CHAPTER I

INTRODUCTION
Leptospirosis is a widespread zooanthroponosis caused by *Leptospira interrogans*, the discovery of which was made a little more than 100 years back. The clinical spectrum of infection ranged from subclinical to severe illness with high mortality rate. Strong rodent–man association has given the inappropriate term ‘rat-fever’ to the illness, as bigger animals (cattle, horses, goats, dogs etc.), by virtue of shedding litres of infected urine into the environment play a much bigger role in dampening and contaminating the soil, ground waters, streams, and rivers.

Leptospirosis has been reported from many countries including India, China, Japan, Korea, Australia, Continental Europe and a large number of tropical countries. It is known by different names in different parts of the world - seven-day fever or autumn fever (Akiyami) in Japan, cane cutter’s disease in Australia, rice-harvest jaundice in China, rice-field fever of Indonesia, Fort Bragg in America and swineherd’s disease, all syndromes associated with season, occupation or environment.

Though more prevalent in the tropics there are varying reports of endemicity from different parts of the world (Feresu *et al*., 1994; Alexander *et al*., 1957; Ball, 1966; Machang’u, 1992; Everard and Everard, 1993; Tsai & Sulzer, 1971) and India (Dalal, 1960; Joseph, 1966; Ratnam *et al*., 1993; Madhusudhana *et al*., 1980; Sehgal, 1995; Kuriakose *et al*., 1998). As noted by Thiermann (1984) only a small numbers of serovars are endemic in any geographic location. A region with rich and varied fauna will support a larger number of serogroups than one with few animal hosts. Laboratory defined precise identification and classification of *Leptospira*
species is important for epidemiological and public health surveillance, as different serovars can exhibit different host specificities (eg. *icterohaemorrhagiae* and *ballum* in rats, *canicola* in dogs, *hardjo* in bovine, *pomona* in pigs etc.) and may be associated with a particular clinical form of infection (eg. *icterohaemorrhagiae* with severe icterogenic syndrome, *grippotyphosa* with benign meningitis syndrome etc) [Faine, 1982; Perolat, 1990; Michna, 1970; Brown and Levett 1997]. Even the serovars most commonly associated with severe, fatal form of the disease frequently cause very mild form of infection. (Faine, 1994).

Early reports in India were from Andamans around 1929, with reports of Andaman haemorrhagic fever, a severe form presenting with pulmonary haemorrhage. There was a lull of many years after which revived reports started coming in around the 1980s. Now many states in India like Kerala, Tamil Nadu, Maharashtra, Orissa, and Karnataka report sporadic or epidemic outbreaks following rains. The increased reporting may be due to higher incidence of the disease due to poor drainage, increased agricultural activities, changed crop cycling patterns, increasing infestation of maintenance hosts, close proximity of animals with the human population, or rise in the number of people seeking medical aid. It may also be attributed to availability of better diagnostic facilities that can diagnose and confirm the cases.

**Epidemiology**

Leptospirosis occurs wherever there is a risk of direct or indirect contact with infected urine or animals. Pathogenic leptospires can survive outside the animal body for 3 weeks or longer in environments that are moist and warm with neutral or slightly
alkaline pH. Leptospires are usually well adapted to a particular host with which they establish a commensal relationship. The natural host, mainly the resident animal and rodent population form an infection reservoir in which the leptospiral population is maintained. The spirochete has wide host range such as rodents, dogs, livestock animals including cattle and swine etc., wild animals and man (Harrington, 1975; Nielsen et al., 1992; Om, 2000). A particular species of host may be a transient carrier (incidental host) of some serovars but a maintenance host for others. Almost every mammal, small marsupials, including aquatic animals has been known to be carriers of leptospirosis. Infection may also spread from wild animals to pets and laboratory animals. Common species of rodents include Rattus rattus, Rattus norvegicus, Mus musculus, Bandicota indica, Bandicota benghalensis among others. Proximal renal tubules are the natural habitat of the host although carrier conditions are seen in other organs also.

An occupational hazard, it gains public health importance when transmitted by their natural hosts to other animals or man with whom the reservoirs share the same ecological niche during agricultural practices, veterinary association, sewer works, washing, bathing and other recreational water-associated activities. Other animals and man are accidental hosts, as the infecting leptospires are not adapted to the host and cannot colonize their kidneys. In such hosts, leptospirosis is transient. The leptospires are usually pathogenic to these hosts (Feresu et al., 1994). It is not known to be transmitted from man to man. Its variable prevalence in a specific area depends largely on the land topography, soil pH, moisture and food availability.
Plate 1  Transmission and maintenance of leptospira in the environment
Pathogenesis

Avirulent leptospires fail to multiply and are easily destroyed. Incubation period for leptospirosis varies between 2-30 days. There are two overlapping phases in the course of the illness.

1. Leptospiraemia, followed by rising titres, and

2. Leptospiuria caused by localization of organisms within the kidney tubules.

During the leptospiraemic phase (7-10 days) there is multiplication, spread and establishment of the organism in different parts of the body. They persist longer in the convoluted tubules of the kidney. During the second phase the organism is excreted in the urine and present in other tissues of the body. This is also known as the immune phase.

Clinical manifestations

The disease varies in severity depending on the infecting serovar, age, nutrition and health of the patient. Wide spectrum of distinct clinical manifestations range from mild to influenza-like illness to fulminant disease with jaundice, acute renal failure, septic meningitis, and haemorrhagic diathesis etc. (Farr, 1996; Heath, 1965; Merien et al., 1995). In most of the patients, it presents with febrile illness of sudden onset accompanied by headache, myalgia, abdominal pain, conjunctival suffusion and less often rash. The degree of infection and organ involvement depends on the infecting serovar and other circumstances. (Faine, 1994). The classical characteristics
of Weil’s the severe form of leptospirosis include sudden onset of fever, headache, pains, prostration, relapse, jaundice, conjunctival suffusion and petechial hemorrhages.

**Mortality**

Human leptospirosis causes severe multi organ dysfunction that may end in multi-organ failure and death. Refractory hypotension has very high mortality, so also Acute Renal failure (ARF) and Adult Respiratory Distress Syndrome (ARDS), myocarditis, severe thrombocytopenia leading to haemorrhage in vital organs. In M. O. S. C. Medical College Hospital, Kolenchery, where the study was conducted, the mortality rate due to leptospirosis varied between 5-10%, though others centres in the state report a higher rate of 10-20%. There has been a significant rise in incidence and severity of illness since 1996, with more cases of multi-organ involvement being reported.

**Diagnosis**

Diagnosis is not easy since leptospirosis mimics many diseases like malaria, enteric fever, influenzae, Dengue haemorrhagic fever, other viral fevers, brucellosis etc. Nevertheless with epidemiological information, clinical findings and differential diagnosis to rule out other illnesses with similar presentation, laboratory investigations and microbiological findings, the disease can be diagnosed with confidence. Serological tests including ELISA and MAT are commonly used in the diagnosis of leptospirosis. ELISA is employed as a genus-specific test and MAT still remains the standard test for serovar-detection. Other serological tests include Complement fixation test, Counter immunoelectrophoresis, Macroscopic...
agglutination test, Indirect fluorescent antibody test, Indirect haemagglutination test, Microcapsule agglutination test, and recently developed rapid tests.

**Treatment**

Early treatment with antibiotics and haemodialysis has contributed to the decrease of mortality (Centers for Disease Control, 1981; Sanford, 1984). They have also been shown to shorten the duration of the illness. A variety of antimicrobial agents with the exception of chloramphenicol including penicillins, tetracyclines, erythromycin, and quinolones have been found to be effective. Prompt and apt treatment with supportive therapy is needed to save the critically ill. Non-steroidal anti-inflammatory drugs are potentially nephrotoxic and may cause ARF in otherwise uncomplicated cases.

**The Organism**

Adolf Weil described the severe manifestation of icteric leptospirosis with renal failure known as Weil’s Syndrome in 1886. It was in 1907 that Stimson demonstrated the presence of spirochetes in the kidney tubules of a patient who reportedly died of yellow fever. He named the spirochetes with hooked ends resembling a question mark *Spirochaeta interrogans*. Inada Ido, Hoki and others discovered Leptospira in 1915.
Plate 2: Dark-filled illumination

Plate 3: Diagrammatic representation of the coils

a - outer envelope
b - cell wall
c - endoflagellum
d - flagellar insertion in terminal knobs
Leptospira are thin (approx. 0.1µm), helical, motile organisms, ranging from 6µm to about 21µm long and often hooked at both ends. They are actively motile and constantly spin on their long axis. When magnified thousand times the helix appear as a series of beads with coil amplitude of approximately 0.1µm to 0.15µm and a wavelength of 0.5 µm. Straight variants also occur. There is an outer envelope covering the cell wall in helical shape, with two polar flagella. The endoflagella (also called axial filament, axistyle), encased by the outer membrane, begin at each end of the organism and wind around it, extending to and overlapping at the midpoint.

Classification

The basis for primary classification was the morphology of the organism. The term for genera *Leptospira* was given by Noguchi in 1918 due to its difference from other spirochetes.

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Order Spirochaetales

Family Leptospiraceae
- Leptospira
- Leptonema
- Turneria

Family Spirochaetaceae
- Borrelia
- Treponema
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The genera *Leptospira* was placed in the Family Leptospiraceae among the Order Spirochaetales. Two different classifications have evolved following the development of genetic taxonomy.

1. **SEROLOGICALLY BASED CLASSIFICATION**

   ![Classification Diagram]

   **Leptospira**

   - *L. interrogans* (pathogenic)
   - *L. biflexa* (non-pathogenic)

2. **GENETIC CLASSIFICATION**

   ![Genetic Classification Diagram]

   **Pathogenic Leptospira**

   - *L. interrogans*
   - *L. borgpetersenii*
   - *L. weilii*
   - *L. noguchii*
   - *L. santarosai*
   - *L. inadai*
   - *L. alexanderi*
   - *L. kirschneri*
   - *L. biflexa*
   - *L. meyeri*

   **Non-pathogenic Leptospira**

   - *L. parva*
   - *L. wolbachii*
The serological classification, still recognizes only two species - the pathogenic *L. interrogans* and the non-pathogenic *L. biflexa*. The basic taxon of these organisms is the serovar. Serologically similar serovars are grouped into serogroups that have no formal taxonomic status. Serovars are identified on the basis of cross-agglutinin absorption tests (CAAT), the result of which forms the basis of the serovar concept (Dikken & Kmety, 1978). More than 267 serovars have been recognized (Faine, 1994). *L. interrogans* includes 202 serovars divided in 23 serogroups of antigenically similar serovars (Kmety & Dikken, 1988; Johnson et al., 1988). *L. biflexa* includes 65 serovars in 38 serogroups (Kmety & Dikkens, 1988). Methods of serovar identification are very complex and require the maintenance of all the reference strains and corresponding rabbit hyperimmune sera (Dikken and Kmety, 1978). Time taken for typical identification an unknown strain is two months.

The genus *Leptospira* has been reorganized to include at least eight pathogenic genomic species *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai*, *L. inadai*, *L. alexanderi* and *L. kirschneri* (Yasuda et al., 1987; Wayne et al., 1987; Ramadass et al., 1992) and 4 non-pathogenic species *L. biflexa*, *L. meyeri*, *L. parva*, and *L. wolbachii*. Many more serovars are being added with further revisions taking place. The genotypic reclassification though correct is confusing. In the genomic classification, the serovars of both *L. interrogans* and *L. biflexa* of the serological classification, are placed in a number of genomes according to their genomic heterogeneity and DNA hybridization studies.
Past work

The endemicity of this region was evident from our earlier studies (Kuriakose et al., 1990, Kuriakose et al. 1994, Eapen et al., 2001) carried out from 1989 onwards both by culture and serology, and our isolation rate from culture ranged between 10-14%. This is quite high compared to that of ~3% as reported by Merien et al. 1995 but much lower than the serological evidence of the disease in this region which was between 25-35% by ELISA. Though culture and serological tests can confirm the suspected leptospira cases, their lack of sensitivity can strongly influence the course of disease and severe damage at an early stage can lead to death. (Merien et al., 1995). Comparing culture and ELISA it was clearly evident that about 15-20 % cases were being missed by culture. The study was also directed at selecting a simple, sensitive and rapid test for routine diagnosis.

The serogroups identified by culture earlier in our hospital from 1989 onwards till 2000 from patients diagnosed of having leptospirosis includes Australis, Autumnalis, Bataviae, Canicola, Grippotyphosa, Javanica, Louisiana, Pomona, Pyrogenes and Sejroe. Since leptospira culture from humans is not being done in most hospitals in Kerala there is practically no input from other areas in the state.
Plate 4: MAT reactions

a. Negative MAT reaction

b. Positive MAT reactions

On the subspecies level, serogrouping and identification techniques, based on antigenic traits, is still the recognized and generally accepted method of classification for members of the genus *Leptospira*. 
The most prevalent and efficient serogrouping is the Microscopic Agglutination Test (MAT), which uses live or killed leptospira as antigens. The serogroups identified by MAT at CDC, Atlanta, Amsterdam and Lisbon on serum samples prior to 1994 gave an indication of the serogroups likely to be encountered in this area. The list of serovars from this area identified by MAT at these centres included *australis, autumnalis, bataviae, canicola, grippotyphosa, javanica, louisiana, pomona, pyrogenes, sejroe, djasiman, forthragg, bim, cynopteri, mankarso, ballum, hebdomadis, panama, icterohaemorrhagiae* etc.

Not all serogroups may be retrieved from humans by culture since several factors contribute to their transmission and diagnostics. There are some serogroups listed in the serological MAT above, that have not been isolated from clinical cases. This could mean that either these are extremely fastidious, or that these caused only some subclinical infections in the field workers which did not necessitate their coming to the hospital for treatment so may have gone unnoticed or that these were cross-reactions between different serovars which is a common phenomenon in MAT. Cross-reaction is a major drawback in MAT waylaying the pinpointing of the causative infecting serovar.

**Genomic identification methods**

The serovar concept though still applicable in many situations is not always fully satisfactory. It may delineate closely related strains to distinguish between antigenically similar strains that have different ecological niches. A serovar status
should be issued by the recognized standard method and be complemented by a genetic method, so as to contribute to serovar identification and intraserovar differentiation notably for cases in which serological test fails to differentiate between genetically different leptospires (Feresu et al., 1994).

Recently, a variety of modern methods for genetic analysis have become available (Gravekamp et al., 1993; Herrmann et al., 1992; Perolat et al., 1990; Perolat et al., 1993; Ramadass et al., 1992; Yasuda et al., 1987). Molecular methods have given a new approach to Leptospira taxonomy.

The search for alternate diagnostic method has focused on specific DNA amplification by PCR. The PCR is an invitro method for selectively amplifying target DNA sequences by more than 10 fold (Saiki et al., 1988). It has been used to diagnose infectious diseases caused by fastidious or slow growing bacteria such as Mycobacterium leprae (Woods and Cole, 1989), Mycobacterium tuberculosis (De Wit et al., 1990), Treponema pallidum (Hay et al., 1990) and Borrelia burgdorferi (Ross and Schwann, 1989).

The majority of the methodologies involving molecular studies are not ideally suited to clinical laboratories and small hospitals because of the cumbersome procedures and relatively large amount of DNA required as for DNA-DNA hybridisation. Genomic methods, such as REA of chromosomal DNA, the use of probes, or restriction analysis by PFGE were demonstrated as being useful for typing leptospiral isolates. Electrophoresis of fragments generated by treating total DNA
with restriction enzymes (REA) differentiates leptospira strains (Ellis et al., 1991). REA, though, requires DNA from purified cultures for analysis and fragments are too numerous to be easily readable, making the comparison difficult. RAPD methods have shown some promise for differentiating individual serovars. However, purified DNA samples are required for RAPD fingerprinting (Ramadass et al., 2002). DNA hybridization with repetitive sequences or serovar-specific fragments simplifies the interpretation, but the method is still cumbersome. However, PFGE of restricted chromosomal DNA proved to be very useful for the identification of leptospires at the serovar level but still requires appreciable amount of DNA. Some of these methods though highly discriminatory, require DNA from purified cultures for analysis.

These methods are not only more laborious than PCR-derived methods but also do not allow unambiguous assignment of an isolate to a given species. (Perolat et al., 1994) Moreover PCR-based characterization methods are rapid, use much less DNA, which is an important consideration when studying such slow-growing bacteria such as leptospires (Ramadass et al., 2002). Besides PCR-based profiles are easy to compare and the technique does not require high-resolution electrophoresis, which makes it an attractive proposal. Also both purified and crude DNA produces consistently reproducible patterns unaffected by the amount of target DNA (Brown & Levett, 1997).
**Polymerase Chain Reaction**

Due to these reasons, for effecting a rapid and simple identification which can be easily adapted to small research and clinical laboratories, we selected *Multiplex PCR* using two sets of primers, used previously, for identifying the pathogenic leptospiral isolates from clinical human cases and *Arbitrarily primed PCR* for characterising them.

With the introduction of PCR, rapid detection of small numbers of leptospires in clinical samples has become practical due to specific amplification of leptospiral DNA (Gravekamp *et al.*, 1993). PCR works with intact or broken DNA pieces. The PCR products or amplicons consist of a fragment (or fragments) of DNA, which is normally of a length defined by the boundaries of the PCR primers. The combined sets of primers G1 and G2 and B64-I and B64-II used in the Multiplex-PCR study, amplifies DNA from all pathogenic *Leptospira* species. DNA from strains of *L. interrogans, L. borgpetersenii, L. weilii, L. noguchii, L. santarosai* and *L. meyeri* (strain ICF) is amplified by G1 and G2, whereas DNA from strains of *L. kirschneri* is amplified by B64-I and B64-II (Brown *et al.*, 1995).

Normally, the detection and identification of the amplified products is electrophoresis of an aliquot of the PCR product on an agarose or polyacrylamide gel and visualisation by staining with ethidium bromide, which is a fluorescent dye that intercalates into DNA. After staining, ultraviolet transillumination allows visualisation of the DNA in the gel.
The most potentially useful approach for clinical identification so far proposed is that of arbitrarily primed PCR, because of its simplicity, the fact that only nanogram quantities of Leptospira DNA are required, and its sensitivity to genomic variation (Ralph et al., 1993). The method uses randomly selected primers designed to anneal to the synthetic oligonucleotide extension and to the regions in which they are collocated to amplify anonymous regions of the Leptospira genome. The amplified products are highly polymorphic and appear to constitute serovar-specific PCR fingerprints.

Although the genomic classification establishes a firm scientific basis for the knowledge of leptospira, the identification of a pathogenic species to with one of the seven species is an insufficient step for the epidemiologist. AP-PCR has been found to be a useful and reasonably simple and quick method for typing of leptospires, when compared to cross absorption, nucleic acid probe technology and RAPD fingerprinting method. AP-PCR fingerprinting may contribute to the knowledge of the molecular epidemiology of leptospiral infections due to the clonal nature and the level of genomic heterogeneity of strains isolated inside a delimited enzootic focus. This could be applied to the analysis of leptospira serovars of public health importance. With this method straight clinical samples can be used directly for PCR and AP-PCR amplification. Amplified products are size fractionated by agarose gel electrophoresis. The resultant DNA pattern produced could be used for the typing of the leptospires.

Perolat et al. (1994) opined that resolution of the AP-PCR fingerprints on the sequencing gels makes possible significant intraspecies comparisons, for molecular
epidemiology studies of leptospires. The detailed study of AP-PCR profiles allows an accurate comparison of strains isolated in different geographical areas. They also serve as effective tools for the rapid determination of the genospecies of *Leptospira* isolates, which is the first step in bacterial identification.

**Study area**

Kolenchery is situated between 10° 10' N and 76° 10' to 76° 45' E.

Kerala, with food availability all year-round and moist soil due to efficient irrigation canal networking for its agricultural activities, is a state with severe rodent infestation, which acts as reservoirs for the maintenance of leptospira. The major factors responsible for higher incidence include water-logging in urban areas following rains and poor drainage systems, and active agricultural and water activities in rural. The study area confined to around 50 km. radius of the M.O.S.C. Medical College and Hospital in Kolenchery, Ernakulam District, Kerala, India. This hospital serves 2-lakh population from the surrounding areas. The study area is rural central Kerala which is irrigated throughout the year and also has year-round cultivation of paddy, pineapple, tapioca, rubber etc. providing ideal conditions for the maintenance of leptospira in the environment with its fair share of domestic animals and rodents.
Fig 1 Map of the study area