2.1. Causative organism

Leptospirosis is a zoonotic disease with world-wide distribution. It is little more than 100 years since Weil; Professor of Medicine at Heidelberg (1886) whose name has been given to this disease (Weil’s disease) in humans first described the disease as "acute infectious disease with enlargement of spleen, jaundice and nephritis".

Leptospires were seen as early as 1907, but were not cultured and were named *Spirocheta interrogans* by Stimson in silver stained preparations of liver from a patient believed to have died of yellow fever, the origins of which were then unrecognized. The spirochetes had hooked ends, and Stimson named them *Spirochaeta interrogans* because of their resemblance to the question mark. The contagious nature and microbial origin of the disease were proved independently, in 1913, Inada and Ido found for the first time, that a guinea pig inoculated intraperitoneally with the venous blood of a patient suffering from Weil’s disease showed symptoms of conjunctival congestion, jaundice, and hemorrhage. In 1914, they demonstrated similar pathological observations in the next generation of guinea pigs inoculated with heart blood from an infected guinea pig. Following this they consistently obtained positive results by the inoculation into guinea pigs with blood from patients affected with Weil’s disease, or of liver tissue from patients who died of the disease. At the end of 1914, they detected a spirochete in the liver tissue of a guinea pig injected with the blood of a patient suffering from Weil’s disease, and they could get four isolates, names as Nishimura, Yamasaki, Matsumoto, and Sakamoto. They have concluded in 1915 that, this spirochete was responsible for the pathogenic cause of Weil’s disease, and they again named it *Spirochaeta icterohaemorrhagica japonica* sp.2. In 1914, they renamed the agent *Spirochaeta icterohaemorrhagiae japonica* according to the Latin nomenclature.

After the announcement of the detection of a spirochete from a patient suffering from Weil’s disease in Europe, in 1915, the name of the causative agent of Weil’s disease was changed to *Spirochaeta icterohaemorrhagiae*, deleting “japonica,” and Inada and colleagues proposed substituting the name “spirochaetosis icterohaemorrhagica” Both the groups isolated, cultivated and
described it as pathogenic Leptospires. Another saprophytic *Leptospira* found in fresh water was described in 1914 and was named as *Spirochaeta biflexa*. Within the next few years, two more spirochetes, *Spirochaeta hebdomadis* and *Spirochaeta autumnalis* were discovered by the colleagues of Inada. These were casual organisms for mild febrile illness, that is, nanukayami (7-day fever) and akiyami (autumn fever) in 1917 and 1918, Noguchi studied the spirochaeta *icterohaemorrhagiae* of Inada. He considered that the morphology to be sufficiently characteristic to justify for the creation of a new genus, which he named ‘*Leptospira*’, to be included in the order *spirochaetales*, along with *spirochaeta*, saprospira, cristispira, spironema, and treponema.

Two species of *Leptospira interrogans* namely *L. hardjo* and *L. pomona* were responsible for most of the bovine leptospirosis occurring in Australia. *L. pomona* is primarily a pig pathogen but also causes haemolytic disease in cattle. This species is a well known agent of bovine abortion and also causes fatal haemolytic disease in calves. *L. hardjo* which has adapted cattle as a primary host, is maintained within the bovine population, and has a relatively low pathogenicity. However, serological and bacteriological surveys of 197 bovine abortion cases in Victoria indicated that *L. hardjo* was not responsible for any substantial proportion of bovine abortion in contrast to the situation in Northern Ireland where the genotype *hardjo prajitno* is the prevailing organism (Palaniappan et al., 2005). Ramadass et al. (1992) proposed to remove the prefix hardjo from the strain name *hardjobovis* and call it as *L. borgpetersoni* serovar *hardjo* strain *bovis*, because they did not find any genetical relationship between strains *hardjo bovis* and *hardjo prajitno* as determined using DNA hybridization. Many animals act as carriers or vectors. Human infection results from accidental contact with carrier animals or environment contaminated with animal urine containing the organism. Epidemics of leptospirosis result from poor sanitation in urban areas and are aggravated following natural calamities (Picardeau, 2013). The majority of leptospiral infections are either subclinical or result in very mild illness and patients recover without complications. In a few cases it may manifest as multiorgan failure where the mortality can go up to 40%. Infection in pregnant women may be grave leading to severe fetal and maternal morbidity and mortality (Puliyath and Singh, 2012; Martins and Lilienbaum, 2013).
2.2. Clinical diagnosis

Clinical symptoms of leptospirosis are not highly specific so that a conclusive diagnosis can be made without laboratory confirmation. Therefore, laboratory procedures are very important tools in the diagnosis of leptospirosis. Based on the aims and needs, different techniques can be chosen to confirm the diagnosis. However, the faster and more reliable diagnostic methods would allow the clinical personnel and the farmer to commence appropriate treatment procedure and vaccination regimens with minimal delay.

The common techniques that are available and often used for the diagnosis of leptospirosis include cultural isolation of the organism, serological tests, microscopic examination of urine, fluorescent staining, the hamster inoculation test, the growth inhibition test and molecular biology techniques. During the septicaemic stage, leptospires are present only in the blood. There is laboratory evidence of acute haemolytic anaemia and increased erythrocyte fragility and often haemoglobinuria. Leucopenia has been observed in cattle while in other species there is moderate leucocytosis. However, the only positive diagnostic measure at this stage of the disease would be performing the culture of the blood. If abortion occurs, the kidney, lung and pleural fluid of the aborted foetus should also be examined for the presence of the organism. Serological testing at the time of abortion may lead to inaccurate results (Ellis et al., 1982). In the stage immediately after the normalization of fever, antibodies begin to develop and the leptospires disappear from the blood and appear in the urine. The leptospiruria is accompanied by albuminuria to varying extent and persists for indefinite lengths of time depending upon the species (Radostits et al., 1994).

2.3. Epidemiology of leptospirosis

Weil’s disease, which is one of the severe forms of leptospirosis disease, occurs in many countries, including India and other South-East Asian Countries, China, continental Europe and England. Leptospirosis is present in all the five inhabited continents and in a large number of countries. It occurs in tropical, subtropical and temperate zones. In 1961, an outbreak of leptospirosis occurred among 186 US Army personnel who were engaged in a jungle exercise.
Epidemiological investigations (1975 to 1977) carried out in Barbados revealed the seroprevalence of leptospirosis in the various occupational groups to be 29.8% (highest among sanitation workers – 42.7% followed by sugarcane workers – 39.4%). In a hospital survey 28.7% were seropositive for leptospirosis and 15% were seropositive among healthy individuals. In a survey made in northern Trinidad between mid 1977 and mid 1978 leptospiral infection was found to be widespread among the general population, and among occupational groups the highest prevalence of antibodies was found in sugarcane workers (45% infected). From 1977 to 1982, sera samples were collected from fever affected cases in Trinidad which showed that 9% were confirmed as current cases and 23% showed evidence of previous infection (Damude and Jones, 1973). Therefore, Leptospirosis is one of the most widespread zoonoses in the world (Robertson et al., 2012). More than 300 serovars have been identified among leptospires, including species *Icterohaemorrhagiae*, the most frequent in human infections. Leptospirosis remains a major public health issue in many developing countries, one century after discovering the causative agent. Leptospirosis is expected to become more important due to a rapid urbanization in developing countries (slums), global warming, and extreme climatic events (floods) (Goris et al., 2013; Picardeau, 2013).

A high prevalence of leptospiral antibodies in humans was reported from Somalia in 1982. Another survey in 1987 in Italy showed prevalence 11.34% leptospiral antibodies in rural areas while it was 3.08% in urban areas of central Italy. In 1987 seroprevalence as high as 25% (14/56) was reported in patients hospitalized in Karachi, Pakistan. Symptomatic leptospirosis is particularly frequent and severe in the Seychelles; 80 cases were reported over a two-year period during 1989 to 1990, 65 cases during 1993 to 1994, and 75 cases during 1995 to 1996. In October, 1995, epidemic hemorrhagic fever, without jaundice or renal manifestations, was reported to be caused by *Leptospira* in rural Nicaragua, following heavy flooding. In 1995, 90 out of 295, (30.5%) of apparently healthy individuals tested positive for anti-*Leptospira* antibodies by MAT in the Cordillera province of Bolivia. In Turkey, screening of 1,440 people for *Leptospira* antibodies using MAT revealed 5.48% positive cases (Perolat et al., 1998; Cacciapuoti et al., 1982 and Cacciapuoti et al., 1987). Throughout December 2010 and January 2011, Queensland experienced widespread flooding due to unusually protracted and heavy
rainfalls. In mid-January 2011, four individuals from a small community in Central Queensland were hospitalized with leptospirosis. A further five cases were subsequently identified from around Central Queensland, bringing the total to nine (Smith et al., 2012). Lupi et al. (2013) reported two cases of leptospirosis in military personnel in southeastern Brazil. The cases were hospitalized following field training exercises, and presented with acute meningoencephalitis, respiratory illnesses, and skin rash. *Leptospira interrogans* serovars *icterohaemorrhagiae, hebdomadis, patoc,* and *cynopteri* were identified in the cases by microscopic agglutination test and PCR.

### 2.3.1. Indian situation

The serological study of Leptospirosis with respect to man has been limited in India. In 1931, an extensive survey of the disease outbreak in the Andaman Islands was made and two organisms, *L. andamans* and *L. grippotyphosa* were isolated. Several investigators have confirmed the prevalence of leptospirosis in India by isolating leptospires from human subjects. In 1960, serological evidence of *L. icterohaemorrhagiae* and *canicola* antigen was found in five cases affected by jaundice (Dalal, 1960). In 1966, out of 93 sera from PUO cases, three were positive by the agglutination lysis test, one against *L. icterohaemorrhagiae* and two for *L. canicola* and out of 43 cases of jaundice, two were positive for *L. icterohaemorrhagiae* and one for *L. icterohaemorrhagiae* and *L. pomona* (Joseph and Kalra, 1966). In 1967, in Bombay, one among 150 sera collected from infective hepatitis cases showed evidence of *Leptospira* infection due to *L. pyrogenes*. *Leptospira* agglutinins at significant titres were demonstrated in 5 out of 17 sera from suspected cases of leptospirosis and in 6 cases out of 11 sera from workers of animal farms and piggeries (Bhatnagar et al., 1967). In 1983, in Madras, the seroprevalence of leptospirosis in jaundice affected patients was 18% and it was 24% in PUO cases (Ratnam et al., 1983). In 1983, a serological study was made of a population that consisted mainly of children in a village near Madras city in Tamil Nadu State, India, following an outbreak of disease in cattle; 35 of 75 (47%) human sera gave positive antibody titres (Ratnam et al., 1983). During 1984 to 1985, acute renal failure due to leptospirosis in 19 human patients was reported in Madras (Muthusethupathi and Shivakumar, 1987). In 1988, during the peak of the monsoon
season, serum and urine samples from 40 patients, with a history of fever, vomiting, jaundice, abdominal pain and renal failure, from various hospitals in Madras city and MAT revealed that 33 (82.5%) had specific *Leptospiral* antibodies (Anon, 2000). In 1993, a serosurvey of conservancy workers in Madras (using MAT) revealed a prevalence rate of 32.9% (Ratnam et al., 1993). An outbreak of acute febrile illness with hemorrhagic manifestations and pulmonary involvement occurred in Diglipur of North Andamans during October to November 1993; 66.7% of the victims had significant titres of antibodies against *Leptospira* (Sehgal et al., 1995). In 1994, an increase in the number of individuals with uveitis was noted at Aravind Eye hospital, Madurai, India after an epidemic of leptospirosis in South India; the epidemic followed severe flooding of Tamil Nadu District in the autumn of 1993 in which 37/46 patients (80%) had *Leptospira* DNA and 33/46 patients (72%) had positive serology. In 1995, a seroprevalence rate of 12% leptospirosis was found among febrile and jaundice patients in Pondicherry (Prabhakar et al., 1995). Thirty-eight acute renal failure cases with clinical suspicion of leptospirosis were screened from July to November, 1996 and 27 (71%) seropositive cases were diagnosed by MAT (Saravanan et al., 1998).

2.4. Culturing of the organism

Definitive diagnosis of leptospirosis can be obtained by performing the culture of the infecting organism. Leptospires are commonly isolated from urine or kidney of infected animals. The major advantage during the culture is that leptospires of any serovar can be detected and subsequently can be identified (Zhang and Dai, 1992). The disadvantages are, bacteriologic culture procedures are highly expensive and very slow for routine use, because fresh samples are necessary and 4 to 6 months may be required for getting conclusive results. It has been reported that even in a fresh foetus the positive identification of leptospirae in lesions is very difficult, especially with *L. hardjo* which is very fastidious in its cultural requirements. Isolation of leptospires from the urine of vaccinated cows will usually provide correct result. This is because of the presence of antibodies or other substances in the urine of vaccinated cows which restrict with the growth of leptospires.
For selecting proper tissues or samples for isolation, it is important to first determine the stage of the disease. In cases where the disease is acute isolation should be done from blood samples. In the chronic form after the development of serological responses, isolation should be performed from the urine. In the case of clinical disease or with aborted foetus, isolation should be done from either kidney, liver or the aqueous humour (Levett et al., 2005 and Levett et al., 2006).

2.5. Serological tests

The diagnosis of bovine leptospirosis is based primarily on serological tests because isolation of leptospires is difficult and time consuming. The bacterin induces an anamnestic response in cattle with prior natural exposure, and the resulting titres persist for such long time it is possible that they may be confused for titres induced by active infection. However, serology gives only limited information on the prevalence of leptospirosis because bovine leptospirosis often occurs in the absence of detectable serologic titres. The common serological tests used are the enzyme-linked immunosorbent assay (ELISA) and microscopic agglutination test (MAT), formerly known as the agglutination-lysis test (Chaudhry et al., 2013).

2.5.1. Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) is popular and in this several assays are available. They can be performed either by using commercial kits or with "in house" produced “antigen”. A broadly reactive so-called genus-specific antigen is generally used to detect IgM, and sometimes also IgG antibodies. The presence of IgM antibodies indicate current or recent leptospirosis (Ellis et al., 1988). It has a number of advantages over the MAT like it uses a dead antigen. Results can be read objectively rather than subjectively, and it can measure different immunoglobulin classes without prior fractionation of sera. Only a single antigen is used, namely the genus-specific antigen, which is shared by pathogenic and saprophytic leptospires alike. Culture preparation of leptospires in the local laboratory to provide the antigen is not required if a commercial source of kits is available. Disadvantages of ELISA are some ELISA test systems are less specific than MAT and weak cross reactions due to the presence of other diseases may be observed. ELISA result should therefore be confirmed by MAT. This may require testing a follow-up sample if the
initial sample was taken at an early stage of the infection when the ELISA test may prove positive, but the MAT may prove negative. Since it is based on genus-specific antigen, the ELISA test does not give any indication about the infecting serovar (Hartleben et al., 2013).

2.5.2. Microscopic agglutination test

The Microscopic agglutination test (MAT) which was originally described by Galton et al. (1965) and modified by Cole et al. (1973) is the most widely used serological test for leptospirosis. The MAT test determines agglutinating antibodies in the serum of a patient by mixing it in various dilutions with live or dead, formolized leptospires. Antileptospiral antibodies present in the serum make leptospires to stick together to form clumps. This clumping process is called agglutination which could be observed using dark-field microscopy. Agglutinating antibodies can be of both IgM and IgG classes. The major advantage of MAT is its high specificity. The disadvantages of MAT are the need for facilities to culture and maintain panels of live leptospires. The test is both technically demanding and time consuming, particularly when the panel is large. The antibodies may not be detectable if the causative strain is not represented in the panel. The completeness of the panel cannot to assured. Since new, unidentified leptospires may cause disease. Hence it is advisable to go for genus specific screening test such as ELISA using a broadly reactive antigen.

2.6. DNA hybridisation

DNA hybridisation with genomic probes is widely used for rapid, specific and sensitive diagnosis of many infectious diseases. The use of such probes for diagnosis of leptospirosis has been explored. Detection of leptospires in clinical samples using a DNA probe was described that identifies L. hardjobovis in cattle. Later, Zuerner and Bolin, (1988) cloned a repetitive DNA sequence from L. hardjo bovis, and found that it is a sensitive and specific probe for the diagnosis of L. hardjo bovis in cattle. In a comparative study, Bolin et al. (1989) showed that DNA hybridisation was more sensitive than culture techniques for the diagnosis of bovine Leptospirosis. In a more recent serological study in 2012, two new serovars of Leptospira interrogans, Australis and Tarassovi have been reported from Iran (Hamali et al., 2012).
2.7. Polymerase chain reaction

The Polymerase chain reaction (PCR) is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. It is useful to diagnose infectious diseases caused by fastidious bacteria. It is rapid, reliable and sensitive test for the diagnosis of leptospirosis.

In a comparative study, PCR was found to be more sensitive than either MAT or culture methods in the diagnosis of human leptospirosis. This study also indicated that PCR can be an efficient tool for early diagnosis of the disease, especially when the clinical expression of the disease leads to confusion. The main concern with PCR assay is the sometimes it leads false-positive results caused by contamination with previously amplified DNA or target DNA. Chemical or biological reagents may reduce the sensitivity of the PCR assay. The need for rapid diagnostics at the time of admission has led to the development of numerous PCR assays. Their advantage lies in the ability to obtain a definitive diagnosis during the acute stage of the illness prior to antibodies are detectable, while treatment may be effective. PCR detects DNA in blood in the first 5–10 days after the onset of the disease and up to the 15th day. The bacterial load in serum/blood ranges from $10^5$ to $10^9$ leptospires/l (Musso and Scola, 2013).

The $rpoB$ gene encodes the β-subunit of DNA dependent RNA polymerase (Severinov et al., 1996; Ko et al., 2002), and rifampicin resistance is related to mutation in a specific region of $rpoB$. Recently, $rpoB$ sequences were used as an alternative tool either for determining the phylogeny or for identification of enteric bacteria (Mollet et al., 2003), mycobacterium (Kim et al., 1999), and spirochetes including *Borrelia* (Lee Dong-hun, 2000; Renesto et al., 2000). The $rpoB$ gene has been shown to be useful and more discriminating alternative to the 16S rRNA gene for inferring phylogenetic relationship. It was earlier been demonstrated to be a suitable target to base species identification for the genera (Case et al., 2007).

PCR is advantageous because, it can amplify within few hours any specific sequence of pathogen present at low concentration. PCR detects nucleic acid of microbes irrespective of their capacity to cause infection (Vinodh et al., 2008). This
technique is the most simple, specific and rapid method for the detection as well as
differentiation between pathogenic and non-pathogenic Leptospira. Several PCR
based methods and also various regions targeting different genes have been
developed recently for the detection of Leptospira (Sugathan and Varghese, 2005).
By the use of real-time PCR, it is possible to quantify the amount of DNA/template.
Many researchers have attempted to design PCR primers specifically for Leptospira
sp. The sensitivity of PCR repeatedly precludes the need for isolation and culturing,
thus making it a rapid tool to detect the organism involved in acute infection
(Smythe et al., 2002).

While PCR-based protocols offer several advantages over standard culture
techniques, the risk of cross-contamination is a major drawback. Detailed studies on
diagnosis and epidemiology of leptospirosis have not been done and as such no
reports are available from India. Sensitive and specific confirmative diagnostic
technique for Leptospira has not been developed. In the present study an attempt
has been made to assess the usefulness of RNA polymerase β-subunit encoding gene
(rpoB) sequence and tried to use as an alternative tool for Leptospira detection and
comparison of rpoB sequences with other country isolates, which have been used for
phylogenetic analyses. Recent work clearly illustrates that rpoB sequence analysis is
a powerful tool for the identification of Leptospira species (Scola et al., 2006).

A simple and reliable tool for the early diagnosis of leptospirosis is urgently
needed. Lee et al. (2011) reported the development of a lyophilized reagent-based
PCR assay targeting LipL32 gene, which is present only in pathogenic leptospires.
To determine the effectiveness of the newly developed assay in the early diagnosis
of leptospirosis, the sensitivity and specificity was evaluated. In simulated clinical
samples, the assay was able to detect 102 and 103 leptospires/ml in urine and blood
samples, respectively. In experimentally infected animals, leptospiral DNA could be
detected in blood and lung samples as early as Day 1 post infection. This assay was
also shown to be stable and remained sensitive for up to five months at ambient
temperature. Hence, this lyophilized reagent-based PCR assay with high specificity,
sensitivity and stability would provide a simple, rapid and reliable method in
diagnosing acute leptospirosis, especially in the field of veterinary medicine. The
multiplex PCR assay (mPCR) developed in this study can be used for the early
detection of leptospirosis. The LipL32 gene could also serve as another target to aid in the efficient detection of leptospiral infection because using 2 sets of primers in mPCR increases the sensitivity and specificity of the test (Ahmed et al., 2012).

PCR, more specifically the real-time detection of the amplified PCR product, is a methodology that can provide a diagnosis in a definite time as compared to culture and serology. There are a limited number of real-time PCR assays for detecting Leptospira and not all of them can distinguish pathogenic from nonpathogenic species. In addition, there are a variety of probe technologies. Stoddard (2013) presented qPCR assay that targeted lipL32, a gene which is present only in pathogenic Leptospira spp. PCR methods use either 16S ribosomal RNA (rRNA) or 23S rRNA gene primers for the detection of different Leptospira interrogans serovars (D’Andrea et al., 2012). The performance of these two methods was assessed using DNA extracted from bovine tissues previously inoculated with several bacterial suspensions. PCR was performed on the same tissues before and after the formalin-fixed, paraffin-embedding procedure (FFPE tissues). The 23S rDNA based PCR detected all fresh and FFPE positive tissues while the 16S rDNA-based protocol detected primarily the positive fresh tissues. Both methods are specific for pathogenic L. interrogans. The 23S-based PCR detected Leptospira in four dubious cases of human leptospirosis from archival tissue specimens and one leptospirosis-positive canine specimen. Any sensitive method for leptospirosis identification in FFPE tissues would also be useful to screen histological specimen archives and to get a better understanding of human leptospirosis prevailing, especially in tropical countries.

2.8. Current diagnostic trend

For the past several decades, laboratory diagnosis of leptospirosis was based on the isolation of the organism and/or on the detection of specific antibodies. Laboratory diagnosis of leptospirosis was conventionally performed by demonstration of antibodies of leptospires in the sera. The traditional serologic test and microagglutination tests have the disadvantage that they are tedious to test when there is a large number of serovars. Other serological tests are performed to diagnose leptospirosis. Dark field microscopy is not recommended because it requires more than $10^4$ cells/ml.
Leptospira isolation is difficult, time consuming, and potentially biohazardous. The polymerase chain reaction (PCR), first described in 1985, is a highly sensitive and specific technique used in the detection of nucleic acids. Polymerase chain reaction (PCR) was developed for the diagnosis of leptospirosis by Eys et al. (1989). Primers for polymerase chain reaction (PCR) were synthesized from clones derived from a Leptospira hardjo (type hardjo bovis) library. Little or no amplification was observed with DNA from other serovars of this group. As also no amplification was evident with DNA from other serogroups, other bacteria, or eukaryotic organisms. Bao and Dai, (1990) detected the microquantity DNA of Leptospira interrogans by polymerase chain reaction. Zhang and Dai, (1992) developed a nucleotide sequence of 23S rRNA gene from Leptospira interrogans species canicola strain. Eight different species of Leptospira interrogans were detected by PCR, but not the DNA from L. biflexa.

Merien et al. (1992) developed a sensitive assay for Leptospira DNA based on amplification of the Leptospira ribosomal RNA 16S (rrs) gene. rRNA genes are very conserved throughout the bacterial kingdom. Partial rRNA sequences were analyzed in terms of oligonucleotide, which allowed the definition of major subdivisions of the eubacteria and among them was the spirochete "phylum". A 331-bp sequence from the Leptospira interrogans species canicola rrs (16S) gene was amplified, and the PCR products were analyzed by DNA-DNA hybridization by using a 289-bp fragment internal to the amplified DNA. Specific PCR products were also obtained with DNA from the closely related nonpathogenic Leptospira biflexa but not with DNA from other spirochetes, such as Borrelia burgdorferi, Borrelia hermsii, Treponema denticola, Treponema pallidum, Spirochaeta aurantia, or more distant organisms such as Escherichia coli, Staphylococcus aureus, Mycobacterium tuberculosis, and Proteus mirabilis (Natarajaseenivasan et al., 2005). Using quantitative polymerase chain reaction, Desvars et al. (2012) showed that 29.8% of rats carried leptospires in their kidneys. The sequencing of 16S rRNA (rrs) gene sequences of Leptospira found in black rat kidneys identified four genomospecies (Leptospira borgpetersenii, Leptospira interrogans, Leptospira kirschneri, and L. borgpetersenii group B), which established black rats as the major source of leptospirosis transmission to humans.
Zuerner et al. (2000) developed a PCR-based assay for typing *L. interrogans sensu lato* serovars. The assay was designed to exploit the presence of many copies of the leptospiral insertion sequence IS1533 and IS1533-like sequences present in the genomes of most leptospiral serovars. The PCR primers were designed to amplify DNA of unknown sequence between closely placed IS1533 or IS1533-like sequences. Amplification reactions primed with IS1533-based primers generated products of different sizes. When few copies of IS1533 were present in the genome, amplification of a few products was still detected. These results suggested that IS1533 elements may be found close together. Analysis of DNA amplified from different serovars showed the presence of differently sized products, thus enabling the serovars to be identified. Genetic variation among isolates within the same serovar was also demonstrated with the IS1533-based primers.

In 1997, rapid and specific method for the detection of pathogenic *Leptospira* species in bovine semen using the polymerase chain reaction was developed. The primers used were derived from an *EcoR1/BamH1* fragment that hybridized strongly to chromosomal DNA from the *hardjo bovis* serovar (Merien et al., 1992). Bolin et al. (2000) compared sensitivity and specificity of various polymerase chain reaction assays for detection of *Leptospira borgpetersenii* serovar *hardjo* in bovine urine and compared results of the optimal PCR assay with that of immunofluorescence, nucleic acid hybridization, and bacteriologic culture results. Sensitivity and specificity of five PCR assays were compared to determine the optimal assay to be used with bovine urine samples. The optimal PCR assay was then compared with results of bacteriologic culture, nucleic acid hybridization, and immunofluorescence. A PCR assay with the best combination of specificity (100%) and sensitivity (91%) was selected for comparison with the other diagnostic tests. Sensitivity of nucleic acid hybridization was 55%, whereas sensitivities of bacteriologic culture and immunofluorescence were 89 to 93% (Ellis et al., 1984).

*LipL32* is also a prominent immunogen in human leptospirosis. The sequence and expression of *LipL32* is highly conserved among pathogenic *Leptospira* species. These findings indicate that *LipL32* may be important in the pathogenesis, diagnosis, and prevention of leptospirosis (Cousins et al., 1985). Leptospirosis was diagnosed in the early stage of infection by using *flaB*-PCR by
Koizumi et al. (2003). Nassi et al. (2003) developed nested-PCR method for diagnosis of leptospirosis. Primers were designed to amplify a 264 bp region within the LipL32 gene. The sensitivity and specificity of the assay were evaluated using seven saprophytic serovars and 35 pathogenic serovars. This technique was found to be very specific for pathogenic serovars, but it lacked sensitivity. In order to enhance the sensitivity, another primer pair was designed to amplify 183 bp region within the 264 bp region in LipL32 gene, and used in Nested-PCR assay.

A PCR for the detection of Leptospira in urine was described by Ramadass et al. (1992). No amplification was observed in acidic urine, therefore neutralization of the sample immediately after collection is strongly recommended. PBS gave better results than Tris or NaOH as neutralizing reagent. Freezing and thawing of samples before processing yielded negative results. Elimination of epithelial cells, leukocytes and crystals by centrifugation at 3,000 rpm at room temperature increased sensitivity. A real-time PCR assay was developed using a 423 bp target on the LipL32 gene, which was conserved among pathogenic serovars of Leptospira by Levette et al. (2005). Positive results were obtained with all pathogenic leptospiral serovars, with the exception of Leptospira fainei species hurstbridge (Marshall et al., 1981).

Cheemaa et al. (2007) detected pathogenic leptospires in animals by PCR based on LipL21 and LipL32 genes. Efficacy of primers capable of amplifying conserved outer membrane protein (OMP) genes i.e., LipL21 and LipL32 of Leptospira strains was tested for rapid and early diagnosis of the leptospirosis using polymerase chain reaction. These OMP genes were conserved in various leptospiral serovars like, canicola, pomona, icterohaemorrhagiae, pyrogenes, sejroe, grippotyphosa, ballum and tarassovi as PCR products of 561 bp and 756 bp and were obtained by PCR employing LipL21 and LipL32 based primers respectively, in all these serovars. Absence of such amplicons in DNA extracted from Pasteurella, Campylobacter and Brucella confirmed the specificity of the primers.

There is need for some method for the identification of Leptospira species, the important reemerging pathogen, with accurate identification up to the species level. 16S rRNA gene sequence analysis is at present widely used method for identification of fastidious bacteria, including Leptospira species. The general
molecular identification of *Leptospira* relies on nucleotide sequence determination and comparison. Though DNA sequencing has become a common technique now-a-days, but the equipment itself is still costly, especially when dealing with large number of samples or strains in bionetwork and biodiversity studies. A simple and comparative study of PCR which combined with SSCP was applied to the analysis of *Leptospira* species to reveal the sequence polymorphism, differentiation and sequencing of the PCR products (Orita *et al.*, 1989; Stach *et al.*, 2001).

The taxonomy of the genus *Leptospira* has now been clarified from genetics and leptospirosis point of view and can now be studied using genetic tools, when isolates are available (Levett, 2007; Cerqueira *et al.*, 2009). Similarly, Leptospirosis diagnosis increasingly relies on PCR results (Goarant *et al.*, 2009), where a single positive sample provides a certainty diagnosis before serological conversion (WHO, 2003). This frequently results in the loss of the serology based identification of the infecting strains, which is epidemiologically important to identify the reservoirs. Therefore, the increased use of PCR has greatly improved the early diagnosis of leptospirosis, but paradoxically restricts the data needed for epidemiological surveillance. However as because the genetic tools implemented provide an insight into the genome of the infecting strain, epidemiologically relevant information could be deduced from sequence polymorphisms of the diagnostic PCR products. This approach was notably suggested and evaluated by Victoria *et al.* (2008) while studying the phylogeny of the S10-spc-a locus- these authors demonstrated that this locus is highly conserved and is a useful phylogenic target. They additionally suggested a short 245 bp region of secY as a suitable target for diagnosing leptospirosis by PCR, the sequence of the diagnostic PCR product then becomes epidemiologically informative. Later on, a diagnostic PCR using this target was designed, and validated according to international guidelines and confirmed to provide an epidemiologically relevant phylogeny (Ahmed *et al.*, 2009).

The quantitative PCR technique used for leptospirosis diagnosis amplifies a 331pb DNA fragment within the lfb1 gene, and this sequence polymorphism allows the identification of the species of the infecting *Leptospira* strain using melting curve analysis (Merien *et al.*, 2005). The Multi Locus Sequence Typing (MLST) technique uses sequence polymorphisms of multiple housekeeping genes for
characterization of isolate and to investigate evolutionary relationships among closely-related bacteria. It is increasingly considered as the gold standard typing method at least in species where sufficient sequence polymorphisms exist in housekeeping genes, because it relies on sequence data that are exchangeable and independent of the analytical platform (Maiden et al., 1998; Urwin and Maiden, 2003). This technique was successfully applied to a number of bacterial pathogens and recently it was applied to the study of leptospires (Ahmed et al., 2006; Thaipadungpanit et al., 2007; Leon et al., 2010).

Several molecular techniques have been evaluated for the identification of Leptospira species or serovars. These include random amplified polymorphic DNA, arbitrarily primed PCR, use of insertion sequences in PCR-based assays, restriction length polymorphism, specific probes, variable number tandem repeat analysis and pulsed-field gel electrophoresis (Barocchi et al., 2001; Levett, 2001; Majed et al., 2005). However, none of these techniques were based on sequence analysis. This may lead to problems as far as reproducibility of results among different laboratories is concerned. The genes coding for outer membrane proteins have been sequenced for six Leptospira pathogenic species. These genes transfer horizontally from one species to the other. This makes these genes unsuitable for the identification of various Leptospira species (Haake et al., 2004).

Using rpoB sequence analysis, Scola et al. (2003) separated 11 L. interrogans serovars and placed those under seven genotypes. As in the case with rrs (Postic et al., 2000), this technique separated several genogroups. However, it failed to distinguish clearly the member of one serovar from that of the other. This confirms the belief that the same serovar can be found in different genetic groups/species (Herrmann et al., 1992; Brenner et al., 1999; Levett, 2001). Analyses of the most commonly encountered serovars may be useful in understanding the contribution of rpoB sequencing to the molecular screening of Leptospira isolates. Such information may prove invaluable for the determination of various serovars of Leptospira. Alternatively, analysis of a segment of rpoB may be useful as an initial screening test for the identification of a new isolate of Leptospira using a system of similarity cut-off to define species (Scola et al., 2006). If the partial rpoB similarity of a test isolate is lower than 92%, it should be considered as a new species. On the
other hand, if the value goes above 97%, the isolate under scrutiny should be taken as representative of a known species. However, for routine identification of *Leptospira*, these cut-offs should be validated on large collections of isolates, as it was done for *Corynebacterium* sp. (Khamis et al., 2005). Finally, this technique can become useful for the detection as well as identification of *Leptospira* in clinical or environmental samples, if the specificity of the primers had been verified earlier.

Gene sequence analysis is an important technique for the delineation of *Leptospira* species. The genes used earlier in such analyses were 16S rRNA (*rrs*) (Boonsilp et al., 2011), DNA gyrase subunit B (*gyrB*) (Kawabata et al., 2006; Slack et al., 2006; Slack et al., 2009; Villanueva et al., 2010) and RNA polymerase subunit B (*rpoB*) (Scola et al., 2006). It is not still clear as to what constitutes a new *Leptospira* species based on genetic differences at one or a small number of genetic loci. No doubt that a *Leptospira* species may be genetically distinct from the other species of the genus based on a large degree of genetic diversity. Nucleotide distances between pathogenic and intermediate species evaluated in this study were more than 4% when compared using the nearly entire *rrs* gene (> 3% based on the 443-nucleotide *rrs* region).

Spirochetes are emerging pathogens for which culture and identification are not fully unresolved. In fact, 16S rRNA-based sequencing is by far the most widely used PCR methodology that is able to detect such uncultivable pathogens. However, it has some limitations with respect to problems of contamination, which hampers diagnosis. To overcome this, Renesto et al. (2000) have devised a simple PCR strategy involving targeting of the gene encoding the RNA polymerase β-subunit (*rpoB*), a highly conserved enzyme. The complete sequence of the *Leptospira biflexa* (serovar *patoc*) *rpoB* gene was determined and compared with the published sequences of *Borrelia burgdorferi* and *Treponema pallidum*. From the results, degenerate nucleotide primers were designed and tested for their ability to amplify a portion of the *rpoB* gene from various spirochetes. Using two different pairs of these primers, they obtained specific *rpoB*-amplified fragments for all members of the genera *Leptospira*, *Treponema*, and *Borrelia*. Their findings have significant implications in development of a new technique for the identification of spirochetes,
especially, if clinical samples are contaminated or when the infecting strain is un cultivable.

Thousands of bacterial species have been isolated and studied so far and the infections continuous to get transmitted from animals to man as well as the parasitic diseases. Infectivity has the ability to overcome the hosts defenses and virulence and variability has the capacity to harm the host by the pathogen (Elizabeth, 2001). India is a vast country having vide diversity in climatic conditions, botanical and mineral wealth, flora and fauna, and well practiced knowledge of traditional herbal medicines. Herbal medicines are still mainstay of 75-80% of the world population, mainly in the developing countries for primary health care. It has been accepted the part of culture and has compatibility with the human body causing lesser or no side effects (Shah, 1981). Leptospirosis is a ubiquitous and zoonatic disease of worldwide in distribution which affects internal organs producing multiorgan dysfunction to multiple organ failure. It is basically an occupational disease and man gets the infection mostly by virtue of his occupation (Ciceroni et al., 2000). In 1888, this disease was reported from agricultural workers with some febrile illness. It also affects other occupational groups who have close proximity with animals and water bodies. Human infection is accidental, usually occurring by direct or indirect contact with urine from infected animals (Barwick et al., 1997). Other mechanisms of transmission include animal bites and handling of infectious tissues.

An effective course of treating leptospirosis still remains as an unsolved problem. Leptospirosis usually responds to treatment with the antibiotics, provided they are properly treated in the early stages of infection (Burne and Canine, 1996). Benzyl penicillin should be administered intravenously for up to 7 days with daily dose of 6-8 mega units (3.6-4.8 g) but penicillin may cause a temporary exacerbation of the symptoms. Tetracycllin should be administered if there is evidence of renal failure. Continuous renal replacement therapy is supposed to be superior to conventional haemodialysis in leptospirosis (Emmanouilides et al., 1994). Vaccines are currently available in a very limited extent outside certain geographical areas and few have been licensed to produce in developed countries. In recent years, there has been a global trend towards the use of natural phytochemicals present locally, such as herbs, fruits and vegetables, as sources of antioxidant. To overcome the adverse
reaction by the above drugs, herbal-based therapeutics are being used in treating leptospirosis (Chen, 1986; Hubbert, 1997).

Modern medicine has evolved from folk medicine and traditional system only after thorough chemical and pharmaceutical screening. The use of synthetic compounds led to a decline in the use of plant based products in modern medicine. However, synthetic medicines can cause side effects and as a result people are more favorable to use natural compounds obtained from plants. Thus, plants remain a major source of medicinal compounds. About 20,000 plant species are used for medicinal purposes (Penos, 1983). Seventy four percent of 119 plant derived drugs were discovered as a result of chemical studies to isolate the active substances responsible to cure the ailment (Farnsworth and Soejarto, 1991). So plants, especially the higher plants contain a variety of substances, which are useful as food additives, perfumes, and in the treatment of various diseases due to their versatile therapeutic properties (Mukherjee and Wahile, 2006). The active secondary metabolites possess various medicinal applications as drugs or as model compounds for drug synthesis. Phytochemical analysis of plants, used in folklore has yielded a number of compounds with various pharmacological activities. In view of the increasing development of resistant microorganisms, treatment of various diseases caused by microorganisms has become a major challenge in the human medical field. This may be due on the one hand, to the synthetic nature of these substances, and on the other to their known side effects and in some cases to their unpleasant smell, taste or the burning sensation felt on the skin. Medicinal plants are important sources for the study of their traditional uses through the verification of pharmacological effects and can be natural composite sources that act as new anti-infectious agents. About 3,000 active ingredients from 2,764 plant species have been screened for their pharmacological and chemotherapeutic properties (Anon, 1988). India has yielded an incredible array of plant products that have drawn the attention of ethno pharmacologists from around the world. Traditionally used medicinal plants produce a variety of compounds having known therapeutic properties (Iyengar, 1976; Harborne, 1989; Chopra et al., 1992).

_Eclipta alba_ L. Hassk. (Asteraceae), commonly known as _Bhringaraja_ (Sanskrit), _Maka_ (Marathi) and _Bhangra_ (Hindi) has been reported to show protective effect on experimental liver damage in rats and mice (Dhaka, 2013; Singh
The plant has been used for the treatment of liver cirrhosis and infective hepatitis (Chopra et al., 1966). The uses of many traditional herbs in the treatment of many diseases, are usually free from side effects, are economical and also easily accessible to humans. Plants are the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and even more continue to provide mankind with new remedies (Heinrich et al., 2004). The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, lack of side effects and economic viability (Karthikumar et al., 2007). In Ayurveda, the root powder of *E. alba* is used for treating hepatitis, enlarged spleen and skin disorders. Mixed with a little oil when applied to the head, the herb relieves headache. The extract of leaves is mixed with honey and given to infants, for the expulsion of worms. *E. alba* is also given to children in case of urinary tract infections. The methanolic extract of *E. alba* was subjected to analysis for the antioxidant and antimicrobial activities (Prabhu et al., 2008).

*Phyllanthus amarus* is a member of the Euphorbiaceae family, which groups over 6500 species in 300 genera. Euphorbiaceae is a large family of upright or prostrate herbs or shrubs, often possessing milky acrid juice (Lewis and Elwin-Lewis, 1977) and is mainly a pantropical family with some species belonging to more or less temperate. Numerous species of this family are native to North, Central and South America (Unander et al., 1995). The plants are monoecious or homogamous; leaves are simple, alternate or opposite, some are leathery; flowers are very small and diclinous, they cluster in cup-shaped structures, greenish, often with glands. The fruit is a three-lobed capsule extending from the cup and commonly the long stalk pendant (Wessels et al., 1976). The name ‘*Phyllanthus*’ means “leaf and flower” because the flower as well as the fruit seem to become one with the leaf (Cabieses, 1993). Plants in the genus *Phyllanthus* can be found around all tropical regions of the world: from Africa to Asia, South America and the West Indies. *Phyllanthus* comprises of 550 to 750 species in 10-11 subgenera.

Various biological activities have been listed for by *Eclipta alba*, such as memory disorders treatment, general tonic, edema, fevers, rheumatic joint pain treatment, indigestion, hepatitis, enlarged spleen, antioxidant property and skin
disorders (Karnick and Kulkarni, 1990; Karthikumar et al., 2007). Wedelolactone is the active principle compound of *E. alba* for liver disorder treating (Wagner et al., 1986). It also exhibits trypsin inhibitor activity (Samiulla et al., 2003; Syed et al., 2003), suppresses LPS-induced caspase-11 expression in cultured cells by directly inhibiting the I kappa B kinase (IKK) complex (Kobori et al., 2004), curative effect on cirrhosis of the liver and infectious hepatitis. The shoot extract of *E. alba* shows antimicrobial (Wiart et al., 2004), antifungal activity (Venkatesan and Ravi, 2004) and weak cytotoxicity against M-109 cell lines by alkaloids verazine (Abdal et al., 1998), antiviral activity against Ranikhet disease virus (Khin et al., 1978) and antibacterial activity (Kumar et al., 2007). *E. alba* is a weed/herb growing in damp, moist puddles distributed in tropical and subtropical regions of the world. Besides ethnobotanical evidence, it can be hypothesized that plants which survive in soils rich in microbes also will have most likely possessing antimicrobial principles.

In many countries around the world plants in the genus *Phyllanthus* is used in folk remedies; therefore this genus is of great importance in traditional medicine (Foo, 1993). The genus *Phyllanthus* has a long history of use in the treatment of liver, kidney and bladder problems, as also diabetes and intestinal parasites. Some related species in this region with medicinal significance are *P. epiphyllanthus*, *P. niruri* *P. urinaria*, *P. acuminatus* and *P. emblica* (Tirimana, 1987). *P. amarus*, *P. nururi* and *P. urinaria* are used in the treatment of kidney/gallstones, other kidney related problems, appendix inflammation, and prostate problems (Heyde, 1990).

*Phyllanthus amarus* has also shown to work as an antifungal, antibacterial and antiviral agent (Houghton et al., 1996). Foo and Wong (1992) reported that in India this plant is used in traditional medicine to treat liver diseases, asthma and bronchial infections. Chevallier (2000) notes that *P. amarus* is also used traditionally in India to treat cardiovascular problems. This popular medicinal herb is also a remedy around the world for influenza, dropsy, diabetes and jaundice (Foo, 1993; Yuandani et al., 2013).

The standard methods to be followed for the study of efficacy of drugs against leptospiral members are tube dilution technique (TDT) and micro dilution technique (MDT). On comparing with tube dilution technique, micro dilution was found to be better that it cleared the leptospires even during the study period of
30 min, which might make this method to be better suited for performing antileptospiral studies (Prabhu et al., 2008)

Heyde (1990) and Nanden-Amattaram (1998) noted that in traditional medicine herbal decoction along with other herbs is used to treat bladder and kidney disorders, cramps and uterus complaints. This plant decoction can also be used as an appetizer. Extract of the roots is used for jaundice. Three roots are boiled in ½ liter of water and two cups are drunk daily. Decoctions for the other described ailments can be prepared by extracting two fresh plants or five dried plants in ¾ liter water drinking one cup of tea four times day.

The secondary metabolites present in Phyllanthus amarus are alkaloids, flavanoids, hydrolysable tannins, major lignans and polyphenols. Several investigations were reported where the structures of most of the phytochemicals were determined by UV, IR, Mass and NMR spectroscopy (Foo and Wong, 1992; Foo, 1993; Foo, 1995). Houghton et al. (1996) isolated securinega type alkaloids by Column Chromatography (CC) and preparative Thin Layer Chromatography (TLC). They did qualitative analysis by using TLC, and spots were detected by UV radiation (254 nm and 365 nm). The unknown compounds were determined by UV, IR, mass and NMR spectroscopy.

The problem of increasing resistance of microorganisms to the antibiotics felt the need for the development of novel antimicrobial agents (Travis, 1994). Peptides are the most versatile bioactive molecules, but they are not fit as drugs, because of their quick degradation or get modified in the body. A number of studies indicated that most antibacterial peptides exerted their activities by enhancing the permeability of pathogenic cell membranes and these kind of antibacterial peptides are difficult to induce resistant strains of pathogens compared to normal antibacterial agents (Park et al., 2003). Over the past decade, many defence antimicrobial peptides counter were isolated and characterized from a variety of natural sources (Zasloff, 1987) such as magainins and a class of antimicrobial peptides and Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor (Orita et al., 1989).
However, even in spite of high potentials, there are still some limitations for peptides to be used as drugs. Main disadvantages are short half-life, rapid metabolism and poor oral bioavailability. Nevertheless, pharmacokinetic properties of peptides can be improved by different types of modifications (Adessi and Soto, 2002). Peptidomimetic modifications or cyclization of linear peptides are frequently done to provide more conformationally constrained and thus more stable bioactive peptides (Davies, 2003) Pseudo-peptides or peptide bond surrogates, in which peptide bonds have been replaced with other chemical groups have now become especially attractive. Such approaches create an amide bond surrogate with defined three dimensional structures similar to natural peptides, but with significant differences in polarity, hydrogen bonding capability and acid-base character. The structural and stereochemical integrities of the adjacent pair of α-carbon atoms in these pseudo-peptides remain unchanged.

The high synthetic nature and the ease with which chemical functionality is introduced indicated the high potentiality of artificial pseudo-peptides. In addition, their high bio stability has enabled successful applications in both in vitro and in vivo studies. The design, synthesis and application of peptidomimetic compounds have taken many years of research to develop the therapeutics with peptide like activity. In the past 10 years, many defence antimicrobial peptides to counter infection by microbes were isolated and characterized from a variety of natural sources (Hultmark et al., 1980; Zasloff, 1987). Most antimicrobial peptides acted through the lipid membrane of pathogenic cells and had low level of toxicity against mammalian cells (Saberwal and Nagaraj, 1994; Maloy and Kari, 1995; Matsuzaki et al., 1995). As the membrane-active peptides have different mode of action compared to classical antibiotics, these peptides can be considered as alternative agents for fighting resistant strains of pathogen. However, the antimicrobial peptides, like other biologically active peptides, have a limitation that is poor in vivo stability. In addition, they have also the risk of degradation by enzymes released by the pathogens (Vunnam et al., 1998; Dalhammar and Steiner, 1984). Peptide modifications including reduced amide (Sasaki et al., 1987), peptoid (Horwell, 1995), carbamate (Cho et al., 1993) and azatide (Han and Jenda, 1996) have been developed and applied for improving the bioavailability of various active peptides (Doulut et al., 1992; de Solms et al., 1998; Guichard et al., 1995). These
peptide backbone modifications can mimic the conformation of the parent peptide without side chain modification or the change of chirality and can be applied easily the peptides regardless of primary amino acid sequence.

Membrane-active antimicrobial peptides, isolated and developed from natural sources, have antibacterial and antifungal activities. Although these membrane-active peptides have poor bioavailability for using as therapeutic agents, still they have received attention because of their low toxicity against mammalian cells and the unique mechanism of perturbing the membrane of the pathogen. The different types of peptidomimetics and other pseudo-peptides having drug properties include peptoids (N-substituted oligoglycines) β-peptides, γ-peptides, pyrrole-imidazole polyamides, DNA-like peptide nucleic acids, alpha-helical peptide nucleic acids, DNA-cleaving pseudo-peptides in DNA binders as well as peptide nucleic acids. Peptides are the most versatile bioactive molecules, but they are not good drugs, because of their quick degradation or modification taking place in the body. Thus, drug discovery has turned to the novel field of peptidomimetics to design non-peptide compounds mimicking the pharmacophore and thus the activity of the original peptide. These novel compounds open up new perspectives in drug design by providing an entire range of highly specific pharmaceuticals that have a high bioavailability.

Peptides are among the most versatile bioactive molecules, but they are quickly degraded or modified in the body. The novel field of peptidomimetics deals in designing non-peptide compounds that mimics the pharmacophore and thus has the activity of the original peptide. These novel compounds open up new perspectives in drug design by providing an entire range of highly specific pharmaceuticals that have a high bioavailability (Cheng et al., 1992). Barros and coworkers in 2010 described the synthesis of a novel class of pseudo-peptides derived from isomannide and several oxazolones as potential inhibitors of serine proteases with preliminary pharmacological assays for hepatitis C. Hepatitis C, Dengue and West Nile fever are flaviviruses that share one important serine protease enzyme. Serine proteases are the most studied proteolytic enzymes and much attention is bestowed the drug development field. Several pseudo-peptides were
obtained in good yields by the reaction of isomannide with oxazolones and their anti-HCV potential was shown the HCV replicon-based assay.

Incorporation of aza-β3-amino acids into an endogenous neuropeptide from mollusks with weak antimicrobial activity allowed the design of new antimicrobial peptide (AMPs) sequences. Depending upon the nature of substitution, this can make the pseudo-peptides either inactive or may drastically enhance the antimicrobial activity without high cytotoxicity. Structural studies of the pseudo-peptides carried out by NMR and circular dichroism show the impact of aza-β3-amino acids on peptide structure. The first three-dimensional structures of pseudopeptides containing aza-β3-amino acids in aqueous micellar SDS were determined and demonstrated that the hydrazino turn can be formed in aqueous solution. Thus, AMP activity can be modulated through structural modifications induced depending upon the nature and the position of amino acid analogues in the peptide sequences (Laurencin et al., 2012).