Introduction

Leptospirosis has long been considered as a rare zoonatic disease in India with only sporadic cases being recorded. Since 1980’s the disease has been reported from various states during monsoon months. The disease is endemic in Karnataka, Kerala, Tamil Nadu, Gujarat, Andaman and Maharastra. Leptospirosis has been variously called as Weil's disease, canicola fever, cane field fever, nanukayami fever, 7-day fever and many more. It is a bacterial zoonotic disease caused by spirochaetes of the genus *Leptospira* that affects humans and wide range of animals including mammals, birds, amphibians and reptiles (Bharti *et al.*, 2003; Levett, 2001; Matthias *et al.*, 2008). The spectrum of human disease caused by leptospires is extremely wide, ranging from subclinical infection to severe syndrome of multi organ infection with high mortality. This syndrome called icteric leptospirosis with renal failure was first reported almost 100 years ago by Adolf Weil in Heidelberg. However, an apparently identical syndrome occurring in sewer workers was described several years earlier.

Leptospirosis has been under reported and under diagnosed from India due to a lack of awareness of the disease and lack of appropriate laboratory diagnostic facility in most parts of the country. Combining clinical expertise and awareness with confirmatory laboratory back up dramatically increases the recognition of patients with leptospirosis (Shivakumar, 2008). Leptospirosis is recognized as the world's most common zoonoses. The infection is commonly transmitted to humans by allowing fresh water that has been contaminated by animal urine to come in contact with unhealed breaks in the skin, eyes or with the mucous membranes. Outside tropical areas, leptospirosis cases have a relatively distinct seasonality with most of them occurring during August-September or February-March (Zhang and Dai, 1992).

The earliest recognized accounts of leptospirosis were descriptions of severe illness with jaundice and renal involvement in man published by Landouzy (1883) and Weil (1886), these were clinically distinct from other icteric and nephritic illnesses, but the exact cause was not known (Turner, 1967). The first report of bovine leptospirosis was published by former USSR
in 1935 where *L. grippotyphosa* was isolated from calves with acute infection (Amatredjo and Campbell, 1975). The first isolation of *L. hardjo* from cattle was made by Roth and Galton (1960) in America, later in Canada by Robertson *et al.* (1964). In Australia, the first isolation of *L. hardjo* was made by Sullivan and Stallman, (1969). Since then, many serovars were discovered throughout the world and now all pathogenic leptospires are classified into one species called *Leptospira interrogans* containing 223 serovars arranged into 23 serogroups (Ramadass *et al.*., 1992; Woodward and Redstone, 1994). Clinical expertise and awareness combined with confirmatory laboratory back up give a long way in increasing the identification of patients with leptospirosis. Clinical trait of leptospirosis may vary from mild ailment to severe life threatening illness. Leptospirosis can be diagnosed only through laboratory tests as the clinical features are nonspecific. But the laboratory tests are complex and hence definite guidelines are necessary for proper diagnosis of leptospirosis.

Leptospirosis was certainly recognized as an occupational hazard of rice harvesting in ancient China and the Japanese name akiyami or autumn fever still persists in modern medicine. With hindsight, clear descriptions of leptospiral jaundice can be recognized as having appeared earlier in the 19th century, some years prior to the description given by Weil. The etiology of leptospirosis was demonstrated independently in 1915 in Japan and Germany. In Japan, Inada and Ido detected both spirochetes and specific antibodies in the blood of Japanese miners with infectious jaundice and two groups of German physicians studied German soldiers afflicted by “French disease” in the trenches of northeast France (Inada *et al.*, 1916).

Uhlenhuth and Fromme (1915) and Hubener and Reiter (1915) detected spirochetes in the blood of guinea pigs inoculated with the blood of infected soldiers. Given the initial controversy over nomenclature, it is ironic that the organism was first described almost 10 years before. Stimson by silver staining demonstrated the presence of clumps of spirochetes in the kidney tubules of a patient who reportedly died of yellow fever (Stimson, 1907). The spirochetes had hooked ends and Stimson named them Spirochaeta *interrogans* because of their resemblance
to a question mark. Unfortunately, this sentinel observation was overlooked for many years. The importance of occupation as a risk factor was recognized early. The role of the rat as a source of human infection was discovered in 1917 while the potential for leptospirosis in livestock was identified some years later (Alston and Broom, 1958). Several monographs provide extensive information on the early development of knowledge on leptospirosis (Wolff, 1954; Faine, 1994 and Faine et al., 1999). Leptospira originated from the Greek word leptos meaning fine or thin and Latin word spira meaning saprophytic species was first observed in 1907 in kidney tissue slices of a leptospirosis victim who was described as having died of “yellow fever” (Faine, 1994).

**Serological taxonomy**

Each species is divided into serogroups on the basis of serological cross-reactivity and these are subdivided into serovars. On the other hand, the term serovar is a description of a *Leptospira* whose homologous rabbit antiserum agglutinates it, but not other strains of leptospires. Serovars that show 10% cross-agglutination are collected into one serogroup. Based on variations in the carbohydrate side chains of lipopolysaccharide (LPS) of the cell membrane, more than 269 different antigenic types or serovars of pathogenic *Leptospira* have been recognized (Kmety and Dikken, 1993).

**Genetic taxonomy**

Classification and identification of leptospires using serology is a difficult and long procedure. Recently molecular biology techniques have been introduced into the field of leptospirosis. These techniques are first used to facilitate the study of epidemiology of leptospirosis. Genetic taxonomy involves DNA/DNA hybridisation and the guanine-plus-cytosine percentages (G+C) content of DNA. Genotypic classification based on DNA hybridization defined 21 genomo species of *Leptospira* that included 29 serogroups and 269 serovars. Yasuda et al. (1987) proposed a new classification of the genus *Leptospira* based on DNA homology study of 46 pathogenic and non pathogenic serovars. Woodward and Redstone
(1994) developed a polymerase chain reaction (PCR) combined with restriction fragment length polymorphism which can be used for differentiation of *Leptospira* serovars. These authors suggested that the PCR combined with restriction fragment length polymorphism can be used as a technique useful for rapid detection and preliminary differentiation of leptospires. The Committee on the Taxonomy of *Leptospira* of the International Union of Microbiological Societies in its 2002 meeting approved the following nomenclature for serovars of *Leptospira* (Zhang and Dai, 1992). Leptospires are phylogenetically related to other spirochetes. The Leptospiral genome is approximately 5,000 kb in size, although smaller ones have been reported. The genome of pathogenic *Leptospira* consists of two chromosomes, a 4,400 kb chromosome and a smaller 350 kb chromosome. Other plasmids have not been reported. Physical maps have been constructed from species *pomona* subtype *kennewicki* and *icterohaemorrhagiae* (Levett, 2001).

**Morphology**

Although more than 269 serovars of *Leptospira* have been described, all members of the genus possess similar morphology. *Leptospira* are spiral-shaped bacteria that are 6-20 µm long and 0.1 µm diameter. One or both ends of the spirochete are usually hooked. As they are very thin, live *Leptospira* are best observed under dark field microscope. The bacteria have a number of freedom degrees; when ready to proliferate via binary fission, the bacterium noticeably bends at the place of the possible split (Levett *et al.*, 2005).

**Cellular structure**

*Leptospira* have gram-negative-like cell envelope consisting of cytoplasmic and outer membrane. However, the peptidoglycan layer is associated with the cytoplasmic rather than the outer membrane, an arrangement that is unique only to spirochetes. The two flagella of *Leptospira* extend from the cytoplasmic membrane at the ends of the bacteria into the periplasmic space and are necessary for the motility. The outer membrane of *Leptospira*, like most other gram-negative bacteria contains lipopolysaccharide (LPS). Differences in the highly immunogenic LPS structure account for the numerous serovars of *Leptospira*. Consequently, immunity is species specific; current Leptospiral vaccines, which consist of single or several
serovars of *Leptospira* endemic in the population to be immunized, protect only against those serovars that are contained in the vaccine preparation. Leptospiral LPS has low endotoxin activity.

### Habitat

*Leptospira*, both pathogenic and saprotrophic can occupy diverse, habitats and life cycles; it is generally recognized that these bacteria are virtually ubiquitous in terms of geographic distribution (present everywhere except Antarctica). Most *Leptospira*, however, are hydrophilic - high humidity and neutral (6.9-7.4) pH are essential for their survival in the environment with stagnant water reservoirs, shallow lakes, ponds, puddles, etc., being natural places for the bacteria (Ratnam, 1994).

### Nutrition

*Leptospira* are aerobes whose major carbon and energy sources during *in vitro* growth are long-chain fatty acids, which are metabolized by beta-oxidation. Fatty acids are provided in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium in the form of Tween. Fatty acid molecules are bound by albumin in EMJH and are released slowly into the medium to prevent their toxic accumulation (Levett *et al.*, 2005). *Leptospira* are typically cultivated at 30°C in EMJH medium, which can be supplemented with 0.2-1% rabbit serum to enhance growth of fastidious strains. Growth of pathogenic *Leptospira* in an artificial nutrient environment such as EMJH becomes noticeable in 4-7 days; growth of saprophytic strains occurs within 2-3 days. The minimum growth temperature of pathogenic species is 13-15°C. As the minimum growth temperature of the saprophytes is 5-10°C, this temperature difference for growth is used to distinguish saprophytic from pathogenic *Leptospira* species. The optimal pH for growth of *Leptospira* is 7.2-7.6. The most widely used medium in current practice is based on the oleic acid-albumin medium EMJH (Ellinghausen and McCullough, 1965).

Like most bacteria, *Leptospira* require iron for growth, *L. interrogans* and *L. biflexa* have the ability to acquire iron in different forms. A TonB-dependent receptor requiring ferrous form of the iron for utilization has been identified in *L. biflexa*, and an ortholog of the receptor is encoded in the genome of
*L. interrogans*. *L. interrogans* can also obtain iron from heme, which is bound to most of the iron in the human body. The HbpA hemin-binding protein, which is involved in the uptake of hemin, has been identified on the surface of *L. interrogans*. Although other pathogenic species of *Leptospira* and *L. biflexa* lack HbpA, yet another hemin-binding protein, *LipL41*, may account for its ability to use hemin as a source of iron. Although *Leptospira* do not secrete siderophores, still *L. biflexa* and *L. interrogans* are capable of getting iron from siderophores secreted by other microorganisms (Levett et al., 2005).

**Epidemiology: Source of infection**

The urine of infected animals or healthy carriers, which may contaminate soil, pasture, drinking water and feed, is the main source of infection. In the case of *Leptospiral* abortion, infection can spread by the aborted foetus and uterine discharges. An infected foetus can carry the infection for up to seven weeks after birth. The semen of an infected bull may carry leptospires and transmission from such a bull to heifers by coitus and artificial insemination has been observed (Thiermann, 1984). Field observations of herd outbreaks of *L. hardjo* infection have frequently implicated bull as the source of infection.

In cattle, excretion of leptospires in the urine may take up to 542 days. *L. hardjo* is excreted from the genital tract of aborting cows for as long as eight days after abortion or calving and is detectable in the oviducts and uterus for up to 90 days after experimental infection and in naturally infected cows. Ellis et al. (1986) concluded that the genital tract appeared to be as important a site of *L. hardjo* localisation as the urinary tract. Infection may also persist in the mammary gland, as organisms have been isolated from milk of an experimentally infected cow even 91 days after infection. Leptospiral infection has been known to persist for up to 142 days in the pregnant bovine uterus and for 96 days in the non-pregnant uterus (Abdollahpour, 1990).

**Modes of transmission**

Leptospiral organisms enter into the body most often through cutaneous or mucosal abrasions. Oral transmission may occur when animals are feeding on contaminated pasture or feedstuffs, or drinking or while standing in contaminated
water. However, oral dosing is not a satisfactory method for experimental transmission as compared to injection and instillation into the nasal cavities, conjunctival sac and vagina (Amatredjo and Campbell, 1975). The finding of *L. hardjo* in the genital tract, specially the vagina of non-pregnant cattle, indicates that venereal transmission may also play a part in the epidemiology of bovine leptospirosis. Transplacental transmission is not common but neonatal infection, probably contracted in the uterus, has been recorded. It is also commonly found in the genital tract of bulls and venereal spread of the infection occurs in cattle. Leptospires have been isolated from bull semen for 18 to 38 days after infection, and Leptospiral antibodies have been detected in the semen of 54% of 71 bulls examined in Brazil (Thiermann, 1984 and Ellis *et al*., 1985).

**Pathogenesis**

Leptospires enter into the body of a susceptible host through mucous membrane or abraded skin. After 4 to 10 days, the host becomes bacteraemic, this period lasting from hours to 7 days, and may be characterised by pyrexia, presence of leptospires in milk and anorexia. However, clinical symptoms will depend on the susceptibility of the host, the serotype of *Leptospira* and the virulence of the organisms (Thiermann, 1984). After the number of leptospires in the blood and tissues reach a critical level, lesions due to the action of leptospiral toxin and consequent symptoms appear. Capillary damage is common to all species and during the septicaemic phase, petechial haemorrhages in mucosae are a common expression of this. Immunity appears to be solely humoural with circulating antibodies opsonizing leptospires, causing cessation of bacteraemia. With the appearance of circulating antibodies, leptospires localise and persist in a number of organs, especially in the proximal renal tubes and in the female genital tract. The duration of such localisation and urinary shedding will depend on host-serovar adaptation. When leptospires are infecting a definite host, infection of these organs will be long-term, perhaps even for life. In the case of accidental host infections, colonisation is short-term and urinary shedding may not occur (Ellis *et al*., 1984).

In the case of *L. pomona* infection, intravascular haemolysis and interstitial nephritis are important parts of the disease, whereas *L. hardjo* neither produces haemolysin nor causes interstitial nephritis. *L. hardjo* grows in the pregnant and
non-pregnant uterus and lactating mammary gland producing initially septicaemia and then mastitis and/or abortion. The pathology of the milk drop syndrome in *L. hardjo* infection has not been studied, but appears to be associated with the bacteraemic phase and an increase in leucocytes in the milk. *L. hardjo* has been recovered from the milk of an experimentally infected cow on day 30 and day 91 post infection (Hathaway and Marshall, 1980; Radostits *et al*., 1994). Leptospirosis in cattle may appear as acute, subacute or chronic forms. The number of animals clinically affected in a cattle herd depends on the host-serovar adaptation, and the susceptibility of the herd to the infecting serovar. In all animals the incubation period will be from 3 to 7 days (Thiermann, 1984).

Acute leptospirosis in cattle is characterised by one or more of these symptoms: fever, haemolytic anaemia, haemoglobinuria, hepatitis, jaundice, interstitial nephritis, meningitis and agalactia. Ellis (1986) enumerated the following features for diagnosis of acute leptospirosis: a) Sudden onset of agalactia in adult cattle b) icterus in young animals; and c) meningitis. *L. hardjo* causes a milk drop syndrome associated with "flabby udder mastitis" in the absence of other symptoms. Agalactia has also been observed in *L. hardjo* infected sheep and is characterised by starving of lambs in the first week of infection (Ellis *et al*., 1986).

Animals which have recovered from acute leptospirosis may develop a carrier condition in which leptospires grow and remain in the renal tubes for days to years. The excretions of animals are the central points of distribution of leptospires to other animals or people. However, *L. hardjo* can also persist in other organs, notably the genital tract of cattle. There is strong presumptive evidence that the chronic form of leptospirosis in horses causes periodic ophthalmia (Hathaway *et al*., 1981; Radostits *et al*., 1994).

**Clinical manifestation in human beings**

Humans are considered "accidental hosts" and become infected by coming into contact with the urine of infected animals. This is either through direct contact with urine or through contact with soil, water, or plants that have been contaminated by animal urine. *Leptospira* can survive as long as six months outdoors under favorable conditions. *Leptospira* can enter the body through cuts or other skin
damages or through mucous membranes (such as those inside the mouth and nose) or even through intact skin. Once pass through the skin barrier, the bacteria enter the blood stream and rapidly spread throughout the body. The infection causes damage to the inner lining of blood vessels. The liver, kidneys, heart, lungs, central nervous system, and eyes may be affected. There are two stages in the disease process. The first stage is ‘bacteremic phase’ that lasts from three to seven days and presents typical flu-like symptoms. During this phase, bacteria can be found in the patient's blood and cerebrospinal fluid. The second stage, or ‘immune phase’, occurs either immediately after the bacteremic stage or after one to three day symptom-free period. The immune phase can last up to one month. During the immune phase, symptoms are milder but meningitis (inflammation of spinal cord and brain tissues) is common. Bacteria can be isolated only from patient's urine during this second phase.

Risk factors includes, occupational exposure like farmers, ranchers, abattoir workers, trappers, veterinarians, loggers, sewer workers, meat inspectors, rodent control workers, rice field workers, military personnel, septic tank cleaners, fish farmers, miners. Recreational activities like fresh water swimming, canoeing, kayaking and trail biking in warm areas. Household exposure like pet dogs, domesticated live stock, rainwater catchment systems, and infestation by infected rodents. In humans the clinical signs are usually non specific (flu-like symptoms) and the disease may remain undetected. The first symptoms include: High fever, headache, chills, muscle pains, nausea, vomiting and diarrhea. If the patient does not recover at this stage, the condition can deteriorate and the following signs can be seen: Headaches, muscle tenderness, conjunctival suffusion, lymphadenopathy, rash, meningitis, kidney failure, liver failure, hemorrhage, myocarditis, Icterus (Weil's disease). Mortality rate can range from 5 to 40% depending upon the serovars involved and the patient's immunity status (Zuerner and Bolin, 1990).

**Economic importance of leptospirosis**

The economic importance of bovine leptospirosis includes direct or indirect costs of abortion, loss of milk production and related veterinary costs and human infection. There are also costs associated with vaccination and surveillance, including testing of serum samples before international transport, and in the case of
human infection, medical treatment, loss of productive working time and capacity. The economic importance of \( L. \) hardjo abortion, mastitis and human infection has recently been recognized in many countries. Although it is difficult to determine the actual rate of economic loss due to leptospirosis, there are some reports that show the importance of these losses. Economic losses due to leptospirosis can be caused by any of the acute, subacute or chronic forms of the disease. It is impossible to assess the total cumulative losses in an individual herd. There is a report which indicated that in a 6 years study with a split herd, vaccination experiments with \( L. \) hardjo bacterin, conducted in the dry tropics of Northern Queensland caused a significant reduction of prenatal losses but not perinatal or postnatal losses. In Venezuela, 40.8% of 1526 bovine abortions were attributed to leptospirosis. In Northern Ireland, \( L. \) hardjo infections were diagnosed in 49.7% of 348 bovine abortions. In Australia 50% of a 400-cow unit aborted within 60 days following \( L. \) pomona infection (Holroyd, 1980; Arbiol et al., 2013).

Aside from a few serological and epidemiological studies on leptospirosis, there is still a paucity of information and knowledge gaps on the economic burden of leptospirosis in India, particularly on the value that the society places on disease prevention. The importance of being able to determine the economic burden is that it allows the quantification of costs that could be avoided in reducing the rate of disease incidence. It also enables policymakers to make informed decisions when formulating cost-effective health programs, and to prioritize health investments.

The present study was undertaken with the following objectives, after conducting a thorough literature review;

**Chapter II:** This chapter includes the review of literature pertaining to the topic.

**Chapter III: Collection and isolation of Leptospira samples from both animal and plant sources (Sewage water plants),** includes collection of samples (i.e. biological materials like serum, plasma, urine and others) from different places of Karnataka state and isolates were also obtained from serum institutes and other reference laboratories like PD_ADMAS, Bangalore and Three sewage water plants located in Mysore and Bangalore were selected for the study (A, B and C). Bacterial sample were subcultured in a semisolid medium, the cultures were examined under a
dark field microscope every week. The presence of leptospires was confirmed by conducting ADMAS Leptospira staining technique.

Chapter IV: Development of molecular diagnostics for the specific detection of *Leptospira*. As the course of Leptospirosis varies from mild to severe fatal forms, laboratory based techniques gain prominence for the precise diagnosis. Culturing of the organism is the most demonstrative approach, but due to its laborious work and prolonged time, alternatively Polymerase Chain Reaction (PCR) based molecular technique potentially rapid and specific for the diagnosis of leptospirosis especially in situations of outbreaks. To overcome the above limitations associated with cultivation and serology, we developed PCR assay targeting partial *LipL32*, *LipL41* and *rpoB* gene of pathogenic leptospires using in-house designed primers. The SSCP analysis is designed for the detection of minor sequence changes in DNA amplified by PCR of single base substitution because of its simplicity and rapidity, ease and generally high sensitivity. This study demonstrates that the SSCP with our own designed primer readily distinguish eight species in the genus *Leptospira*. SSCP analysis also can aid in *Leptospira* related disease diagnosis in several aspects.

Chapter V: Genetic typing of recent *Leptospira* isolates from India. In the present study an attempt was made to assess the usefulness of RNA polymerase β-subunit encoding gene (*rpoB*) sequence which is used as an alternative tool for *Leptospira* detection and comparison of *rpoB* sequences with the other country isolates that are being used for phylogenetic analyses. The present study shows that *rpoB* sequence analysis can be a powerful tool in the identification of *Leptospira* species.

Chapter IV: *In vitro* and *In vivo* evaluation of medicinal plants and pseudo-peptides against *Leptospira*. The present study comprises the use of traditional medicines to overcome the side effects caused by chemotherapeutics. In the present study an attempt has been made to use the herbal medicine to cure leptospirosis either by direct killing or by inhibiting the growth of *Leptospira*. In this study the antioxidant activity and radical scavenging activity of methanolic and aqueous extracts of *Eclipta alba* and *Phyllanthus amarus* were evaluated for anti-oxidant activity. DNA damaging studies on *Leptospira* were also investigated. *In vitro* and *in vivo* antileptospiral and antioxidant activities were performed by using the
isolated compounds from *Phyllanthus amarus*. The spectral characteristics (IR, $^1$H-NMR, $^{13}$C-NMR and Mass Spectroscopy) of three biologically active compounds isolated from *Phyllanthus amarus* and their efficacy against *Leptospira* were determined.