EVALUATION OF OUTER MEMBRANE PROTEIN BASED IgM DOT ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE RAPID DIAGNOSIS OF CANINE LEPTOSPIROSIS

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DECLARATION

I hereby declare that this thesis, entitled **"EVALUATION OF OUTER MEMBRANE PROTEIN BASED IgM DOT ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE RAPID DIAGNOSIS 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.

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Certified that this thesis, entitled **"EVALUATION OF OUTER MEMBRANE PROTEIN BASED IgM DOT ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE RAPID DIAGNOSIS OF CANINE LEPTOSPIROSIS"** is a record of research work done independently by ***G. ABHINAY*****,** under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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1. INTRODUCTION

Leptospirosis is one of the most widespread zoonoses, which has become an important global human and veterinary health problem (Adler and Moctezuma, 2010). It is a major threat in tropical and subtropical countries as well as in temperate countries (Faine *et al*., 1999). Many places in south India, including Kerala, are known to be endemic for leptospirosis. The disease affects many animal species including cattle, horses, sheep, goats, pigs, dogs and wild rats, which are the sources of infection to humans. Dairy cattle affected with the disease lead to great economic losses as a consequence of agalactia, abortion, stillbirth, birth of weak calves and reduced fertility (Thiermann and Garrett, 1983; Yan *et al*., 1999). The important manifestations in horses include abortions and uveitis, while in dogs the disease is characterized by fever, vomiting, dehydration, collapse, hepatitis, nephritis and death (Faine, 1982).

Diagnosis of the disease in its early course is important to initiate appropriate treatment and prophylactic measures. Doxycycline therapy initiated soon after the onset of the disease can prevent severe complications and deaths due to leptospirosis (Faine *et al*., 1999). The diagnosis of leptospirosis, especially in its early phases, is difficult, because of the varied symptomatology, often making it indistinguishable from other febrile illnesses (Levett, 2003). Diagnosis of leptospirosis in an endemic region is further complicated by the presence of under - current antibodies, either due to prior infection or vaccination (Wolff and Bohlander, 1966). Thus, the need for rapid and appropriate diagnostic tests has become ever more urgent to aid clinical case identification, implementation of appropriate medical measures and to conduct seroprevalence studies in the risk population.

The laboratory diagnosis of leptospirosis can be achieved with bacteriological, molecular and serological methods. The bacteriological methods include isolation and dark field microscopy (DFM). Isolation of the organism is definitive, but time consuming and cumbersome. Approximately 10^4 leptospires per milli litre are necessary for one cell per field to be visible by dark field microscopy (Ahmad *et al.,* 2005). The molecular diagnostic techniques developed so far are mostly based on

polymerase chain reaction (PCR) assays and *in situ* hybridization, requiring well-established laboratory facilities and highly skilled personnel (Levett, 2003). The confirmative diagnosis of leptospirosis is mostly dependent on serological methods.

Serological assays described in the past included the macroscopic agglutination (Mailloux *et al.,* 1974), microcapsule agglutination (Arimitsu *et al.,* 1982) and indirect hemagglutination tests (Levett and Whittington, 1998), all of which suffered from low sensitivity and identified less than 50 per cent of patients presenting the early phase of leptospirosis (Guerreiro *et al.,* 2001).

The gold standard reference test, microscopic agglutination test (MAT) has many disadvantages, *viz.,* requires expertise for interpreting results, paired sera collected at acute and convalescent phase of the disease for the confirmatory diagnosis and handling of live cultures, restricting its use to specialized laboratories (Guerreiro *et al*., 2001). The MAT detects both IgG and IgM antibodies (Chernukha *et al.,* 1976; Romero *et al.,* 1998). Moreover, MAT is serovar specific and so may not be of true diagnostic value (Champagne *et al.,* 1991).

Genus specific tests like enzyme linked immunosorbent assays (ELISAs) (Adler *et al.,* 1980; Pappas *et al.,* 1985) and latex agglutination test (LAT) (Ramadass *et al*., 1999; Senthilkumar *et al.*, 2007) were tested and proved to be useful diagnostic approaches. Earlier, ELISAs employed whole-cell leptospiral antigens (Terpstra *et al*., 1985; Yersin *et al*., 1999), but these tests suffered from low specificity because of the broadly reactive immunodominant moiety (Matsuo *et al*., 2000 a, b). Later, the outer membrane proteins (OMP) were employed as they were genus specific and showed high sensitivity and specificity (Singh *et al.,* 2004; Srivastava *et al*., 2006). However, ELISAs require costly equipment and need technical expertise, making them unfit for routine field level diagnosis.

Therefore, to make the screening of leptospiral antibodies easier, with much lesser effort, LAT was tried. Though, LAT proved to be a cheap and easy diagnostic test which enables rapid disease diagnosis, it may not differentiate the truly infected animals from among vaccinates and those which harbour antibodies of prior infection.

Therefore, there is an ever more urgent need to develop a rapid, economical and simple diagnostic test that can detect acute reactors in leptospirosis endemic areas. The diagnostic tests which can detect leptospiral IgM antibodies, present in the acute infection can serve as a better diagnostic approach (Silva *et al.,* 1995; Winslow *et al.,* 1997). Thus IgM dot ELISA has been tried as a simple, rapid and economical test to diagnose acute human leptospirosis (Pappas *et al*., 1985; Sehgal *et al*., 1999; Tansuphasiri *et al*., 2005), but its potential as a diagnostic tool has not been explored in canine cases. Therefore, a study was designed to evaluate the potential of OMP based IgM dot ELISA for diagnosing acute canine leptospirosis, with the following objectives:

1. Preparation of OMP from *Leptospira interrogans* serovar Australis

2. Development of OMP based IgM dot ELISA for the detection of leptospiral antibodies in canine sera

3. Comparison of the results of OMP based IgM dot ELISA test with that of MAT.

2. REVIEW OF LITERATURE

Leptospirosis is the general term that denotes all infections of man and animals by *Spirochates* of the genus *Leptospira*. The disease characterized by icterus and renal failure was first reported by Weil (1886). The term Weil's disease was first used by Goldschmidt (1887) to denote a severe febrile illness with jaundice and renal abnormalities in humans.

Stimson (1907) named the organism *Spirochaeta interrogans* on the basis of its morphological characters. Later on, *Spirochaeta interrogans* was named as *Leptospira interrogans* which included both parasitic and potentially pathogenic strains. *Spirochaeta biflexa*, which was saprophytic and non-pathogenic strain, was later renamed as *Leptospira biflexa*. Noguchi (1917), isolated leptospires from rat and human and suggested a generic name "*Leptospira*" (fine coil) to these organisms.

2.1 CULTURAL CHARACTERISTICS

Growth of leptospires in media containing serum or albumin with polysorbate and 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). The most widely medium used is Ellinghausen, Mc Cullough, Johnson and Harris (EMJH) medium (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967). 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. Growth of contaminants from clinical specimens could be inhibited by addition of fluorouracil (Johnson and Rogers, 1964). Other antibiotics like polymyxin B, rifampicin and vancomycin have been added to media for culturing of veterinary specimens, in which contamination is more likely to occur (Myers and Varela-Diaz, 1973; Adler *et al*., 1986). Growth of leptospires was often slow on primary isolation and culture bottles were retained for 13 weeks before being discarded, but pure cultures in liquid media usually grew within 14 days. The addition of two per cent pooled rabbit serum to semi-solid commercial EMJH medium with EMJH enrichment and 0.5 mg of 5-fluorouracil per milli litre was found to enhance the growth rate and success of isolation of *Leptospira interrogans* serovar Hardjo from bovine urine (Brugge and Louw, 1985). In semi-solid media (0.1 - 0.2 per cent agar), growth

reaches 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, which is related to the optimum oxygen tension (Faine, 1982). Leptospiral cultures could be maintained either by repeated sub-culturing (Waitkins, 1984) or preferably by storage in semisolid agar containing hemoglobin (Faine *et al*., 1999).

2.2 SEROPREVALENCE

2.2.1 Abroad

The studies on the prevalence of leptospirosis in the entire world have shown the presence of this disease globally. 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).

Alonso *et al.* (2001) investigated the 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 from cows in Brazil revealed that serovars Hardjo and Bratislava were the predominant cause of seroconversion (Oliveira *et al.,* 2001).

A sero-epidemiologic study on canine leptospirosis was conducted in Trinidad (Adesiyun *et al*., 2006). Among a total of 419 serum samples tested, 61 (14.6 per cent) were positive for *Leptospira* agglutinins.

Leptospirosis is reported to be the most widespread zoonosis in the world with increasing global incidence (Adler and Moctezuma, 2010).

2.2.2 India

The prevalence of leptospirosis in animals and humans has been reported based on sero-surveillance studies conducted in different 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 (5.9 per cent) of 68 buffaloe, 12 (7.1 per cent) of 169 sheep, 5 (6 per cent) of 80 goats and 3 of 7 wild animals were found positive.

Autumnalis and Pomona were found to be the predominant serovars in a study conducted in Tamil Nadu employing sera of different domestic animals and humans (Ratnam *et al*., 1994).

In a seroprevalence study conducted during 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 buffaloe, 14.3 per cent of 271 goats and 19.1 per cent of 166 swine, in various states of the country.

Soman (2004), in a seroprevalence study conducted in Thrissur, Kerala, reported that Pomona and Australis were the most prevalent serovars among canines.

Koteeswaran (2006) carried out a seroprevalence study in Tamil Nadu and 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. The predominant serovar among domestic animals was Australis (20.28 per cent), followed by the other serovars *viz.*, Hebdomadis (14.31 per cent), Sejroe (12.85 per cent), Pomona (12.51 per cent), Pyrogenes (8.23 per cent), Tarassovi (7.89 per cent), Autumnalis (6.42 per cent), Canicola (5.41 per cent), Ballum (4.28 per cent), Icterohaemorrhagiae (3.72 per cent), Grippotyphosa (2.37 per cent) and Javanica (1.75 per cent).

Swapna *et al*. (2006) conducted a seroprevalence study in Kerala, among agricultural workers, hospital sanitary workers, veterinarians and labourers. The most common serovar identified in the high risk groups was Pomona (26.5 per cent),

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 buffaloes by Mariya *et al*. (2006) 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 Tamil Nadu.

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

Sariprabha (2010) conducted a seroprevalence study of canine leptospirosis in Thrissur, Kerala and reported that the serovar Australis was the most prevalent serovar (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).

2.3 ANTIGENS OF LEPTOSPIRES

Research has involved identification of antigens of leptospires which could be of importance for diagnostic and prophylactic use. So far, lipopolysaccharides (LPS), surface proteins and outer membrane proteins of several leptospiral serovars have been identified as putative candidates (Srivastava and Tiwari, 2000).

2.3.1 Whole cell antigens

Whole cell antigens were tried in various serological assays like, macroscopic agglutination (Mailloux *et al.,* 1974), microcapsule agglutination (Arimitsu *et al.,* 1982) and ELISA (Terpstra *et al*., 1985; Yersin *et al*., 1999). The disaccharide epitope in the immunodominant moiety of whole cell preparations was present in non pathogenic leptospires as well as a diverse group of non leptospiral species that resulted in cross-reactivity (Matsuo *et al*., 2000 a, b). As a result these tests suffered from low sensitivity and identified less than 50 per cent of patients presenting the early phase of leptospirosis (Guerreiro *et al.,* 2001).

2.3.2 Outer membrane proteins

An important area of current *Leptospira* research is identification and characterization of outer membrane components that are essential in molecular understanding of bacterial structure and function. Outer membrane proteins were described as trans-membrane proteins, lipoproteins and peripheral membrane proteins (Hata *et al*., 1988; Brown *et al*., 1991; Haake *et al*., 1991; Zuerner *et al*., 1991; Nicholson and Prescott, 1993; Haake *et al*., 2000).

Nicholson and Prescott (1993), reported that the OMPs of 66, 59.5, 44, 42, 35.5 and 18 kDa were common in serovars Grippotyphosa, Hardjo, Autumnalis, Bratislava, Canicola, Icterohaemorrhagiae and Pomona.

The proteins of six leptospiral serovars *viz.*, Hardjo (Hardjobovis), Hardjo (Hardjoprajitno), Pomona, Icterohaemorrhagiae, Canicola and Grippotyphosa were extracted by Triton X-114 treatment and observed that the major proteins of 32, 41 and 45 kDa were common to all serovars (Alves *et al*., 1999).

Cullen *et al.* (2002) analyzed the outer membrane proteins by fractionating into LipL32, LipL36, LipL41, LipL48 and identified eight novel proteins *i.e.,* pL18, pL21, pL22, pL24, pL45, pL47/49, pL50 and pL55.

Outer membrane proteins were employed in various assays as they were genus specific and showed high sensitivity and specificity with minimal background reactions (Singh *et al.,* 2004; Srivastava *et al*., 2006; Sharma *et al.,* 2007).

The outer membrane of *Leptospira* facilitates direct interaction with the environment where the cells survives and express important constituents involved in infection, transmission, survival and adaptation to environmental conditions, besides generating immunity (Nally *et al*., 2005).

Studies conducted by Joseph (2007) revealed the presence of major bands at 77, 43, 36, 29 and 21 kDa in the protein profile of sarcosyl insoluble part of OMP and 41, 32 and 25 kDa bands in the sarcosyl soluble portion of OMP.

2.4 DIAGNOSIS OF LEPTOSPIROSIS

Bacteriological diagnosis of leptospirosis is achieved by methods like isolation and DFM. Confirmatory diagnosis can be provided by isolation of the organism, but it was reported to be time consuming and cumbersome (Levett and Whittington, 1998). Dark field microscopy required the presence of a minimum of 10^4 leptospires per milli litre, to observe one cell per field, making it less sensitive (Ahmad *et al.,* 2005). Molecular diagnostic techniques like PCR required well-established laboratory facilities and highly skilled personnel (Levett, 2003). Serological methods are highly useful in providing confirmatory diagnosis of leptospirosis.

2.4.1 Serological diagnosis

Many serological screening tests for leptospiral antibodies have been developed in earlier times which include complement fixation test (Pot and Dornickx, 1936), microcapsule agglutination test (Arimitsu *et al*., 1982) and indirect haemagglutination test (Levett and Whittington, 1998). All these tests were proved to be less sensitive than the gold standard test, MAT (Guerreiro *et al*., 2001).

2.4.1.a Microscopic agglutination test

The antigenic variability of leptospiral lipopolysaccharide (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 most likely based on the seroreactivity with LPS antigens. Microscopic agglutination test is a complex test to control, perform, interpret and required maintenance of live cultures (Turner, 1968). Being serovar specific, MAT may not be of true diagnostic value in

diagnosing the disease (Champagne *et al.,* 1991). The microscopic agglutination test was reported to detect both IgG and IgM antibodies (Chernukha *et al.,* 1976; Romero *et al.,* 1998). The titers are affected by the culture medium in which the antigens are grown (Myers, 1976).

The repeated weekly subculture of large numbers of strains presents hazards for laboratory workers (Pike, 1976). Formalinized antigens have been used in the MAT to overcome some of the difficulties associated with the use of live antigens. Titers obtained with these antigens were lower and more cross-reactions could be detected (Sulzer and Jones, 1978; Faine, 1982).

Paired sera from the suspected individuals are required to confirm a diagnosis with certainty. Moreover, patients with fulminant leptospirosis may die before seroconversion occurs (Ribeiro *et al*., 1994).

Serological observations made by MAT were reported to be insensitive in early acute-phase specimens (Cumberland *et al*., 1999). 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).

In an epidemiological study conducted by Ooteman *et al.* (2006) in Brazil, MAT was used for confirmatory diagnosis. Confirmation was done by observing a four-fold increase in titer between paired serum samples or a titer greater than 800 in a single serum sample.

2.4.1.b Latex agglutination test

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, to facilitate rapid screening of samples. A number of reports are available on the use of macroscopic agglutination 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).

Srivastava *et al*. (1989) standardized a LAT for the rapid screening of animal and human sera for leptospirosis using a partially purified antigen of *Leptospira biflexa* serovar Patoc for coating the latex beads.

Ramadass *et al*. (1999) standardized a rapid semi-quantitative LAT using pooled sonicated antigen from different *Leptospira* serovars *viz*., Icterohaemorrhagiae, Australis, Autumnalis and Canicola. 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.

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.

Dey *et al*. (2007) developed a rapid rLipL32 OMP LAT to detect specific anti-leptospiral antibodies from human and dog sera. The test was found to be sensitive, specific and accurate as compared to the MAT.

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

Sariprabha (2010) developed an OMP based LAT, whose sensitivity and specificity were 95.65 per cent and 86.21 per cent, respectively.

2.4.1.c Enzyme linked immunosorbent assay

Different types of ELISA have been tested and proved to be promising for detecting leptospiral antibodies (Adler *et al.,* 1980; Pappas *et al.,* 1985). Enzyme

linked immunosorbent assays have been described for the detection of serovar Hardjo in sheep (Adler *et al*., 1981), serovar Pomona (Thiermann and Garrett, 1983; Cousins *et al*., 1985) and Hardjo infection in cattle (Thiermann and Garrett, 1983; Yan *et al*., 1999).

The earlier ELISA tests were performed using the whole-cell antigen of *Leptospira* (Terpstra *et al*., 1985; Yersin *et al*., 1999). However, these tests suffered from low specificity because of the broadly reactive immunodominant moiety in whole cell antigen (Matsuo *et al*., 2000 a, b).

Flannery *et al*. (2001) evaluated the diagnostic utility of five recombinant antigens *viz.,* rLipL32, rLipL41, rHsp58, rOmpL1 and rLipL36 for the diagnosis of leptospirosis in humans. They found rLipL32 as the most sensitive antigen for the detection of the disease in acute (56 per cent) as well as the convalescent (94 per cent) phase of the disease. Singh *et al*. (2004) developed an OMP based ELISA for the diagnosis of bovine leptospirosis.

Srivastava *et al*. (2006) evaluated rLipL32 and rLipL41 OMPs of *Leptospira interrogans* serovar Canicola as antigens in ELISA for screening bovine serum samples. The sensitivity and specificity of rLipL32 ELISA as against MAT was 100 per cent and 78.8 per cent, respectively, whereas using rLipL41 ELISA it was 100 per cent and 83 per cent.

2.4.1.d Immunoblotting

In humans, Natarajaseenivasan *et al.* (2004) reported that IgM recognition was 32.6, 32.6, 30.2 and 37.2 per cent in acute phase sera and 32.6, 37.2, 44.2 and 41.9 per cent in convalescent sera for leptospiral proteins of p14, p25, p32 and p41/42 respectively.

Niwetpathomwat and Doungchawee (2006) studied the efficacy of western blotting for diagnosis of human leptospirosis using a mixed antigen from ten serovars. They reported that proteins of the molecular weight 15-20, 23-24, 41 and 45 kDa were the common reactors detected.

Doungchawee *et al.* (2008) evaluated the accuracy of IgM immunoblot test by using the reactivity to leptospiral proteins ranging from the molecular weights of 14 to18 kDa. The utility of 19-23, 24-30, 35/36, 37 and 41/42 kDa antigens for leptospiral diagnosis was also studied. They opined that high sensitivity and specificity of IgM-immunoblot test would provide greatly improved diagnostic accuracy for identifying acute human leptospirosis.

2.4.1.e IgM dot ELISA

Enzyme linked immunosorbent assays require costly equipment and technical expertise making them unfit for routine field level diagnosis. So, they have been applied with a number of modifications. Simple versions of ELISA such as dot ELISA have been developed in many laboratories (Pappas *et al*., 1985).

Most of the tests for diagnosing acute leptospirosis are aimed at the detection of leptospiral IgM which is detectable from about second to fifth day after infection (Silva *et al.,* 1995; Winslow *et al*., 1997).

Gussenhoven *et al*. (1997) developed a whole-cell based lepto-dipstick assay using antigen from *L .biflexa* serovar Patoc, which showed a sensitivity of 86.8 per cent and specificity of 92.7 per cent.

Silva *et al.* (1997) developed an IgM specific dot ELISA, in which polyvalent leptospiral antigen was dotted on to nitrocellulose filter disks. Further modifications of this approach were used to detect IgG and IgA, in addition to IgM.

The IgM lepto-dipstick had high sensitivity during the second, third and fourth weeks of illness (87·6 per cent). Sensitivity during the first week and after four weeks were relatively low, but acceptable (Sehgal *et al*., 1999).

Tansuphasiri *et al.* (2005) developed an IgM dot ELISA with antigens from prevalent serovars, which showed a sensitivity of 98.96 per cent and specificity of 93.93 per cent. The high negative predictive value of 99.57 per cent made the assay highly suited for rapid screening.

In an IgM dot ELISA for human leptospirosis, developed by Sharma *et al.* (2007), outer membrane proteins gave lesser background reactions and were proved to be better antigens than the whole cell moieties.

Shekatkar *et al*. (2010) developed an IgM dot ELISA using locally prevalent *Leptospira* strains which showed high sensitivity, specificity and negative predictive values of 97.33 per cent, 96.00 per cent and 96.00 per cent, respectively. The assay was reported to be simple, inexpensive and easy to perform.

3. MATERIALS AND METHODS

All the chemicals used in the study were of molecular biology grade, obtained from Sisco Research Laboratories (SRL) private limited. Some of the reagents used in the study were procured from Merck Genei, Sigma-Aldrich and Immunology Consultants Laboratory (ICL), USA. The source for these reagents has been provided in the appropriate places of the text. Ready-made medium from Difco Laboratories and culture medium supplement from HiMedia laboratories private limited were used. Glassware of Borosil brand and plasticware of Tarsons brand were used in the study.

3.1 MATERIALS

3.1.1 Reference strains of *Leptospira*

The following strains of *Leptospira*, representing nine different serogroups procured from the National *Leptospira* Reference centre, Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands and maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, were used in the study.

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

3.1.2 Media

3.1.2.1 Leptospira culture medium base (Difco)

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

3.1.2.2 Supplement

3.1.2.2 a Leptospira Culture Medium Supplement (HiMedia)

3.1.2.2 b Albumin supplement

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

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 (SRL) in 100 milli litre 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 milli litre with distilled water and then sterilized by filtration (0.2 μm).

3.1.2.2 c Hemolysed rabbit serum

Hemolysed serum collected from rabbits of New Zealand White strain was used in the present study.

3.1.2.2 d Reconstituted Bovine Serum

Serum was collected from bovine calves reared at University Livestock Farm, Mannuthy and screened employing MAT for the presence of leptospiral antibodies. Later, vitamin B_{12} (20mg/ml) was added at one per cent level to the medium.

3.1.2.3 Preparation of 5-Fluorouracil solution (5-FU)

One hundred mg of the 5-FU was added to 5 milli litre of the sterile triple distilled water. To this, 0.2 milli litre of 0.1N NaOH was added and the volume was made up to 10 milli litre with sterile triple distilled water. The solution was sterilized by filtration through a 0.2 μm membrane filter and stored at -20^0C. One milli litre of this solution was added to 100 milli litre of liquid or semi-solid medium to obtain a final concentration of 100 μg/ml of medium.

3.1.2.4 Leptospira culture medium

The *Leptospira* culture medium was prepared by dissolving 2.3 g *Leptospira* culture liquid medium base in 900 milli litre of triple distilled water. The medium was autoclaved at 121^{o}C for 15 min at 15 lbs and allowed to cool to 40-50^{o}C. Supplement was added at 10 per cent level to the medium base. To avoid contamination, 5 – FU (100μg / ml final concentration) was added to the culture medium.

Medium was dispensed in three to five milli liter quantities in screw capped tubes. The tubes were checked for sterility by incubating at 37^0C for 48 h and then stored at 4^0C.

3.1.2.5 Leptospira semi-solid medium

Leptospira semi-solid medium was prepared by adding 0.5 per cent bacteriological agar to *Leptospira* liquid medium. The medium was autoclaved at 121^0 C for 15 min at 15 lbs pressure and was allowed to cool. Supplement and 5-FU were added as described above (3.1.2.4).

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 h at 37^0C and then stored at 4^0C.

3.1.3 Buffers and reagents

3.1.3.1 Buffers and reagents for OMP extraction

3.1.3.1.a Phosphate Buffered Saline (PBS, 1 x solution), pH 7.2

Sodium chloride	8g
Potassium chloride	0.2g
Disodium hydrogen phosphate (Na_2HPO_4)	1.133g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2g
Distilled water	1000 ml

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

3.1.3.1.b 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.

3.1.3.1.c Sodium lauroyl sarcosinate (0.5 per cent)

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.

3.1.3.2 Buffers and reagents for Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

3.1.3.2.a 30 per cent Acrylamide – bisacrylamide mix

Acrylamide	29.0 g
Bisacrylamide	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.

3.1.3.2.b 1.5 M Tris pH 8.8

Tris base	181.7 g
Distilled water up to	1000 ml

pH was adjusted to 8.8 with 1 N HCl and stored at 4^0C.

3.1.3.2.c 0.5 M Tris pH 6.8

Tris base	60.6 g
Distilled water up to	1000 ml

pH was adjusted to 6.8 with 1 N HCl and stored at 4^0C.

3.1.3.2.d Resolving gel (12 per cent)

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

3.1.3.2.e Stacking gel (5 per cent)

30 per cent Acrylamide: bisacrylamide mix	310 µl
Tris base (0.5 M), pH 6.8	622 µl
Sodium dodecyl sulphate (10 per cent)	25 µl
Ammonium per sulphate (10 per cent)	25 µl
TEMED	4 µl
Distilled water	1.5 ml

3.1.3.2.f Tris Glycine Buffer (5 x)

Tris base	15.1g
Glycine	54.0 g
Sodium dodecyl sulphate	50.0 g

Final volume was adjusted to 1000 ml and pH to 8.2.

3.1.3.2.g Sample preparation buffer (2 x)

Tris hydrochloride (1M, pH 6.8)	6.2 ml
Glycerol	5.0 ml
Sodium dodecyl sulphate (10 per cent)	1.0 ml

2 – Mercaptoethanol	0.25 ml
Bromophenol blue	0.25 mg

The volume was adjusted to 25 ml using distilled water, mixed and stored at 4^0C.

3.1.3.2.h Coomassie brilliant blue staining solution

Coomassie brilliant blue (R 250)	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 up to	200 ml

3.1.3.2.i Destaining solution

Methanol	150 ml
Glacial acetic acid	50 ml
Distilled water	300 ml

3.1.3.3 Buffers, reagents and materials for IgM Immunoblotting

3.1.3.3.a Nitrocellulose membrane (NCM) (Amersham private limited)

3.1.3.3.b Whatmann filter paper no.1

3.1.3.3.c PBS, pH 7.2 (prepared as in 3.1.3.1.a)

3.1.3.3.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

3.1.3.3.e Tris buffered saline (TBS)

Tris hydrochloride	100 mM
NaCl	0.9 per cent
Stored at 4^0C	

3.1.3.3.f Tris buffered saline with tween-20 (TTBS)

0.1 per cent Tween – 20 in TBS

3.1.3.3.g Ponceau S stain (Merck Genei)

3.1.3.3.h Blocking buffer

Dried skim milk powder	5 g
PBS	100 ml

3.1.3.3.i Conjugate

Horse radish peroxidase conjugated goat anti-canine immunoglobulin M (ICL) was diluted 1:2500 in PBS, pH 7.2.

3.1.3.3.j Chromogenic visualization solution

100 mM Tris hydrohloride (pH,7.5)	5 ml
Diaminobenzidene (DAB) stock (40 mg/ ml)	100 µl
Nickel Chloride stock (80 mg / ml)	25 µl
3 per cent H_2O_2	15 µl

3.1.3.4 Buffers, reagents and materials for LAT

3.1.3.4.a 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.

3.1.3.4.b Glycine buffer saline, pH 8.2

Glycine (0.1M) and NaCl (0.17M) prepared in distilled water

pH was adjusted to 8.2 and stored at 4^oC.

3.1.3.4.c Latex beads (0.80µm diameter) (HiMedia)

3.1.3.5 Buffers, reagents and materials for IgM dot ELISA

3.1.3.5.a NCM (Amersham private limited)

3.1.3.5.b PBS, pH 7.2 (prepared as in 3.1.3.1.a)

3.1.3.5.c Carbonate – bicarbonate buffer, pH 9.6 (prepared as in 3.1.3.4.a)

3.1.3.5.d Phosphate buffered saline with tween-20 (PBS-T)

0.05 per cent Tween – 20 in PBS

3.1.3.5.e Blocking buffer (prepared as in 3.1.3.3.h)

3.1.3.5.f Conjugate (as in 3.1.3.3.i)

3.1.3.5.g Chromogenic visualization solution (prepared as in 3.1.3.3.j)

3.2 METHODS

3.2.1 Maintenance of leptospiral cultures

The different *Leptospira* serovars used in the present study were maintained by regular sub-culturing in the liquid *Leptospira* culture medium at 28-30^{0}C at 14 - 21 day intervals. The stock cultures were maintained in semi-solid medium and sub-cultured at one month interval.

3.2.2 Identification of leptospires

The live *Leptospira* organisms were identified by their morphology and motility. In order to check the purity of the liquid cultures, occasionally they were streaked on to blood agar plates, and observed for contaminants. The cultures which were found contaminated were discarded or in some cases were purified by filtration through 0.2 µ syringe type membrane filters (Whatmann) of 25 mm diameter.

3.2.3 Serum samples

A total of 114 canine serum samples collected from clinical cases during the period of September 2009 to January 2011, presented at University Veterinary Hospitals at Mannuthy and Kokkalai, Thrissur, were used in the study. The samples included those collected from 22 healthy vaccinated animals and 28 healthy unvaccinated animals. The rest of the samples were taken from cases suspected for acute leptospirosis.

3.2.4 Extraction of outer membrane proteins

The OMP of *L. interrogans* serovar Australis strain Ballico was extracted using sarcosyl detergent as described by Nicholson and Prescott (1993), with some modifications. Briefly, leptospires were grown in 500 milli litre *Leptospira* culture medium for about 10-11 days. When good growth was observed by visual turbidity, they were harvested by centrifugation at 40,000 x g for 20 min, followed by three

washings with 0.01 M PBS, pH, 7.2. The cells were re-suspended in 10mM HEPES buffer and disrupted by sonication at 60µ for 15 sec each for four times, interrupted by a five sec pause, while cooling in an ice bath. Cellular debris was removed by centrifugation at 2000 x g for 10 min at 4^0C. The supernatant was collected and centrifuged at 1, 00, 000 x g for 60 min at 4^0C. The pellet was re-suspended in two milli litre of one per cent sodium lauryl sarcosinate detergent (Sigma-Aldrich), prepared in 10mM HEPES buffer, pH, 7.4 and incubated at room temperature overnight, with gentle rotation. The sarcosyl insoluble (SI) fraction was sedimented by centrifugation at 1, 00, 000 x g for 60 min at 4^0C and suspended in 125 µl of 0.06 M carbonate-bicarbonate buffer, pH, 9.6. The sarcosyl soluble (SS) and the sarcosyl insoluble (SI) 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), using the commercial protein estimation kit (Merck Genei).

3.2.5 Analysis of OMP by SDS - PAGE

The OMP profile of *L. interrogans* serovar Australis was analysed by using a one-dimensional SDS - PAGE as per the method described by Laemmli (1970), in a vertical electrophoresis apparatus (Hoefer, USA), with relevant modifications.

Glass plates were cleaned and set in the gel moulding tray of electrophoresis apparatus and 6 milli litre of 12 per cent resolving acrylamide gel solution was poured and then 1 milli litre of water was layered over the gel and allowed to polymerize. Then, 2.5 milli litre of five per cent stacking gel was poured over the resolving gel and a suitable comb was inserted. The gel sandwich was mounted on to the electrophoresis chamber and buffer reservoirs were filled with tris- glycine buffer, after removing the comb. Before loading, 20 µl of each of the samples, solubilized in 2 x gel loading buffer, was boiled for 5 min and spun for 30 sec. The standard protein medium range molecular weight marker (Merck Genei) 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 100V till the dye front reached end of the gel. The current was disconnected and the gel was removed from the glass plate. The stacking gel was snipped off and the resolving gel was subjected to the Coomassie brilliant blue staining for one hour and then destained 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.

3.2.6 IgM immunoblotting of the OMP

The proteins fractionated in the SDS-PAGE gel were transferred onto a NCM as per the technique described by Towbin *et al*. (1979), with some minor modifications. The sensitivity of OMP was tested by IgM immunoblotting with the MAT confirmed sera collected from cases of acute canine leptospirosis and specificity was checked with MAT negative sera. The MAT negative sera were collected from cases confirmed with diseases showing high pyrexia *viz.,* ehrlichiosis and trypanosomiasis.

3.2.6.a Manual transfer of proteins to NCM

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 (3.2.5) and other portion was kept in transfer buffer for 5-10 min. Transfer membrane was prepared by cutting NCM to same size as that of gel. Membrane was then placed into distilled water slowly at 45^0 angle. When fully wet, it was equilibrated for 15 min in transfer buffer. Eighteen Whatmann No. 1 filter paper sheets were cut to gel size and soaked in transfer buffer. A large Petri plate was taken and a microtitre plate was placed in its centre. Blot buffer was filled in the Petri plate to a level just below the microtitre plate. A large glass plate was placed above the microtitre plate and a large Whatmann No. 1 filter paper of size larger than the gel wetted with transfer buffer was placed above the glass plate with ends immersed in blot buffer. Nine gel-sized equilibrated filter papers were stacked above this large filter paper and NCM was placed above this stack. The gel portion soaked in transfer membrane was placed over this NCM and nine equilibrated filter papers were stacked over this assembly. A glass rod was then rolled over the assembly to ensure that there were no air bubbles trapped between the gel and NCM. On the top of this, a glass plate was kept and a weight of two kilograms was placed. This assembly was left for overnight period at 4^oC to ensure complete transfer.

3.2.6.b Identification of immunodominant proteins

The transfer unit was disassembled and the membrane was removed after marking the orientation and the gel was subjected to staining and destaining as described previously to verify the transfer efficiency. Occasionally, the transferred proteins were reversibly stained by putting the membrane in Ponceau S solution for 2 min at room temperature and destaining for 10 min in distilled water. Subsequently, the membrane was placed in 5 milli litre blocking buffer and incubated at 37^0C for 2 h, followed by washing twice with TTBS for 10 min each. Primary antibody *i.e.*, the serum was diluted in blocking buffer (1:100). Diluted primary antibody was added to the membrane and it was incubated for 45 min at 37^0C, with constant agitation. The membrane was washed four times by agitation, 15 min each, with sufficient amount (100 - 200 milli litre) of TTBS.

Membrane was then incubated at 37^0C in suitably diluted goat anti-canine horse radish peroxidase IgM conjugate (1:2500, in blocking buffer) for one hour, with constant agitation. The membrane was washed four times in TTBS 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.

3.2.7 Serological tests

3.2.7.a Microscopic agglutination test

The MAT was carried out using live *Leptospira* organisms as described by Faine (1982). A 1:100 diluted serum was serially diluted two fold times in PBS, to obtain dilutions of 1: 100 to 1: 25600, in 96 well U-bottom microtitre plates. To 30 µl each of the serum dilution, 30 µl of 7-8 day old live antigen was added. Appropriate antigen controls were set with 30 µl PBS and 30 µl of antigen and the plates were incubated at 37^0C for 3 h. After incubation, the result was read by examining a drop of serum-antigen mixture from each well under low power (10 x) of DFM for agglutination of leptospires. The antibody titer was the highest dilution of serum showing agglutination of 50 per cent or more leptospiral organisms. Reciprocal

agglutination titers of greater than or equal to 100 were considered as positive reactions, according to OIE recommendations. The serogroup reacting at the highest titer was presumed to be the infecting serogroup. Sera samples showing same agglutination titers to more than one serogroup were considered as mixed equals.

3.2.7.b Latex agglutination test

The test was performed as per the techniques described by Srivastava *et al*. (1989) and Smits *et al*. (2000), with relevant modifications. The latex beads (0.80µm diameter, HiMedia) were washed in carbonate - bicarbonate buffer (pH, 9.6) and finally suspended as a 10 per cent suspension in glycine buffered saline (pH, 8.2). One milli litre of the antigen (containing 1.2 mg/ml OMP) was used to coat on 300 µl of the washed latex beads. The suspension was incubated at 37^{o}C in shaker incubator for four hours and subsequently kept for overnight at 4^{o}C. Simultaneously a negative control *i.e*., washed latex beads incubated without antigen was also processed. The latex agglutination test was performed by placing two 20 µl drops of serum sample on a clean grease free glass slide and mixing with an equal volume of the test and control antigens with help of a sterile disposable wooden tooth pick. The glass slide was then rotated gently against a light source for four min. Samples were considered as negative, if no agglutination was observed within four min.

3.2.7.c IgM dot ELISA

The test was performed as described by Sharma *et al.* (2007), with minor modifications. The concentration of leptospiral OMP was adjusted to 100 µg/ml in carbonate - bicarbonate buffer (pH, 9.6). This antigen was coated onto the centre of the NCM strips. The strips were allowed to dry at room temperature. The unbound sites of the NCM strips were incubated with blocking buffer at 37°C for one hour. The blocked NCM strips were rinsed in PBS-T, dried and kept in the refrigerator for further use. Later, the strips were incubated in the serum samples (1:100 dilution in blocking buffer) at the room temperature for one hour and washed with PBS-T for 10 min with five to six changes of wash buffer. Further, the NCM strips were incubated with horse radish peroxidase conjugated goat anti-canine immunoglobulin M at a dilution of 1: 2500 in blocking buffer for one hour at room temperature. The strips were washed four times in PBS-T and were put into the chromogenic visualization

solution at room temperature, with mild rocking for three min. The reaction was terminated by washing NCM strips with distilled water. The NCM strips were air dried and reaction was observed for the development of a brown spot.

3.2.8 Statistical analysis of results

Statistical analysis was done with Fisher's exact test with *GraphPad* statistical software. The two-tailed P value was calculated to statistically analyze the association between LAT and MAT.

Sensitivity was defined as the capacity of the LAT to identify positive serum samples which were also positive with the MAT, while specificity was defined as the capacity of the LAT to identify negative sera samples which were also negative with the MAT. The relative sensitivity, specificity, accuracy and predictive values of the LAT for serodiagnosis of canine leptospirosis were evaluated in comparison to the MAT using a two-into-two contingency table, as described by Thrusfield (2005).

Sensitivity = [a / (a +c)] x 100 where ‘a’ is the number of sera positive by LAT and MAT and ‘c’ the number of sera positive by MAT but negative by LAT.

Specificity = [d / (b + d)] x 100 where ‘d’ is the number of sera negative by LAT and MAT and ‘b’ the number of sera negative by MAT but positive by LAT.

Accuracy = [(a + d) / (a + b + c + d)] x 100

Positive Predictive Value = a / (a + b) x 100 where ‘a’ is the number of sera positive by LAT and MAT and ‘b’ the number of sera negative by MAT but positive by LAT.

Negative Predictive Value = d / (c + d) x 100 where ‘d’ is the number of sera negative by LAT and MAT and ‘c’ the number of sera positive by MAT but negative by LAT.

Prevalence of acute leptospirosis cases was calculated as the ratio of number of IgM dot ELISA positive cases to the population at risk *i.e.,* positive reactors as shown by MAT and LAT (Thrusfield, 2005).

4. RESULTS

4.1 MAINTENANCE OF REFERENCE STRAINS OF *Leptospira*

Leptospira serovars were maintained in *Leptospira* culture medium with different supplements *viz.,* readymade supplement (HiMedia), albumin supplement, hemolysed rabbit serum and reconstituted bovine serum. The best growth was observed with hemolysed rabbit serum. The culture attained optimum concentration of 2 X 10^8 cells per milli litre within five days. Readymade supplement, albumin supplement and reconstituted bovine serum took a comparatively longer period for achieving optimum growth. Henceforth, hemolysed rabbit serum was used for regular maintenance of cultures and reconstituted bovine serum was used in bulk cultures.

4.2 SDS-PAGE ANALYSIS OF LEPTOSPIRAL OMP

The OMP concentration was estimated using the commercial protein estimation kit (Merck Genei) and found to be 1.2 mg/ml. (Fig. 1)

SDS-PAGE analysis of OMP from *Leptospira interrogans* serovar Australis revealed many fractions of the protein. Standard protein molecular weight marker of medium range (Merck Genei) was used which revealed six distinct bands ranging from 97.4 kDa to 14.3 kDa.

The SI portion of the OMP revealed five distinguishable bands located at 21, 32, 36, 41 and 66 kDa. In SS portion, three bands could be found at 29, 41 and 66 kDa. (Fig. 2)

4.3 IgM IMMUNOBLOTTING

The sensitivity of OMP was tested by IgM immunoblotting with MAT confirmed sera collected from cases of acute canine leptospirosis. Similarly, specificity was checked with MAT negative sera from cases confirmed with diseases showing high pyrexia *viz.,* ehrlichiosis and trypanosomiasis. Outer membrane proteins

of molecular weight 36, 32 and 21 kDa were found to be the major reactors, while those of 66 and 41 kDa were observed to be the minor reactors. (Fig. 3)

4.4 SEROLOGICAL TESTS

4.4.1 Microscopic agglutination test

One hundred and fourteen canine sera samples were subjected to the MAT, out of which the sera from 28 healthy unvaccinated animals were negative with MAT and the remaining 86 samples, which included serum from 22 healthy vaccinated animals were positive with MAT. The MAT titers varied from 100 to 6400 (Table 1). The most predominant serovar in the region was found to be Australis followed by Pomona, Grippotyphosa, Icterohaemorrhagiae, Autumnalis, Javanica, Pyrogenes, Canicola and Patoc and in vaccinated cases agglutination was observed against vaccinal serovars of Canicola, Pomona, Grippotyphosa and Icterohaemorrhagiae. The seroprevalence results are summarized in table 2 and represented diagrammatically in fig. 4.

Additionally, MAT was carried out for 8, of the 13 IgM dot ELISA positive samples, with the convalescent sera collected after 14 - 21 days of first collection. All of them were observed to show a four-fold increase in MAT titer. Convalescent sera collected from 15 random cases which were negative by IgM dot ELISA but positive with MAT and LAT, did not show any increase in the MAT titer.

4.4.2 Latex agglutination test

One hundred and fourteen serum samples screened by MAT were tested with LAT. Out of 86 MAT positive cases, 81 cases were diagnosed as positive and 5 cases as negative by LAT. Among 28 MAT negative cases, 23 cases were shown as negative by LAT, but 5 cases were diagnosed as positive by LAT. The results are summarized in table 3. The representative positive and negative samples are shown in fig. 5.

4.4.2.a Statistical analysis of results

Statistical analysis was done with Fisher's exact test with *GraphPad* statistical software. The two-tailed P value was less than 0.0001. Therefore, the association between LAT and MAT was considered to be extremely statistically significant.

Sensitivity was defined as the capacity of the LAT to identify positive serum samples which were also positive with the MAT, while specificity was defined as the capacity of the LAT to identify negative sera samples which were also negative with the MAT. The relative sensitivity, specificity, accuracy and predictive values of the LAT for serodiagnosis of canine leptospirosis were evaluated in comparison to the MAT.

Sensitivity = [a / (a +c)] x 100 where 'a' is the number of sera positive by LAT and MAT and 'c' the number of sera positive by MAT but negative by LAT.

Specificity = [d / (b + d)] x 100 where 'd' is the number of sera negative by LAT and MAT and 'b' the number of sera negative by MAT but positive by LAT.

Accuracy = [(a + d) / (a + b + c + d)] x 100

Positive Predictive Value = a / (a + b) x 100 where 'a' is the number of sera positive by LAT and MAT and 'b' the number of sera negative by MAT but positive by LAT.

Negative Predictive Value = d / (c + d) x 100 where 'd' is the number of sera negative by LAT and MAT and 'c' the number of sera positive by MAT but negative by LAT.

The results are summarized in table 4.

4.4.3 IgM dot ELISA

The positive reactors in both MAT and LAT were subjected to test with IgM dot ELISA, to find out the acute cases. Prevalence of acute leptospirosis cases was calculated as the ratio of number of IgM dot ELISA positive cases to the population at risk *i.e.,* positive reactors as shown by MAT and LAT.

Among 81 samples tested, 13 were found positive with IgM dot ELISA, showing a prevalence of 16.04 per cent. Fig. 6 shows the representative positive and negative samples. The results of IgM dot ELISA in comparision with MAT and LAT are given in table 1.

Table 1. Comparision of results of MAT, LAT and IgM dot ELISA

S.no	Australis	Autumnalis	Canicola	Grippotyphosa	Icterohaemorrhagiae	Javanica	Patoc	Pomona	Pyrogenes	LAT	IgM-dot ELISA
1.		1:100*								+	
2.	1:200*		1:100	1:100						+	
3b.				1:200*	1:100					+	
4#			1:100						1:100	+	
5.	1:800*		1:200	1:100						+	+
6.	1:100*									+	
7b.				1:100*						-	
8.	1:200*							1:100		+	
9.	1:100*									+	
10a.										-	
11a.										-	
12.	1:200*				1:100			1:100		-	
13b.			1:200*					1:100		+	
14.	1:100		1:100					1:200*	1:100	+	
15.								1:800*		+	+
16.	1:200		1:100			1:3200*				+	
17.						1:100*				-	
18.	1:200*									+	
19b.					1:200*					+	
20a.										-	
21.							1:100*			+	
22.		1:100*								+	
23a.										+	
24.	1:3200*									+	
25.							1:200	1:800*		+	+
26a.										-	
27.							1:200*			+	
28#	1:100				1:100					-	
29a.										-	
30.	1:100				1:400*					+	

* : The serovar reacting at the highest titer (presumed to be the infecting serovar) a : Healthy unvaccinated animals b : Healthy vaccinated animals

: Mixed equals

Table 1. Comparision of results of MAT, LAT and IgM dot ELISA (contd.)

S.no	Australis	Autumnalis	Canicola	Grippotyphosa	Icterohaemorrhagiae	Javanica	Patoc	Pomona	Pyrogenes	LAT	IgM-dot ELISA
31b.					1:200			1:400*		+	
32a.										+	
33a.										-	
34.									1:200*	+	
35b.								1:100*		+	
36a.										-	
37a.										-	
38b.								1:200*		+	
39b.								1:200*		+	
40.	1:100*									+	
41.	1:100*									+	
42.			1:3200*							+	+
43.	1:100			1:100				1:200*		+	
44a.										-	
45a.										-	
46.								1:800*		+	+
47#	1:100					1:100				+	
48a.										+	
49.	1:3200*	1:400	1:400							+	
50.				1:800*	1:400					+	+
51b.				1:200*						+	
52a.										-	
53b.			1:100					1:200*		+	
54a.										-	
55.									1:400*	+	
56.	1:200*									+	
57.			1:800*							+	+
58a.										-	
59b.				1:100*						+	
60.									1:200*	+	

* : The serovar reacting at the highest titer (presumed to be the infecting serovar) a : Healthy unvaccinated animals b : Healthy vaccinated animals

: Mixed equals

Table 1. Comparision of results of MAT, LAT and IgM dot ELISA (contd.)

S.no	Australis	Autumnalis	Canicola	Grippotyphosa	Icterohaemorrhagiae	Javanica	Patoc	Pomona	Pyrogenes	LAT	IgM-dot ELISA
61b.					1:100*					-	
62.	1:200*							1:100		+	
63#b.				1:100	1:100			1:100		+	
64.	1:400*									+	
65b.								1:200*		+	
66.	1:400*									+	+
67.	1:200*									+	
68.	1:200*									+	
69a.										-	
70.	1:800*									+	+
71a.										+	
72.	1:100							1:1600*		+	+
73b.								1:200*	1:100	+	
74a.										-	
75a.										-	
76.				1:200*						+	
77a.										-	
78.					1:200*					+	
79b.								1:200*		+	
80b.				1:100		1:100		1:200*		+	
81.					1:100			1:100	1:200*	+	
82b.								1:100*		+	
83b.								1:100*		+	
84.	1:200*							1:100		+	
85.	1:100*									+	
86.								1:800*		+	+
87a.										-	
88a.										-	
89.		1:100*								+	
90a.										-	

* : The serovar reacting at the highest titer (presumed to be the infecting serovar) a : Healthy unvaccinated animals b : Healthy vaccinated animals

: Mixed equals

Table 1. Comparision of results of MAT, LAT and IgM dot ELISA (contd.)

S.no	Australis	Autumnalis	Canicola	Grippotyphosa	Icterohaemorrhagiae	Javanica	Patoc	Pomona	Pyrogenes	LAT	IgM-dot ELISA
91.	1:200*			1:100						+	
92.	1:100							1:100	1:200*	+	
93.	1:200*				1:100			1:100		+	
94a.										-	
95.		1:200*						1:100		+	
96.		1:200*								+	
97a.										+	
98.		1:100*								-	
99.	1:200*							1:100		+	
100.			1:400*							+	+
101a.										-	
102.				1:100*						+	
103.										+	
104.	1:400*			1:100						+	+
105#	1:100							1:100		+	
106#b.					1:100			1:100		+	
107.		1:100*								+	
108.						1:100*				+	
109a.										-	
110.	1:3200*	1:200								+	
111.	1:200*									+	
112.	1:800*		1:400			1:200		1:400		+	
113.					1:6400*	1:200				+	
114.	1:800*				1:400					+	

* : The serovar reacting at the highest titer (presumed to be the infecting serovar) a : Healthy unvaccinated animals b : Healthy vaccinated animals

: Mixed equals

Table 2. Seroprevalence of Leptospirosis by MAT

Serogroup	Number of positive cases	Per cent positivity
Australis	32	28.07
Autumnalis	7	6.14
Canicola	5	4.38
Grippotyphosa	8	7.01
Icterohaemorrhagiae	8	7.01
Javanica	7	6.14
Semaranga (serovar Patoc)	3	2.63
Pomona	18	15.78
Pyrogenes	6	5.26

Table 3. Comparision of MAT and LAT results

	MAT positive	MAT negative	Total
LAT positive	81 (a)	5 (b)	86(a + b)
LAT negative	5 (c)	23 (d)	28(c + d)
Total	86 (a + c)	28 (b + d)	114

Table 4. Results of statistical analysis between MAT and LAT results

Characteristic	Value
Sensitivity	94.19 per cent
Specificity	82.14 per cent
Accuracy	91.23 per cent
Positive Predictive Value (PPV)	94.19 per cent
Negative Predictive Value (NPV)	82.14 per cent
Two-tailed P value	< 0.0001

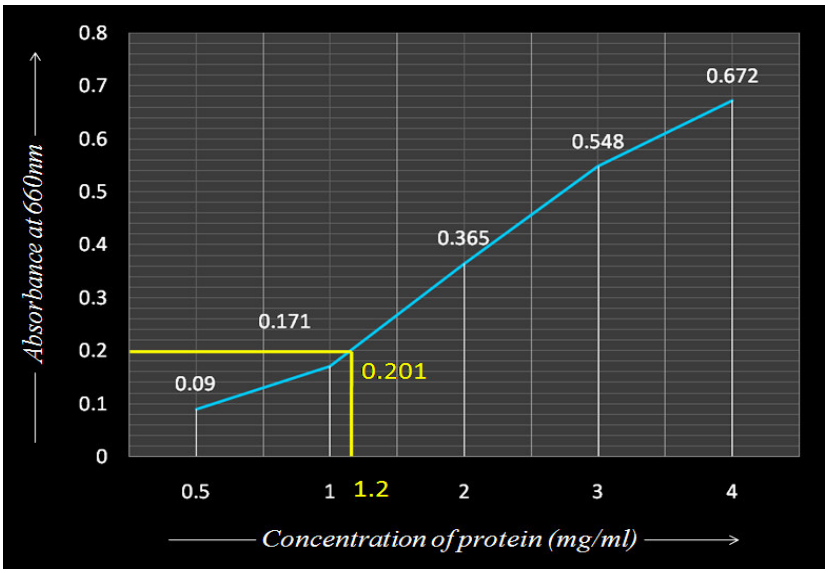
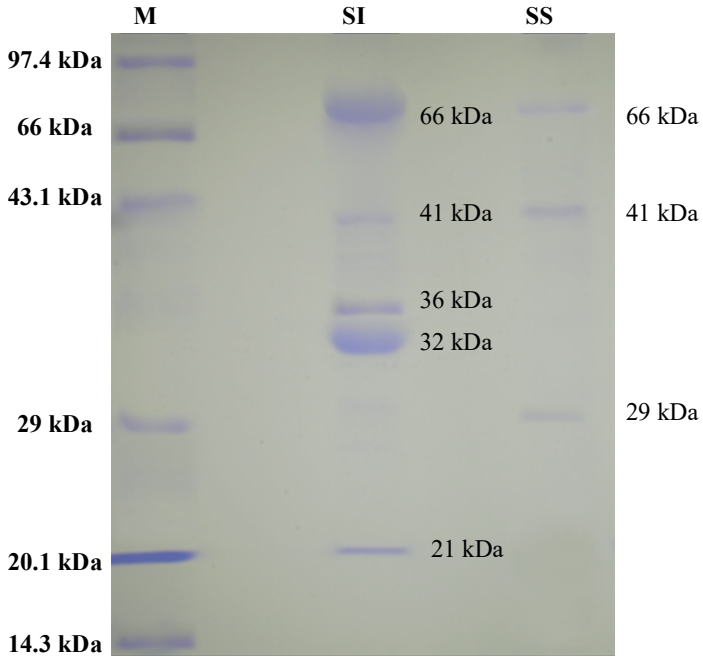
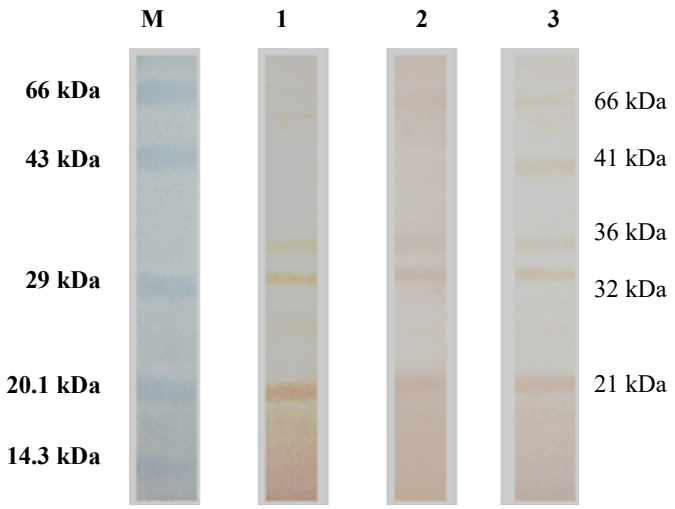


Fig. 1: Estimation of OMP concentration by Lowry's method



Lane M: Standard protein marker
Lane SI: Sarcosyl insoluble OMP
Lane SS: Sarcosyl soluble OMP

Fig. 2: SDS-PAGE analysis of OMP



M: Standard protein marker
1, 2, 3: Representative positive samples

Fig. 3: IgM Immunoblotting of leptospiral OMP

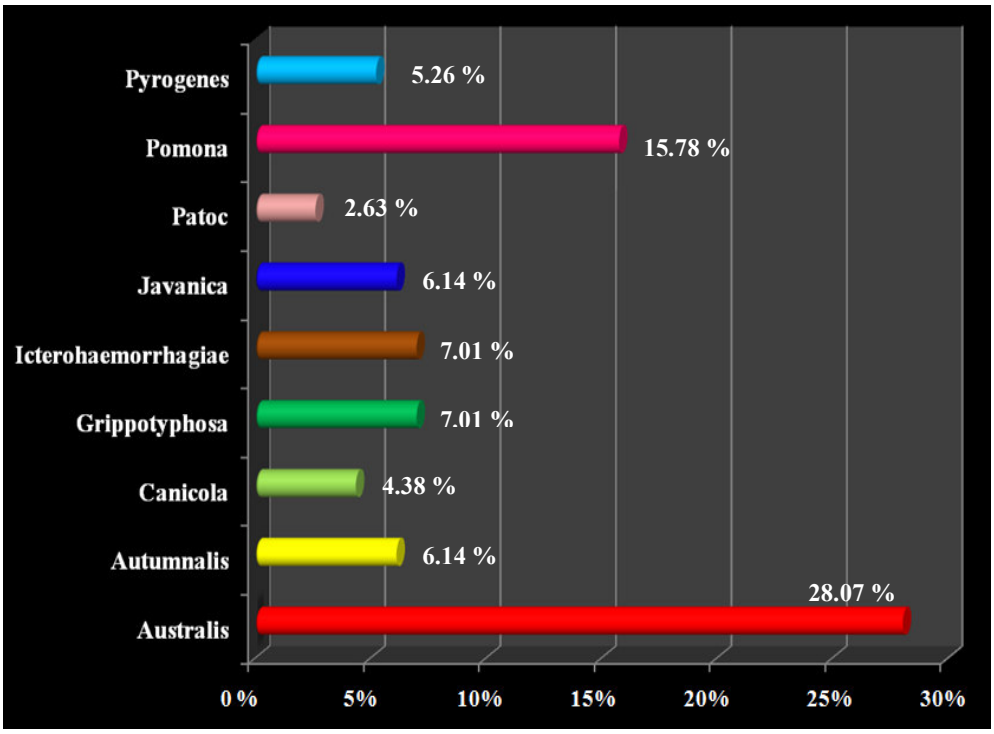


Fig. 4: Seroprevalence of leptospirosis by MAT

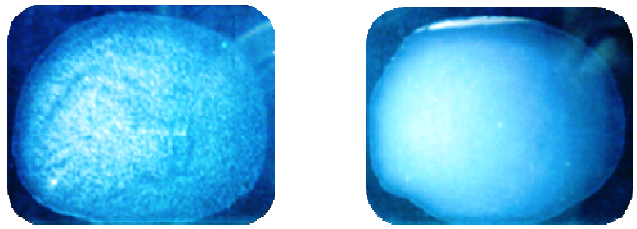
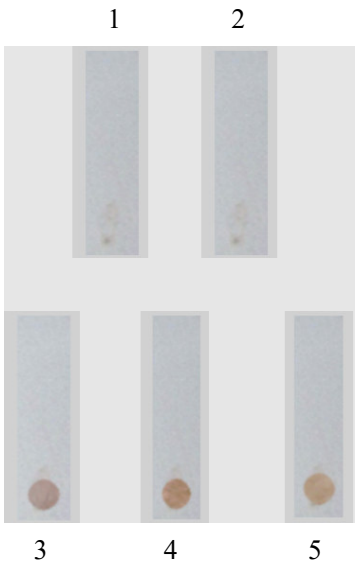


Fig. 5: Latex agglutination test



1: Control negative (with PBS)
2: Vaccinate serum
3, 4, 5: Representative positive samples

Fig. 6: IgM dot enzyme linked immunosorbent assay

5. DISCUSSION

Leptospirosis being an important zoonotic disease of global distribution requires serious attention of the researchers for achieving its effective diagnosis. Leptospirosis affects many organ systems, often making it indistinguishable from that of other febrile illnesses causing meningitis or hepatitis. Therefore, rapid and appropriate diagnostic tests are needed to aid clinical case identification and to facilitate rapid outbreak investigations, for adopting optimal treatment and timely prophylaxis.

The conventional methods of diagnosis like isolation and molecular techniques like PCR although available, are either highly time consuming or can be performed only in well equipped laboratories (Levett, 2003). Laboratory confirmation of leptospirosis relies mainly on serological assays aimed at detection of specific antibodies in serum samples. In leptospirosis, antibodies usually appear within three to seven days after infection and persist in detectable quantities for many months (Faine, 1982).

The MAT is considered the standard serological test which is serovar-specific and provides useful epidemiological data in the form of presumptive serovars. However, this assay is not suitable for routine laboratories since it is technically demanding, costly and requires the maintenance of live, hazardous stock cultures. A confirmatory diagnosis can be made with MAT by showing the seroconversion of a four-fold increase in titer with paired serum samples collected at two to three week intervals, which delays diagnosis and render it unfit for acute case management. The procedure is time consuming and delays the time for initiating proper treatment and control measures. Moreover, MAT being a serovar-specific test, can detect only the specific antibodies and may not be of true diagnostic value.

Ideally, a diagnostic test should be easy to perform, rapid and employ only a single specimen from the clinical case presented (Silva *et al*., 1997). Many genus specific, potentially useful tests like ELISAs and LAT have been proposed. Earlier,

whole cell moieties were employed as antigens, which were observed to give non-specific reactions (Matsuo *et al*., 2000 a, b). Later, OMPs were successfully employed in various diagnostic assays, with high specificity and sensitivity and were proved to be promising antigens (Singh *et al.,* 2004; Srivastava *et al.,* 2006; Sharma *et al.,* 2007).

Latex agglutination test was tried as a useful diagnostic approach, but failed to differentiate the acute carriers of the infection from among chronic cases and vaccinates. Moreover, the interpretation of LAT is often complicated by the presence of antibodies due to regular vaccination or prior infection, especially in an endemic region.

Most of the tests for the detection of acute leptospirosis are aimed at the detection of leptospiral-IgM antibodies, which are detectable from about two to five days after infection (Silva *et al*., 1995; Winslow *et al.,* 1997). Many ELISAs for detection of leptospiral IgM antibodies have been tested and proved to be promising for effective diagnosis (Adler *et al.,* 1980). However, the requirement of technical expertise and costly equipment in ELISAs, make them unfit for routine field level diagnosis. Therefore, recently the research on leptospiral diagnosis was focused on evolving of cost effective, rapid, sensitive, preferably genus specific serological tests, which could diagnose acute infection. The efficiency of IgM dot ELISA, which could serve as a suitable alternative serological assay to diagnose acute canine leptospirosis was evaluated in the present study.

A seroprevalence study was made in and around Thrissur, screening canine serum samples with MAT. The stock cultures were maintained in EMJH medium with different supplements. The best growth was observed with hemolysed rabbit serum. Readymade supplement, albumin supplement and reconstituted bovine serum required longer time for achieving optimum growth. *Leptospira* was observed to grow well with reconstituted bovine serum, which was comparatively easy to procure and cheap. Therefore, hemolysed rabbit serum was used for regular maintenance of cultures and reconstituted bovine serum was used in bulk cultures.

The most predominant serovar in the region was found to be Australis, followed by Pomona, Grippotyphosa, Icterohaemorrhagiae, Autumnalis, Javanica,

Pyrogenes, Canicola and Patoc. The serovar Australis was reported to be the emerging serovar, especially in southern states of India (Koteeswaran, 2006; Balakrishnan *et al*., 2008). These results were similar to the observations made in Thrissur, by Sariprabha (2010).

The OMP extracted from *Leptospira interrogans* serovar Australis strain Ballico was used in this study. The OMP was analyzed by SDS-PAGE to reveal bands located at 21, 32, 36, 41 and 66 kDa in the SI portion of the OMP and at 29, 41 and 66 kDa in the SS portion of the OMP. Similar banding pattern was observed by Joseph (2007).

The sensitivity and specificity of the OMP were checked by IgM immunoblotting with serum from known cases of acute canine leptospirosis and negative sera, respectively. Leptospiral OMPs of molecular weight 36, 32 and 21 kDa were found to be the major reactors, while those of 66 and 41 kDa were observed to be the minor reactors. Similar findings were reported by Doungchawee *et al.* (2008) with IgM immunoblot studies in humans.

Latex agglutination test is an extremely simple and inexpensive diagnostic test which requires no sophisticated equipment and specific expertise. Latex agglutination test was tested to show a sensitivity, specificity and accuracy of 94.19 per cent, 82.14 per cent and 91.23 per cent, respectively as compared to MAT. Hence, LAT could prove useful as a convenient screening test, in agreement with the findings of Ramadass *et al.* (1999), Dey *et al.* (2007) and Senthilkumar *et al.* (2007). Five MAT negative samples were diagnosed as positive by LAT, which could be explained by the fact that LAT is genus specific and suggests the possibility of presence of more infecting serovars prevalent in the region other than the nine serovars employed in the study.

A titer of 1:100 and above is considered as positive in MAT according to OIE recommendations. However, in an endemic region like Kerala, where vaccination is routinely practiced, this titer has to be increased to confirm the presence of infection (Ooteman *et al.,* 2006). So, LAT could give false positives, if the MAT titer of 1:100 is considered as positive for infection. Moreover, LAT could not differentiate antibodies of infection from those of vaccination and prior infection.

Therefore, to circumvent these problems, an IgM dot ELISA was devised with an aim of detecting the acute cases of canine leptospirosis in an endemic region. A total of 13 cases were diagnosed positive with IgM dot ELISA, out of 81 cases which were regarded as positive by both MAT and LAT, showing a prevalence of 16.04 per cent. Serum samples from 22 healthy vaccinated animals and 28 healthy unvaccinated animals, gave negative results with IgM dot ELISA, showing the high specificity of the test. Additionally, 8 cases could be traced back from among 13 cases which were diagnosed as positive by IgM dot ELISA to collect the sera at convalescent stage *i.e.,* after an interval of 14 - 21 days. The paired sera were tested with MAT to observe a four-fold increase in the titer, confirming the presence of infection in these cases and thereby, sensitivity of the test. Microscopic agglutination test was performed with convalescent sera from fifteen randomly collected cases which were negative by IgM dot ELISA but positive with both, MAT and LAT. None of these showed an increase in the MAT titer, confirming the absence of infection. These observations were supported by the findings of Tansuphasiri *et al*. (2005), Sharma *et al.* (2007) and Shekatkar *et al*. (2010).

The simple and convenient form of ELISA, IgM dot ELISA reported in the study would thus be of great value in finding the acute reactors, especially in an endemic region or during outbreaks. The test is also rapid and economical, not requiring any special equipment or technical expertise, making it suitable as a field level test. In future, research has to be carried out to identify the immunogens expressed in the acute phase of infection, so as to produce recombinant antigens to evolve better diagnostics for acute canine leptospirosis.

6. SUMMARY

A total of 114 canine serum samples collected from clinical cases during the period of September, 2009 to January, 2011, presented at University Veterinary Hospitals at Mannuthy and Kokkalai, Thrissur were employed in the study. The samples included those collected from 22 healthy vaccinated animals and 28 healthy unvaccinated animals. The rest of the samples were taken from cases suspected for acute leptospirosis.

The serum samples were screened with MAT using the most prevalent serovars in the region. Eighty six samples which included 22 healthy vaccinated animals were positive with MAT. The serum samples from 28 healthy unvaccinated animals were negative with MAT. The most predominant serovar in the region was found to be Australis followed by Pomona, Grippotyphosa, Icterohaemorrhagiae, Autumnalis, Javanica, Pyrogenes, Canicola and Patoc.

The leptospiral OMP, which was proved to a promising antigen in many diagnostic assays, was employed as the antigen in the study. Outer membrane protein of *Leptospira interrogans* serovar Australis was extracted and analyzed by SDS-PAGE. The sensitivity and specificity of the OMP were checked by IgM-immunoblotting with serum from known cases of acute canine leptospirosis and negative sera, respectively. Latex agglutination test was used as a screening test and compared with the results of MAT.

Among 114 serum samples screened with MAT, 86 were positive cases out of which 81 gave positive reaction with LAT. Among the 28 cases shown as negative by MAT, 23 showed a negative reaction with LAT. Latex agglutination test used in the study showed a sensitivity, specificity and accuracy of 94.19 per cent, 82.14 per cent and 91.23 per cent, respectively with MAT and so could prove useful as a convenient screening test for leptospirosis.

Among 81 cases diagnosed as positive by both, MAT and LAT, 13 samples showed positive results with IgM dot ELISA, showing a prevalence of 16.04 per cent.

The serum samples from the healthy vaccinated animals and the healthy unvaccinated animals were shown as negative on being tested with IgM dot ELISA, proving the specificity of the test. Convalescent sera, collected from 8 cases among the 13 positive reactors of IgM dot ELISA were screened with MAT to show a four-fold increase in the MAT titers, proving the sensitivity of the test. There was no observable increase in MAT titers for the serum collected from among negative reactors of the test.

The IgM dot ELISA reported in the study would be of great value in diagnosing acute cases of canine leptospirosis. The test being rapid, economical and not requiring any special equipment or technical expertise, make it a reliable field level test.

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EVALUATION OF OUTER MEMBRANE PROTEIN BASED IgM DOT ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE RAPID DIAGNOSIS OF CANINE LEPTOSPIROSIS

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ABSTRACT

Leptospirosis is an important public health threat in many places of south India, including Kerala. Diagnosis of leptospirosis is mostly dependent on serological methods. The undercurrent antibodies caused due to prior infection or vaccination, complicate the confirmation of disease, especially in an endemic region. Therefore, tests based on detecting leptospiral IgM antibodies can serve as a useful approach in diagnosing acute canine leptospirosis. The potential of IgM dot ELISA for diagnosis of acute canine leptospirosis was evaluated in this study.

A total of 114 canine serum samples collected from clinical cases during the period of September, 2009 to January, 2011, presented at University Veterinary Hospitals at Mannuthy and Kokkalai, Thrissur were employed in the study. The samples included those collected from 22 healthy vaccinated animals and 28 healthy unvaccinated animals. The rest of the samples were taken from cases suspected for acute leptospirosis.

Among 114 samples screened with MAT, 86 were positive cases, out of which 81 gave positive reaction with LAT. Among the 28 cases shown as negative by MAT, 23 showed a negative reaction with LAT.

Among 81 cases diagnosed as positive by both, MAT and LAT, 13 samples showed positive results with IgM dot ELISA, showing a prevalence of 16.04 per cent. The serum samples from the healthy vaccinated animals and the healthy unvaccinated animals were shown as negative, when tested with IgM dot ELISA, proving the specificity of the test. Convalescent sera, collected from 8 cases among the 13 positive reactors of IgM dot ELISA when screened with MAT showed a four-fold increase in MAT titers, proving the sensitivity of the test.

Latex agglutination test used in the study could prove useful as a convenient screening test for leptospirosis, under field conditions. However, LAT failed to differentiate between acute and chronic carriers of the infection. The OMP based IgM dot ELISA reported in the study would thus be of great value in diagnosing acute cases of canine leptospirosis. The test being rapid and economical, not requiring any special equipment or technical expertise, makes it suitable as a field level test.