RESEARCH ARTICLE

Seroprevalence and Molecular Detection of Canine Leptospirosis in and around Navsari, South Gujarat, India

Sunayana Kanthala[1], Dharmesh R. Patel[1]*, V. Balamurugan[2], Pushpa M. Makwana[1], Dixit K. Parasana[1], Prahlad S. Chaudhary[3], Irsadullahkhan H. Kalyani[1]

Abstract

Leptospirosis, an anthropozoonotic disease, is a growing global public health concern due to its increasing prevalence in both developing and developed countries caused by serovars of Leptospira. The present study was conducted to estimate the seroprevalence, and associated risk factors of leptospirosis in and around Navsari district (Surat and Valsad) of Southern Gujarat,India . The data was collected through a pretested questionnaire to detect the risk factors associated with disease occurrence. Samples were collected from 410 dogs (410 sera samples, 66 blood and 66 urine samples). Microscopic Agglutination Test (MAT) was conducted to determine the seroprevalence of predominant leptospirosis serovars. Out of 410 sera samples, 45 were found positive with seroprevalence of 10.98%, and serovar Pyrogenes followed by Shermani and Djasiman were found to be predominant. In ImmunoComb dot assay, out of 108 samples 53 (49.07%) were found positive for leptospiral antibodies. Overall seroprevalence was found to be 15.37% with MAT and ImmunoComb dot assay. A total of 6/66 (9.09%) blood and 4/66 (6.06%) urine samples were found positive by PCR using Primers lep1 & lep2 and LipL32 F and R showing amplicon band length at 331 bp and 242 bp, respectively. The overall prevalence of leptospirosis was found to be 16.59% by MAT, ImmunoComb and PCR. The risk factor analysis using a multivariable logistic model revealed that animals' clinical status ($\chi 2=7.7$, $p<0.01$ with odds ratio[OR] 0.45), walking and other activities in the surrounding forest ($\chi 2=11.7$, $p<0.01$ with OR 5.49), sanitation of dog and housing area ($\chi 2=14.04$, $p<0.01$ with OR 5.69) and owners own house/land ($\chi 2=4.42$, $p<0.05$ with OR 2.68), were significantly associated risk factors with the occurrence of the disease. Further investigation must be taken to determine the serovars responsible for occurrence of the disease and its associated risk factors

Key words: Canine leptospirosis, Molecular detection, Seroprevalence, South Gujarat.

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Introduction

Leptospirosis is a growing global public health concern due to its increasing prevalence in developing and developed countries (Ahmad *et al*., 2005; Vijayachari *et al*., 2008; Pratt *et al*., 2017). It is a significant zoonotic disease brought on by pathogenic Leptospira spirochetes, which are distributed worldwide in various hosts. Numerous mammalian species, including feral, farm and pet animals serve as maintenance hosts (natural carriers) for this disease (Levett, 2001), while humans serve as incidental hosts (Ko *et al*., 2009). Domestic animals such as cattle, pigs, goats, sheep, dogs and horses have been infected with the disease. The reservoir hosts, such as rodents, spread the disease either directly or indirectly through contaminated urine in water, feed, and soil (Schneider *et al*., 2015). This disease affects farmers, veterinarians, butchers, abattoir workers, rodent control workers and other vocations that need frequent contact with animals. As a result, Leptospirosis is an occupational threat for them.

Leptospira organism does not multiply outside of host. Their survival is very crucial and depends on the environmental conditions in which they are found. Leptospira are sensitive to drying, high temperature and pH changes can be fatal (Bierque *et al*., 2020). In India, leptospirosis is known to be an endemic illness (Vijayachari *et al*., 2008; Himani *et al*., 2013). The majority of leptospirosis outbreaks are recorded from the coastal areas of West Bengal, Orissa, Kerala, Tamil

[1]Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Navsari, Gujarat, India.

[2]ICAR-NIVEDI, Ramagondanahalli, Post Box No. 6450, Yelahanka, Bengaluru-560064, Karnataka, India

[3]Nandini Veterinary Hospital, Opp. Children Traffic Training Park, Ghod Dod Road, Surat – 395001, Gujarat, India.

Corresponding Author: Dharmesh R. Patel, Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Navsari, Gujarat, India, e-mail: vetdharmesh74@yahoo.com

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Nadu, Karnataka, Maharashtra, Gujarat and the Andaman Islands (Himani *et al*., 2013). In these areas, the highest rates occur during the monsoon season. Canine leptospirosis is more common during the rainy season, when there is a lot of stagnant water and swampy situations. Direct touch, stagnant water, polluted urine, contaminated water, plants, soils, and contaminated food are the most common ways for dogs to become infected. To prevent the illness, vaccines for dogs are available (Desai *et al*., 2020), though vaccinated dogs were typically diagnosed with leptospirosis caused by non-vaccinal serovars (Abhinay *et al*., 2012). Prior to 1960, most clinical cases of canine leptospirosis were thought to be caused by *L. interrogans* serovars *icterohaemorrhagiae* and *canicola,* characterized by acute haemorrhagic diathesis, icterus, or uraemia (Brown *et al*., 1996).

An early diagnosis of the disease allows for the development of a more effective treatment plan and increases the likelihood of recovery. Due to the ambiguity of disease recurrence patterns, disease may go untreated and dog owners may be unaware of vaccination requirements, resulting in a lack of complete immunization coverage (Desai *et al*., 2020). However, there is paucity of research data on prevalence as well as sero-incidence study in dogs in Southern Gujarat. Thus, keeping in view on global impact, paucity of published reports and clinical significance, it was planned to investigate the occurrence of leptospirosis in dogs in and around Navsari district of Southern Gujarat.

Materials and Methods

Selection of Animals

Dogs presented to Veterinary Clinical Complex (VCC), College of Veterinary Science & A.H., Navsari and Nandini Veterinary Hospital, Surat (South Gujarat, India) with clinical history of any one of the clinical signs of leptospirosis mentioned in the questionnaire, and information regarding age, sex, breed of dog, owners' details, locality and presence of rodents etc. were also filled in the proforma.

Collection and Processing of Samples

Blood samples were collected aseptically from cephalic or saphenous vein of dogs in EDTA vials (K_2E Vacuette) and clot activator vials (BD Vacutainer). Blood samples were stored at 2 to 8°C refrigerated temperature till further use. The serum was collected from clot activator vials in screw capped plastic vials and was stored at -20°C temperature till further use. The sera samples were collected from all 410 dogs under study and were subjected to Microscopic Agglutination Test (MAT) and ImmunoComb test at NIVEDI, Bengaluru.

Urine samples were collected with commercially available sterile container (5 mL). Voided midstream urine was collected in container through catheterization or manually from males and females, and transported to laboratory as quickly as possible. Approximately 1 mL of phosphate buffered saline was added to urine sample to maintain alkalinity of the sample. Samples were then stored at -20°C temperature till further use.

Microscopic Agglutination Test (MAT) and ImmunoComb Test

The MAT was performed for the detection of Leptospira agglutinating antibodies as per the procedure of Faine *et al*. (1999) using a battery of 20 reference Leptospira serovars covering 18 serogroups preserved at NIVEDI, Bengaluru, It was performed in doubling dilutions, starting from a dilution of 1 in 25. Positive samples were titrated up to end titres. A MAT titre of ≥ 1:50 was considered as positive reactor to monitor associated epidemiological factors.

The MAT seropositive samples (1:50) of dogs were further tested for ImmunoComb - a modified ELISA - assay to determine the antibody titre to different pathogenic serovars of Leptospira interrogans *i.e., L. icterohaemorrhagiae* (Copenhageni and RGA), *L. canicola, L. pomona* and *L. grippotyphosa*. It was done by following the manufacture's instruction manual.

Molecular Assay

Genomic DNA was extracted from 66 clinical samples (66-each blood and urine) by using DNeasy® Blood & Tissue Kit, Qiagen by following manufacture's protocol. PCR was carried out in a final reaction mixture of 20 µL with 10 µL (2X) Master mix, 5 µL DNA template, 1 µL each forward and reverse primer and 3 µL Nuclease Free Water. The leptospira genus specific (Lep 1/2) and pathogenic primers (LipL32 F/R) were used for the detection of Leptospira (Table 1) (Merien *et al*., 1992; Stoddard *et al*., 2009). The protocol used for the PCR amplification is given in Table 2. Four µL of the PCR product was mixed with 1 µL of 5X gel loading buffer and checked on 2.0% agarose gel containing ethidium bromide along with 100 bp DNA molecular weight marker (Gelipilot® Cat. No. 239045, Qiagen) at constant 80V for 30 min in 0.5X TBE buffer. The amplified product was visualized as a single compact

Table 1: Details of the primers used for conventional PCR

Target gene	Primer sequence 5'-3'	Primer length	Product length (bp)	Reference
16s *rRNA*	**(Lep1)** GGCGGCGCGTCTTAAACATG	20	331 bp	(Merien *et al*., 1992)
(Genus specific)	**(Lep2)** TTCCCCCCATTGAGCAAGATT	21		
LipL32	**(LipL32-F)** AAG CAT TAC CGC TTG TGG TG	20	242 bp	(Stoddard *et al*., 2009)
(Pathogenic gene specific)	**(LipL32-R)** GAA CTC CCA TTT CAG CGA TT	20		

band of expected size under UV light and documented by gel documentation system.

Table 2: Steps and conditions of the thermal cycling for 16s RNA andLipL32 gene PCR

Stage	Temperature	Time	Cycles
Initial denaturation	94°C	5 min	1 cycle
Denaturation	94°C	1 min	
Annealing	57°C	1 min	40 cycles
Extension	72°C	1 min	
Final extension	72°C	10 min	1 cycle

Statistical Analysis

Data generated from laboratory investigations and questionnaire surveys were coded and recorded using Microsoft® Excel 2016. The Chi-square test and odds ratio were calculated using the IBM SPSS Statistics for Windows, version 22 (IBM Corp., Armonk, NY, USA) to determine the associated risk factors. Differences among groups of each factor were considered significant at $p< 0.05$ and highly significant at $p< 0.01$ for all parameters tested.

Results and Discussion

Among 410 sera samples of dogs tested, 45 samples had antibodies against at least one serogroup of Leptospira (MAT Titre ≥ 1:50). Serovars found in our study were Pyrogenes (24.40%), Shermani (22.20%), Djasiman (20.00%), Hurstbridge (17.80%), Canicola (15.60%), Grippotyphosa (15.60%), Panama (13.30%), Ballum (11.10%), Javanica (4.40%), Icterohaemorrhagiae (2.20%), and Lai (2.20%). Seroprevalence of leptospirosis by MAT was found to be 10.98%, whereas Godhani (2021) observed 53.13% seroprevalence in Navsari. Many authors reported a wide range of seroprevalence in different cities of the country with lowest overall prevalence (7.07%) in Izatnagar, Delhi, Paramilitary forces and Chennai (Kumar *et al*., 2009), and highest (75.44%) in Thrissur, Kerala (Abhinay *et al*., 2012). The predominant serovars present in our study were Pyrogens, Shermani and Djasiman, which were in accordance with Patil *et al*. (2014), who reported that Pyrogenes was found to be most prevalent serovar followed by the Icterohaemorrhagiae in Mumbai, while Godhani (2021) reported Pomona (70.58%) and Grippotyphosa (11.76%) predominant in and around Navsari. Amrutha *et al*. (2019) summarized that serovar Pyrogenes was highly prevalent followed by Australis, Grippotyphosa, Icterohaemorrhagiae, Tarasovi and Javanica. Contrary to this, Meera (2016) reported Javanica as the most predominant serovar in Tiruchirappalli, Tamil Nadu, while Abdullathief *et al*. (2018) observed serovars *L. interrogans* serovar Australis followed by serovar Autumnalis and Canicola in Mannuthy, Kerala. The difference might be due to difference in the amount of sample size, geography, location of kennel or its (dog) location and the number of serovars included in the MAT.

In this study, 108 MAT positive as well as some apparently healthy dogs samples were further analysed by ImmunoComb test for antibody where 49.07 % (53/108) were found positive. Desai *et al*. (2020) noted 36.95% seroprevalence in dogs in and around Navsari, Gujarat, whereas Tuemmers *et al*. (2013) found 21.30 % seropositivity in Temuco city, Chile, and Ojha *et al*. (2018) reported 11.40 % seroprevalence in Kathmandu valley, Nepal. Seroprevalence rate in our study is considerably higher than previous studies. This might be due to incorporation of more clinically suspected and MAT positive samples in our study. Overall seroprevalence was assessed in and around Navsari district by Microscopic Agglutination Test (MAT) and Canine Leptospira Antibody Test (IgG based Immuno Comb Antibody test) (Positive in either of the test is considered) and said to be 15.37% (63/410).

Further 66 clinically suspected samples (66 each of blood and urine) were subjected to PCR, in which six out of 66 blood samples (9.09%) and four of 66 urine samples (6.06%) were found positive with lep1/lep2 primers having amplicon size of 331 bp (Fig. 1). All PCR positive samples tested with lep1/lep2 primers were screened with pathogenic specific LipL32 primers having amplicon size of 242 bp (Fig. 2) meant for pathogenic leptospires.

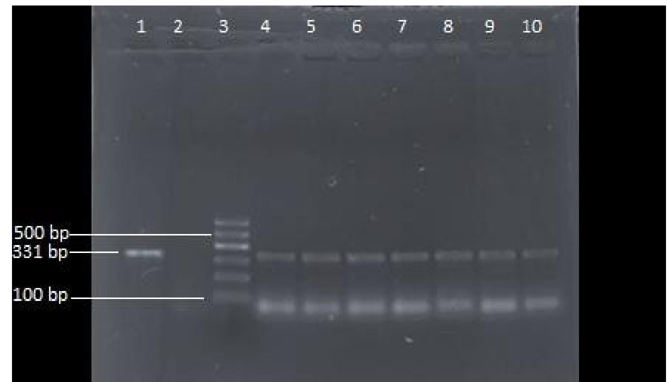

Fig. 1: Agarose gel electrophoresis showing 16S rRNA gene specific PCR product (331 bp) amplified with Lep1 & Lep2
Lane 1 Positive control, Lane 2 Negative control, Lane 3 DNA Ladder of 100 bp, Lane 4-10 Field samples

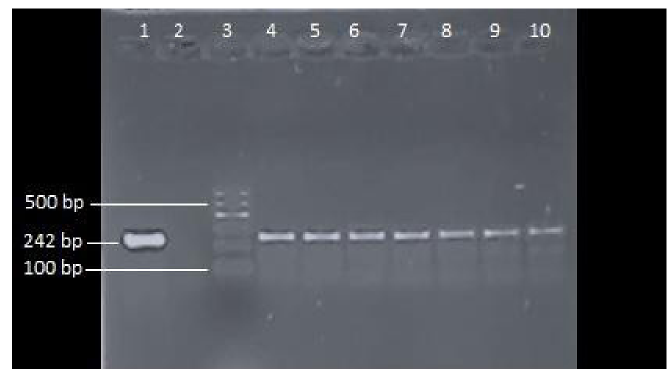

Fig. 2: Agarose gel electrophoresis showing LipL32 gene specific PCR product (242 bp) amplified with LipL32 F and LipL32 R primer
Lane 1 Positive control, Lane 2 Negative control, Lane 3 DNA Ladder of 100 bp, Lane 4-10 Field samples

This is the first comprehensive study of overall prevalence of canine leptospirosis in and around Navsari in Gujarat. Overall prevalence of Leptospirosis in dogs was assessed in and around Navsari district by Microscopic Agglutination Test (MAT), Canine Leptospira Antibody Test (IgG based Immuno Comb Antibody test) and PCR. Out of 410 samples tested 68 samples were found positive in either of the test. Risk factor analysis using a multivariable logistic model (Table 3) in the present study revealed that statistically age, breed, sex, district and vaccination status of dogs has no correlation with occurrence of the disease. Whereas, animal clinical status (χ^2=7.7, $p<0.01$ with odds ratio 0.45), walking and other activities in the surrounding forest (χ^2=11.7, $p<0.01$ with odds ratio 5.49), sanitation of dog and housing area (χ^2=14.04, $p<0.01$ with odds ratio 5.69) and owners own house/land (χ^2=4.42, $p<0.05$ with odds ratio 2.68) were found to be significantly associated risk factors with the occurrence of the disease according to the data collected from the owners.

In the present study, Pyrogenes was found to be the most predominant serovar followed by Shermani and Djasiman. Though the dogs were vaccinated, the serovar present in vaccine didn't provide cross protection against other leptospiral serovars. So, the serovars' which are endemic in a particular geographic area must be incorporated in the

Table 3: Multivariable logistic regression model to identify factors associated with leptospira positivity for dogs (n = 410)

Variable	Categories	Negative No. (%)	Positive No. (%)	OR (95% CI)	χ^2	P value
Age group	0-1 Yr	11 (100)	0 (0)	Ref	4.87	0.3
	1-3 Yr	101 (85.59)	17 (14.41)	2.08 (0.76-5.67)		
	3-6 Yr	112 (84.21)	21 (15.79)	1.87 (0.7-4.97)		
	6-9 Yr	98 (80.99)	23 (19.01)	1.49 (0.56-3.95)		
	9 Yr or more	20 (74.07)	7 (25.93)	-		
Sex	Female	117 (84.17)	22 (15.83)	Ref	0.09	0.77
	Male	225 (83.03)	46 (16.97)	0.92 (0.53-1.6)		
District	Navsari	161 (85.64)	27 (14.36)	Ref	1.73	0.42
	Surat	179 (81.74)	40 (18.26)	2.24 (0.2-25.28)		
	Valsad	2 (66.67)	1 (33.33)	2.98 (0.26-34.03)		
Location/Area	Rural	59 (83.10)	12 (16.90)	Ref	0.01	0.94
	Urban	283 (83.48)	56 (16.52)	0.97 (0.49-1.93)		
Water bodies in the vicinity	No	271 (83.13)	55 (16.87)	Ref	0.09	0.76
	Yes	71 (84.52)	13 (15.48)	0.9 (0.47-1.74)		
Owners own house/land	No	60 (92.31)	5 (7.69)	Ref	4.42	0.04*
	Yes	282 (81.74)	63 (18.26)	2.68 (1.03-6.95)		
Condition of area surroundings the owners house	Dry	289 (84.26)	54 (15.74)	Ref	1.08	0.3
	Wet	53 (79.10)	14 (20.90)	1.41 (0.73-2.73)		
Rivers/Streams nearby	No	320 (84.21)	60 (15.79)	Ref	2.38	0.12
	Yes	22 (73.33)	8 (26.67)	1.94 (0.83-4.56)		

(Table continued)

(Table continued)

Variable	Categories	Negative No. (%)	Positive No. (%)	OR (95% CI)	χ^2	P value
House/Land location	High Land	80 (83.33)	16 (16.67)	Ref	0.01	0.93
	Low land	262 (83.71)	51 (16.29)	0.99 (0.54-1.83)		
Rat/ Rodents infestation/ presence	No	72 (83.72)	14 (16.28)	Ref	0.01	0.92
	Yes	269 (83.28)	54 (16.72)	0.97 (0.51-1.84)		
Housing pattern of the animal	Indoor	221 (84.67)	40 (15.33)	Ref	0.82	0.36
	Outdoor	121 (81.21)	28 (18.79)	1.28 (0.75-2.18)		
Sanitation of the dog and housing area	High	1 (50.00)	1 (50.00)	Ref	14.04	<0.001**
	less	11 (55.00)	9 (45.00)	1.22 (0.07-22.4)		
	Moderate	330 (85.05)	58 (14.95)	5.69 (0.35-92.25)		
Dog Housing area condition	Dry	332 (83.84)	64 (16.16)	Ref	1.51	0.22
	Wet	10 (71.43)	4 (28.57)	2.08 (0.63-6.82)		
Contact with other animal/ Presence of other animals in house	Yes (dogs)	58 (86.57)	9 (13.43)	Ref	0.52	0.47
	No	283 (82.99)	58 (17.01)	6.44 (0.37-112.46)		
Exposure to rain water/ flood water	No	162 (84.82)	29 (15.18)	Ref	0.51	0.48
	Yes	180 (82.19)	39 (17.81)	1.21 (0.72-2.05)		
Walking and other activities in forest area	No	335 (84.60)	61 (15.40)	Ref	11.7	<0.001**
	Yes	7 (50.00)	7 (50.00)	5.49 (1.86-16.21)		
Activities in public park or gardens	No	149 (82.32)	32 (17.68)	Ref	0.26	0.61
	Yes	192 (84.21)	36 (15.79)	0.87 (0.52-1.47)		
Recent Visits to veterinarian/clinic	No	220 (85.94)	36 (14.06)	Ref	3.14	0.08
	Yes	122 (79.22)	32 (20.78)	1.6 (0.95-2.71)		
Previous Leptospirosis incidence history to the animal	No	338 (84.08)	64 (15.92)	Ref	3.64	0.06
	Yes	4 (57.14)	3 (42.86)	3.96 (0.87-18.12)		
Status of Leptospirosis vaccination	Not Vaccinated	40 (80.00)	10 (20.00)	Ref	0.48	0.49
	Vaccinated	302 (83.89)	58 (16.11)	1.38 (0.65-2.92)		
Clinical status of animal	Apparently Healthy	281 (85.93)	46 (14.07)	Ref	7.7	0.01*
	Symptomatic/ Suspected	60 (73.17)	22 (26.83)	0.45 (0.25-0.8)		

(Table continued)

Variable	Categories	Negative No. (%)	Positive No. (%)	OR (95% CI)	χ^2	P value
Months since vaccinated	0-6	10 (12.99)	67 (87.01)	Ref	2	0.37
	6-12	45 (16.36)	230 (83.64)	2.87 (0.64-12.96)		
	>12	3 (30.00)	7 (70.00)	2.19 (0.55-8.79)		
Sample collected on which day of symptom	0-3 days	18 (12.24)	129 (87.76)	Ref	4.99	0.17
	4-7 days	40 (18.10)	181 (81.90)	7.17 (0.43-119.68)		
	8-21 days	7 (23.33)	23 (76.67)	4.53 (0.28-73.88)		
	>22 days	1 (50.00)	1 (50.00)	3.29 (0.18-59.6)		

χ^2: Chi-Squared *p-value at 5 % significance **p-value at 1% significance, OR – Odds Ratio, CI – Confidence Interval.

vaccine to decrease the occurrence of the disease in that area. The symptoms of leptospirosis in animals are nonspecific. Early identification of carrier animals as well as information on the shedding condition are key to prevent the transmission of leptospiral infection to other animals and human beings.

Conclusions

Leptospira seropositivity prevalence with inclusions / involvement of emerging serovars in dogs suggest that leptospirosis could be a major public health and animal health problem that needs appropriate attention in respective areas of study in Southern Gujarat to reduce the occurrence of disease. Isolation and identification of infecting serovars should be emphasized along with serodiagnosis to better understand the prevalent serovars for appropriate control strategies. Awareness of leptospirosis disease among health communities and public is much needed.

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