EVALUATION OF WHOLE CELL ANTIGEN AND OUTER MEMBRANE PROTEIN BASED LATEX AGGLUTINATION TEST FOR SERODIAGNOSIS OF CANINE LEPTOSPIROSIS

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DECLARATION

I hereby declare that this thesis, entitled **"EVALUATION OF WHOLE CELL ANTIGEN AND OUTER MEMBRANE PROTEIN BASED LATEX AGGLUTINATION TEST FOR SERODIAGNOSIS OF CANINE LEPTOSPIROSIS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy,

SARIPRABHA P.

CERTIFICATE

Certified that this thesis, entitled **"EVALUATION OF WHOLE CELL ANTIGEN AND OUTER MEMBRANE PROTEIN BASED LATEX AGGLUTINATION TEST FOR SERODIAGNOSIS OF CANINE LEPTOSPIROSIS"** is a record of research work done independently by **Sariprabha P.,** under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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CERTIFICATE

We, the undersigned members of the Advisory Committee of **Dr. Sariprabha, P.,** a candidate for the degree of Master of Veterinary Science in Veterinary Microbiology, agree that this thesis entitled **"EVALUATION OF WHOLE CELL ANTIGEN AND OUTER MEMBRANE PROTEIN BASED LATEX AGGLUTINATION TEST FOR SERODIAGNOSIS OF CANINE LEPTOSPIROSIS"** may be submitted by Sariprabha, P., in partial fulfillment of the requirement for the degree.

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Sariprabha P.

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Introduction

INTRODUCTION

Leptospirosis is a widespread and re - emerging zoonosis and has become an important global human and veterinary health problem. It is an acute febrile illness caused by spirochaete belonging to genus *Leptospira,* which comprises of at least 268 serovars, which have been genetically classified under 17 genomospecies of *Leptospira* and *Leptonema* (Brenner *et al.,* 1999). These species are *L. interrogans, L. borgpetersenii, L. santarosai, L. inadai, L. noguchii, L. weilli, L. kirschneri, L. biflexa, L. meyeri, L. wolbachii, L. illini, Turneria parva* (proposed) and 5 others unnamed species *viz.*, *L.* genomospecies 1, *L.* genomospecies 2, *L.* genomospecies 3, *L.* genomospecies 4 and *L.* genomospecies 5. These different *Leptospira* strains have been allotted to 31 serogroups (Levett, 2001).

Most of these serovars can infect different animal species, but there is a primary host reservoir for each serovar, which ensures the survival and dissemination of the organisms. For example, the dog is the maintenance host for *L. interrogans* serovar Canicola and is considered as an incidental host for a variety of other serovars. The rodents and domestic animals are the most important reservoir hosts, although pathogenic *Leptospira* species have been isolated from essentially every known mammalian species.

Transmission generally occurs after a susceptible animal is directly exposed to leptospires in an infected host's urine or contaminated water, mud, or moist soil. In their reservoir host, leptospires escape the immune system in the proximal convoluted renal tubules allowing the infected animal to become a persistent shedder. Following shedding in urine, these bacteria may survive for some months, given appropriately temperate, moist or wet environments; however, survival is very poor in dry or cold environments. Shedding animals pose a public health risk. Leptospires can be transmitted to humans through

contact with urine-contaminated environments, particularly water. Human leptospirosis is usually associated with direct contact with shedding companion animals, occupational exposure (sewage and agricultural workers, veterinarians, for example) or outdoor recreational activities such as swimming, boating, or endurance competitions.

Seroprevalence of leptospirosis in apparently healthy and diseased animals had been reported in various parts of India (Natarajaseenivasan and Ratnam, 1997; Varma *et al.,* 2001). Common *Leptospira* serovars detected were Icterohaemorrhagiae, Patoc, Australis, Autumnalis, Grippotyphosa, Pyrogenes, Pomona and Canicola.

Leptospira interrogans serovars Canicola and Icterohaemorrhagiae were mainly responsible for causing canine leptospirosis (Forrest *et al*., 1998). But canine leptospirosis may also be associated with other serovars, *viz*. Pomona, Bratislava, and Grippotyphosa (Prescott *et al*., 1996; Birnbaun *et al*., 1998). Acute renal failure is associated with serovars Pomona and Grippotyphosa.

Early diagnosis of leptospirosis is important, since the mortality rate is high among patients with the most severe presentations. But, the clinical recognition of leptospirosis is very difficult in early stages of the disease because the initial symptoms are often indistinguishable from that of other febrile illnesses (Levett *et al*., 2000). The disease is evident in different forms, with a wide spectrum of clinical symptoms. In case of dogs the symptoms include vomiting, dehydration, collapse, fever, hepatitis, nephritis and death. However, Doxycycline therapy initiated soon after the onset of the disease can prevent severe complications and deaths due to leptospirosis (McClain *et al*., 1984; Faine *et al*., 1999).

Traditionally, diagnosis of leptospirosis depends on the demonstration of leptospires in clinical samples by culturing of leptospires; by dark field

microscopy (DFM) or by demonstration of leptospiral antibodies by microscopic agglutination test (MAT).

For diagnostic purpose, the culture of leptospires from body fluids is the most demonstrative approach. But, this technique can take up to two months and is very laborious (Cousins *et al.,* 1985). Also detection of leptospires in blood and urine by DFM is not always possible and the possibilities of false positive results are greater with inexperienced workers. Thus, there is a need for a rapid and appropriate diagnostic test to help clinical case identification, adoption of appropriate medical measures and to conduct seroprevalence studies in risk population.

Considering the above mentioned drawbacks in the isolation or demonstration of leptospires in clinical samples, emphasis has been largely laid on serodiagnosis. Anti - leptospiral antibodies are detectable by the seventh day of illness.

Serological diagnosis includes detection of specific antibody response of the host that can be assessed by MAT, the most widely accepted test with unsurpassed specificity. Demonstration of leptospiral antibodies by MAT requires good laboratory facilities for maintenance and culturing of different leptospiral strains. Moreover, the test uses several leptospiral serovars in their active growth phase. Maintenance and standardization of such live antigens is difficult, expensive, tedious and time consuming (Thiermann and Garret, 1983; Cousins *et al.,* 1985; Bolin *et al.,* 1989). The danger of posing a considerable risk to laboratory technicians while handling the live leptospiral antigens, the cumbersome mechanisms of recording the results and need of paired serum samples to confirm the disease have frustrated many researchers to adopt it as a routine test.

Enzyme Linked Immuno Sorbent Assay (ELISA) has been found to be a sensitive method for detection of antibodies (Pappas *et al.,* 1985; Vijayachari *et*

al., 2001), however, elaborate laboratory facilities like incubators and ELISA readers are required. Recently, DNA based methods like nucleic acid probes, polymerase chain reaction and Random Amplified Pplymorphic DNA methods have been developed for the diagnosis of leptospirosis. However, these techniques require well established laboratory facilities and highly skilled personnel. Hence, there is an urgent need for the development of new diagnostic strategies for leptospirosis.

Rapid serologic tests based on whole cell leptospiral antigen preparations have been developed as an alternative method to screen leptospiral infection (Terpstra *et al*., 1985; Yersin *et al.,* 1999; Smits *et al*., 2000). But, these have not been of diagnostic value since the immunogenic moiety in whole cell antigen preparations appears to be broadly reactive *i.e*. present in both pathogenic and non-pathogenic species (Matsuo *et al*., 2000 a, 2000 b). So, development of rapid, sensitive and appropriate diagnostic test that could be used in a routine diagnostic laboratory to detect antibodies against canine leptospirosis is the need of the hour.

Recently the focus of research on leptospirosis diagnosis has been on the standardization of outer membrane protein (OMP) based serological tests. These tests may achieve high sensitivity and specificity because of the lack of non-specific moieties present in whole cell preparations. More widely acceptable approaches have been developed, which take advantages of presence of cross-reactive antigens in crude extracts, which are shared among diverse leptospiral serovars (Guerreiro *et al.,* 2001). These cross-reactive antigens include the proteins present in the outer membrane of the organisms. The OMPs seem to play an important role in pathogenicity of bacteria. Utilizing a small number of different serovars, outer membrane preparations have been shown to provide heterologous protection. Use of these cross-reactive antigens *i.e.,* OMPs, which are shared among diverse leptospiral serovars, could be a major development in an effective serodiagnosis of the disease. OMP based Latex agglutination test

(LAT) is a suitable technique for the examination of a large number of sera as it involves immunodominant antigen and lacks non-specific moieties present in whole-cell preparations. This test is extremely simple and rapid and can be performed in situations where facilities or resources to perform more complicated tests are not available. To explore this presumed link, a study was conducted with the following objectives.

1. preparation of whole cell antigen and outer membrane protein (OMP) from *Leptospira interrogans* serovar Australis.

2. development of whole cell antigen / OMP based latex agglutination assay for the detection of leptospiral antibody in canine sera.

3. comparison of the results of whole cell antigen and OMP based latex agglutination tests with that of microscopic agglutination test.

Review of Literature

2. REVIEW OF LITERATURE

Leptospirosis is considered to be the most wide spread zoonosis in the world. Organisms belonging to the genus *Leptospira* cause the disease. These are capable of infecting most mammalian species through either direct contact with infected animals or indirectly by contaminated soil and water.

2.1 HISTORY

The earliest records of suspected leptospirosis are available through the reports of Alston and Broom (1958) and Gsell (1984) who have reviewed the epidemics of infectious jaundice during the 19th century.

Leptospirosis as a distinct clinical entity was first recognized in Heidelberg on the basis of description given by Adolf Weil in 1886. He investigated cases of spirochaetal jaundice in man that was later named as Weil's disease by Gold Schmidt in 1887. Within next few years, the disease was reported from Britain (Young, 1889) and several other European countries (Jaegar, 1892). Stimson (1907) described the morphological characteristics of the organism and named it *Spirocheta interrogans*. Later on Wolbach and Binger (1914) recovered an organism from pond water by filtration method and called it *Spirochaeta biflexa.* The causative agent of Weil's disease was discovered independently by Inada *et al.* (1916) and Uhlenhuth and Fromme (1916) and confirmed by Stokes and Ryle (1916) in Britain. The organism was grown in culture and was named *Spirochaeta icterohaemorrhagiae* by Japanese workers and as *Spirochaeta nodosa* and *Spirochaeta icterogenes* by the German groups (Penn and Pritchard, 1990).

Noguchi isolated leptospires in the year 1917 from rat and human and gave the name leptospira (fine coil). Ido *et al*. (1917) discovered the role of rat as a source of human infection and isolated *L. Icterohaemorrhagiae* from a patient in Japan with jaundice and haemorrhagic manifestations.

The leptospirosis in domestic animals was first recognized in a jaundiced dog (Krumbein and Frieling, 1916). The first serovar isolated from cattle was Grippotyphosa from USSR in 1928. Later, Canicola from Netherlands in 1933, Australis and Pomona from Ballice in 1937, Sexkoebing and Ballum from Denmark in 1944 and other strains of different serogroups were subsequently isolated in different parts of the world from animals and reservoir hosts (Ratnam, 1994). In 1948, Tarassovi was found to be carried by pigs and produced disease in human. Serovar Hardjo was isolated from cattle in USA in 1957 and was slowly recognized as a major serovar causing disease in cattle in Australia and the UK. Penn and Pritchard (1990) reported that the commonest cause of leptospirosis in people exposed to cattle in most parts of the world is *L. hardjo*.

2.2 PREVALENCE

2.2.1 India

The prevalence of leptospirosis in animals and humans based on the demonstration of antibodies in sera samples had been reported from time to time from different states of the country. Taylor and Goyle (1931) were probably the first to report the prevalence of *Leptospira* jaundice amongst human beings from Andaman Islands. The strains isolated from the cases were identified as *L. andamana* and *L. grippotyphosa* (Dasgupta, 1938; Lahiri, 1941). The report was followed by those of Ayyar (1932) in dogs, Dasgupta (1940) among rodents and Dasgupta and Sen (1945) in dogs. Ball and Sheikh (1958) reported the prevalence of disease in sheep, goats and horses in Bombay. Pande and Sekariah (1960) studied the seroprevalence of the disease in cattle and buffalo and found that 4.4

per cent of sera were positive for *Leptospira* antibodies. The presence of leptospiral infection among cattle population of a farm in Uttar Pradesh was reported by Adinarayanan *et al.* (1960). During an outbreak of leptospiral infection among bovines in Madras city, the infected animal's sera showed antibody titres ranging from 1:100-1:300 against serovars Poi, Javanica and Hyos (Venkataraman and Jaganathan, 1961). In a report of occurrence of leptospirosis in Uttar Pradesh among sheep and goats, *L. icterohaemorrhagiae* was found to be a predominant serovar (Mukherjee *et al.,* 1962). Paragaonker and Ramakrishna (1964) reported that strains of Pomona and Hebdomadis serogroups were responsible for infection in sheep and goats from Andhra Pradesh.

In Madhya Pradesh serovars Icterohaemorrhagiae, Sejroe, Canicola and Grippotyphosa were predominant in cattle (Sawhney and Saxena, 1967). In a report of abortion associated leptospiral infections in cattle in Kerala, titres of 1:100 against serovars Hebdomadis, Medinensis, Hardjo, Sexkoebing and Grippotyphosa were observed (Sivadas *et al.,* 1970). In later years, Rao and Surendran (1970), Rajasekhar and Nanjiah (1971) and Palit and Sharma (1971) reported seroprevalence of leptospiral infection in cattle and buffalo from different parts of India. Sheep and goat sera samples were found positive for Autumnalis, Pomona and Wolfii. Arora and Baxi (1978) detected agglutinins in sera of donkeys with a history of abortion. Further, the disease prevalence has been reported by Srivastava *et al.* (1983) and Ahmed *et al.* (1985). These workers screened the sera of domestic animals and found the predominance of serovars Autumnalis, Patoc, Pomona, Canicola, Icterohaemorrhagiae, Australis and Hardjo in various parts of the country. Srivastava *et al.* (1983) used MAT to detect antibodies to 16 serovars of *L. interrogans* and *L.biflexa* in serum samples throughout India. A total of 36 (10.1 per cent) of 355 cattle, 4 (5.8 per cent) of 68 buffaloes, 12 (6 per cent) of 169 sheep, 5 (6 per cent) of 80 goats and 3 (42.3 per cent) of 7 wild animals were found positive.

Vaid *et al.* (1991) reported that 8.17 per cent of cattle in Himachal Pradesh harboured antibodies against serovars Autumnalis, Hardjo and Australis. Serovars Autumnalis and Pomona were found to be predominant in a study conducted in Tamil Nadu involving sera of different domestic animals and humans (Ratnam *et al.,* 1994). Natarajaseenivasan and Ratnam (1996) investigated leptospirosis in a laboratory animal house and 79.2 per cent of albino mice, 90 per cent of the wistar rats, 71.4 per cent of guinea pigs and 81.8 per cent of rabbits had antibodies to one or more serogroups of *L. interrogans.* Natarajaseenivasan and Ratnam (1997) further reported 61.5 per cent, 56.3 per cent, 75 per cent, 52.9 per cent and 72.5 per cent seroprevalence of leptospirosis among cattle, sheep, goats, rats and agricultural workers, respectively. Indu (1997) found 32.67 per cent prevalence of canine leptospirosis in Thrissur.

In Andhra Pradesh, 10.58 per cent animals were found positive for subclinical leptospirosis. A seroprevalence of 10.52 per cent in cattle, 11.4 per cent in buffalo and 9.34 per cent in sheep was reported with a predominance of serovars Hardjo and Pomona (Mrunalini and Ramasastry, 2000). Varma *et al*. (2001) reported 14.81 per cent, 25.46 per cent, 26.66 per cent and 20.43 per cent seroprevalence of leptospirosis in crossbred cattle, non descript cattle, buffalo and goat, respectively, in Andaman and Nicobar island, Grippotyphosa being the predominant serovar.

Singh (2003) studied the prevalence of a leptospiral serovars in 638 sera samples from different animal species from various parts of India and reported an overall seropositivity of 19.12 per cent for leptospiral antibodies. The prevalence of 25.4 per cent, 15.4 per cent, 10.5 per cent and 6.5 per cent was reported in buffalo, cattle, sheep and goats respectively with predominance of serovar Hardjo (8.4 per cent) followed by Javanica (7.4 per cent), Hebdomadis (2.3 per cent) and Pomona (2.0 per cent) predominated. In a seroprevalence study conducted during a period of 1990-2000 by Srivastava and Kumar, (2003), leptospiral agglutinins

were detected in 15.8 per cent of 2601 cattle, followed by 9.9 per cent of 756 equine, 15.2 per cent of 551 sheep, 2.7 per cent of 443 buffalo, 14.3 per cent of 271 goat and 19.5 per cent of 166 swine in various states of the country.

Recently, Sivaseelam *et al.* (2003) collected 2164 and 344 serum samples from sheep and goats, respectively, from Madurai district of Tamil Nadu and subjected them to MAT. Seroevidence of leptospirosis was reported in 13.08 per cent of goats and 7.0 per cent of sheep, with a predominance of serovars Grippotyphosa and Pomona in sheep and Grippotyphosa and Icterohaemorrhagiae in goat. Srivastava and Kumar (2003) investigated the seroprevalence of leptospirosis in animals and human beings, using sera collected from various states of the country, during a period of 10 years beginning 1990. Most of these animals were reported to have suffered with fever, jaundice, abortions and repeat breeding. A total of 4992 sera collected from domestic animal (4348), wild animals (112) and human beings (532) were subjected to MAT using a panel of 18 leptospiral antigens. Maximum positivity was detected in sera received from Andhra Pradesh and Gujarat. A total of 2601 cattle, 414 buffalo, 271 goat, 551 sheep, 756 equine, 204 dog and 166 swine sera collected from various states were tested and 15.8 per cent 2.7 per cent, 14.3 per cent, 15.2 per cent, 9.9 per cent, 19.1 per cent, 9.0 per cent, respectively, were positive. Seroprevalence in human beings and wild animals was reported to be 14.6 per cent and 12.5 per cent, respectively. *Leptospira* serovars predominantly responsible for seropositivity among animals and man were identified as Icterohaemorrhagiae, Hardjo, Patoc, Australis, Canicola, Grippotyphosa, Pyrogenes, Pomona, Tarassovi and Ballum. In a study conducted in Thrissur, Kerala, MAT was found to be the most appropriate test for epidemiological surveillance as it could identify the infecting leptospires up to the serogroup level (Manju, 2004). In a study conducted in Uttaranchal state, a total of 290 serum samples of cattle, buffaloes and goats were tested using five serovars of

Leptospira viz., Australis, Canicola, Hardjobovis, Javanica and Pomona. The prevalence was higher in buffalo (14.7 per cent) followed by cattle (11.9 per cent) and goats (4.6 per cent) (Agarwal *et al*., 2005).

Koteeswaran (2006), in a seroprevalence study of leptospirosis in man and animals in Tamil Nadu, reported a seropositivity of 57.47 per cent among domestic animals, 72.73 per cent in wild animals in captivity and 37.03 per cent in rodents with an overall percent positivity of 56.68 per cent. Seghal. (2006) commented that the core determinants of epidemiology of leptospirosis were often influenced by various socio-cultural, occupational, behavioral and environmental factors prevailing in a community and hence the epidemiology of the disease could be quite complex and dynamic. Swapna *et al*. (2006) conducted a study in Calicut, Kerala, among agricultural workers, fishermen and fisher folk, hospital sanitary workers, sewage workers, laboratory staff, veterinarians and construction workers. The highest prevalence in the study was found in hospital sanitary workers (56.2 per cent) and fishermen and fisher folk (52.8 per cent) followed by construction workers (40 per cent), agricultural workers (30 per cent), sewage workers (28.2 per cent), veterinarians (13.3 per cent) and laboratory staff (3.3 per cent). Healthy controls had a prevalence of 24 per cent, which was comparatively high suggesting leptospirosis was predominantly an environmental disease, with an added component in the form of occupational activity. The most common serovar identified in the high risk groups (26.5 per cent) was Pomona, followed by Shermani (19.5 per cent), Canicola (16 per cent), Bataviae (13.5 per cent), Autumnalis (11 per cent), Djasiman (10.5 per cent), Tarassovi (10 per cent), Icterohaemorrhagiae (7 per cent), Australis (6.5 per cent), Hebdomadis (4.5 per cent), Hardjo (3 per cent), Ballum (2.5 per cent), Cynopteri (2.5 per cent), Sarmin (2.5 per cent) and Patoc (2.5 per cent). A seroprevalence study was conducted among the cattle and buffalo by Maria *et*

al (2007) in Uttaranchal, Tamil Nadu and Uttar Pradesh. A total of 68 sera samples came positive among the 321 samples tested and serovar Sejroe was found to be predominant (6.2 per cent). Balakrishnan *et al*. (2008) reported *L. interrogans* serovar Australis as an emerging serovar in Tamilnadu.

Sumathi *et al.* (2008) revealed that leptospirosis occurs throughout the year although the number may increase during the monsoon season (June to January). This emphasizes the importance of a polluted environment which is an important epidemiological risk factor.

2.2.2 Abroad

Leptospirosis in animals had been reported from most of the countries of the world. Bovine leptospirosis was first recognized in northern Caucasus in USSR when leptospires were demonstrated as the cause of infectious jaundice or icterohaemoglobinuria in calves (Michin and Azinov, 1935; Semskow, 1941). Ryu, (1962) detected antibodies against several leptospiral serotypes in buffalo sera in Taiwan, Malaysia and Thailand. Higgins *et al*. (1980) conducted a sero-surveillance study in Canada during 1977-79 and found that majority of the sera samples reacted with serovar *Pomona.* The seroprevalence of leptospirosis was monitored by Schoenberg *et al.* (1987) in German Federal Republic and observed the predominance of serovar Hardjo in cattle and sheep. Feresu (1987) tested cattle sera samples in Zimbabwe during 1981-82 and found the predominance of Sejroe and Tarassovi serovars.

Jeblawi (1984) tested serum samples of cattle and goat from 8 regions of Syria using 13 serovars of *L. interrogans*. In cattle, 34 per cent of positive samples reacted to serovar Poi and in goats 12 per cent of samples positive to *L. autumnalis* were reported. Draghi *et al.* (1986) tested bovine serum samples from 73 farms in Argentina and found that 12.4 per cent were positive at an agglutination titre of 1:200 and 4 per cent with titres of 1:200- 1:10,000. Bahaman *et al.* (1987) conducted sero survey in animals and reported that serovar

Sejroe was prevalent in cattle and buffalo and Pomona in sheep and goats. Collares-Pereria (1991) surveyed for bovine leptospirosis in Portugal and reported a significantly higher prevalence (13.3 per cent) of infection in coastal regions. The prevalence of leptospirosis was examined in 286 stray dogs from Turkey and leptospiral antibodies were present in 26.9 per cent of serum samples as detected using Grippotyphosa, Icterohaemorrhagiae, Ballum and other *L. interrogans* strains (Ozdemir and Diker, 1999).

The studies on the prevalence of leptospirosis in the entire world have shown the emergence of this disease globally. Juliano *et al.* (2000) showed the seroprevalence of 81.9 per cent of leptospirosis by testing serum samples collected from cattle in the microregion of Goianio-Go, Brazil. The commonest serovars were Wolfii (36.1 per cent), Icterohaemorrhagiae (20.5 per cent), Hardjo (5.2 per cent) and Tarassovi (4.9 per cent). Alonso *et al.* (2001) investigated seroprevalence of leptospirosis in herds and individual cattle in Spain and found that 43 per cent of the herds and 8 per cent of the individual animals were positive against one or more of the serovars studied, with maximum prevalence for *L. bratislava.* The prevalence of various *L. interrogans* serovars in dairy cattle as determined by analyzing 464 serum samples form cows in Brazil revealed that serovars Hardjo and Bratislava were the predominant cause of seroconversion (Oliveira *et al.,* 2001).

In a report on seroprevalence of leptospirosis in Nigeria, serovars Icterohaemorrhagiae (18.13 per cent), Hardjo (15.7 per cent), Grippotyphosa (13.0 per cent), Hamptoni (13.0 per cent), Pomona (12.2 per cent) and Ballum (10.4 per cent) were found predominant by Agunloye *et al.* (2001). Ozdemir and Erol (2002) serosurveyed leptospirosis in animals in Turkey. Of a total of 574 cattle, 200 sheep and 150 human serum samples tested, 257, 16 and 37 samples, respectively, were positive. The results showed that serovar Hardjo was dominant (72 per cent) among the positive samples. A total of 512 cattle sera samples

collected from Turkey during April-July 2003 were tested for antibodies against 3 different serovars (Grippotyphosa, Hardjo and Icterohaemorrhagiae), using MAT and ELISA. 45 and 72 of 512 serum samples were found positive with MAT and ELISA respectively, the predominant serovar being Grippotyphosa (Aslantis and Ozdemir, 2005). A serological survey was conducted among sows in South Vietnam in 1999 to 2004 to investigate variation in leptospiral seroprevalence (Boquist *et al*., 2005). In this study the serology of 6 *Leptospira* serovars was analyzed by MAT using sera samples from 429 sows and variations were found for serovars Bratislava and serovar Icterohaemorrhagiae. A sero-epidemiologic study on canine leptospirosis was conducted in Trinidad (Adesiyun *et al*., 2006). Of a total of 419 serum samples tested, 61 (14.6 per cent) were positive for *Leptospira* agglutinins.

Among equines, leptospiral antibodies have been reported against serovars Pomona and Grippotyphosa in USA and Europe by Damude *et al*. (1979). Kitson Piggut and Prescott (1987) examined 557 horse sera samples in south western Ontario, Canada against 7 leptospiral serovars and found that serovar Bratislava was predominant. Lopez *et al*. (1998) detected antibodies to *L.interrogans* in horses used for production of hyperimmune serum and found that 83 per cent horses were positive to different serovars *viz.,* Autumnalis, Australis and Pomona.

2.3 MORPHOLOGY OF LEPTOSPIRES

Leptospira cells are tightly coiled spirochetes, usually 0.1μm by 6 to 0.1 by 20 μm, but occasionally cultures may contain much longer cells. The helical amplitude is approximately 0.1 to 0.15 nm and wavelength 0.5 nm (Faine *et al.,* 1999). The cells have pointed ends, either on one or both of which are usually bent into a distinctive hook. Two axial filaments (periplasmic flagella) with polar insertions are located in the periplasmic space. Leptospires have a typical double

membrane structure in common with other spirochaetes, in which cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlaid by an outer membrane (Haake, 2000).

Leptospires exhibit two distinct forms of movement, translational and non-translational. Lipopolysaccharide has a lower endotoxic activity than other Gram negative bacteria (Shimizu *et al*., 1987).

They produce both catalase and oxidase (Smibert, 1977). They are obligate aerobes with an optimum growth temperature of 28-30°C. The most widely used medium in current practice is Ellinghausen McCullough Johnson Harris (EMJH) medium supplemented with Tween-80 and bovine serum albumin (Faine, 1982).

2.4 CULTURAL CHARACTERISTICS

Growth of leptospires in media containing serum or albumin with polysorbate and also in protein free synthetic media had been described (Turner, 1970). Several liquid media containing rabbit serum were described by Fletcher, Korthoff, Noguchi and Stuart (Turner, 1970). Some strains are more fastidious and require the addition of either pyruvate (Johnson *et al*., 1973) or rabbit serum (Ellis *et al.,* 1976) for initial isolation.

The most widely used is EMJH (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967).

Growth of contaminants from clinical specimens could be inhibited by addition of a fluorouracil (Johnson and Rogers, 1964). Other antibiotics have been added to media for cultures of veterinary specimens, in which contamination is more likely to occur (Myere and Varela-Diaz, 1973; Adler *et al*., 1986). Growth of leptospires is often slow on primary isolation and culture

bottles are retained for 13 weeks before being discarded but pure cultures in liquid media usually grow within 10-14 days. The addition of 2 per cent pooled rabbit serum to semi-solid commercial EMJH medium with EMJH enrichment and 0.5 mg of 5-fluorouracil per ml was found to enhance the growth rate and success of isolation of *Leptospira interrogans* serovar Hardjo from bovine urine (Brugge and Louw, 1985).

In semisolid media (0.1 per cent-0.2 per cent agar) growth reaches a maximum density in a discrete zone beneath the surface of the medium which becomes increasingly turbid as incubation proceeds. This zone is known as Dinger's ring (Dinger, 1932), which is related to the optimum oxygen tension (Faine *et al*., 1999).

Leptospiral cultures may be maintained by repeated subculture (Waitkins, 1984) or preferably by storage in semisolid agar containing hemoglobin (Faine *et al*., 1999).

2.5 OUTER MEMBRANE PROTEINS OF LEPTOSPIRA

The outer membrane of *Leptospira* facilitates direct interactions with the environment and likely contains important constituents involved during infection, transmission, survival and adaptation to environmental conditions, including putative vaccinogen and diagnostic candidates (Nally *et al*., 2005).

Based on the studies conducted, OMPs were described as transmembrane protein, lipoprotein and peripheral membrane protein (Hata *et al.,* 1988; Brown *et al*., 1991; Haake *et al*., 1991; Zuerner *et al*., 1991; Nicholson and Prescott, 1993; Haake *et al*., 2000). The common OMPs shared among different *L. interrogans* serovars were found at 110, 100, 43, 41, 38, 34, 32, and 21kDa (Gitton *et al*. 1992). The OMPs act as cross-protective immunogen as they share many of the polypeptides with several pathogenic *Leptospira* strains and are immunogenic in

laboratory animals (Haake *et al*., 1993). Recently, Luo *et al*. (2005) reported that the LipL32 and LipL41 genes were extensively carried and frequently expressed by different serogroups of *L. interrogans* and their expression products exhibited cross-antigenicity and also homogeneity of nucleotide and the putative amino acid sequence of LipL32/1-LipL41/1 fusion gene were 99.9 per cent and 99.8 per cent in comparison with the reported sequences.

Surface exposed moieties or structures are presumably the factors that mediate adherence of the leptospiral pathogen to mammalian host cells. Like other spirochetes, leptospires have a large repertoire of lipoproteins; subgroups of which may be involved in mediating the host cell infections. A high level of amino acid sequence conservation was found with 98.9 per cent and 97.3 per cent average amino acid sequence identity for LipL32 and LipL41, respectively (Haake *et al*., 2000).

An inverse relationship between expression of transmembrane OMPs and virulence was demonstrated (Haake *et al*., 1991). Outer membrane lipoprotein LipL36 was down regulated *in vivo* and was not recognized by the humoral immune response to host adapted leptospirosis in hamster (Haake *et al*., 1998). In contrast, LipL41 was demonstrated on organisms colonizing the lumen of proximal convoluted tubules. Thus, outer membrane components might be important in the pathogenesis of interstitial nephritis (Barnett *et al*., 1999).

Global analysis of OMPs, carried out using two-dimensional gel electrophoresis (2-DE) identified proteins that included the outer membrane lipoproteins LipL32, LipL36, LipL41 and LipL48. No known proteins from any cellular location other than the outer membrane were identified (Cullen *et al*., 2002). Recently outer membrane vesicles (OMVs) were purified and characterized by 2-DE. These contained the previously described outer membrane proteins $OMPL_1$, Qlp42, LipL32, LipL41, LipL36 and Loa22 (Nally *et al*., 2005).

The LipL32 and LipL41 OMPs appeared to be expressed constitutively by all pathogenic *Leptospira* species under all conditions i.e. during cultivation and infection, while LipL36 and a fourth lipoprotein OMP, LipL48, were subjected to differential expression (Barnett *et al.,* 1999). LipL32 is also a prominent immunogen during human leptospirosis. These findings indicate that LipL32 may be important in the pathogenesis, diagnosis and prevention of leptospirosis (Haake *et al*., 2000).

Recently, recombinant lipoproteins of *L. interrogans* were investigated for the immune-functional epitopes and inflammation inducing effects. Xu *et al*. (2005) reported that the expressed LipL32 or LipL41 of *L. interrogans* contained similar immune functional epitopes. The recombinant clones *viz.* LipL32/1, LipL32/2, LipL41/1 and LipL41/2 were able to directly induce inflammatory reaction in human umbilical vein endothelial cell line, EVC-304, which was measured by ELISA.

2.6 SEROLOGICAL DIAGNOSIS

Serological approaches have been developed taking advantage of cross-reactive antigens in purified or crude extracts that are shared among diverse leptospiral serovars. These cross-reactive antigens could include proteins and/or components of LPS. Serological assay described in the past included the macroscopic agglutination (Mazzonelli *et al*., 1974), microcapsule agglutination (Arimitsu *et al.,* 1982) and indirect hemagglutination (Levett and Whittington, 1998), all of which were less sensitive than the MAT and identified less than 50 per cent of patients presenting the early phase of leptospirosis (Guerreiro *et al.,* 2001).

Above-mentioned tests were performed using whole cell antigens and the immunodominant moiety was found to be a broadly relative antigen that resulted in cross reactivity (Matsuo *et al.,* 2000 a ; 2000 b). Recombinant protein-based

serological tests might achieve high sensitivity and specificity because of the high concentration of immunoreactive antigens which could be used in assays and the lack of nonspecific moieties present in whole-cell preparations (Flannery *et al*., 2001).

2.6.1 MICROSCOPIC AGGLUTINATION TEST (MAT)

The antigenic variability of leptospiral LPS was considered to be one of the limiting factors for serodiagnosis of the disease. The MAT has been the "gold standard" confirmatory test for the past several years and is based on the seroreactivity with LPS antigens. The need to assess agglutination by dark field microscopy and to maintain a large battery of live leptospiral antigens in culture, restricts the use of the MAT to a few reference laboratories worldwide (Guerreiro *et al*., 2001).

The MAT is a complex test to control, perform, and interpret (Turner, 1968). Live cultures of all serovars required for use as antigens must be maintained and the range of antigens used should include serovars representative of all serogroups (Turner, 1968; Faine, 1982). Also the repeated weekly subculture of large numbers of strains presents hazards for laboratory workers (Pike, 1976). Other drawbacks of MAT include the continuous risk of cross contamination of the antigen cultures, necessitating periodic verification of each serovar. The MAT titers are affected by the culture medium in which the antigens are grown (Myers, 1976).

Interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serogroups, especially in acute-phase samples (Tjalma and Galton, 1965). The broad cross-reactivity in the acute phase, followed by relative serogroup specificity in convalescent-phase samples, results from the detection in the MAT of both IgM and IgG antibodies (Tong *et al*., 1971; Morris and Hussaini, 1974; Awad - Masalmeh and Willinger, 1983) and

the presence of several common antigens among leptospires (Chapman *et al*., 1987; Lin *et al*., 1997). Therefore, paired sera from the suspected individuals are required to confirm a diagnosis with certainty. Moreover, patients with fulminant leptospirosis might die before seroconversion occurred (Ribeiro *et al*., 1994). Therefore, MAT serology is considered as insensitive, particularly in early acute-phase specimens (Cumberland *et al*., 1999).

Formalized antigens have been used in the MAT to overcome some of the difficulties associated with the use of live antigens. But the titers obtained with these antigens were somewhat lower, and more cross-reactions were detected (Sulzer *et al*., 1978; Faine, 1982). However, for laboratories lacking expertise to maintain live antigens, formalin-treated and lyophilized antigens might present a good alternative (Levett, 2001). Locally isolated strains, which often increase the sensitivity of the test compared with reference strains, can also be included in the battery of antigens.

2.6.2 SLIDE AGGLUTINATION TESTS

In order to facilitate a rapid screening of the serum samples, macroscopic slide agglutination tests have been tried by many workers as a practical adjunct to the serological tests like MAT and ELISA in resource-poor settings where the availability of trained personnel and expensive equipment are scarce. The test has been used nowadays as a reliable screening test for the detection of acute and recent infections by many laboratories (Faine *et al*., 1999**)**. Considerable reports are available on the use macroscopic agglutinantion tests for detecting leptospiral antibodies in humans as well as in animals.

A macroscopic slide agglutination test was described in which 12 serovars were combined into four pools for the rapid screening of sera from humans and animals (Galton *et al*., 1965). Despite the use of an expanded antigen range, false-negative results were reported with sera from populations in areas of

endemic leptospirosis (Wolff and Bohlander, 1966). The test was modified by employing single serovar as antigen, usually serovar Patoc (Mailloux *et al*., 1974; Marı´n-Le´on *et al*., 1997). Animal serum samples were tested in a macroscopic slide agglutination test using a single antigen, a thermo resistant antigenic fraction of *L.biflexa* serovar Patoc and found satisfactory for the diagnosis of the disease (Mailloux *et al*., 1974). Nicolescu *et al*. (1982) have tested 571 domestic animal sera suspected of leptospirosis by macroscopic agglutination test employing Patoc antigen and concluded the test as a valuable screening test for veterinary investigations, although some studies had reported that test was insensitive (Wanyangu *et al.*, 1987; Marı´n-Le´on *et al*., 1997). In a commercial slide agglutination assay, it was found that Patoc slide test was as sensitive and specific as an IgM-ELISA **(**Brandao *et al*., 1998).

2.6.3 LATEX AGGLUTINATION TEST

Srivastava *et al*. (1989) standardized a latex agglutination test (LAT) for the rapid screening of animal and human sera for leptospirosis. They have used partially purified antigen of *Leptospira biflexa* serovar Patoc for coating the latex beads. The test was found to have a sensitivity and specificity of 85.5 per cent and 76.3 per cent, respectively for animal sera as compared to MAT. Similarly for the human sera it was 76.5 per cent and 89.4 per cent.

Ramadass *et al*. (1999) standardized a rapid semi-quantitative latex agglutination test for the detection of leptospiral antibodies in serum samples of man and animals. For sensitizing the latex beads, pooled sonicated antigen from different *Leptospira* serovars *viz*., Icterohaemorrhagiae, Australis, Autumnalis and Canicola was used. A total of 276 human sera samples were analyzed by both LAT and ELISA and percentage positives were 84.8 and 85.9 per cent, respectively. Similarly, of 65 animal samples tested, 63.1 and 69.2 per cent positivity were observed in LAT and ELISA, respectively. They had concluded

that even though the ELISA test was slightly more sensitive than LAT, the rapidity, simplicity and economics of the LAT were found to fulfill the requirements of a screening test for leptospiral antibodies.

A latex agglutination test using the antigen prepared from the pathogenic strain Ley 607 of the serovar Hardjo was evaluated for the detection of genus-specific antibodies in human sera (Smits *et al*., 2000). The overall sensitivity and specificity of the test was 82.3 per cent and 94.6 per cent, respectively.

Sukone and Janya (2006) evaluated a simple latex agglutination test for the serodiagnosis of acute human leptospirosis. Using a total of 380 serum samples they observed a sensitivity and specificity of 94.1 per cent and 97.0 per cent, respectively.

Dey *et al*. (2007) developed a rapid recombinant antigen-based latex agglutination test to detect specific anti-leptospiral antibodies from human and dog sera. The recombinant LipL32 antigen developed and used for detecting the antibodies was specific in detection of the pathogenic serovars of *Leptospira* as the expression of the LipL32 antigen was restricted only to the pathogenic leptospires. The test was found to be sensitive, specific and accurate as compared to the MAT.

Senthilkumar *et al.* (2007) evaluated the OMP rLipL41 Latex Agglutination Test and compared the results with that of MAT. The sensitivity of LAT was 95.83 per cent to canine serum samples, when compared with standard MAT and the kappa value (k=0.92) also showed perfect agreement between the tests.

Senthilkumar *et al.* (2008) opined that LAT and flow through test were potential format for rapid large scale screening of samples in endemic areas without any sophisticated equipment. The sensitivity and specificity of the LAT

were 89.70 and 90.45 per cent and that of Flow through assay were 89.09 and 77.70 per cent.

Materials and Methods

3. MATERIALS AND METHODS

Glassware of Borosil brand and Tarsons brand plasticware were used in this study. All chemicals used were of Molecular biology grade, obtained from Bangalore Genei and Sisco Research Laboratories Private Limited (SRL). Ready-made media were procured from Hi-Media Laboratories Private Limited.

3.1 MATERIALS

3.1.1 REFERENCE STRAINS OF *Leptospira*

The following strains of Leptospira, representing nine different serogroup were procured from National Leptospira Reference centre, Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands, India and maintained in Leptospira culture medium (Himedia).

Serogroup	**Serovar**	**Strain**
Australis	Australis	Ballico
Autumnalis	Rachmati	Rachmati
Canicola	Canicola	Hondutrecht IV
Grippotyphosa	Gryppotyphosa	Moskava V
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
Javanica	Poi	Poi
Semaranga	Patoc	Patoc I
Pomona	Pomona	Pomona
Pyrogenes	Pyrogenes	Salinem

3.1.2 GLASSWARE

Screw capped test tubes of Borosil brand were used for the study. The glassware were washed using mild soap solution, rinsed in running tap water and then in double glass distilled water, dried and sterilized in hot air oven at 160^0C for one hour.

3.1.3 MEDIA

3.1.3.1 *Leptospira* culture medium base

Leptospira culture medium base with supplement (Himedia) was employed for growth and maintenance of the leptospires.

3.1.3.2 Supplement

3.1.3.2 a Leptospira Culture Medium Supplement (Himedia)

3.1.3.2 b Haemolysed rabbit serum

Haemolysed rabbit sera were procured from Krishi Vigyan Kendra, Thrissur. Prior to collection, syringes were flushed with single distilled water and blood was collected from ear vein using 24 gauge scalp vein set. About 20 ml of blood collected from each rabbit was kept undisturbed for about half an hour at room temperature and then shifted to 4^0C for overnight. The sera were separated by centrifugation at 2000 rpm for 20 min and preserved at -20^0C until use.

3.1.3.2 c Albumin supplement

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

Zinc sulphate 7 H_2O	0.1
Calcium Chloride 2 H_2O	1.0
Magnesium Chloride 6 H_2O	1.0

Ferrous Sulphate 7 H_2O	0.5
Copper Sulphate $5H_2O$	0.3
Tween 80	10.0
Vitamin B_{12}	0.02

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

Zinc sulphate 7 H_2O	2 ml
Calcium Chloride 2 H_2O	2 ml
Magnesium Chloride 6 H_2O	2 ml
Ferrous Sulphate 7 H_2O	20 ml
Copper Sulphate $5H_2O$	0.2 ml
Tween 80	25.0 ml
Vitamin B_{12}	2.0 ml

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

3.1.3.2 d Foetal Bovine Serum (Sigma, USA)

3.1.3.3 Preparation of 5-Fluorouracil solution

One hundred mg of the 5-FU was added to 5 ml of the sterile triple distilled water. To this 0.1 to 0.2 ml of 0.1N NaOH was added and the volume was made up to 10 ml with sterile triple distilled water. The solution was sterilized by filtration through a 0.2 μm membrane filter and stored at -20^0C.

One ml of this solution was added to 100 ml of liquid or semisolid medium to obtain a final concentration of 100μg/ml of medium.

3.1.3.4 *Leptospira* culture liquid Medium

The *Leptospira* culture liquid Medium was prepared by dissolving 2.56 g *Leptospira* culture liquid Medium Base in 900 ml of triple distilled water. The pH was adjusted to 7.5 using 1N NaOH. After sterilization by autoclaving at 121^0 C for 15 min at 15 lbs, the base medium was allowed to cool to 40-50^0C . To the cooled medium, heat inactivated, filtered, haemolysed rabbit serum at 10 per cent level was added with sterile precautions. To avoid contamination 5 - Fluorouracil (100µg / ml final concentration) was added into the culture medium. Medium was dispensed in three to five milliliter quantities in screw capped tubes. The tubes were checked for sterility by incubating at 37^0C for about 48 hours and then stored at 4^0C.

3.1.3.5 *Leptospira* semisolid Medium

Leptospira semisolid Medium was prepared by adding 0.1-0.5 per cent bacteriological agar to Leptospira liquid Medium and sterilized by autoclaving at 121^0 C for 15 min 15 lbs pressure. After sterilization, the medium was allowed to cool. After adding supplement and 5-Fluorouracil as described above, the medium was distributed in aliquots of three to five milliliter in screw–capped tubes. Sterility of the medium was tested by incubating the medium for 48 hours at 37^0C and then stored at 4^0C.

3.1.4 BUFFER AND REAGENTS

3.1.4.1 Phosphate Buffered Saline (PBS 1x solution)

Sodium chloride	8g
Potassium chloride	0.2g
Disodium hydrogen phosphate (Na2HPO4)	1.133g
Potassium dihydrogen phosphate (KH2PO4)	0.2g
Distilled water	1000 mL

pH was adjusted to 7.2 by 1N NaOH and sterilized by autoclaving at 121^0C for 15 min at 15 lbs pressure and stored at 4^0C until use.

3.1.4.2 Carbonate – bicarbonate buffer (0.06M) pH 9.6

Sodium Carbonate	1.59 g
Sodium Bicarbonate	2.93 g
Distilled water	1000 ml

pH was adjusted to 9.6 by 1N NaOH and sterilized by autoclaving at 121^0C for 15 min at 15 lbs pressure.

3.1.4.3 Buffers and reagents for outer membrane protein extraction

3.1.4.3 a Phosphate buffered saline (PBS) pH 7.2 (prepared as in 3.1.4.1)

3.1.4.3 b Tris – HCl 20 mM (pH 7.2)

Tris – HCl 3.15 g

Distilled water to 1000 ml

pH was adjusted to 7.2, autoclaved and stored at 4^0C.

3.1.4.3 c HEPES (N-2-hydroxyethyl Piperazine N-2 ethane sulphonic acid) buffer, 10mM, pH 7.4

HEPES buffer	0.238 g
Distilled water to	100 ml

pH was adjusted to 7.4, autoclaved and stored at 4^0C.

3.1.4.3 d Sodium Lauroyl sarcosinate (0.5 %)

Five hundred mg of Sodium Lauroyl sarcosinate was dissolved in 100 ml of sterile 10 mM HEPES buffer (pH 7.4) just prior to use.

3.1.4.4 Buffers and reagents for SDS- PAGE

3.1.4.4 a 30 % Acrylamide–bisacrylamide mix

Acrylamide	29.0 g
Bis–acrylamide	1.0 g

The volume was made upto 100 ml using distilled water.

Filtered through Whatmann no.1 filter paper and stored at 4^0C.

3.1.4.4 b 1.5 M Tris pH 8.8

Tris base	181.7 g
Distilled water to	1000 ml

pH was adjusted to 8.8 with 4 N HCl and stored at 4^0C

3.1.4.4 c 0.5 M Tris pH 6.8

Tris base	60.6 g
Distilled water to	1000 ml

pH was adjusted to 6.8 with 4 N HCl and stored at 4^0C

3.1.4.4 d Resolving gel (12 %)

30% Acrylamide : bisacrylamide mix	2.5 ml
Tris hydrochloride (1.5 M) pH 8.8	1.4 ml
Sodium Dodecyl sulphate (10%)	50 µl
Ammonium per sulphate (10%)	50 µl
N, N, N- tetra methyl ethylenediamine (TEMED)	4 µl
Distilled water	2 ml

3.1.4.4 e Stacking gel (5 %)

30 % Acrylamide Bisacrylamide mix	622 µl
Tris hydrochloride (0.5 M) pH 6.8	310 µl
Sodium dodecyl sulphate (10 %)	25 µl
Ammonium per sulphate (10 %)	25 µl
N, N, N- tetra methyl ethylenediamine (TEMED)	4 µl
Distilled water	1.5 ml

3.1.4.4 f Tris Glycine Buffer (5x)

Tris base	15.1g
Glycine	54.0 g
Sodium dodecyl sulphate (10 %)	50.0 g

Finally the volume was adjusted to 1000 ml and pH 8.2

3.1.4.4 g Sample preparation buffer (2x)

Tris hydrochloride (1M, pH 6.8)	6.2 ml
Glycerol	5.0 ml
Sodium dodecyl sulphate (10 %)	1.0 ml
2 – mercaptoethanol	0.25 ml
Bromophenol blue	0.25 mg

The volume was adjusted to 25 ml using distilled water. Mix and store at 4^0C.

3.1.4.4 h Coomassie brilliant blue staining solution

Coomassie brilliant blue	1.0 g
Methanol	250 ml

Mixing was done by stirring for 30 min and the volume of methanol was re-adjusted.

Glacial acetic acid	50 ml
Distilled water to	200 ml

3.1.4.5 Buffers and reagents for Western blot

3.1.4.5 a Nitrocellulose membrane (Amersham private limited)

3.1.4.5 b Whatmann filter paper no.1

3.1.4.5 c PBS pH 7.2 (prepared as in 3.1.4.1)

3.1.4.5 d Transfer buffer / blot buffer

Tris base	18.2 g
Glycine	86.5 g
Methanol	1.2 l
Distilled water up to	6.0 l

3.1.4.5 e TBS

Tris chloride	100 mM
NaCl	0.9 %
Stored at 4^0C	

3.1.3.5 f TTBS

0.1 % Tween – 20 in TBS

3.1.3.5 g Blocking buffer

TTBS	100 ml
Dried skim milk powder	5 g

3.1.3.5 h Chromogenic visualization solution

100 mM Tris Chloride (pH 7.5)	5 ml
DAB stock (40 mg/ ml)	100 µl
Nickel Chloride stock (80 mg / ml)	25 µl
3 % H_2O_2.	15 µl

3.1.3.5 i Conjugate

Anti- immunoglobulin G horse raddish peroxidase (anti – Ig G HRP) conjugate against rabbit raised in goat (Banglore Genei) was diluted 1: 2500 in PBS, pH 7.2.

3.2 METHODS

3.2.1 MAINTENANCE OF LEPTOSPIRAL CULTURES

The leptospiral strains were maintained at room temperature in liquid *Leptospira* culture medium by routine subculture at 14 - 21 days interval. The usual interval of subculture in semisolid medium was one month. The contaminated leptospiral cultures were filtered under pressure through 0.2µ syringe type membrane filters (Whatmann syringe filter) of 25 mm diameter

3.2.2 IDENTIFICATION OF LEPTOSPIRES

3.2.2.1 Dark Field Microscopy (DFM)

Leptospira organisms were identified on the basis of their typical morphology and motility under the dark field microscope. In the liquid medium leptospires appeared to rotate along their long axis, moving forward with no apparent polar identification.

3.2.3 COLLECTION OF CANINE SERUM SAMPLES

A total of 60 canine serum samples were screened. Canine blood samples were collected from suspected dogs presented at University Veterinary Hospitals at Mannuthy and Kokkalai. About 5ml of blood was collected taking aseptic precautions and then centrifuged at 2000 rpm for 20 min for the separation of serum. Serum samples were stored at -20^{0}C until use.

3.2.4 MICROSCOPIC AGGLUTINATION TEST (MAT)

The MAT was carried out using live *Leptospira* organism as described by Faine (1982), with minor modifications. A 1:100 diluted serum was serially diluted two fold times in PBS, to provide dilutions of 1: 100 to 1: 25600, in 96 well U bottom microtiter plates. To 10 µl each of the serum dilution, 10 µl of 7-9 days old live antigen was added. Appropriate antigen controls were set with 10 µl PBS and 10 µl of antigen and the plates were incubated at 37^{0}C for 2 to 3 hrs. After incubation, the result was read by examining a drop of serum-antigen mixture from each well under low power of DFM for agglutination of leptospires. The antibody titer was the highest dilution of serum showing agglutination of 50 per cent or more leptospiral organism. Reciprocal agglutination titers of greater than or equal to 100 were considered as positive reactions. The serogroup reacting at the highest titer was presumed to be the infecting one. Sera samples showing same agglutination titers to more than one serogroup (excluding serogroup Patoc) were considered as mixed equals.

3.2.5 EXTRACTION OF OUTER MEMBRANE PROTEIN (OMP):

The OMP of *L. interrogans* serovar Australis was extracted using sarcosyl detergent as described by Nicholson and Prescott (1993) with some modifications. The Leptospires were grown in 500 ml *Leptospira* culture medium for about 7–10 days and harvested, when good growth was observed by visual

turbidity, by centrifugation at 40,000 x g for 20 min, followed by 3 washings with 0.01M PBS, pH 7.2. The cells were resuspended in 10mM HEPES buffer and disrupted by sonication at 60µ for 15 sec each for four times, as interrupted by a five sec pause, while cooling in ice bath. Cellular debris was removed by centrifugation at 2000 xg for 10 min at 4^0C. The supernatant was collected and centrifuged at 1,00,000 xg for 60 min at 4^0C. The pellet was resuspended in two ml of one per cent Sodium Lauryl Sarcosinate detergent (Sigma, USA), prepared in 10mM HEPES buffer, pH 7.4 and incubated at room temperature overnight, with a gentle rotation. The sarcosyl insoluble (SI) fraction was sedimented by centrifugation at 1, 00, 000xg for 60 minutes at 4^0C and suspended in 125µl 0.06M Carbonate-bicarbonate buffer, pH9.6. The supernatant *i.e.* sarcosyl soluble (SS) and the sarcosyl insoluble fractions were stored at -20^{o}C till further use. Protein estimation in extracted samples was carried out by the method of Lowry *et al.* (1951).

3.2.6 LEPTOSPIRAL WHOLE CELL ANTIGEN

Laboratory strains of L. interrogans serovar Australis were cultured in Leptospira liquid medium (Himedia) for about 7–10 days. Once good growth was observed by visual turbidity of the medium, the leptospires were pelleted at 10,000 rpm for 20 min at room temperature and washed and resuspended in 0.06M carbonate buffer, pH 9.6. The cells were disrupted by sonication at 20Hz for two periods, each of 15 sec (Adler *et al*., 1981).

3.2.7 ANALYSIS OF OUTER MEMBRANE PROTEINS AND WHOLE CELL ANTIGEN BY SDS - PAGE:

The protein profile analysis of *L. interrogans* serovar Australis whole cell antigen and the two fractions of OMP obtained after the sarcosyl extraction *i.e.* SS and SI was carried out by performing a one – dimensional SDS - PAGE as per the method described by Laemmli (1970) in a vertical electrophoresis apparatus (Axygen) with relevant modifications.

Briefly, glass plates were cleaned and set in the gel molding tray of electrophoresis apparatus and a 10 ml volume of 12 per cent resolving acrylamide gel solution was poured and then 1ml of water was layered over the gel and allowed to polymerize. After polymerization, 2.5 ml of five per cent stacking gel was poured over the resolving gel and a suitable comb was placed over it. After removing the comb from the polymerized gel wells, the gel sandwich was mounted on to the electrophoresis chamber and buffer reservoirs were filled with Tris- glycine buffer. Before loading, 20- 40µl of each of the samples, solubilized in 2x gel loading buffer, was boiled for 5-10 min and spun for 30 sec. The standard protein molecular weight marker (GENEI, Bangalore) was also run along with the samples in a separate well. The samples were initially run at a constant voltage of 50V till the dye front crossed the stacking gel. Then the voltage was increased to 100 V till the dye front reached end of the gel. The current was discontinued and the gel was then removed from the glass plate, the stacking gel was snipped off and subjected to the Coomassie Brilliant blue staining for one hour and then destaining was carried out for six to eight hours with three to four changes of destaining solution at intervals, till the background became clear. The gel was then transferred to Distilled water and viewed in white light and photographed.

3.2.8 RAISING HYPER IMMUNE SERA AGAINST *L. interrogans* serovar Australis

Two Newzealand White male rabbits weighing approximately 2kg were obtained from Small animal breeding Station, College of Veterinary and Animal Sciences, Mannuthy. Live *Leptospira* culture, grown to a density of 2 x 10^8 organisms per ml was injected intravenously at weekly intervals. The weekly injected doses were respectively 1, 2, 4, 6 and 6 ml. One week after the last injection seroconversion were checked by LAT. The rabbits were bled by cardiac puncture one week after the last injection.

3.2.9 WESTERN BLOTTING OF THE OMP

The leptospiral whole cell antigen and outer membrane proteins were subjected to western blot analysis using the hyperimmune sera raised in rabbits against the OMPs of *L. interrogans* serovar Australis, in order to find out the immunodominant proteins and to check the immunogenicity of OMP. The proteins fractionated in the SDS- PAGE gel were transferred electrophoretically onto a nitrocellulose membrane using semi- dry western blotting apparatus following the protocol described by Towbin *et al*. (1979) with some minor modifications as described below.

Transfer of proteins to NCM

Electrical method

After SDS-PAGE, the gel sandwich was disassembled and half of the gel containing one portion of the duplicate protein samples was cut apart and used for Coomassie Brilliant blue staining and destaining as described above (3.2.7) and other portion was kept in transfer buffer for 5-10 min. Transfer membrane was prepared by cutting to same size as gel. Membrane was then placed into distilled water slowly at 45^0 angle. When fully wet, it was equilibrated for 10-15 min in transfer buffer. Nine one mm Whatmann filter paper sheets were cut to gel size and were placed onto anodal side of the semi- dry blotting apparatus after saturation with transfer buffer. Equilibrated transfer membrane was then put on the top of the filter paper stack and the gel was placed over it. Onto it nine equilibrated one mm filter paper sheets were put. Entrapment of bubbles between filter paper, membrane and gel layers was prevented by rolling a glass pipette over the surface of the stack. Top electrode was then placed on to the transfer stack. A constant current (0.8 mA/cm^2 of the gel area) was applied to initiate the protein transfer for 90 min.

Manual transfer

A large Petri plate was taken, in the center of which was placed one micro-titration plate. Over the plate a glass plate was placed. The Petri plate was filled with blot buffer. On the glass plate a Whatmann No. 1 filter paper of size larger than the gel, wetted with blot buffer was placed, whose ends were immersed in the blot buffer. The gel was placed on the filter paper. The NC membrane soaked in blot buffer for five minutes was placed over the gel. Over the NCM was placed a small sheet of wet Whatmann No 1 filter paper. A glass road was then rolled over this to ensure that there were no air bubbles trapped between the gel and NCM. Over the filter paper, stacks of filter paper were placed. On the top of this a glass plate was kept and on which a weight of two kilograms was placed. This set up was left as such overnight for complete transfer.

Identification of OMP

After the transfer, the unit was disassembled and the membrane was removed from the transfer stack after marking the orientation and the gel was subjected to staining and destaining as described previously to verify the transfer efficiency. Membrane was placed in 5 ml blocking buffer and incubated at 37^0C for 2 h, followed by washing twice with PBS-T for 10 min each.

Primary antibody *i.e*., the hyperimmune sera raised against the *Leptopsira interrogans* serovar Australis OMP was diluted in blocking buffer (1:100). Diluted Primary antibody was added to the membrane and it was incubated for 1 h at 37^0C with constant agitation. The membrane was washed 4 times by agitation with sufficient (100-200 ml) of PBS – T, 10-15 min each.

Membrane was then incubated at 37^0C in suitably diluted (1:2500) goat anti–rabbit HRPO IgG conjugate (diluted in blocking buffer) for 1hr with constant agitation. The membrane was washed four times in PBS-T as described earlier and the blots were developed by putting into the chromogenic

visualization solution at room temperature, with mild rocking, until colour developed. The reaction was terminated by washing the membrane with distilled water. Membrane was air–dried and photographed.

Cross reaction was checked by using hyperimmune sera against *Pasteurella multocida* and *Mycoplasma gallisepicum* as primary antibody.

3.2.10 SENSITIZATION OF LATEX BEADS

For the preparation of latex beads and performance of the latex agglutination test, the method of Dey (2003) was followed, with few modifications. Latex beads (0.88 μ, Sigma, USA) were washed in carbonate–bicarbonate buffer, 0.06M and pH 9.6, twice by centrifugation at 6800g for 2 min each time. The final latex beads were made one per cent with carbonate–bicarbonate buffer and mixed with an equal volume of OMP and whole cell leptospiral antigen for whole cell antigen based LAT and OMP based LAT respectively. This mixture was incubated at 37°C for 6 h with constant shaking at 250 xg. The sensitized beads were centrifuged at 6800 xg for 3 min and the pellet resuspended as a 1 per cent suspension in phosphate buffered saline (PBS) containing 5 mg/ml of bovine serum albumin (BSA). The latex beads were left at 37°C overnight and centrifuged as before. The pellet was resuspended in PBS containing 0.5 mg/ml of BSA and 0.1 per cent Sodium azide, as a 0.25 per cent suspension. The sensitized latex beads were stored at 4°C until use.

3.2.11 LATEX AGGLUTINATION TEST

The LAT was performed on glass slides by mixing equal volumes of serum (25 μl) and sensitized latex beads. The slide was rocked briefly for two to five min to mix the coated beads and serum samples. Phosphate buffered saline and normal rabbit serum were used as negative controls and rabbit anti-leptospiral hyperimmune serum was used as a positive control.

The results were read well within two min by the naked eye. The test was positive if agglutination had occurred, which was indicated by the formation of fine granular particles, which tended to settle at the edges of serum- bead mixtures. If the suspension remained homogenous, the test was declared negatives.

Results

4. RESULTS

4.1 Maintenance of reference strains of *Leptospira*

Leptospira serovars were maintained in *Leptospira* culture medium with different supplements. The best supplement was found to be haemolysed, filtered, heat inactivated rabbit serum. When haemolysed rabbit serum was used, the culture attained optimum concentration of 2 X 10^8 cells per ml within 3-4 days. Moreover, rabbit serum was easy to procure and cheap and hence it was used in medium for maintenance of *Leptospira*. Readymade supplement and Foetal bovine serum were costly and took a longer period for achieving optimum growth. In case of Albumin supplement, Bovine serum albumin fraction V (SRL) was costly. Hence these supplements were not used for routine subculturing, but they were used for bulk culturing of *L. interrogans* serovar Australis.

All the *Leptospira* serovars for maintenance and MAT was subcultured in liquid medium. Stock cultures of all the nine serovars were prepared in semisolid medium. In semisolid medium, Dinger's ring (Figure 1) was formed at the subsurface level by about one month. All the cultures were maintained at room temperature.

4.2 Microscopic Agglutination Test

Sixty clinical samples were tested by MAT, of which 46 were positive and 14 were MAT negative. A battery of nine serovars was used throughout the study. Agglutination of leptospires by MAT is shown in Fig. 2.

The results of MAT are summarized in Table 1.

The Table 2 shows the percentage seropositivity to different *Leptospira* serovars. The most predominant serovar found was Australis followed by Grippotyphosa, Pomona, Canicola, Icterohaemorrhagiae, Javanica, Patoc,

Autumnalis and Pyrogenes. Seroprevalence of leptospirosis by MAT is represented diagrammatically in Fig. 3.

4.3 SDS-PAGE analysis of OMP and Whole cell antigen

SDS-PAGE analysis of whole cell antigen and OMP of *L.interrogans* serovar Australis was done as per the method of Laemmli, (1970).

Medium range protein molecular weight marker (GENEI, Bangalore) was used which revealed six distinct bands ranging from 97.4 kDa to14.3 kDa.

Whole cell antigen revealed several bands which are less intensely stained. However, 17kDa, 38kDa, 43kDa, 92kDa and 100kDa bands could be identified in the protein profile of whole cell antigen (Fig. 4).

The SI portion of the OMP revealed nine distinguishable bands located approximately at 17kDa, 21kDa, 32kDa, 38kDa, 43kDa, 66kDa, 77kDa, 92kDa and 100kDa (Fig. 4). In SS portion, only 4 bands could be found at 17kDa, 38kDa, 66kDa and 77kDa (Fig. 4).

The protein concentration was estimated as per the method of Lowry *et al.* (1951). The OMP concentration was found to be 7mg/ml and that of Whole cell antigen was found to be 8.5mg/ml.

4.4 Western Blotting

The specificity of OMP was analysed by western blotting using rabbit hyper immune sera raised against *L. interrogans* serovar Australis. Manual method was found to be more efficient in transferring protein from gel to NCM than electrical method. Three distinct bands *viz.* 38kDa, 92kDa and 100kDa, were obtained on immunoblot (Fig. 5). The immunogenic protein bands present in both OMP and whole cell antigen were 38kDa and 100kDa. Cross reaction was checked by using hyper immune sera against *P. multocida* and *Mycoplasma*

gallisepticum, where no reactions could be detected on immunoblot of OMP, while the whole cell antigen immunoblot showed some non specific reactions.

4.5 LAT

The slides showing granules at the periphery were taken as positive and negative cases yielded a uniform solution. The intensity of agglutination had been graded to make semi-quantitative evaluation as 1+, 2+, 3+ and 4+, depending upon the intensity and speed with which agglutination occurred (Fig. 6).

1+ - Mild agglutination in 5-10 min

2+ - Moderate agglutination in 5min

3+ - Heavy agglutination in 3-5 min

4+ - Very heavy agglutination in 2-3 min

4.5.1 Whole cell antigen based LAT

Among the 46 MAT positive samples, 45 were positive and one was negative in whole cell antigen based LAT. The LAT showed a positive result for five among the 14 MAT negative samples. In addition, out of the 15 known negative control sera, two were found positive in LAT. The results are summarized in Table 3 and 4.

4.5.2 OMP based LAT

Out of 46 MAT positive samples LAT detected 44 as positive and 2 as negative. LAT showed a positive result for 4 samples among the 14 MAT negative sera samples tested. All the 15 known negative control sera were found to be negative by LAT also. The hyper immune sera showed 2+ agglutination on LAT. The results are summarized in Table 3 and 5.

4.6 Statistical analysis of results

The relative sensitivity and specificity of the OMP based LAT for the detection of anti-leptospiral antibodies in dog sera were determined in comparison to the MAT. From the two – by - two contingency tables (Table 4 and Table 5) the following parameters were calculated.

Sensitivity = $[a / (a + c)] \times 100$, where a was the number of sera positive by LAT and MAT and c was the number of sera positive by MAT but negative by LAT.

Specificity = $[d / (b + d)] \times 100$, where d was the number of sera negative by LAT and MAT and b was the number of sera negative by MAT but positive by LAT.

Accuracy = $[(a + d) / (a + b + c + d)] \times 100$

The results obtained from the tests were analysed for the percentage agreement with MAT with the use of the kappa statistics. The kappa statistics is a decimal measure of agreement between two tests and is defined as kappa or κ.

$$\kappa = a + d - P / (1 - P)$$

where $P = (a + b)(a + c) + (c + d)(b + d)$; P was the probability, a was the number of samples positive by both LAT and MAT, b was the number of samples positive by MAT but negative by LAT, c was the number of samples negative by MAT but positive by LAT and d was the number of samples negative by both MAT and LAT. A kappa value greater than 0.81 indicated perfect agreement.

An intuitive method for calculating predictive values (in per cent) for positive and negative test results was done as below:

PV+ = a / (a + b) x100
PV- = d / (c + d) x 100
The results are summarized in Table 6.

Table 1. MAT titers to different leptospiral serogroups

SL. NO.	MAT								
	Australis	**Autumnalis**	**Canicola**	**Grippotyphosa**	**Icterohaemorrhagiae**	**Javanica**	**Patoc**	**Pomona**	**Pyrogenes**
1	1:200		1:100			1:3200*			
2				1:200*	1:100				
3	1:200*		1:100	1:100					
4		1:100*							
5	1:3200*	1:200							
6	1:800*		1:200	1:100					
7									
8			1:100*						1:100*
9				1:100*					
10	1:100*								
11									
12	1:200*							1:200*	
13	1:100*								
14									
15	1:200							1:800*	
16	1:200*				1:100			1:100	
17								1:800*	
18			1:200*					1:100	
19	1:100			1:100				1:200*	1:100
20	1:800*		1:400			1:200		1:400	
21					1:200*				
22	1:200*						1:100		
23							1:100*		
24		1:100*							
25									
26									
27	1:3200*								
28							1:200	1:800*	
29							1:200*		
30	1:200*								
31									
32	1:100*				1:100*				
33	1:100				1:400*				
34					1:200			1:400*	
35									

Table 1. (contd.)

SL. NO.	MAT								
	Australis	**Autumnalis**	**Canicola**	**Grippotyphosa**	**Icterohaemorrhagiae**	**Javanica**	**Patoc**	**Pomona**	**Pyrogenes**
36									
37									1:200*
38						1:100*			
39						1:200*			
40						1:200*			
41									
42									
43	1:100*			1:100*					
44	1:100*								
45			1:3200*						
46	1:100			1:100				1:200*	
47									
48	1:800*								
49							1:800*		
50	1:100*					1:100*			
51									
52	1:800*			1:400					
53	1:3200*	1:400	1:400						
54				1:800*	1:400				
55				1:6400*	1:200				
56			1:100					1:200*	
57									
58									
59				1:200*					
60									1:400*
TOTAL	23	4	9	11	8	6	5	11	4

* The serogroup reacting at the highest titer, presumed to be the infecting one

Mixed equals – one each for Australis and Grippotyphosa, Australis and Icterohaemorrhagiae, Australis and Javanica, Australis and Pomona and Canicola and Pyrogenes.

Table 2. Seroprevalence of Leptospirosis by MAT

Serogroup	No of positive cases	Per cent
Australis	23	38.33
Grippotyphosa	11	18.33
Pomona	11	18.33
Canicola	9	15.0
Icterohaemorrhagiae	8	13.33
Javanica	6	10.0
Patoc	5	8.33
Autumnalis	4	6.67
Pyrogenes	4	6.67

Table 3. Results of Whole cell antigen based LAT and OMP baesd LAT

	WHOLE CELL ANTIGEN-LAT	OMP-LAT	MAT
1	++	++	+
2	++	+	+
3	+	+	+
4	+++	+++	+
5	+++	+++	+
6	+++	++	+
7	–	–	–
8	–	+	+
9	++	+	+
10	++	++	+
11	+	–	–
12	++	+	+
13	+	+	+
14	–	–	–
15	+++	+++	+
16	++	–	+
17	++++	+++	+
18	++	+	+
19	++	+	+
20	+++	++	+
21	+	+	+
22	+	+	+
23	+	+	+
24	–	+	+
25	–	–	–
26	++	++	–
27	++++	+++	+
28	++++	++++	+
29	++	+	+
30	++++	++++	+
31	+	+	–
32	+	–	+
33	++	++	+
34	+++	++++	+
35	++++	++++	–
36	–	–	–
37	++++	++++	+
38	++++	++++	+
39	++	+	+
40	++	++	+
41	+	–	–
42	–	–	–
43	+	++	+
44	+	++	+
45	++++	++++	+
46	+	+	+
47	–	–	–
48	++	+++	+
49	++	++	+
50	+	+	+
51	+++	++++	–
52	+	+	+
53	++	+	+
54	+++	++++	+
55	+	+	+
56	++	+	+
57	+	–	–
58	+	–	–
59	+	+	+
60	+	+	+
TOTAL POSITIVE	52	48	46

Table 4. Results of Whole cell antigen based LAT

		MAT		TOTAL
		+	_	
Whole cell antigen based LAT	+	45(a)	7 (c)	52
	_	1 (b)	22(d)	23
TOTAL		46	29	75

(a) is the number of samples positive by both LAT and MAT, (b) is the number of samples positive by MAT but negative by LAT, (c) is the number of samples negative by MAT but positive by LAT, and (d) is the number of samples negative by both MAT and LAT.

Table 5. Results of OMP based LAT

		MAT		TOTAL
		+	_	
OMP-based LAT	+	44(a)	4(c)	48
	_	2(b)	25 (d)	27
TOTAL		46	29	75

(a) is the number of samples positive by both LAT and MAT, (b) is the number of samples positive by MAT but negative by LAT, (c) is the number of samples negative by MAT but positive by LAT and (d) is the number of samples negative by both MAT and LAT.

Table 6. Results of statistical analysis in comparison with MAT

	Whole cell antigen LAT	**OMP based LAT**
Sensitivity (%)	97.83	95.65
Specificity (%)	75.86	86.21
Accuracy (%)	86.7	97.33
K	0.98	0.97
PV+ (%)	86.54	92.59
PV- (%)	88.0	91.67
$\chi 2$	48.24*	42.02*

* Significant at 1 degree of freedom.

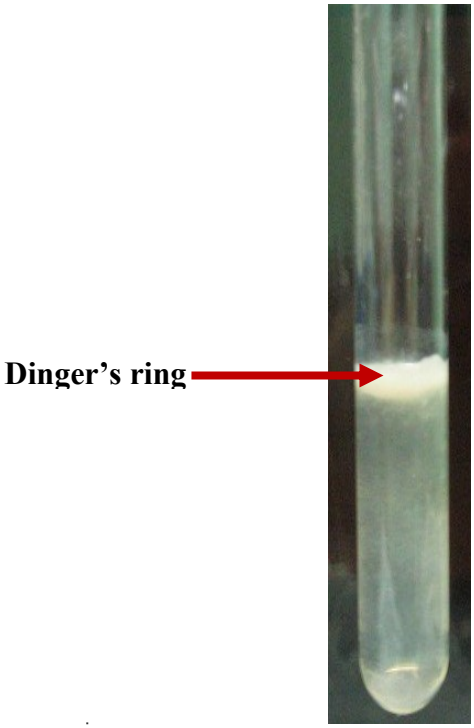


Fig 1. Culture in semisolid medium

Negative **Positive**

Fig 2. Agglutination of leptospires in MAT by DFM (450 x)

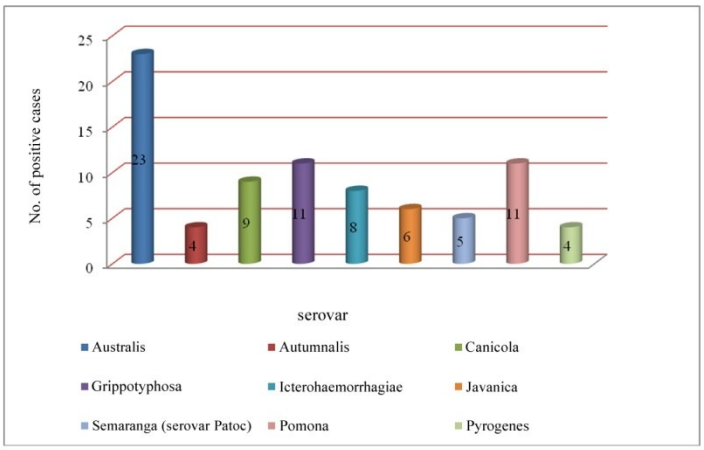


Fig. 3. Seroprevalence of leptospirosis by MAT

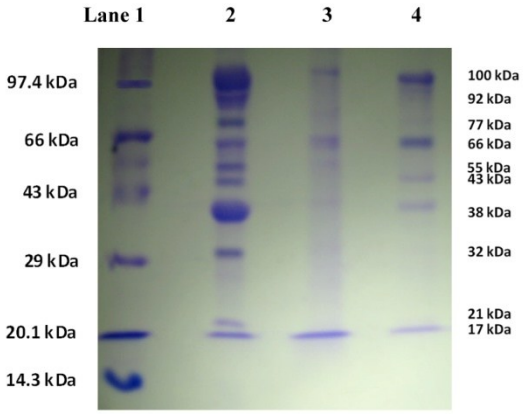


Fig. 4. SDS-PAGE analysis of OMP and Whole cell antigen

Lane 1 - Protein marker

Lane 2 - Sarcosyl insoluble OMP

Lane 3 - Sarcosyl soluble OMP

Lane 4 - Whole cell antigen

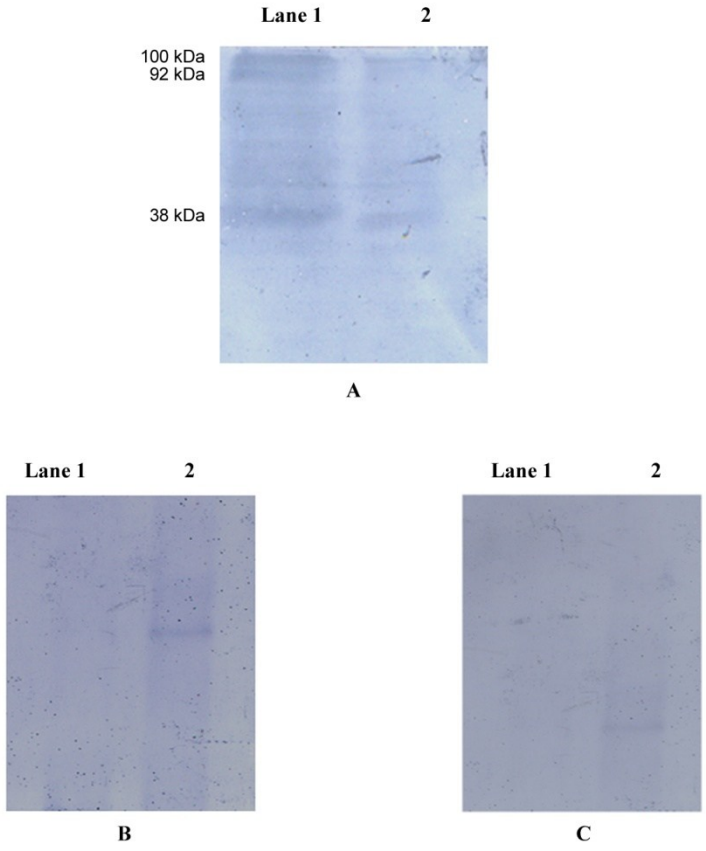


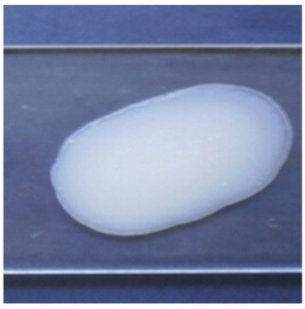
Fig. 5. Western Blotting of OMP and Whole cell antigen

Lane 1 - OMP **Lane 2 - Whole cell antigen**

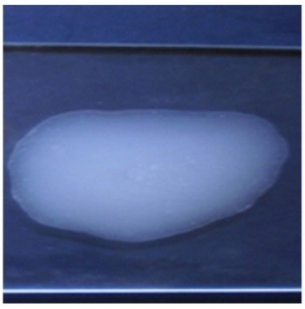
A - Immonoblot developed with hyper immune sera against *L. interrogans* serovar Australis

B - Immunoblot developed with hyper immune sera against *P. multocida* DP1

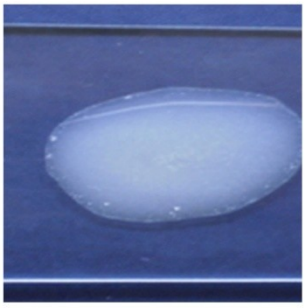
C - Immunoblot developed with hyper immune sera against *M. gallisepticum*



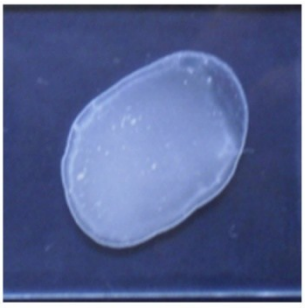
Negative control with PBS



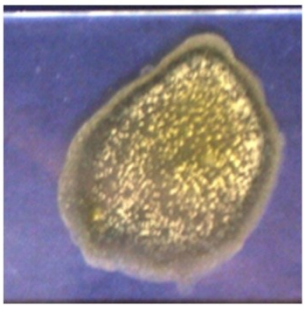
Negative sera



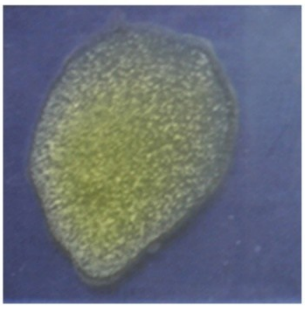
1+ Agglutination



2+ Agglutination



3+ Agglutination



4+ Agglutination

Fig. 6. Latex Agglutination Test

Discussion

5. DISCUSSION

Leptospirosis, a ubiquitous zoonosis associated with sinister complications and fatalities, has been recognized in India since 1931 (Taylor and Goyle**,** 1931). It is especially rampant in southern, central, eastern and western India, where heavy monsoon, animal rearing practices, unplanned urbanization and agrarian way of life predispose to this infection (Sehgal, 2006, Swapna *et al.,* 2006 and Sumathi *et al.,* 2008).

The diagnosis of leptospirosis is very difficult in early stages of the disease because leptospires can affect many organ systems, resulting in a wide variety of clinical presentations, and these initial symptoms are often indistinguishable from that of other febrile illnesses. But for a favourable clinical outcome timely diagnosis is needed for early and prompt initiation of specific treatment. From a clinical point of view, the early recognition of the disease is very important for initiating appropriate treatment to avoid severe complications. The diagnosis of leptospirosis cannot be easily made owing to the lack of less cumbersome diagnostic tools. Hence, leptospirosis is often not recognized or is erroneously mistaken for other diseases showing similar symptoms. As a result, this serious disease may be left untreated or treated improperly.

The laboratory diagnosis of acute *Leptospira* infection is usually dependent on serological methods. In leptospirosis, antibodies usually appear within five to seven days after the onset of the symptoms and in a significant proportion of patients, antibodies persist in detectable quantities for many months (Faine, 1982). The “gold standard” Microscopic Agglutination Test (MAT), which is serovar and serogroup specific, requires paired specimens, considerable

technical resources and training and is not useful for acute case management (Champagne *et al*., 1991). The drawback of a serovar or serogroup specific test is that it can detect only the specified antibodies, so may not be of a true diagnostic value. The conventional methods of diagnosis like isolation techniques, other serological tests like Indirect haemagglutination, serum adsorption as well as the molecular techniques like the PCR although available, are either highly time consuming or can be performed only in well equipped laboratories. Therefore, currently the research on leptospirosis diagnosis is focused on to evolve a serological test which is cost effective, rapid, sensitive and preferably genus specific. The Latex Agglutination Test (LAT) is an extremely simple and inexpensive diagnostic test which requires no sophisticated equipment and specific expertise (Ramadass *et al*., 1999). Considering all these beneficial points, LAT was opted for detection of leptospiral antibodies.

Ramadass *et al*. (1999) prepared sonicated whole - cell pooled leptospiral antigen, where the LAT method detected fewer samples when compared to plate ELISA. Smits *et al*. (2000) used heat stable antigen for conducting the LAT, but there were very few positives when a set of known highly positive and negative sera were screened. Antigens associated with the outer membrane fractions of strains of *Leptospira* elicit immune response in hosts suggesting that these antigens could be potential candidates for diagnostic and prophylactic use against leptospirosis (Haake *et al*., 2000). The major OMPs, LipL32 and LipL41, based LAT results showed good sensitivity and specificity, when compared with standard MAT (Dey *et al*., 2007 and Senthilkumar *et al*., 2007). Thus, in the present study, whole cell antigen and OMP based latex agglutination tests (LAT) have been developed for detection of leptospiral antibodies from canine samples.

The MAT was conducted on 60 samples of which 46 samples were positive and 14 were negative. All the samples were tested using nine *Leptospira interrogans* serovars *viz*. Australis, Autumnalis, Canicola, Grippotyphosa, Icterohaemorrhagiae, Javanica, Patoc, Pomona and Pyrogenes at their active

phase of growth in *Leptospira* liquid culture medium. All the serovars showed good growth in liquid medium supplemented with 10 per cent haemolysed, filtered and heat inactivated rabbit serum. This might be due to the reason that, rabbit serum contains highest concentration of bound vitamin B_{12} which is an essential factor for the multiplication of leptospires (Brugge and Louw, 1985). The most predominant serovar was Australis (38.33 per cent) followed by Grippotyphosa (18.33 per cent), Pomona (18.33 per cent), Canicola (15.0 per cent), Icterohaemorrhagiae (13.33 per cent), Javanica (10.0 per cent), Patoc (8.33 per cent), Autumnalis (6.67 per cent) and Pyrogenes (6.67 per cent). The emergence of Australis other than vaccinal serovars in dogs might be due to epizootic changes in canine leptospirosis as a result of widespread use of canine vaccines and possibly increased contacts between dogs and rodents, which serve as maintenance host for leptospires (Balakrishnan *et al*., 2008).

In this study, the whole cell antigen and OMP were prepared from the *L. interrogans* serovar Australis strain Ballico. The serovar Australis was reported to be the emerging serovar, especially in southern states of India (Koteeswaran, 2006 and Balakrishnan *et al*., 2008) and the seroprevalence conducted during the present study also revealed Australis as the predominant serovar involved in canine leptospirosis.

The protein profile analysis of OMP and Whole cell antigen by SDS-PAGE exhibited an array of protein bands ranging from 100kDa to 17kDa. The protein profile revealed 100kDa, 38kDa, 32kDa and 21 kDa protein bands. Western blot analysis of OMP revealed three immunodominant bands *viz*. 100kDa, 92kDa and 38kDa and that of whole cell antigen had two bands *viz.*100kDa and 38kDa. These results show agreement with the reports by Gitton *et al*. (1992) and Nicholson and Prescott (1993). The immunoblots of OMP could not develop any bands when hyper immune sera against *Pasteurella multocida* and *Mycoplasma gallisepticum* were used as primary antibody, thus proving the

specificity of OMP antigen. On the other hand, whole cell antigen proved less specific as it developed some non specific bands on immunoblot, when *P. multocida* and *M. gallisepticum* hyper immune sera were used as primary antibody and this finding is in accordance with Matsuo *et al*. (2000 a and 2000 b).

In the present study, the whole cell antigen and OMP sensitized latex beads were used to detect the antibodies against the pathogenic serovars of *Leptospira* species. The samples were considered negative if no agglutination was observed within 5 - 10 min and positive results were recorded with the naked eye on a +1 to +4 scale depending on the extent of agglutination and the time taken for the development of agglutination (Ramadass *et al*., 1999). Thus the test is simple and inexpensive and at the same time requires no specific expertise and expensive or sophisticated equipment.

The sensitivity, specificity, accuracy and predictable value of LAT relative to MAT were evaluated. The sensitivity and specificity of OMP based LAT were 95.65 per cent and 86.21 per cent respectively, whereas the corresponding values with Whole cell antigen were 97.83 per cent and 75.86 per cent respectively. As the kappa values of both LATs were greater than 0.81 the tests indicated perfect agreement. The results were supported by the findings of Ramadass *et al.* (1999), Dey *et al.* (2007) and Senthilkumar *et al.* (2008).

Four MAT negative samples (sample no. 26, 31, 36 and 51) were turned positive in both whole cell and OMP based LAT. It is well established that MAT is highly serovar or serogroup specific, whereas LAT is only genus specific which could detect infection cases irrespective of serovar. The MAT negatives were only negative for the nine serovars used in this study and there might be the possibility of them to be positive for serovars other than the nine tested serovars, which could have been solved by including all the locally isolated strains in the battery of antigens, thus increasing the sensitivity of the MAT (WHO, 2003).

Even though sensitivity of whole cell LAT was higher than that of OMP based LAT, the specificity was lower than that of the latter as the whole cell LAT detected two known negative samples as positive. This might be due to the presence of non-specific moieties present in whole-cell preparations (Flannery *et al.,* 2001). Thus it is proved that, OMP, which is the single unit containing all the immunoreactive antigens, is a better antigen candidate for sensitizing latex beads than whole cell antigen.

The results of this study demonstrated equally high sensitivities of the latex agglutination assay for detecting infections with strains of the serogroups Australis, Autumnalis, Canicola, Grippotyphosa, Icterohaemorrhagiae, Javanica, Pomona, and Pyrogenes, showing that the assay was independent of serovar specificity and was rather genus specific. The information on the serogroup of the infecting strain could not be obtained from the results of the latex agglutination assay, which indeed had no clinical implications and was mainly of epidemiological interest as opined by Smits *et al.* (2000).

The current study revealed that the OMP based latex agglutination assay would be a valuable tool in the diagnostic armament against leptospirosis. The assay was extremely simple and rapid to perform, used stabilized components and can be performed in resource-poor settings by investigators with only limited training. Further, the assay had good sensitivity, specificity, and acceptable predictive values in comparison with those of the MAT. This assay had some potential advantages, like portability and limited amount of generated biomedical waste. The assay could be performed quickly enabling the rapid screening of large number of sera samples. Given the high specificity of the OMP based latex assay and its ease of use, this would be an appropriate test for use in field studies, particularly in outbreak investigations. Further studies on the applicability of the test under field conditions are required to be undertaken in future.

Summary

SUMMARY

Leptospirosis, a direct anthropozoonosis has gained importance both in man and animals and is assuming greater dimension as a re-emerging disease. In India, various workers have reported the disease from various parts of the country. Many places in India are known to be endemic for leptospirosis with heavy casuality, during the post monsoon periods. Early diagnosis is the key to the treatment of leptospirosis. But, the wide spectrum of symptoms confuses the clinical diagnosis and makes it undependable. The laboratory diagnosis of leptospirosis, a prerequisite for treatment, is usually achieved either by isolation of the causative organisms or by serological evidence. Several methods have been developed for use in diagnosis of leptospirosis as an alternative to MAT, of which Latex agglutination test is a promising approach and detects genus-specific antibodies.

In the present study, in order to develop a simple rapid immunodiagnostic assay, whole cell antigen and outer membrane protein were employed as antigen candidates in Latex agglutination test and their efficacy was compared with the standard reference test i.e., Microscopic agglutination test (MAT).

A total of 60 canine serum samples were screened. Canine blood samples were collected from suspected dogs presented at Veterinary Hospitals of Mannuthy and Kokkalai, during the period of June 2009 - January 2010. About five ml of blood was collected taking aseptic precautions and then centrifuged at 2000 rpm for 20 min for the separation of serum. Serum samples were stored at -20^0C until use.

The serum samples were subjected to MAT using a battery of nine serovars *viz., Leptospira interrogans* serovar Australis, Autumnalis, Canicola, Grippotyphosa, Icterohaemorrhagiae, Javanica, Pomona and Pyrogenes and Patoc

one strain of *L. biflexa* serovar Patoc. All strains were obtained from National Leptospira Reference centre, Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands, India. The cultures were maintained at Leptospira liquid culture media (Himedia).

On MAT, 76.67 per cent seropositivity could be detected. Among the reacted serogroups, Australis was observed as the predominant one with a seropositivity of 38.33 per cent followed by Grippotyphosa (18.33 per cent), Pomona (18.33 per cent), Canicola (15.0 per cent), Icterohaemorrhagiae (13.33 per cent), Javanica (10.0 per cent), Patoc (8.33 per cent), Autumnalis (6.67 per cent) and Pyrogenes (6.67 per cent). This current information warrants immediate attention to incorporate this emerging serovar in the vaccines against Leptospirosis.

L. serovar Australis had been selected for whole cell antigen and OMP preparation, which were used for sensitization of latex beads in LAT. The Whole cell antigen was prepared by sonication followed by centrifugation. The OMP was extracted by sonication followed by sarcosyl treatment and Ultracentrifugation. Protein profile of both Whole cell antigen and OMP was analyzed by SDS – PAGE and specificity was checked by Western blotting. Later, they were used to sensitize latex beads.

The samples were screened using whole cell antigen and OMP based LAT. Among the 46 MAT positive samples, 45 were positive and one as negative in whole cell antigen based LAT where as, OMP based LAT detected 44 as positive and 2 as negative.

When MAT negative samples were tested, Whole cell based LAT showed a positive result for five among the 14 MAT negative samples. In addition, out of the 15 known negative control sera, two were found positive in LAT. In OMP based LAT, 4 samples were found as positive among the 14 MAT negative sera samples tested. All the 15 known negative control sera were found to be negative

by OMP based LAT also. The hyper immune sera showed 2+ agglutination on LAT.

The sensitivity and specificity of OMP based LAT were 95.65 per cent, and 86.21 per cent respectively. On the other hand, the sensitivity and specificity of Whole cell antigen were 97.83 per cent and 75.86 per cent respectively. As the kappa values of both LATs are greater than 0.81, the tests indicate perfect agreement with the MAT.

Thus, on the basis of the results presented above, it could be concluded that OMP – based LAT should be considered as an alternative to MAT or even a replacement for MAT for laboratory screening of leptospirosis, since it requires fewer laboratory facilities and enables a faster diagnosis and earlier therapeutic intervention.

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EVALUATION OF WHOLE CELL ANTIGEN AND OUTER MEMBRANE PROTEIN BASED LATEX AGGLUTINATION TEST FOR SERODIAGNOSIS OF CANINE LEPTOSPIROSIS

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ABSTRACT

A study was undertaken to evaluate the efficacy of whole cell antigen and OMP based Latex Agglutination Test (LAT) for the serodiagnosis of canine leptospirosis. Serum samples were collected from University Veterinary Hospitals of Mannuthy and Kokkali. These samples were subjected to serologic testing by Microscopic Agglutination Test (MAT) and LAT and the results were compared.

A total of 60 serum samples were screened in this study. MAT detected a prevalence rate of 76.67 per cent. The most predominant serovar was Australis (38.33 per cent) followed by Grippotyphosa (18.33 per cent), Pomona (18.33 per cent), Canicola (15.0 per cent), Icterohaemorrhagiae (13.33 per cent), Javanica (10.0 per cent), Patoc (8.33 per cent), Autumnalis (6.67 per cent) and Pyrogenes (6.67 per cent). The sensitivity and specificity of whole cell antigen were 97.83 per cent and 75.86 per cent respectively. On the other hand, the sensitivity and specificity of OMP based LAT were 95.65 per cent and 86.21 per cent respectively. As the kappa values of both LATs were greater than 0.81, the tests indicate perfect agreement with MAT. From this results, OMP–based LAT developed for the detection of leptospiral antibodies was proved to be a very useful rapid test for immunodiagnosis. OMP–based LAT is an extremely simple and inexpensive test that does not require expertise or sophisticated equipments and could also be used for the detection of leptospiral antibodies in place of MAT, which requires live leptospiral cultures, expertise, time.