified from 500 microliters of blood collected from experimentally infected gerbils.

Savio *et al.* (1994) developed an assay for the detection and identification of *Leptospira interrogans* serovars, based on PCR coupled with restriction enzyme analysis of amplified DNA fragment. They reported that a direct PCR on biological samples such as kidney samples demonstrated that preliminary isolations and culturing of leptospires were not required for efficient detection.

Brown *et al.* (1995) evaluated PCR for the early diagnosis of leptospires in clinical samples from patients with acute leptospiral infection. Blood and urine samples form 71 patients were examined by PCR, culture and serology. Polymerase chain reaction detected 44 cases (62 per cent) while culturing detected only 34 (48 per cent) of the cases. Polymerase chain reaction was a rapid, sensitive and specific means of diagnosing leptospiral infection, especially during the first few days of the disease.

In a study on 200 patients with various clinical syndromes compatible with leptospirosis, PCR was compared with culture and microagglutination test for diagnosis (Merien *et al.*, 1995). They reported that PCR was an efficient tool for diagnosis of Leptospirosis, especially during the first ten days of disease.

An IS 1533 based PCR assay was developed for identification of *Leptospira interrogans sensulato* serovars (Zuerner *et al.*, 1995). They concluded that this assay was selective enough to be used for typing leptospiral serovars from clinical material, and for attaining leptospiral typing without isolation of bacteria in pure culture.

Usefulness of PCR for rapid diagnosis of leptospirosis in animals and man was evaluated (Ramadass *et al.*, 1997).They observed 27 to 37 per cent positivity with dark field microscopy and 65 to 81 per cent positivity with PCR and indicated the PCR technique as a highly sensitive method as compared to DFM.

Magnetic immuno capture PCR (MIPA) for the detection of *Leptospira borgpetersenii* serovar *hardjo* was developed (Taylor *et al.*, 1997). Monoclonal

antibody based magnetic immunoseparation coupled with PCR amplification resulted in a marked improvement on previous detection methods.

Romero *et al.* (1998) detected leptospires in cerebrospinal fluid of patients with aseptic meningitis by PCR, and compared the results with those of microscopic agglutination test (MAT) and an enzyme-linked immunosorbent assay for detection of immunoglobulin M (ELISA-IgM). A positivity of 39.80, 8.74 and 3.88 per cent were observed by PCR, MAT and ELISA-IgM respectively.

Heinemann *et al.* (1999) compared the PCR, culture/isolation and serology to detect leptospiral infection in bovine semen. Eighty per cent of semen samples were found to be positive by PCR and leptospires could not be isolated from any of the samples examined. Polymerase chain reaction was found to be a method of great potential for the detection of leptospires.

Faber *et al.* (2000) detected Leptospira DNA in the aqueous humor of horses with naturally acquired recurrent uveitis, by polymerase chain reaction. Serological results did not correlate well with the presence of organism in the aqueous humor and indicated the PCR assay as a more reliable tool for detecting leptospirosis in equine recurrent uveitis.

Heinemann *et al.* (2000) applied PCR to 26 serovars of *Leptospira interrogans* to determine the detection threshold in semen samples and observed a threshold of 100 bacteria/ml in semen samples of bulls and concluded that PCR was a method of great potential for the detection of *Leptospira* spp. at bovine artificial insemination centres.

Senthilkumar *et al.* (2001) used PCR for the detection of leptospires in clinical samples like, blood, urine, CSF and milk and compared the results with those of DFM and concluded that PCR assay was highly sensitive and specific for detection of leptospira infection.

Venkatesha and Ramadass (2001) indicated that DNA based methods like slot blot hybridization and PCR were more rapid and sensitive in diagnosis of leptospirosis over conventional methods like isolation and culturing.

Harkin *et al.* (2003b) employed polymerase chain reaction as a diagnostic tool for leptospirosis in dogs and compared the results with those of microagglutination test. Positive PCR test results prior to seroconversion could be valuable in establishing an early diagnosis.

Harkin *et al.* (2003a) in their study on prevalence of urinary shedding of leptospires in dogs compared PCR assay, bacteriologic culture of urine and serological testing. Irrespective of the health status, urinary shedding of leptospires was observed in 8.2 per cent of dogs and concluded the serological testing as a poor predictor of urinary shedding.

2.7 RESTRICTION ENDONUCLEASE ANALYSIS (REA)

Restriction endonuclease analysis was the first molecular method used to differentiate serovars (Marshall *et al.*, 1981; Robinson *et al.*, 1982).

Savio *et al.* (1994) differentiated *L. interrogans* serovars and *L. borgpetersenii* serovars by restriction patterns of PCR products. The best polymorphic patterns were obtained by digestion with *Hinf* I and *Dde*I. The sizes of the digested fragments were the sum to that of the amplified band for most serovars.

Polymerase chain reaction – Restriction endonuclease analysis (PCR-REA), of the 285-bp product generated by G1/G2 primed amplification, yielded seven patterns among 29 serovars of pathogenic leptospira. A reasonable agreement between PCR-REA profile and species could be observed (Brown and Levett, 1997).

The DNA restriction enzyme analysis was used for typing of leptospiral serovars. Clear differentiations were obtained between different serovars with the

enzyme *Hae* III and leptospiral restriction enzyme analysis using enzyme *Hae* III could be an effective method for identifying various serovars (Senthilkumar *et al.*, 1997).

Heinemann *et al.* (2000) evaluated the polymerase chain reaction and restriction fragment length polymorphism for the possibility of differentiation among serovars, using 19 restriction endonucleases. They concluded that the PCR-RFLP strategies might represent a useful tool for the rapid detection and differentiation of leptospira spp. serovars.

2.8 SEQUENCING

The identity of the polymerase chain reaction products may be confirmed more specifically as target DNA by restriction enzyme digestion or direct sequencing of the fragment (Murphy *et al.*, 1999).

Fukunaga *et al.* (1990) reported the nucleotide sequence of a 16S rRNA gene for *Leptospira interrogans* serovar *canicola* strain Moulton.

A broad range bacterial PCR targeting rRNA genes (rDNAs) was used to directly analyse clinical samples form patients. The molecular diagnosis based on DNA sequencing of the PCR product was compared to that obtained by bacterial culture. Molecular approach was proved to be superior to bacterial culture in two clinical situations: infection caused by bacteria with unusual growth requirements and specimens taken during antimicrobial treatment of the patient (Rantakokko-Jalava *et al.*, 2000).

Materials and Methods

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacterial Cultures

3.1.1a Reference Strains of Leptospires

The following reference strains of leptospires representing 12 different serogroups were procured from National Leptospirosis Reference Centre, Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands, India.

	Serogroup	Serovar	Strain
1.	Australis	*australis*	Ballico
2.	Autumnalis	*rachmati*	Rachmati
3.	Canicola	*canicola*	Hond Utrecht IV
4.	Grippotyphosa	*grippotyphosa*	Moskva V
5.	Hebdomadis	*hebdomadis*	Hebdomadis
6.	Icterohaemorrhagiae	*icterohaemorrhagiae*	RGA
7.	Javanica	*poi*	Poi
8.	Pomona	*pomona*	Pomona
9.	Pyrogenes	*pyrogenes*	Salinem
10.	Sejroe	*hardjo*	Hardjoprajitno
11.	Tarassovi	*tarassovi*	Peripeletsin
12.	Semerenga	*patoc*	Patoc1

3.1.1b Other Bacterial Strains

The following bacterial strains maintained in the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, were used in the present study.

(i) *Pasteurella multocida,*

(ii) *Klebsiella pneumoniae,*

(iii) *Escherichia coli,*

(iv) *Steptococcus zooepidemicus,*

(v) *Pseudomonas aeruginosa.*

3.1.2 Media

3.1.2.1 Fletcher's Medium

Fletcher's leptospira medium base was prepared by autoclaving at 121°C for 15 min, at 15 lbs pressure. After cooling the base medium was added with heat inactivated rabbit serum to 10 per cent level. The medium was dispensed in three to five milliliter quantities in screw-capped tubes. The tubes were checked for sterility by incubating at 37 °C for about 48 h and then stored at 4°C.

3.1.2.2 Ellinghausen McCullough Johnson Harris (EMJH) Medium

The EMJH medium (Johnson and Harris, 1967) was employed for the growth and maintenance of the leptospires and is a modification of Tween 80 albumin medium of Ellinghausen and Mc Coullough (1965).

3.1.2.2a EMJH Base

The EMJH base medium was procured from DIFCO laboratories (USA) for culture work.

3.1.2.2b EMJH Albumin Supplement

The following stock solutions were prepared in distilled water (expressed in grams per 100 ml)

Zinc sulphate $7H_2O$	0.1
Calcium chloride $2H_2O$	1.0
Magnesium chloride $6H_2O$	1.0
Ferrous sulphate $7H_2O$	0.5
Copper sulphate $5H_2O$	0.3
Tween 80	10.0
Vitamin B_{12}	0.02

The supplement was prepared by adding 20 g of Bovine serum albumin, fraction V in 100 ml of distilled water. The following quantities of stock solutions were added slowly to the albumin solution while it was being stirred.

Zinc sulphate	2.0 ml
Calcium chloride	2.0 ml
Magnesium chloride	2.0 ml
Ferrous sulphate	20. 0 ml
Copper sulphate	0.2 ml
Tween 80	25.0 ml
Vitamin B_{12}	2.0 ml

The pH of the albumin solution was adjusted to 7.4 using 1N NaOH and the final volume made upto 200 ml with distilled water and then sterilized by filtration (0.2 µm).

3.1.2.2c EMJH Liquid Medium

The EMJH liquid medium was prepared by dissolving 2.3 g of EMJH base in 900 ml of triple glass distilled water. pH was adjusted to 7.5 using 1N NaOH. After sterilization by autoclaving at 121°C for 15 min at 15 lbs pressure, the base medium was added with the EMJH albumin supplement at 10 per cent level with sterile precautions. Medium was dispensed in three to five milliliter quantities in screw-capped tubes. The tubes were checked for sterility by incubating at 37 °C for about 48 h and then stored at 4°C.

3.1.2.2d EMJH Semisolid Medium

EMJH semisolid medium was prepared by adding 1.5 per cent bacteriological agar to EMJH base medium and sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure, to that added EMJH albumin supplement at 10 per cent level with sterile precautions. The medium was distributed in three to five milliliter quantities in screw-capped tubes. Sterility of the medium was tested by incubating the medium for 48h at 37°C, and then stored at 4°C.

3.1.2.2e Medium for Isolation

Fletcher's medium and/or EMJH semisolid medium with 5-Fluorouracil at a concentration of 100µg per ml was used for isolation.

3.1.3 Buffers and Reagents

3.1.3.1 Phosphate Buffered saline (PBS) Stock Solution (10x)

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 by 1N NaOH and sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure. The stock solution was diluted to 1x before use.

3.1.3.2 ***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 at 15 lbs pressure.

3.1.3.3 ***EDTA Stock Solution*** **(0.5 M) pH 8.0**

Sodium EDTA. $2H_2O$	186.1 g
Distilled water	800 ml

The pH was adjusted to 8.0 with 10N NaOH. Distilled water was added to make up the volume to one liter. The solution was sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure.

3.1.3.4 ***Tris Borate EDTA Buffer (TBE)*** **pH 8.2**

Stock solution (10x)

Tris base	108.0 g
Boric acid	55.0 g
EDTA (0.5 M, pH 8.0)	40 ml

Triple distilled water to make 1 litre.

The stock solution was diluted to 1x before use.

3.1.3.5 *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°C.

3.1.3.6 *Gel Loading Buffer* (6x)

Bromophenol blue	0.25 per cent
Xylene cyanol	0.25 per cent
Sucrose	40 per cent (w/v) in water

3.1.3.7 *Chloroform: Isoamyl Alcohol Mixture*

Chloroform and isoamyl alcohol mixture was prepared in the ratio of 24:1 (v/v).

3.1.3.8 Sodium *Acetate* (3 M) (pH 4.8)

Sodium acetate	408.0 g
Triple distilled water	800.0 ml

The pH was adjusted to 4.8 with glacial acetic acid. Autoclaved at 121°C for 15 min at 15 lbs pressure and stored at 4°C.

3.1.3.9 *Alcohol*, 70 per cent

Absolute alcohol 70 ml was added with 30 ml of triple glass distilled water, and stored at 4°C.

3.1.3.10 *Reagents for PCR*

3.1.3.10a *Primers*

Two sets of primers were used in the present study. The primers were selected from published reports (Merien *et al.*, 1992 and Gravekamp *et al.*, 1993) and were custom synthesized (Alpha DNA, Canada).

The sequences of the primers were as follows.

First set, A, 5'-GGC GGC GCG TCT TAA ACA TG-3'

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

(Merien *et al.*, 1992)

Second set, G1, 5'-CTG AAT CGC TGT ATA AAA GT – 3'

G2, 5'-GGA AAA CAA ATG GTC GGA AG – 3'

(Gravekamp *et al.*, 1993)

3.1.3.10b PCR Reaction Buffer (10x)

This includes 500 mM KCl, and 100 mM Tris hydrochloride.

3.1.3.10c *Magnesium Chloride*

Magnesium chloride with strength of 25mM.

3.1.3.10d *Taq DNA Polymerase*

The *Taq* DNA polymerase enzyme with a concentration of 3 U/µl

3.1.3.10e *Deoxy Nucleotide Triphosphates*

Deoxy nucleotide triphosphates (dNTPmix) 10 mM (2.5 mM of each dGTP/dCTP/dATP/dTTP)

Urine samples from the dogs were collected by catheterization. In case of humans and bovines midstream urine was collected. About five to ten milliliter of urine samples were collected in vials containing equal volume of sterile PBS (3.1.3.1).

Bandicoots and rats caught from different places were anesthetized and opened; urine was collected from the bladder and used for DFM and PCR. The kidney was exposed, capsule removed carefully, then tiny portions of kidney tissues were collected with finely drawn out sterile Pasteur pipette and used for culturing.

A total of 192 samples were collected, this includes 107 blood and three urine samples from human beings, 22 blood and five urine samples from dogs, 30 bovine urine samples from suspected cases of leptospirosis and 25 rodents samples.

3.2.3 Dark Field Microscopy (DFM)

3.2.3.1 *Blood*

Two milliliter of blood samples collected in buffered anticoagulant (3.1.3.2) was processed immediately for microscopic examination. The blood was centrifuged at 1000 x g for 15 min, and the plasma was separated. A drop of plasma was placed on a clean, grease free glass slide and applied a cover slip (18mm square). This wet mount preparation was examined under low (10x) and high (45x) power objective of the dark field microscope. Utmost care was taken to examine as many microscopic fields as possible with a minimum of 100 high power fields. The plasma was centrifuged again at 3000 x g for 20 min, the supernatant was discarded and then the sediment re-examined in the same way.

3.2.3.2 *Urine*

Five to six milliliter of urine samples collected with equal quantity of sterile PBS (3.1.3.1) were immediately centrifuged at 3000 x g for 10 min. A

drop of sediment was placed on a clean, grease free glass slide, and applied a cover slip (18mm square). The slide was examined under low (10x) and high (45x) power objective of the dark field microscope to demonstrate the presence of leptospires. While examining utmost care was taken to observe as many microscopic fields as possible with a minimum of 100 high power fields.

3.2.4 Culturing of the Clinical Samples

Blood and urine samples were cultured on Fletcher's and/or EMJH semisolid media containing 5-Fluorouracil.

3.2.4.1 *Blood*

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

3.2.4.2 Urine

A drop of sediment obtained after centrifugation of the urine at 3000 x g for 10 min was inoculated into culture media with aseptic precautions, mixed thoroughly and then inoculated into two more tubes as described for blood.

3.2.4.3 *From Rodents*

The method described by Cruickshank *et al.* (1975) was followed for the isolation of leptospires from rodents. The tiny portions of kidney tissues collected with finely drawn out sterile Pasteur pipette was inoculated into culture medium with aseptic precautions, mixed properly, and then inoculated into two more tubes as described for blood.

All the inoculated culture tubes were incubated at 37°C for 24 h and then at 28-30°C for a period of two to three months. All the inoculated tubes were examined at weekly intervals for the presence of leptospires by dark field microscopy. The tubes showing contamination were discarded and those showing growth of leptospires were subcultured into fresh medium.

3.2.5 Polymerase Chain Reaction (PCR)

3.2.5.1 *Preparation of Samples for PCR Analysis*

3.2.5.1a *Reference Strains of Leptospires*

In case of reference strains of leptospires, a loopful of culture was added to an Eppendorf tube containing 1 ml of PBS (3.1.3.1). 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 10 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 and used for PCR reaction.

3.2.5.1b Other Bacterial Strains

To determine the specificity of the primers A, B and G1, G2 they were tested to amplify the DNA from other bacterial strains (3.1.1b) The DNA was prepared as described above for reference strains of leptospires.

3.2.5.1c *Reconstitution Experiments with Biological Samples*

Artificial inoculation of biological samples with leptospires was performed, to evaluate the method of sample preparation for PCR

A loop full of culture was mixed with one milliliter of anticoagulant added whole blood from healthy animals. The tubes containing one milliliter of blood were centrifuged at 1000 x g for 15 min at room temperature, and 0.5 ml of plasma was removed. After centrifugation of the plasma at 13,000 x g *for 10 min* at 4°C, the pellets were washed twice in sterile PBS and suspended in 10 µl of

sterile triple distilled water. The sample in Eppendorf tubes were placed in a boiling water bath for 10 min and immediately kept on ice and then used as template DNA for PCR reaction.

One milliliter of urine from healthy animal was mixed with a loop full of culture and tested by PCR. These samples were first centrifuged at 1000 x g for 10 min to eliminate large particles (bladder cells, urinary crystals) and then washed. The supernatant was then treated as described for blood.

3.2.5.1d ***Blood and Urine Samples***

Blood and urine samples collected from the presented cases were processed to prepare DNA.

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 13,000 x g for 10 min at 4°C and the rest of the procedures were same as for reconstitution experiments.

One milliliter of urine was first centrifuged at 1000 x g for 10 min to eliminate larger particles (bladder cells, Urinary crystals). The supernatant was centrifuged at 13,000 x g for 10 min at 4^0C and the rest of the procedures were same as for reconstitution experiments.

3.2.5.2 ***Reconstitution and Dilution of Primers***

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

3.2.5.3 *Setting up of PCR (Test proper)*

The PCR reaction was carried out with two different primer sets as per the methods described by Merien *et al.* (1992) and Gravekamp *et al.* (1993).

PCR was performed in a total volume of 25 µl reaction. A master mix was prepared before setting up the PCR reaction by combining the following reagents in 20µl volume.

PCR reaction buffer	50 mM KCl, 10 mM Tris hydrochloride
Primers	20 pM of each primer
dNTPs	200 µM of each dNTP
$MgCl_2$	2mM
Taq polymerse	one unit

Preparation of 200 µl of mastermix was as follows,

Reagents	Quantity
PCR reaction buffer (10x)	25µl
Forward primer	10µl
Reverse primer	10µl
dNTP mix	20µl
$MgCl_2$	20µl
Taq polymerase	3.3µl
Triple distilled water to make	200µl

To each PCR tube 20µl of master mix and five µl of template DNA were added. One negative control without template DNA was included to monitor contamination, if any. The tubes were spun briefly and placed in the thermal cycler.

The programme of amplification were as follows.

	Primer A and B		Primer G1 and G2
	First cycle	Next 29 cycles	
Denaturation	94°C for 3 min	94°C for 1 min	94°C for 1.5 min
Annealing	63°C for 1.5 min	63°C for 1.5 min	55°C for 1 min
Extension	72°C for 2 min	72°C for 2 min	72°C for 2 min
		Final extension of 72°C for 10 min	
Number of cycles	30		32

Amplification of DNA was performed in Eppendorf mastercycler gradient.

3.2.5.4 *Detection of PCR Products*

3.2.5.4a *Submarine Agarose Gel Electrophoresis*

The PCR products were detected by electrophoresis in a 1.5 per cent agarose gel in TBE buffer (1x) (3.1.3.4). Agarose was dissolved in TBE buffer (1x) by heating and cooled to 50°C. To this ethidium bromide was added to a final concentration of 0.5 µg/ml.

The clean, dry, gel platform edges were sealed with adhesive tape and the comb was kept in proper position before pouring agarose. Once the gel was set,

the comb and adhesive tape were removed gently and placed the gel tray in the buffer tank. Poured TBE buffer (1x) till it covered the top of the gel.

The PCR product (5 µl) was mixed with one microlitre of 6x gel loading buffer (3.1.3.6) and the samples were loaded into the respective slots carefully. The pUC 19 DNA/*Msp*1 digest was used as DNA molecular size marker (3.1.2.12).

Electrophoresis was carried out at 5 V/cm for one hour (or) until the bromophenol blue migrated more than two-third of the length of the gel.

3.2.5.4b *Recording of the Results*

The gel was visualized under UV transilluminator (Hoefer, USA) and the results were documented in a gel documentation system (Bio-rad laboratories, USA).

3.2.6 Analysis of PCR Amplified Product

3.2.6.1 *Restriction Enzyme Analysis of PCR products*

For restriction enzyme analysis the PCR products were digested with the restriction enzymes according to the manufacturer's instructions. Restriction digestion of PCR products was carried out in a total volume of 20 µl. The following components were added to sterile microfuge tubes.

PCR product	10 µl
RE buffer (10x)	2 µl
RE	1 µl (10 U)
Sterile distilled water to make	20 µl

The restriction endonucleases, *Dde* I and *Mnl* I (3.1.3.11) were used with the appropriate enzyme buffer supplied by the manufacturer. The digestion

mixture was incubated at 37°C for 2h. Heating at 65°C for 20 min inactivated the restriction enzyme activity

3.2.6.1a *Separation of Restriction Fragments*

The restriction enzyme digested PCR products were separated by submarine agarose gel electrophoresis in 2 per cent agarose gels in TBE buffer (1x) (3.1.3.4) stained with ethidium bromide at a final concentration of 0.5 µg/ml. The pUC 19 DNA digested with *Msp*I was used as DNA molecular size marker (3.1.3.12). Samples were loaded in the wells after the addition of gel loading buffer (3.1.3.6), electrophoresed at 5V/cm till the bremophenol blue migrated more than two third length of the gel.

At the end of electrophoresis, the gel was visualized under UV transilluminator (Hoefer, USA) and the results were documented in a gel documentation system (Bio-rad laboratories, USA).

3.2.6.2 Sequence Determination and Analysis

3.2.6.2a Purification of PCR Product

Electrophoresis

The PCR products were electrophoresed in 1.5 per cent agarose gel as described for detection of PCR products. The gel was transferred to the UV transilluminator and the band was visualized under preparative UV source. Using a sharp scalpel (or) razor blade, the agarose containing the band of interest was cut with minimum amount of free agarose in the slice.

Elution

A piece of dialysis tubing about seven centimeter was taken and one end was sealed. Gel slice was pushed through the open end down to the sealed end of the dialysis tubing. About 300-500 µl of TBE buffer (3.1.3.4) was added to the dialysis tubing so that the gel slice was completely immersed without any bubbles

and the other end of the dialysis tubing was then sealed. The dialysis tubing containing the agarose slice was immersed in a shallow layer of TBE buffer in an electrophoresis tank. Electroelution was carried out at 4-5 V/cm for two to three hours. During this time the DNA was electroeluted out of the gel into the inner wall of the bag.

The polarity of the current was reversed for one minute to release the DNA from the wall of the bag. After the end of electroelution the bag was opened and the entire buffer surrounding the gel slice was carefully transferred to sterile Eppendorf tube.

DNA cleaning and precipitation

The Eppendorf tube containing the buffer was added with equal volume of chloroform: isoamylalcohol mixture (24:1) [3.1.3.7] and was spun at 12,500 x g for about three min at 4°C. Chloroform: isoamylalcohol will sink and the DNA will be in the aqueous phase. The top aqueous phase, leaving behind any debris at the interphase was transferred to a sterile Eppendorf tube.

Added 1/10 volume of 3 M sodium acetate (pH 4.8) [3.1.3.8] and twice the volume of ethanol [3.1.3.9] to the tube containing the aqueous phase and placed at –20°C for about 15 min. Spun at 12,500 x g for about 15 min at 4°C. The supernatant was decanted and added 500 µl of 70 per cent ethanol (ice cold) [3.1.3.9] was added to the pellet. The tube was spun at 14,000 rpm for 2 min. Supernatant was decanted. The DNA pellet was completely dried and dissolved in sterile triple glass distilled water. This purified PCR product was then directly used for sequencing.

3.2.6.2b Sequencing of PCR Product

The purified PCR product was directly sequenced by dideoxy-chain termination method using ABI PRISM Model 310 version 3.4.1. The primers A and B were used as sequencing primers. Sequencing was carried out at School of Biotechnology, Madurai Kamaraj University, Madurai, Tamil Nadu.

Sequence similarity search was performed using Basic Local Alignment Search Tool (BLAST) network provided by the National Centre for Biotechnology Information (NCBI).

Results

4. RESULTS

4.1 MAINTENANCE OF REFERENCE STRAINS

Fletcher's medium supplemented with 10 per cent rabbit serum and EMJH medium with EMJH albumin supplement could support the growth and maintenance of all the 12 reference strains of leptospires in the laboratory when incubated at 28-30^{0}C. Seven to 10 day-old cultures revealed the growth of leptospires and were recognized by the formation of sub-surface ring in the medium and these cultures provided the template DNA for polymerase chain reaction.

4.2. AMPLIFICATION OF LEPTOSPIRAL DNA BY PCR

4.2.1. Amplification with Primers A and B

The 12 reference strains maintained in the laboratory provided the template DNA for PCR amplification with primers A and B. Thirty cycles of A and B primed amplification produced the PCR product from all the reference strains.

Agarose gel (1.5 per cent) electrophoresis of the amplified PCR product was carried out along with a negative control and a molecular size marker in 1x TBE buffer. Analysis of the electrophoresed gel under UV transilluminator revealed the presence of a 331 base pair (bp) band in all the 12 lanes (reference strains of leptospires) in comparison with pUC19 DNA/*Msp*I digest DNA molecular size marker. In the negative control no amplification product was detected. The amplified PCR products are shown in Fig. 1.

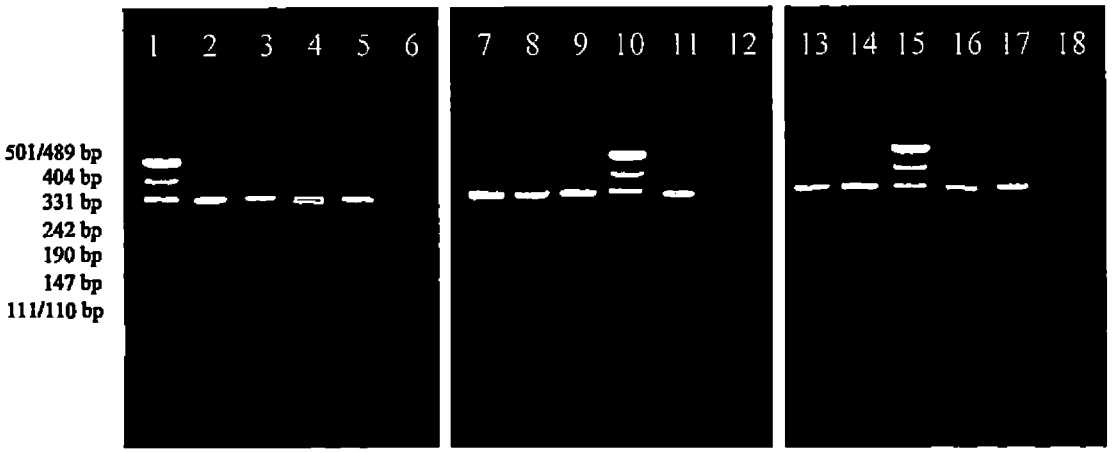

Fig.1. Agarose gel (1.5 per cent) electrophoresis of primer A, B amplified PCR product of leptospira reference serovars

Lane 1, 10, 15	: pUC 19 DNA/*Msp* I digest	Lane 8	: *icterohaemorrhagiae*
Lane 2	: *australis*	Lane 9	: *poi*
Lane 3	: *rachamti*	Lane 11	: *pomona*
Lane 4	: *canicola*	Lane 13	: *pyrogenes*
Lane 5	: *grippotyphosa*	Lane 14	: *sejroe*
Lane 6, 12, 18	: Negative control	Lane 16	: *tarassovi*
Lane 7	: *hebdomadis*	Lane 17	: *patoc*

4.2.2 Amplification with Primers G1 and G2

All the 12 reference strains provided the DNA for PCR amplification with the primers G1 and G2. In 32 cycles of amplification with the G1 and G2 primers an amplified product was observed in all the serovars except serovar *patoc*. In the reaction mix containing G1 and G2 primers the amplified product had a size of 285 base pair on comparison with pUC 19 DNA/*Msp* I digest molecular size marker. No amplification product was observed in the negative control reaction (Fig. 2.).

4.2.3 Specificity of the Primers

No amplification product was detected when the primers A and B were used to amplify the DNA prepared from *Pasteurella multocida, Klebsiella pneumoniae, Streptococcus zooepidemicus, Pseudomonas aeruginosa,* and *E.coli.*

The primers G1 and G2 have not amplified the template DNA prepared from *Pasteurella multocida, Streptococcus zooepidemicus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* However, primers G1 and G2 showed some non-specific amplification, in the form of several different sized fragments with DNA prepared from *E. coli.*

Based on the above findings the primers A and B were selected for the amplification of leptospiral DNA in clinical samples.

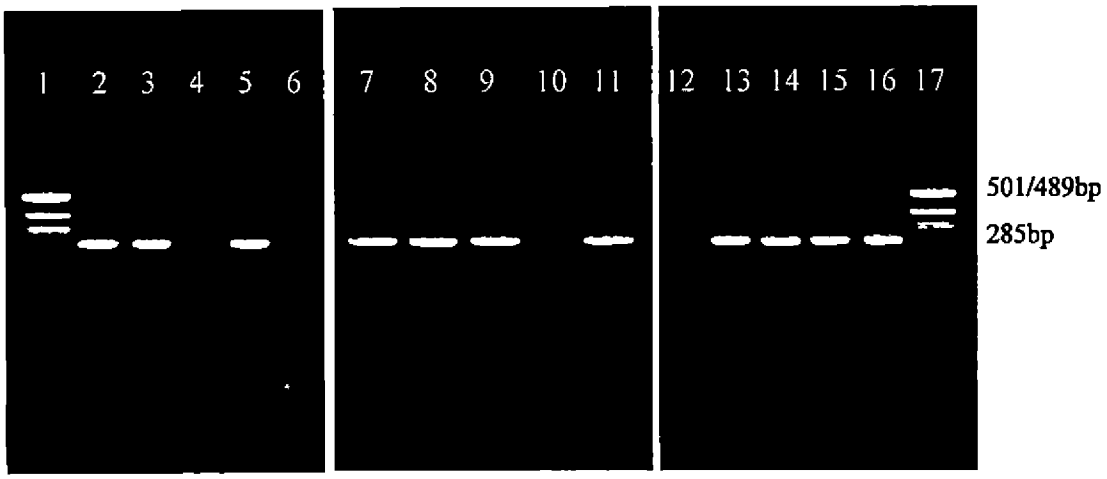

Fig. 2. Agarose gel (1.5 per cent) electrophoresis of primer G1, G2 amplified PCR product of leptospira reference serovars

Lane 1, 17	: pUC 19 DNA/*Msp* I digest	Lane 9	: *icterohaemorrhagiae*
Lane 2	: *australis*	Lane 11	: *poi*
Lane 3	: *rachamti*	Lane 12	: *patoc*
Lane 4, 6, 10	: Negative control	Lane 13	: *pomona*
Lane 5	: *canicola*	Lane 14	: *pyrogenes*
Lane 7	: *grippotyphosa*	Lane 15	: *sejroe*
Lane 8	: *hebdomadis*	Lane 16	: *tarrasovi*

4.2.4 Reconstitution Experiments with Biological Samples

Blood and urine samples seeded with laboratory maintained cultures of leptospires were used in reconstitution experiments. DNA prepared from these samples provided the template DNA for PCR amplification. With primers A and B specific amplification product of 331 bp was observed in both the cases.

4.3 ANALYSIS OF THE AMPLIFIED PCR PRODUCT

4.3.1 Restriction Enzyme Analysis

The 331bp sized PCR products were readily digested with the restriction enzymes, *Dde* I and *MnI* I and they produced clear restriction fragments. Electrophoresis of the restricted DNA was carried out on an agarose gel (1.5 per cent) along with an undigested amplified product and molecular size marker, in 1x TBE buffer. Analysis of the fragments under UV transilluminator revealed fragments of sizes 159, 61, 43 and 41 base pairs in *Dde* I digest (Fig. 3.). The enzyme *MnI* I produced fragments of sizes 124, 84 and 49 base pairs (Fig. 4.).

4.3.2 Sequencing

Electroelution of the amplified product provided the purified PCR product which was then used for sequencing. Electrophoresis of the purified PCR product revealed only the 331 base pair band and no other non-specific bands or oligonucleotide primers could be observed. The dideoxy-chain termination method of sequencing produced the sequence of amplified 331base pair product (Fig. 5.).

In sequence similarity searches performed with Basic Local Alignment Search Tool (BLAST) provided by the National Centre for Biotechnology Information (NCBI), the sequence was found to have 100 per cent identity with *Leptospira interrogans* 16S ribosomal RNA gene.

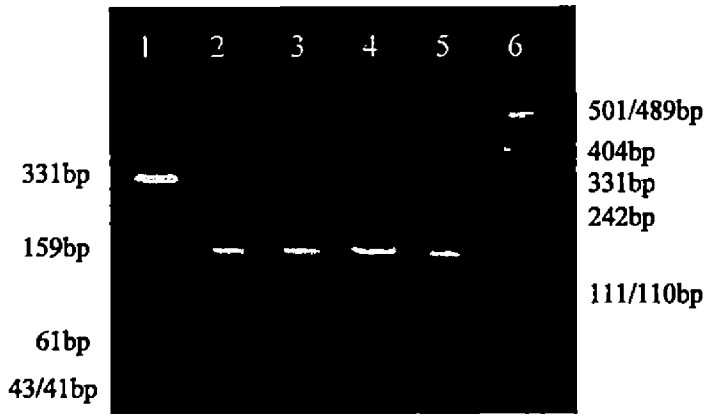

Fig. 3. Agarose gel (2 per cent) electrophoresis of *Dde* I digested PCR product (primer A, B)

Lane 1 : Undigested amplified product
Lane 2, 3, 4, 5 : Restriction fragments of amplified product
Lane 6 : pUC 19 DNA/*Msp* I digest

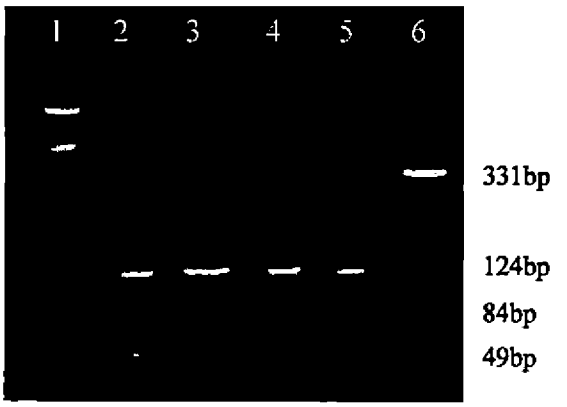

Fig. 4. Agarose gel (2 per cent) electrophoresis of *Mnl* I digested PCR product (primer A, B)

Lane 1 : pUC 19 DNA/ *Msp* I digest
Lane 2, 3, 4, 5 : Restriction fragments of amplified product
Lane 6 : Undigested amplified product

Fig.5. Sequence of primer A and B amplified PCR product

```
  1 ggcggcgcgt cttaaacatg caagtcaagc ggagtagcaa tactcagcgg cgaacgggtg
 61 agtaacacgt gggtaatctt cctctgagtc tgggataact ttccgaaagg gaagctaata
121 ctggatggtc ccgagagatc acaagatttt tcgggtaaag atttattgct cggagatgag
181 cccgcgtccg attagctagt tggtgaggta aaggctcacc aaggcgacga tcggtagccg
241 gcctgagagg gtgttcggcc acaatggaac tgagacacgg tccatactcc tacgggaggc
301 agcagttaag aatcttgctc aatgggggga a 331
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4.4 DARK FIELD MICROSCOPY

Dark field microscopic examination in clinical samples of blood and urine collected from human beings, dogs and rodents could detect the presence of leptospires. The leptospires in the clinical samples were identified by their characteristic motility and structure. The number of samples tested and the results are presented in the Table.1

A total of 125 samples were examined. These include 40 blood and 3 urine samples from human cases, 27 samples (22 blood and 5 urine) from dogs, 30 urine samples from bovines and 25 urine samples from rodents.

Of the 40 blood samples from humans 19 (47.50 per cent) were found to contain leptospires. No leptospires could be demonstrated in any of the human urine samples examined.

Dark field microscopy demonstrated the presence of leptospires in 5 (22.72 per cent) blood samples and one (20 per cent) urine sample among the samples collected from dogs. Of the 30 urine samples collected from bovines, none were found to be positive by DFM. In the rodents, out of the 25 urine samples examined 7 (28 per cent) were found to be positive.

4.5 CULTURE

The growth of leptospires in the culture tubes inoculated with blood or urine from different sources like human beings, dogs and bovines and kidney tissues from rodents was checked by DFM at weekly intervals.

Inoculated tubes were found to be contaminated mostly between third and fifth day of incubation. On dark field examination, contaminants like cocci and bacilli were detected and those tubes were discarded. Contamination was more frequent with samples of urine, compared to those of blood.

Culture tubes without any contamination were examined routinely at weekly intervals for a period of two to three months for the growth of leptospires, before confirming as negative. The tubes in which growth of leptospires was detected were subsequently subcultured and maintained. One of the culture tubes that was inoculated with kidney tissues from a bandicoot, on dark field examination, showed the presence of leptospira like organisms along with other bacteria, but on subsequent subculturing no leptospires could be obtained.

Attempts were made to isolate leptospires from a total of 125 samples, which included 40 blood and 3 urine samples from human cases, 27 samples (22 blood and 5 urine) from dogs, 30 urine samples from bovines, and 25 kidney tissues from rodents (10 bandicoots and 15 rats).

Leptospiral growth could be obtained from only three (12 per cent) samples among the 25 kidney tissues cultured from rodents. These included two from rats and one from bandicoot. Subsurface ring was observed in the tube inoculated with sample from bandicoot by 10^{th} day, and on 7^{th} and 10^{th} day in the tubes inoculated with samples from two rats. Attempts to isolate leptospires from human beings, dogs and bovine samples were unsuccessful.

Table 2. Results of clinical samples cultured

Sl. No.	Sample	Culture		
		No. tested	No. positive	% positive
I	Human			
(i)	Blood	40	-	-
(ii)	Urine	3	-	-
II	Dogs			
(i)	Blood	22	-	-
(ii)	Urine	5	-	-
III	Bovines			
(i)	Urine	30	-	-
IV	Rodents			
(i)	Kidney tissues	25	3	12

4.6 POLYMERASE CHAIN REACTION

The blood and urine samples from the presented cases were processed and amplified with primers A and B. Presence of leptospiral DNA in the clinical samples was observed by the amplification of a 331bp fragment. The negative control kept along with the samples during each amplification had not produced any amplification (Fig. 6.). The number of samples tested and the results are presented in the Table 3.

Fifty eight (54.2 per cent) blood and one (33.3 per cent) urine sample collected from human cases was found to be positive by PCR. Among the 27 samples from dogs subjected to PCR, amplification of leptospiral DNA was observed for eight (36.36 per cent) blood and two (40 per cent) urine samples.

None of urine samples from bovines tested was found to be positive by PCR. The PCR demonstrated presence of leptospiral DNA in 12 (48 per cent) of 25 rodents' urine samples tested.

4.7 COMPARISON OF PCR, DFM AND CULTURE.

A total of 125 samples were tested by PCR, DFM and culture. Of the 125 samples tested 52 (41.60 per cent), 32 (25.60 per cent) and 3(2.4 per cent) were found positive by PCR, DFM and culture respectively (Table.4).

When the results of PCR and DFM were compared, PCR detected 28 samples that were tested positive by DFM. In addition to this PCR demonstrated the presence of leptospiral DNA in 24 samples which were tested negative by DFM. No leptospiral DNA could be amplified by PCR in four samples which tested positive by DFM (Table 5.)

Fig. 6. Agarose gel (1.5 per cent) electrophoresis of amplified leptospiral DNA from suspected cases

Lane 1 : Negative control

Lane 2, 5, 6 : Positive samples

Lane 3 : Negative sample

Lane 7 : Positive control

Lane 4 : pUC 19 DNA/ *Msp* I digest

The three culture positive samples also tested positive by both DFM and PCR. Additionally leptospiral DNA was amplified in 49 samples from which no leptospires could be cultured (Table 6.).

Table 3. Results of clinical samples tested by PCR

Sl. No.	Sample	Polymerase chain reaction		
		No. tested	No. positive	% positive
I	Human			
(i)	Blood	107	58	54.20
(ii)	Urine	3	1	33.30
II	Dogs			
(i)	Blood	22	8	36.36
(ii)	Urine	5	2	40.00
III	Bovines			
(i)	Urine	30	-	-
IV	Rodents			
(i)	Urine	25	12	48

Table 4. Comparison of PCR, DFM and Culture

Sl. No.	Test	No. tested	No. positive	% positive
1.	PCR	125	52	41.60
2.	DFM	125	32	25.60
3.	Culture	125	3	2.40

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Table 5. Comparison of PCR and DFM

DFM	PCR		Total
	Positive	Negative	
Positive	28	4	32
Negative	24	69	93
Total	52	73	125

Table 6. Comparison of PCR and Culture

Culture	PCR		Total
	Positive	Negative	
Positive	3	0	3
Negative	49	73	122
Total	52	73	125

Discussion

5. DISCUSSION

Leptospirosis is a world wide disease which affects wild and domestic animals and human beings. In tropical and subtropical regions the disease is endemic, and exposure to infection is wide spread. The disease gained extreme public health importance especially in countries like India because of huge livestock, rodent and wildlife populations. Leptospiral infections are observed frequently in recent days. In India, an outbreak has been reported during the post cyclone investigation in Orrisa (WHO, 2000). Epidemics of leptospirosis are being increasingly reported from India (Sehgal *et al.*, 1995; WHO, 2000; Bharadwaj *et al.*, 2002).

The disease manifestation varies from subclinical infection to a severe illness; because of the variety of the clinical symptoms it is often misdiagnosed. Accurate diagnosis of leptospirosis is essential to start prompt and specific treatment, as early as possible, to ensure a favourable outcome (Brown *et al.*, 1995).

Currently diagnosis of leptospirosis relies on either isolation of the causative organism from body fluids (or) demonstration of a rise in specific serum antibodies.

Demonstration of the organism by DFM appears to be a simple method. However the number of leptospires in the blood may be too low to allow detection by DFM. In addition, proteinaceous filaments present in the sample may lead to false positive results (Rahman and Macis, 1979). Isolation of leptospires from clinical material is definite proof of infection. But isolation is slow, difficult, and susceptible to contamination and often unsuccessful (Thiermann, 1984).

There are over 230 known serovars in the genus leptospira. Confirmatory serological diagnosis of leptospirosis is usually made using the microscopic agglutination test (MAT) which is able to detect specific antibodies. The MAT

relies on the use of live cultures as source of antigen, often performed using a panel of antigens representative of local serovars. The MAT is generally performed by reference laboratories due to the inherent safety risks of handling cultures of live leptospires, the high cost of commercial media, and the need for ongoing maintenance of representative serovars (Faine, 1982). Microscopic agglutination test (MAT) is not useful for guiding clinical management early in the course of illness (Effler *et al.*, 2000).

Other techniques such as enzyme linked immunosorbent assay (ELISA) and slide agglutination test (SAT), can detect different classes of antibody but there is possibility of false positive reactions and requires confirmation by the MAT (Faine *et al.*, 1999).

Serology cannot be relied for early diagnosis of leptospirosis as antibodies become detectable approximately on the seventh day of disease. More over the presence of serum antibody bears little relationship to current infection (Michna, 1970). It is therefore, preferable to base the diagnosis on the detection of leptospires themselves (Millar *et al.*, 1987).

Immunofluorescence staining and immunoperoxidase staining are useful methods for the detection of leptospires provided the microorganisms are present in relatively large numbers (Gravekamp *et al.*, 1993).

Enzymatic radioimmunoassay (Chappel *et al.*, 1984) and monoclonal antibody based dot-ELISA (Saengjaruk *et al.*, 2002) have been reported for the detection of antigen for the diagnosis of leptospirosis.

Leptospiral DNA has been detected in clinical material by dot-blotting (Terpstra *et al.*, 1986; Millar *et al.*, 1987) and *in situ* hybridization (Terpstra *et al.*, 1987). Probes specific for serovar *hardjobovis* were developed and applied for the detection of leptospires in bovine urine (Bolin *et al.*, 1989). However the sensitivity of ^{32}P-labelled probes was approximately 10^3 leptospires, which is

much lower than the sensitivity of PCR. Moreover, PCR is an efficient and easy tool for the disease diagnosis. Use of probes have not been practiced extensively for the diagnosis since PCR became available (Levett, 2001). Polymerase chain reaction has slight edge over slot blot hybridization as it could be used for more samples and could be completed in one working day (Venkatesha and Ramadass, 2001).

With the introduction of PCR, rapid detection of small numbers of leptospires in clinical samples has become practical, by specific amplification of leptospiral DNA.

Polymerase chain reaction has been used to diagnose infectious diseases caused by viral and bacterial infections, viz., *Mycobacterium tuberculosis* (De wit *et al.*, 1990) and Bovine herpes virus 1 (Moore *et al.*, 2000).

Here in the present study, PCR was standardized to detect leptospires in clinical samples and the results were compared with those obtained by conventional methods, DFM and culturing.

5.1 AMPLIFICATION OF LEPTOSPIRAL DNA BY PCR

Several genus specific primers have been reported for the detection of leptospiral DNA. In the present study two genus specific primers A, B and G1, G2 have been evaluated to amplify by PCR the DNA of 12 reference strains of leptospires representing 12 different serovars.

5.1.1 *Amplification with Primers A and B*

The primers A and B in PCR amplified a 331base pair DNA fragment from the DNA of reference strains belonging to serovars *australis, rachmati, canicola, grippotyphosa, hebdomadis, icterohaemorrhagiae, poi, pomona, pyrogenes, hardjo, tarassovi* and *patoc*.

The broad spectrum of the primers in the detection of different serovars of leptospires could be explained by the fact that the sequence of the leptospira spp. rrs (16S) gene is highly conserved (Merien *et al.*, 1992). In addition to the Leptospira spp. serovars detected in PCR by Merien *et al.* (1992) the serovars *rachmati* and *poi* were also detected in the present study.

Merien *et al.* (1992) detected 20 serotypes and Heinemann *et al.* (2000) detected 26 serotypes with the primers A and B.

The primers A and B amplified the DNA from both pathogenic and non-pathogenic leptospires, irrespective of the serovar. The inability of the primers A and B to distinguish between pathogenic and nonpathogenic leptospires is not of practical value since the non pathogenic serovar *patoc* is usually not found in human and animal samples (Faine, 1982).

5.1.2 *Amplification with Primers G1 and G2*

The primers G1 and G2 in PCR amplified a 285 bp fragment from the DNA of reference strains belonging to the serovars *australis, rachmati, canicola, grippotyphosa, hebdomadis, icterohaemorrhagiae, poi, pomona, pyrogenes, hardjo* and *tarassovi* while the same failed to amplify the DNA from the serovar *patoc*, belonging to the non-pathogenic leptospires.These results were in accordance with the finding of Gravekamp *et al.* (1993).

In another study Venkatesha and Ramadass (2001) amplified 19 serovars of pathogenic leptospires with the primers G1 and G2.

The primers G1 and G2 were used for the characterization of 35 leptospira serovars by low stringency single specific primer PCR and 37 serovars of leptospires were amplified with the same primers (Oliveira *et al.*, 2003).

5.1.3 Specificity of the Primers

The specificity of the primers for Leptospira spp. was confirmed in that no specific amplification product was obtained using the DNA prepared from other bacterial strains, namely *Pasteurella multocida, Klebsiella pneumoniae, Streptococcus zooepidemicus, Pseudomonas aeruginosa* and *E. coli.*

However the primers G1 and G2 produced some non-specific amplification with the DNA of *E. coli.* Several fragments of different sizes were observed, which were either smaller or larger than the specific product and none of them were in the range of 285 base pair. Similarly Wagenaar *et al.* (1991) reported the amplification of *E. coli* DNA with primers derived from 16S rRNA gene.

Other two studies have also reported the specificity of the primers A and B for leptospira spp. (Merien *et al.*, 1992; Romero *et al.*, 1998). Merien *et al.* (1992) tested the specificity of the primers with DNA from other spirochaetes like Borrelia and Treponema species.

The primers A and B were selected for the detection of leptospiral DNA in clinical samples as it detected all the 12 reference strains, compared to G1 and G2. Moreover, the primers G1 and G2 amplified the DNA from *E. coli,* which occurs as a normal contaminant in the laboratory. The primers A and B have been subjected to more clinical evaluation compared to primers G1 and G2 (Levett, 2001). Additionally the primers G1 and G2 did not amplify *L. krischneri* serovars (Gravekamp *et al.*, 1993).

5.1.4 Reconstitution Experiments with Biological Samples

In this present study, PCR amplified the leptospiral DNA in blood and urine samples seeded with cultures of leptospires maintained in the laboratory.

These findings indicate the effectiveness of preparation of samples for PCR from clinical material.

Similar reconstitution experiment with blood and urine samples (Merien *et al.*, 1992) and kidney samples (Savio *et al.*, 1994) were also reported.

5.2 ANALYSIS OF THE AMPLIFIED PCR PRODUCT

5.2.1 *Restriction Enzyme Analysis*

The restriction enzyme digestion of amplified PCR product with the enzyme *Dde* I produced 159, 61, 43 and 41 base pair fragments and the enzyme *MnI* I produced 124, 84 and 49 base pair fragments. The restriction enzyme digestion yielded the expected restriction fragments and this established the identity of the amplified PCR product.

Moore *et al.* (2000) confirmed the PCR amplified fragment to be the expected region of the BHV I tk gene of Bovine herpes virus 1 by restriction enzyme analysis, with the *Taq* I.

Savio *et al.* (1994) differentiated the serovars of leptospires with the different polymorphic profiles obtained by restriction digestion of the amplified DNA with the enzyme *Hinf* I and *Dde* I.

Brown and Levett (1997) and Heinemann *et al.* (2000) employed restriction enzyme analysis of PCR product for the differentiation of leptospira species and serovars.

Harkin *et al.* (2003a) confirmed the identity of PCR amplified leptospiral DNA by restriction enzyme analysis with *Apa* I, which produced fragments of 260 and 220 base pairs.

5.2.2 *Sequencing of Amplified Product*

The purified PCR product was directly sequenced and sequence of amplified product was obtained. In the sequence similarity searches, the sequence of the amplified PCR product was found to have 100 per cent identity with the *Leptospira interrogans* 16S ribosomal RNA gene. Thus the direct sequencing of the amplified fragment established the identity of the primer A and B amplified product.

Moore *et al.* (2000) confirmed the identity of the PCR amplified fragment by partial DNA sequencing, while Pastic *et al.* (2000) used partial sequencing of 16S r DNA gene for comparison of leptospira isolates.

5.3 DARK FIELD MICROSCOPY

Usefulness of dark field microscopy in the early diagnosis of leptospirosis was described as early as 1954. Demonstration of leptospires by DFM appears to be a simple method. However the number of circulating leptospires in the blood may be too low to allow direct detection under microscopy (Vijayachari *et al.*, 2001).

Of the 40 blood samples collected from human beings, leptospires were demonstrated in 47.5 per cent of samples. None of the human urine samples examined was found positive by DFM.

Chandrasekaran *et al.* (1997) demonstrated leptospires in 52.38 per cent of the human blood samples. In another study DFM detected leptospires in 44.4 per cent of blood samples and 38.89 per cent of urine samples collected from human cases (Senthilkumar *et al.*, 2001).

In case of dogs 22.72 per cent of blood samples and 20 per cent of urine samples were found to be positive by DFM. Of the 30 bovine urine samples examined none were tested positive by DFM.

Senthilkumar *et al.* (2001) detected leptospires in 43.47 per cent of blood samples and 50.0 per cent of urine samples collected from dogs.

Leptospires were demonstrated in 28 per cent of urine samples, out of the 25 rodent samples examined. Pargaonker *et al.* (1957) failed to demonstrate leptospires in rodents by urine examination under DFM but detected 10.6 per cent of the cases by kidney smear and staining by Fontana's method.

5.4 CULTURE

The culturing of leptospires from clinical material is of paramount importance, both for definitive diagnosis of individual cases and for epidemiological purposes. Major problem in culturing of leptospires is the contamination with other microorganisms, especially when samples are from non-sterile sources like urine (Venkatesha and Ramadass, 2001).

Of the 125 samples from different sources like human, dogs, bovines and rodents when cultured, leptospires were isolated from 3 rodents only (2 rats and one bandicoot).

Attempts to isolate leptospires from human, bovines and dogs were unsuccessful. Kaveri and Upadhye (1981) isolated leptospires from 2 cases of 100 urine samples of dogs tested. Natarajaseenivasan and Ratnam (1997) attempted to isolate leptospires from urine of cattle and agricultural workers and were found unsuccessful.

Other workers who failed to isolate leptospires from urine of bovine and human (Natarajaseenivasan and Ratnam, 1997), bovine semen (Heinemann *et al.*, 1999) and in dogs urine (Harkin *et al.*, 2003b).

In the study, leptospires could be isolated from only three (12 per cent) samples of the 25 rodents that were cultured. Natarajaseenivasan and Ratnam (1997) isolated leptospires from one of 34 field rats examined, while Vanasco *et al.* (2001) isolated from 24 (20.0 per cent) of the 118 rodents that were tested by culturing.

5.5 POLYMERASE CHAIN REACTION

In the present study, 54.2 per cent of blood samples and 33.3 per cent of urine samples collected from human cases were found positive for leptospires by PCR. The samples such as blood and urine collected from dogs showed positivity of 36.36 per cent and 40.0 per cent respectively.

Ramadass *et al.*, (1997) observed positivity of 65 to 81 per cent with PCR for the detection of leptospires, in clinical samples such as serum and urine. Senthilkumar *et al.* (2001) detected leptospires in 43.47 per cent blood and 50.0 per cent urine samples collected from dogs.

PCR was successfully used for rapid diagnosis of leptospirosis, concerned with detection of leptospiral DNA in body fluids like aqueous humor (Merien *et al.*, 1993) cerebrospinal fluid (Romero *et al.*, 1998) and in semen (Heinemann *et al.*, 1999).

Other two reports of detection of leptospira spp. are concerned with the specific identification of serovar *hardjo* in bovine urine (Van Eys *et al.*, 1989; Gerritsen *et al.*, 1991), whereas this present study reports the detection of Leptospira spp. in clinical samples, irrespective of the serovar.

Forty eight per cent of the urine samples collected from rodents were positive by PCR. Ralaiarijaona *et al.* (2001) used PCR for detection of leptospires

in kidney tissues of rodents, and the PCR analysis revealed negative for all the samples tested.

5.6 COMPARISON OF PCR, DFM AND CULTURE

The results of the 125 samples that were tested by the three techniques viz., PCR, DFM and culture were used for comparison.

The percentage positivity found with DFM was 25.6 per cent while the PCR showed positivity of 41.6 per cent. Among these, PCR detected 24 samples as positive which were tested negative by DFM. This clearly indicated that PCR technique was highly sensitive as compared to DFM.

In the present study, four of the samples, which were tested positive by DFM were found negative by PCR. This might be attributed to the artifacts such as serum proteins, fibrin strands and cell fragments, which might resemble leptospires under dark field illumination (Vijayachari *et al.*, 2001). It is a known fact that DFM has low sensitivity, the present study not only emphasizes this but also shows that even the specificity of the test is low compared to PCR.

In comparing the results of PCR and culture, PCR detected 41.6 per cent of the samples compared to 2.4 per cent by culture. Among these, PCR detected 49 samples as positive which were tested negative by culture. A positive PCR outcome is attributed to the presence of leptospiral DNA, which may originate from either viable and/or dead bacteria in a sample. Successful isolation by culturing requires at least a few viable leptospires, while PCR could amplify the DNA even from a sample containing non-viable leptospires. Since leptospires do not survive long time in clinical samples, more number of positives observed by PCR than by culturing may thus be attributed to the mere presence of DNA from

either live or dead bacteria. Thus PCR can be a more sensitive alternative to culturing for direct detection of leptospires in clinical samples.

Failure of leptospires to grow in culture observed in this study may be due to at least in part, to the fastidiousness of leptospires. In addition, a delay of even just a few hours between collection and inoculation of the culture medium may also have contributed to the failure to culture leptospires.

Van Eys *et al.* (1989) used isolation and PCR to detect leptospires in a group of 21 cows and only 38.1 per cent of the specimens were positive by culture, whereas 61.9 per cent were tested positive by PCR. Merien *et al.* (1992) successfully isolated leptospires from only one of six clinical specimens, while four of six were positive by PCR.

The results of the present study agree with the findings of earlier workers (Bal *et al.*, 1994; Brown *et al.*, 1995; Harkin *et al.*, 2003b) who opined that PCR was more sensitive than culture for the detection of leptospires.

Polymerase chain reaction is a more rapid diagnostic method for confirming infection with leptospires, as the preparation of sample, performance of the test and electrophoresis can be completed within one day and simultaneously many samples can be processed, whereas culturing is laborious.

The 192 samples collected from different sources when tested by PCR, the positivity ranged from 33.3 to 54.2 per cent. In comparing the results of PCR, DFM and culture, PCR detected 41.6 per cent samples, compared to 25.6 per cent by DFM and only 2.4 per cent by culturing. The reason for the low sensitivity obtained by culturing and DFM may be due to the fact that culturing needs viable organisms, while leptospires may rarely be present in sufficient numbers to allow

detection by DFM. The PCR was more sensitive, specific and rapid, when compared to DFM and culturing, for confirmative diagnosis of leptospirosis.

It can be concluded that PCR technique is a potentially useful, quick diagnostic method for confirming infection with leptospires. Similar observations were made by Brown *et al.* (1995), Ramadass *et al.* (1997). Though routine use of PCR technique for diagnosis may be a little expensive, with the semi-sophisticated facilities, it would be worth the expenditure, when leptospirosis could be detected at an earlier stage of infection and successful treatment could be adopted.

Differentiation of the infecting serovars useful for molecular epidemiological studies warrants further investigations with PCR based methods *viz.*, PCR-restriction fragment length polymorphism and random amplified polymorphic DNA finger printing.

Summary

6. SUMMARY

Leptospirosis is an emerging infectious disease, caused by infection with pathogenic leptospira species, affecting a broad spectrum of host species including wild animals. Leptospirosis is a zoonosis of ubiquitous occurrence, and has been reported from many countries, world wide, and also from India. The disease was reported as endemic in many places in South Indian States viz., Tamil Nadu, Kerala, and Karnataka. The disease has been diagnosed by conventional methods such as demonstration of leptospires in DFM, isolation in culture and serological methods. These methods have low sensitivity, and are laborious, time consuming, and does not contribute to an early diagnosis. Recently, DNA based techniques like PCR has been introduced for the detection of leptospires in clinical samples. Such a test has not been applied so far in this part of the country for the diagnosis of leptospirosis. Hence, this study was undertaken to standardize the PCR technique for the diagnosis of leptospirosis and to compare the PCR with DFM and culturing, in the detection of leptospires.

Twelve reference serovars of leptospires, viz., *australis, rachmati, canicola, grippotyphesa, hebdomadis, icterohaemorrhagiae, poi, pomona, pyrogenes, hardjo, tarassovi,* and *patoc* were used in the present study. The reference serovars were maintained in Fletcher's/EMJH semisolid media by subculturing routinely at 8 weeks interval.

The primers A and B derived from *L. interrogans* serovar *canicola* rrs (16S) gene amplified a 331 base pair DNA fragment from all the 12 reference serovars tested. The primers G1 and G2 derived from genomic library of *L. interrogans* serovar *icterohaemorrhagiae* (strain RGA) amplified a 285 base pair DNA fragment from 11 of the 12 reference serovars tested; it failed to amplify the DNA of serovar *patoc.*

The specificity of the primers for leptospira spp. was evaluated by testing the primers to amplify the DNA of bacterial strains viz., *Pasteurella multocida, Streptococcus zooepidemicus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *E. coli*. Both the primers were found to be specific for leptospira spp. as no specific amplification product was obtained with the DNA prepared from other bacterial strains.

The primers A and B were selected for the routine amplification of leptospiral DNA in clinical samples, such as blood and urine.

Restriction enzyme analysis of the primer A and B amplified PCR product was carried out with the enzymes, Dde I and *MnI* I to establish the identity of the amplified product. Both the enzymes produced clear restriction fragments. In addition, direct sequencing of the amplified PCR product was also performed to establish its identity. In the sequence similarly searches the sequence was found to have 100 per cent identity with *Leptospira interrogans* 16S ribosomal RNA gene.

A total of 192 samples were collected from different sources like human, dogs and bovines with suspected history of leptospirosis and from rodents. Among these, 125 samples were processed by PCR, DFM and culture, while all were tested by PCR.

Among the human blood samples, 47.5 per cent were found to be positive by DFM. In case of dogs, 22.72 per cent blood and 20 per cent urine samples were found positive by DFM. None of the urine samples of bovine and human were tested positive by DFM. Twenty eight per cent positivity was obtained for urine samples of rodents.

Attempts for isolation of leptospires from clinical samples were done using Fletcher's/EMJH semi solid medium containing 5-fluorouracil. Three kidney samples collected from rodents yielded leptospires in culture. Attempts to isolate leptospires from human, dog and bovine samples were unsuccessful.

Among the human cases, 54.2 per cent blood and 33.3 per cent urine samples were found positive by PCR. A positivity of 36.36 per cent and 40 per cent were observed for blood and urine samples respectively of dogs. In case of rodents, 48 per cent were positive by PCR.

In comparing the results, 41.6 per cent, 25.6 per cent and 2.4 per cent positivity was obtained by PCR, DFM and culture, respectively. PCR had detected more number of positive samples, compared to DFM and culturing. PCR has proven to be specific, more sensitive and more rapid than the conventional method in the diagnosis of leptospirosis.

More studies with regard to serovar identification should be carried out to get knowledge on the serovars present in the area, which could be helpful for epidemiological studies.

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COMPARISON OF POLYMERASE CHAIN REACTION WITH CONVENTIONAL METHODS FOR THE DIAGNOSIS OF LEPTOSPIROSIS

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ABSTRACT

A study was undertaken to standardize the PCR technique for the diagnosis of leptospirosis and to compare the efficacy of PCR with DFM and culture in the diagnosis of leptospirosis.

Two sets of primers, namely, primers A and B derived from *Leptospira interrogans* serovar *canicola rrs* (16S) gene and G1 and G2 derived from genomic library of *L. interrogans* serovar *icterohaemorrhagiae*, were evaluated to amplify 12 reference strains of leptospires representing the serovars *viz., australis, rachmati, canicola, grippotyphosa, hardjo, hebdomadis, icterohaemorrhagiae, pomona, poi, pyrogenes, tarassovi* and *patoc*. The primers A and B specifically amplified all the serovars tested, while the primers G1 and G2 failed to amplify the serovar *patoc*. The primers A and B which amplified a 331 base pair fragment of leptospiral DNA were used for the routine detection of leptospires in clinical samples.

Restriction enzyme digestion of the primer A and B amplified product with the enzymes *Dde* I and *MnI* I and direct sequencing established the identity of the amplified product.

A total of 192 samples were collected from different sources like human, dogs and bovines with suspected history of leptospirosis and from rodents. All were tested by PCR and the positivity ranged from 33.3 to 54.2 per cent. Of the samples collected 125 samples were tested by all the three techniques viz., PCR, DFM and culture and the results were compared.

The 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 per cent by culture, of the samples tested.