Indian Journal of Animal Sciences **79** (11): 1089–1094, November 2009

Comparative studies on seroepidemiology of canine leptospirosis by micro agglutination test (MAT) and recombinant Lip L32 ELISA

ATUL KUMAR[1], D K SINHA[2], P CHAUDHURY[3], HARI SHANKAR[4] and S K SRIVASTAVA[5]

Indian Veterinary Research Institute, Izatangar, Uttar Pradesh 243 122 India

Received: 5 February 2009; Accepted: 15 June 2009

ABSTRACT

Dogs (806) of 17 breeds were examined serologically by microscopic agglutination test (MAT) for the presence of different serovars of *Leptospira* organism. An overall seroprevalence of 7.07% was observed by MAT. Serovar Canicola was the predominant followed by lcterohaemorrhagiae, Australis, Autumnalis, Tarassovi, Ballum and others. Male dogs were more sero positive (8.31%) than females (5.41%). The highest seropositivity was observed in 3–5 year age group and lowest in under 1 year of age. Breed-wise more number of positive samples were from large sized breed of dogs like Labrador, German shepherd and Doberman. However, Spitz had maximum (26.31%) seroprevalence among all breeds. Sera samples collected from Chennai, Tamil Nadu showed highest seroprevalence (15.21%) with maximum number of serovars (28).

The efficacy of recombinant leptospiral lipoprotein 32 (rLipL32) as an antigen was evaluated using enzyme-linked immunosorbent assay (ELISA) for serodiagnosis of canine leptospirosis. A seropositivity of 10.29% was observed. The relative sensitivity and specificity of ELISA as compared to MAT, was calculated as 100 and 96.52%, respectively. The concordance percentage between MAT and ELISA was 96.77, and the kappa statistics showed a substantial amount of agreement between MAT and ELISA. Results suggested that rLipL32 protein antigen based ELISA could be used as a serodiagnostic test for serodiagnosis of canine leptospirosis.

Key words: Canine, Leptospirosis, MAT, Recombinant lipoprotein 32 ELISA (rLipL 32 ELISA)

Leptospirosis has been recorded in dogs throughout the major continents of the world (Ribotta *et al*. 2000a, Rad *et al*. 2001, Scanziani *et al*. 2002, O'Keefe *et al.* 2002, Ward 2002, Srivastava and Kumar 2003, Batista *et al.* 2004, Adesiyun *et al*. 2006). The disease is mainly caused by *L. interrogans* serovar Canicola and *L. interrogans* serovar Icterohaemorrhagiae. However, infection with many other serovars has also been reported (Kallin *et al*. 1999, Prescott *et al*. 1999, Batista *et al*. 2004). Various factors like suitability of environment for the survival of the organism and behavioral habits, activity and other host characteristics can be the determinants of incidence and prevalence of the disease.

The microscopic agglutination test (MAT) remains the reference method for serodiagnosis of leptospirosis but the cumbersome technique and handling of live culture precludes its usefulness in most clinical settings. The whole-cell or purified antigen preparations used in ELISA possess broadly reactive immunodominant epitopes present in pathogenic as well as non-pathogenic leptospires. Thus the disease status in animals is difficult to ascertain. These difficulties led to the identification of protein antigen useful for diagnosis of leptospirosis, which is present across all the *Leptospira* serovars. Such important outer membrane proteins of *Leptospira* cells are LipL32, LipL41 and ompL1. Recently, recombinant antigens based seroprevalence studies of leptospirosis in human as well as animals using different outer membrane proteins have been reported (Flannery *et al*. 2001, Dey *et al*. 2004, Mariya *et al*. 2006). The present paper deals with a study on seroepidemiology of canine leptospirosis using MAT and recombinant LipL32 protein based ELISA (rLipL32 ELISA).

Present address: [1]Assistant Professor, Department of Veterinary Epidemiology and Preventive Medicine, Dr. G.C. Negi College of Veterinary and Animal Sciences, C S K HPKV, Palampur, Himachal Pradesh 176 062.

[2]Senior Scientist (e mail: sinhaivri@rediffmail.com), [4]Principal Scientist and In-Charge, Epidemiology Section, CADRAD; [3]Senior Scientist, [5]Scientist Emeritus, Division of Bacteriology and Mycology.

MATERIALS AND METHODS

Canine serum samples: The blood samples were collected aseptically from dogs brought to Referral Veterinary

Polyclinic, IVRI, Izatnagar, Veterinary Polyclinic, Madras Veterinary College, Chennai and dog stationed at 3 centres of Paramilitary forces. Sera samples of stray dogs were also collected from various locations in Delhi with the help of a Non-Government Organization (NGO). All the necessary information was collected from dog owners except the dogs of NGO, whose breed and sex were only known.

Sera were separated, kept in sterile microcentrifuge tubes after adding 0.01% sodium azide. The sera samples were stored at –20°C till use.

Micro agglutination test (*MAT*)*:* MAT was performed to assess the antibody titre in canine sera samples using various *Leptospira* serovars as per the method described by Cole *et al*. (1973). A total of 12 *Leptospira* serovars, viz. Canicola, lcterohaemorrhagiae, Pyrogenes, Australis, Grippotyphosa, Ballum, Habdomadis, Hardjo, Javanica, Pomona and Tarassovi were used as antigen. A 5-day-old leptospiral culture containing about 2–4 $\times 10^8$ cells/ml maintained at 28–30°C in liquid EMJH medium by routine subculture at 7–10 days interval was used as an antigen. The antibody titre was the highest dilution of the serum showing agglutination of atleast 50% *Leptospira* cells. MAT titre equals to or higher than 1:100 was considered positive except for serovars Canicola and Icterohaemorrhagiae in dogs which were vaccinated 10 days ago and within 1 year of sampling. In such cases titres of 1:800 or more for Canicola and Icterohaemorrhagiae were considered positive (Scanziani *et al*. 2002).

Production of recombinant LipL32 antigen: *L. interrogans* serovar Canicola was used for the production of recombinant LipL32 antigen protein following the strategy described by Mariya *et al*. (2006) for LipL 41 protein. The expression clone (*Escherichia coli* DH5 α cells) was maintained in the *Leptospira* Laboratory, Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, India. The polyhistidine (6X-His) tagged fusion protein was purified under denaturing conditions by nickel chelating affinity chromatography. Prior to use as antigen the protein was dialyzed in buffer and protein was estimated using Lowry's method.

Indirect enzyme-linked immunosobent assay (*I-ELISA*): The ELISA was done (Flannery *et al.* 2001) by using a recombinant LipL 32 antigen, with slight modifications. The optimum concentration of the antigen for coating the plates as well as serum was attained by checkerboard titration.

The cut off value for positive sample was taken as two standard deviation (SD) more than the known negative sample OD value.

Evaluation of MAT and ELISA

Relative sensitivity, specificity and accuracy: The relative sensitivity, specificity and accuracy of the recombinant LipL32 antigen based ELISA for serodiagnosis of canine leptospirosis were evaluated (Mc Diarmid and Hellstrom 1987) in comparison to the MAT.

Predictive value of positive and negative test results was calculated as per Thrusfield (1995), while concordance percentage was calculated as per Perrin and Sureau (1987).

Kappa statistics: The agreement between the tests was evaluated by applying Kappa statistic (Thrusfield 1995). Kappa values range from 0–1. Arbitrary benchmarks for evaluation observed Kappa values are >0.81=almost perfect agreement; 0.61–0.80=substantial agreement; 0.41–0.60 =moderate agreement; 0.21–0.40=fair agreement; 0.00–0.20= slight agreement and 0.00=poor agreement.

RESULTS AND DISCUSSION

The seroprevalence of canine leptospirosis has been reported throughout the world. Regarding its seroprevalence in India, precise and systemic information is meager. Keeping in view the practical limitations of isolation and identification of leptospires for the diagnosis and surveillance of canine leptospirosis, demonstration of antibodies in the serum samples by MAT and rLipL32 ELISA were resorted. MAT provides serological evidence of leptospiral infection against commonly encountered serovars.

Table 1. Source-wise seroprevalence of canine leptospirosis

Source		Samples tested	Samples positive (%)	
			MAT	rLipL32 ELISA
Ref. Vet. Polyclinic, IVRI, Izatnagar		443	14 (03.16)	20 (04.51)
Vet. Polyclinic, Madras Vet. College, Chennai		184	28 (15.21)	44 (23.91)
Dogs of Paramilitary Forces	A	63	02 (03.17)	02 (03.17)
	B	53	07(13.20)	09 (16.98)
	C	21	02(09.52)	04 (19.04)
NGO, Delhi		42	04 (09.52)	04 (09.52)
Total		806	57 (07.07)	83 (10.29)

Table 2. Prevalence of antibodies in dog serum samples against different serovars of *Leptospira* spp. in MAT

Serivar	Samples positive	Percentage (%)
Canicola	18	31.58
Icterohaemorrhagiae	12	21.05
Australis	08	14.04
Autumnalis	07	12.28
Tarassovi	03	05.26
Ballum	02	03.51
Pyrogenes	02	03.51
Pomona	02	03.51
Hebdomadis	02	03.51
Grippotyphosa	01	01.75
Hardjo	00	00.00
Javanica	00	00.00
Total	57	100.00

Table 3. Source-wise seroprevalence of different serovars of *Leptospira* spp

Source of canine sera sample		Serovar (No.)	Total
Ref. Vet. Polyclinic, IVRI, Izatnagar		Australis (6), Icterohaemorrhagiae (3), Autumnalis (2), Canicola (1), Grippotyphosa (1), Pomona (1)	14
Vet. Polyclinic, Madras Vet. College, Chennai		Canicola (12), Icterohaemorrhagiae (6), Autumnalis (4), Ballum (2), Pomona (1), Pyrogenes (1), Australis (1), Tarassovi (1)	28
Dogs of Paramilitary Forces	A	Icterohaemorrhagiae (1), Autumnalis (1)	2
	B	Canicola (2), Hebdomadis (2), Tarrasovi (1) Australis (1), Icterrohaemorrhagia (1)	7
	C	Canicola (1), Icterohaemorrhagiae (1)	2
NGO, Delhi		Canicola (2), Pyrogenes (1), Tarassovi (1)	5

Dog serum samples (806) were tested, out of which 57 and 83 samples showed a positive reaction as detected by MAT and rLipL32 ELISA giving an overall seroprevalence of 7.07% and 10.29%, respectively (Table 1). This is in agreement with findings of Venkataraman and Nendunchelliyan (1993), however, Srivastava and Kumar (2003) reported a higher seroprevalence rate of leptospirosis in dogs. The *Leptospira* serovars used in MAT were those, which are commonly associated with seroconversion in animals and human (Srivastava and Kumar 2003, Singh 2003).

The screening of serum samples (Table 2) showed the maximum 18 (31.57%) canine sera samples reacted with serovar Canicola followed by Icterohaemorrhagiae 12 (21.05%) and Australis 8 (14.03%). This finding is in agreement with Verma (1982) who worked on canine leptospirosis in Punjab. Antibodies against serovar Ballum was observed probably for the first time in dog serum sample collected from Chennai. Serum samples (137) were collected from kenneled dogs of Paramilitary forces. All of them were vaccinated against leptospirosis. It is of interest that the infecting serovars found in those serum samples were Hebdomadis, Autumnalis, Tarassovi and Australis along with commonly infecting serovar Canicola and lcterohaemorrhagiae, which are contained in commercially available vaccines. The present study suggests that canine leptospirosis is more prevalent than previously thought and infection by other serovars has left vaccinated dog at risk because immunity is serovar specific. Therefore, information on the prevalence of various serovar in different geographical location would help in the selecting particular serovar for use in vaccine for that region.

A serological response, where more than one serovar give a positive reaction in MAT, is called stereotypic serological response. This was observed in the present study as 7 sera samples gave positive reaction to more than 1 serovar (6 sera samples to 2 serovars and 1 serum sample to 3 serovars). The occurrence of multiple titres in positive sera is a common phenomenon, which was reported by many workers in India (Rajasekhar and Nanjiah 1971, Verma 1982) and abroad (Rentko *et al*. 1992, Scanziani *et al*. 1994, Brown *et al*. 1996). This phenomenon is considered a limitation of serology, and is most often assumed to be caused by antigenic similarities between different serovars (Brown *et al*. 1996). However, in the present study it was accepted that in sera samples with multiple serovar, the highest titre is induced by infecting serovar as suggested by Rentko *et al.* (1992) and Bolin (1996).

The humidity, rainfall, temperature, soil condition like alkaline pH etc. play role in maintenance of *Leptospira* infection. These conditions are more prevalent in southern part of our country that could explain the endemicity of canine leptospirosis in Tamil Nadu, which was reflected in present study also as the maximum seroprevalence of leptospirosis by MAT (15.21%) with highest number of serovars (28) were observed in dog serum samples collected from Chennai, Tamil Nadu (Table 3).

The importance of stray dogs as a source of human infection is well recognized (Torten *et al*. 1971). However, pet dogs were also found to be significant risk factors for leptospirosis as observed in present investigation, which is in concordance with the findings of Douglin *et al*. (1997). Many workers (Adin and Cowgill 2000, Ward *et al*. 2002) had reported that large, sporty, hound, or working male dogs as predominant in cases of canine leptospirosis. However, our study revealed that the canine leptospirosis is not only

Table 4. Breed-wise seroprevalence of canine leptospirosis

Breed	Samples tested	Samples positive (%)	
		MAT	rLipL32 ELISA
Non Descript	279	16 (05.73)	27 (09.67)
German Shepherd	167	09 (05.38)	10 (05.98)
Labrador	149	18 (12.08)	27 (18.12)
Pomeranian	79	03 (03.79)	05 (06.32)
Doberman	59	05 (08.47)	05 (08.47)
Great Dane	20	01 (05.00)	02 (10.00)
Spitz	19	05 (26.31)	06 (31.57)
Others*	34	00 (00.00)	01(02.94)

*Others include Dalmatian (04), Boxer (11), Lhasa Apso (02), Cocker Spanial (01), Rampur Hond (01), Golden Retreiver (02), Bhutia (06), Daschound (05), Lion Bedger (01) and Rottweiler (01).

restricted to active, sporty types of dogs but also in small size dogs like spitz, as the maximum seroprevalence (26.31%) of leptospirosis was found among all breeds (Table 4). It might be also due to small sample size but it certainly indicate that spitz and other smaller breeds also play important role in its transmission as reported by Prescott *et al*. (2002) also.

The age-wise distribution of leptospirosis observed in this study (Table 5) indicated that it is a disease of occupationally active age group, i.e. young to middle age adults. Out of 806 canine sera samples, 764 sample's age status was known, which were distributed in 6 age groups. The lowest seroprevalence was found in dogs of less than 1 year and the highest in 3–5 years of age group, which is in concordance with the finding of other workers (Kaveri 1980, Verma 1982) who reported that infection is not usually acquired until dogs reach maturity, as then they have more exposure to environment and other factors related to leptospirosis.

Leptospira infection was more common in males than in females as reported by different workers (Venkataraman and Nendunchelliyan 1993, Scanziani *et al*. 2002). A higher prevalence in males (8.31%) than in females (5.44%) by MAT was observed in present study also, which is due to the habit of sniffing genitals and licking recently voided urine by male (Dunbar 1979).

Table 5.Age-wise seroprevalence of canine leptospirosis

age (year)	Samples tested	Samples positive (%)	
		MAT	rLipL32 ELISA
<1	90	01 (01.11)	04 (04.44)
1–3	247	15 (06.07)	23 (09.31)
3–5	193	21 (10.88)	26 (13.47)
5–7	104	06 (05.76)	13 (12.50)
7–9	78	06 (07.69)	07 (08.97)
>9	52	04 (07.69)	06 (11.53)
Total	764*	53*	79*

*Age status of 42 stray dogs from NGO, Delhi was not known; *4 stray dogs (non-descript; 2 males+2 females) were found positive by MAT and ELISA, whose age status was not known.

Table 6. Comparison of rLipL32 ELISA to MAT

ELISA	MAT		Total
	Positive	Negative	
Positive	57	26	83
Negative	0	723	723
Total	57	749	806

*Sensitivity, 100%; specificity, 96.52%; accuracy, 98.01%; predictive value of positive test result, 68.67%; negative test result, 100%.

The specificity, sensitivity and accuracy of rLipL32 ELISA relative to MAT was calculated to be 96.52%, 100% and 98.01%, respectively (Table 6), which is in agreement with Dey *et al*. (2004) who also found relative specificity and sensitivity of 97.30 and 96.90%, respectively, for diagnosing canine leptospirosis by using MAT and rLipL32 based ELISA. The ELISA had a sensitivity of 100% relative to MAT, suggesting that it was as effective as MAT in detecting true positive animals. Of the 749 MAT negative sera tested, 26 were found positive by ELISA. This could be explained on the basis that all those sera turned out to be MAT negative may not actually be negative as only 12 *Leptospira* serovars were used as antigens for antibody detection. It may be likely that these sera contained non agglutinating leptospira antibodies that were detectable by ELISA but not by MAT, which can only detect agglutinating antibodies (Ribotta *et al*. 2000b, Agunloye *et al*. 2001). Since ELISA can detect both agglutinating and non-agglutinating antibodies, it showed a higher sensitivity than MAT as also reported by other workers (Trueba *et al*. 1990, Surujballi *et al*. 1995).

The predictive value of positive test for ELISA (Table 8) signifies that there is 68.67% probability that a dog serum positive to rLipL32 ELISA is actually diseased. Predictive value of negative test was 100% that signifies that there is 100% probability that a dog serum negative to ELISA is actually healthy, i.e. leptospirosis free, thus signifying its use as a screcning test. A concordance of 96.77% between rLipL32 ELISA and MAT signifies that ELISA can replace MAT effectively particularly in laboratories where MAT is difficult to perform.

For further evaluation of tests, kappa statistics was applied. The kappa value takes into account the observed and expected proportions of agreement and it will give a true picture of agreement existing between the tests. A kappa value of 0.79 was found which denotes a substantial amount of agreement between MAT and rLipL32 ELISA. The results showed that rLipL32 ELISA was able to detect all the samples that came positive by MAT in this study.

The advantage of using recombinant LipL32 antigen in ELISA are that bulk amount of the purified antigen can be obtained without handling the live leptospiral organisms. Secondly, it can detect genus specific antibodies with absolute analytical specificity. The sensitivity, specificity and reproducibility of recombinant LipL32 antigen based ELISA, therefore, has edge over other serodiagnostic techniques. The conserved nature of highly immmunodominant LipL32 protein among more than 200 pathogenic serovars of *Leptospira* (Haake *et al.* 2000) suggests that rLipL32 based ELISA could be a potential alternative technique for the serodiagnosis of canine leptospirosis and may be efficiently utilized as a screening test for large numbers of serum samples for the detection of leptospiral antibodies in dogs.

The findings of present study advocates more careful

analysis of clinical signs suggestive of leptospirosis and must be confirmed by rLipL32 based ELISA followed by MAT. In long term such effort would be fruitful in knowing the extent of leptospirosis problem and to adopt suitable measures for it control.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, IVRI, the Joint Director, CADRAD and the Head, Division of Bacteriology and Mycology for providing necessary facilities for conducting the study.

REFERENCES

Adesiyun A A, Hull-Jackson C, Mootoo N, Halsall S, Bennett R, Clarke N R, Whittington C U and Seepersadsingh N. 2006. Sero-epidemiology of Canine leptospirosis in Trinidad: Serovars. Implications for Vaccination and Public Health. *Journal of Veterinary Medicine Series B.* **53**(2): 91

Adin C A and Cowgill L D. 2000. Treatment and outcome of dogs with leptospirosis: 36 cases (1990–1998). *Journal of American Veterinary Medical Association* **216**: 371–75.

Agunloye C A, Alabi F O, Odemuyiwa S O and Olaleye O D. 2001. Leptospirosis in Nigerians: a seroepidemiological survey. *Indian Veterinary Journal* **78**(5): 371–75.

Batista C D A, Azvedo S S and Alves C J. 2004. Seroprevalence of leptospirosis in stray dogs from Patos city, state of Paraiba, Brazil. *Brazilian Veterinary Research in Animal Science* **41**(2): 131–36.

Bolin C A. 1996. Diagnosis of leptospirosis: a reemerging disease of companion animals. *Sem Veterinary Medicine and Surgery* (*Small Animal*) **11**: 166–71.

Brown C A, Roberts A W, Miller M A, Davis D A, Brown S A Bolin C A, Jarecki-Black J, Greene C E and Miller-Liebl D. 1996. *Leptospira interogans* serovar Grippotyphosa infection in dogs. *Journal of American Veterinary Medical Association* **209**: 1265–67.

Cole J R, Sulzer C R and Pursell A R. 1973. Improved microtechnique for the leptospiral mieroscopic agglutination test. *Applied Microbiology* **25**: 977–80.

Dey S, Mohan C M, Kumar S Ramdass P, Nainar A M and Nachimuthu, K. 2004. Recombinant LipL 32 antigen-based single serum dilution ELISA for the detection canine leptospirosis. *Veterinary Microbiology* **103**: 99–06.

Douglin C P, Jordan C, Rock R, Hurley A and Levett P N. 1977. Risk factors for severe leptospirosis in the pariah of St. Andrew, Barbados. *Emerging Infectious Disease* **3**: 78–80.

Dunbar I. 1979. *Dog behaviour.* T.F.H. Publications. Neptune. 105.

Flannery B, Costa D, Carvalho F P, Guerreiro H, Matsunagar J, Da Silva E D, Ferreira A G, Riley L W, Reis M G, Haake D A and Ko A I: 2001. Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. *Journal of Clinical Microbiology* **39** (9): 3303–10.

Hakke D A, Chao G, Zuerner R L, Barnett J K Barnett D Mazel M Matsunaga J Levett P N and Bolin C A. 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infection and Immunity* **68**(4): 2267–85.

Kallin M, Devaux C, DiFruscia R, Lemay S and Higgins R. 1999. Three cases of canine leptospirosis in Quebec. *Canadian Veterinary Journal* **40**: 187–91.

Kaveri S V. 1980. 'Comparative studies on the diagnosis of canine leptospirosis by MAT and IPT.' M.V.Sc. Thesis, Submitted to University of Agricultural Science, Bangalore, India.

Mariya R, Chaudhary P, Kumar A A, Thangapandian E, Amutha R and Srivastava S K. 2006. Evaluation of a recombinant LipL 41 antigen of Leptospira interrogans serovar Canicola in ELISA for serodiagnoisis of bovine leptospirosis. *Comparative Immunology, Microbiology and Infectious Diseases* **26**: 269–7 7.

Mc Diarmid S C and Hellstrom J S. 1987. An intradermal test for the diagnosis of brucellosis in extensively managed cattle herds. *Preventive Veterinary Medicine* **4**: 361–69.

O'Keefe J S, Jenner J A , Sandifer NC, Antony A and Williamson N B. 2002. A serourvey for antibodies to *Leptospira* on dogs in the lower North Island of New Zealand. *New Zealand Veterinary Journal* **50**(1): 23–25.

Perrin P and Sureau P. 1987. A collaborative study of experimental kit for rapid rabies enzyme immunodiagnosis (RRIED). *Bullettin of the WHO* **65**: 489–93.

Prescott J F, Key D and Osuch M. 1999. Leptospirosis in dogs. *Canadian Veterinary Journal* **40**: 430–31.

Prescott J F, McEwen B, Taylor J, Woods J P, Abrams-Ogg A and Wilcock B. 2002. Resurgence of leptospirosis in dogs in Ontario: recent findings. *Canadian Veterinary Journal* **43**(12): 955–61.

Rad M A, Zeinali A, Yousofi J V and Tabatabai A H. 2001. Seroepidemiologic study of canine leptospirosis in Tehran, Iran. *World Small Animal Veterinary Association World Congress.* Vancouver.

Rajasekhbar M and Nanjiah R D. 1971. Animal leptospirosis in Mysore State. A serological study. *Indian Veterinary Journal* **48**: 1087–98.

Rentko V T, Clark N, Ross L A and Schelling S H. 1992. Canine leptospirosis: A retrospective study of 17 cases. *Journal of Veterinary Internal Medicine* **6**: 235–44.

Ribotta M J, Higgins R, Gottschalk M and Lallier R. 2000b. Development of an indirect enzyme-linked immunosorbent assay for the detection of leptospiral antibodies in dogs. *Canadian Journal of Veterinary Research* **64**(1): 32–37.

Scanziani E, Calcaterra S, Tagliabue S Luini M, Giusti A M and Tomba M. 1994. Serological findings in cases of acute leptospirosis in dog. *Journal of Small Animal Practices* **35**: 257–60.

Scanziani E, Origgi F, Giusti A M, Lacchia G, Vasino A, Piroano G, Scarpa P and Tagliabue S. 2002. Serological survey of leptospiral infection in kenneled dogs in Italy. *Journal of Small Animal Practices* **43**(4):154.

Singh A. 2003. 'Characterisation of outer membrane lipoprotein of *Leptospira borgpetersenii* serovar *Hardjo* type Bovis.' M.V.Sc. Thesis, Deemed University, IVRI, Izatnagar, India.

Srivastava S K and Kumar A A. 2003. Seroprevalance of leptospirosis in animals and human beings in various regions of the country. *Indian Journal of Comparative Microbiology, Immunolology and Infectious Diseases* **24**(2):155–59.

Surujballi O, Henning D, Marenger R and Howlett C. 1955. Development of monoclonal antibnody based competitive enzyme linked immunosorbent assay for the detection of

Leptospira interrogans serovar Hardjo type Hardjobovis antibodies in bovine sera. *Canadian Journal of Veterinary Research* **61**: 267–29.

Thrusfield M. 1995. *Veterinary Epidemiology.* 2nd edn. Blackwell Science Ltd., Oxford.

Torten M, Kaplun A and Shenberg E. 1971. A survey of leptospirosis in stray dogs. *Refuah. Veterinary* **28**: 98–101.

Trueba G A, Bolin C A and Thoen C O. 1990. Evaluation of enzyme linked immunosorbent assay for the diagnosis of bovine leptospirosis caused by *Leptospira interrogans* serovar Hardjo type Hardjobovis. *Journal of Veterinary Diagnostic Investigation* **2**: 323–29.

Venkataraman K S and Nendunchelliyan S. 1993. Seroepidemiology of canine leptospirosis in Madras city. *Indian Journal of Animal Sciences* **63**(2): 150–52.

Verma B B. 1982. Leptospirosis: seroepidemiology in dogs in state of Punjab. *Indian Veterinary Journal* **59**: 169–72.

Ward M P. 2002. Clustering of reported cases of leptospirosis among dogs in the United States and Canada. *Journal of Preventive Veterinary Medicine* **56**: 215–26.

Ward M P, Glickman L T and Guptill L F. 2002. Prevalence of and risk factors for leptospirosis among dogs in the United States and Canada: 677 cases (1997–98). *Journal of American Veterinary Medical Association* **220**: 53–58.