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Original Article

Leptospira in the different ecological niches of the tribal union territory of India

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Abstract

Introduction: Leptospirosis is a widespread zoonotic disease, which has a medical and veterinary importance, and also a commonly re-emerging infectious disease. The main causes of transmission are known; however, the respective prominence of each of the components and the respective environmental risk factors are obscure.

Methodology: Present study was conducted in the different locations (urban and rural/tribal) of the union territory of Dadra and Nagar Haveli (UT of Dadra and Nagar Haveli). Periodical sample collection approach was used to collect the samples from May 2016 to April 2017, to determine the persistence of leptospiral contamination of the environmental sources.

Results: The PCR detection of *Leptospira* DNA revealed that pathogenic leptospires were present in water, soil and animal urine. The highest positivity was reported from the water of household drainage in urban areas and in the water from rice field of rural areas.

Conclusions: The data obtained from the present study may help and guide in developing preventive measures for leptospirosis in the UT of Dadra and Nagar Haveli.

Key words: *Leptospira*; environment; PCR; India; animals.

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Introduction

Leptospirosis is a microbial disease which affects both animals and humans in countries with tropical, humid and sub-tropical climates. It's also an occupational disease and also been found to be associated with the recreational activities, exposure through contaminated water or soil [1,2]. Humans and animals when they encounter a leptospires-contaminated environment become infected; entry is mainly through their skins and mucous membranes [2]. Leptospires can be found in the variety of places depending on the presence of host and type of leptospires. The saprophytic leptospires such as *L. biflexa* does not require any hosts, because they directly feed on the organic matters in water and hence can be seen in the environment such as water surfaces. However, these pathogenic leptospires needs hosts such as rodents and domestic animals (dogs, pigs and cattle) for their survival and growth [3]. Leptospires are found in a wide variety of environmental contexts, industrialized and developing countries, and also in urban and rural contexts [4-5]. It has been also detected from different ecological niches of different geographical areas such as soil, water and urine [6-19]. Thus, this study was designed to reveal the presence of various species of *Leptospira* in different types of reservoirs. The data obtained from the present study may help in guiding to develop the preventive measures for leptospirosis in the UT of Dadra and Nagar Haveli.

Methodology

Study area

The UT of Dadra and Nagar Haveli is located at latitude - 20° 54' 41" N to 20 ° 21' 36" N, Longitude – 72° 54' 41" N to 73° 13' 13" N in the Western Ghats of India. Silvassa is the capital of Dadra and Nagar Haveli. In recent years this area has undergone a large-scale development through urbanization and industrialization. The 487 sq km area is hilly, forested, composed of 72 villages and one town, mainly occupied by tribes and migrants (population 0.342 million) (Figure 1).

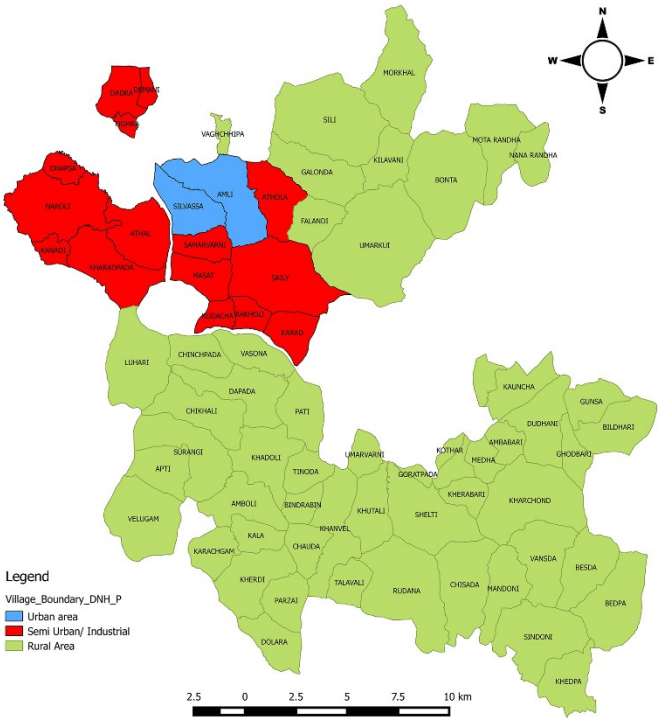
Sample Collection

A total of 467 samples were collected between May 2016 and April 2017. Among them, 216 samples were obtained from water, 60 samples were obtained from soil and 191 urine samples were obtained from animals.

Samples were collected from different sites of urban and rural/tribal areas of the UT of Dadra and Nagar Haveli. Sample collection and areas were divided on the basis of different seasons, among them a total of 119 samples were collected during the pre-monsoon season (January 2017- April 2017), 222 samples were collected during the monsoon season (May 2016- September 2016) and 126 samples were collected during the post-monsoon season (October 2016- December 2016). Random animal urine samples were collected after obtaining permission from its owners.

The water samples were collected from the various ecological niches of urban and rural/tribal areas and transported in sterile 200 to 500 mL glass bottles. Sub-surface samples of water were obtained by using Kemmerrer water sampler (Cole-Palmer, Wildco 1200-E30, 1.2 L) and transferred to sterile glass bottles (250 to 500 mL) immediately after the collection [6]. The soil samples were collected in sterile 50 mL Falcon tubes from wet and shaded urban as well as rural/tribal areas. The collected soil samples were mixed with sterile water and shaken vigorously before transportation. The suspension obtained was allowed to settle down for 5–10 minutes and filtered by using 0.2 μm filter unit (Millipore Cork, Ireland) [6]. Approximately 50 mL of mid-stream urine samples (when the animal started urinating, the flow was alowed up to 2-3 seconds, and then the mid-stream clean catch was collected by holding a sterile plastic container at its edges) were collected and transported to the laboratory maintained at a temperature of 4 °C within 30 minutes. Samples were immediately centrifuged at ~5000×*g* for 15 minutes, the pellets were re-suspended in 1 mL of phosphate buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4(pH 7)], and immediately transferred into a 1.5 mL micro centrifuge tube and centrifuged at ~8000×*g* for 5 min. The supernatant obtained was discarded and the pellets were resuspended in PBS and stored at −20° C until used for testing [20].

Figure 1. GIS map showing the geodemography of the UT of Dadra and Nagar Haveli, India.



Molecular detection

DNA Extraction

Every water sample (10 mL) collected, were centrifuged at 16000×g for 10 minutes. The pellets obtained were resuspended to a total volume of 200 µL in water and immediately lysed to begin the DNA extraction process, as per the manufacturer's instructions, using a QIAamp DNA Mini Kit, (Qiagen, Hilden, Germany) and QIAcube (Qiagen, Hilden, Germany). DNA elution was performed with 100 µL of buffer AE. The quantity of DNA was measured by using QIAxpert (Qiagen, Hilden, Germany).

The filtrate obtained from soil samples were taken and centrifuged at 6000×g for 5 minutes. The supernatant obtained was collected and again centrifuged at 16000×g for 10 minutes. The pellets obtained were finally re-suspended to a total volume of 200 µL in the sterile distilled water and DNA was extracted and quantified by using QIAxpert.

Urine samples collected were immediately centrifuged at ~5000×g for 15 minutes, the pellets obtained were re-suspended in 1 mL of phosphate buffered saline (PBS), then transferred into a 1.5 mL microcentrifuge tube and centrifuged at ~16000×g for 5 minutes. The supernatant produced were discarded and the pellets were resuspended in PBS and stored at −20 °C until its used for DNA extraction. DNA extraction controls were used by spiking the pathogenic *Leptospira* with each kind of sample types during PCR optimization.

PCR amplification of secY gene and 16s rRNA gene

The presence of *Leptospira* in the environmental samples were detected by using 16s rRNA primers [21]. The PCR amplification were performed in a final reaction volume of 25 µL. All reaction mixtures contained 12.5 µL of PCR TopTaq® master mix

Table 1. Showing the summery of molecular detection of leptospiral DNA from samples.

Primer	Forward and reverse primers	PCR Condition	Type	Reference
16s rRNA	5' GAACTGAGACACGGTCCAT 3' 5' GCCTCAGCGTCAGTTTTAGG 3'	95°C for 3 minutes, 35 cycles at 95°C for 30 sec, 55°C for 1 minutes and 72°C for 1 minute and a final extension at 72°C for 10 minutes.	Detection of all leptospiral DNA	[19]
SecY	5' GCGATTCAGTTTAATCCTGC 3' 5'GAGTTAGAGCTCAAATCTAAG 3'	95°C for 3 minutes, 35 cycles at 95°C for 1 minutes, 54°C for 1 minute and 72°C for 1 minute and a final extension at 72°C for 10 minutes.	Detection of pathogenic leptospiral DNA	[20]

(Qiagen GmbH, Hilden, Germany), 2 µL of the primers, 5 µL of DNA and 5.5 µL of dd H_2O. The presence of pathogenic *Leptospira* in the samples were detected by using primers, targeted for the *secY* gene, designed by Ahmed *et al*. [22] (Table 1). The primers of *secY* gene, encoding preprotein translocase for *Leptospira* is located within the *S10-spc-α* locus, containing genes for ribosomal protein. The assay was modified for PCR amplification with the TopTaq® master mix (Qiagen GmbH, Hilden, Germany) by increasing the annealing temperature by 1°C and changing the denaturation, annealing, and extension time to 1 minute. The PCR product was visualized on 1.5 % agarose gel as 430 bp and 202 bp respectively. The *Leptospira interrogans* serovar Bangkinang (Pathogenic Control) and *Leptospira biflexa* serovar Patoc (Non Pathogenic control) obtained from Regional Medical Research Centre, Port Blair was used as positive control and *Escherichia coli* (ATCC 25922) was used as a negative control.

Results

Over the period of one year total 467 samples (60 Soil, 216 Water and 191 Urine) were collected from the various urban and rural/tribal areas of the UT of Dadra and Nagar Haveli, to evaluate the presence of *Leptospira* in various ecological niches. Total 101 water samples were collected from the urban area, out of these 38 (37.6 %) samples were found positive for *Leptospira*, (7 pathogenic and 31 intermediately pathogenic or saprophytic). Highest positivity for *Leptospira* were found from household drainage 13

Table 2. Showing the results of PCR testing for Leptospira from different samples from the UT of Dadra and Nagar Haveli (2016–2017).

Area	Source of sample	Type of sample	No of sample	Positive	Pathogenic Leptospira n (%)	Non-pathogenic Leptospira n (%)
Urban	Sewage	Water	30	14 (46.67)	2 (14.29)	12 (85.71)
	Household drainage		20	13 (65)	4 (30.77)	9 (69.23)
	Market area drainage		30	11 (36.67)	1 (9.09)	10 (90.91)
	Public toilet drainage		10	0 (0)	0 (0)	0 (0)
	Industry drainage water		10	0 (0)	0 (0)	0 (0)
	River		1	0 (0)	0 (0)	0 (0)
	Cattle	Urine	58	8 (13.79)	2 (25)	6 (75)
	Dog		20	1 (5)	1 (100)	0 (0)
	Goats		20	0 (0)	0 (0)	0 (0)
	Industry area	Soil	15	2 (13.33)	1 (50)	1 (50)
	Market area		10	1 (10)	0	1 (100)
Rural	Rice field water	Water	40	19 (47.5)	5 (26.32)	14 (73.68)
	Stream		10	1 (10)	0	1 (100)
	Household drainage		30	9 (30)	2 (22.22)	7 (77.78)
	Cowshed drainage		30	12 (40)	4 (33.33)	8 (66.67)
	Pond		1	0	0	0
	Forest land		3	1 (33.33)	0	1 (100)
	River		1	0	0	0
	Cattle	Urine	63	12 (19.04)	1 (8.33)	11 (91.67)
	Dog		10	1 (10)	0 (0)	1 (100)
	Goat		20	1 (5)	0 (0)	1 (100)
	Soil near stable	Soil	15	3 (20)	2 (66.67)	1 (33.33)
	Forest area		10	0 (0)	0	0 (0)
	Rice field		10	2 (20)	1 (50)	1 (50)

(65.0%), followed by sewage 14 (46.6%), and market area drainage 11 (36.7%). Similarly, 115 water samples were collected from the rural/ tribal areas, out of these, 42 (36.5 %) samples were encountered positive (11 pathogenic and 31 intermediately pathogenic or saprophytic). Highest positivity for *Leptospira* was found from the samples obtained from water present in rice fields 19 (47.5%), followed by animal shed drainage 12 (40.0%), forest land 1 (33.3%), household drainage 9 (30.0%)and slow-moving stream 1 (10.0%).

Among the 60 soil samples, 25 soil samples were collected from the urban area, but only 3 (12%) samples were found positive with *Leptospira* (1 pathogenic and 2 intermediately pathogenic or saprophytic). Among them, the highest positivity for *Leptospira* was found in the samples derived from Industrial area soil 2 (13.3%). Similarly, 35 samples were collected from rural/tribal areas, out of these 5 (14.2%) samples were found positive for *Leptospira* (3 pathogenic and 2 intermediately pathogenic or Saprophytic). The highest positivity for *Leptospira* was found from soil near stable 3 (20%) followed by rice field 2 (20%).

Among the 191 urine samples, 98 urine samples (58 from cattle, 20 from dogs and 20 from goats) were tested for the presence of *Leptospira* in the urban area. 8 (13.7%) cattle samples and 1 (5%) dog sample were positive (2 cattle samples and 1 dog sample was pathogenic and 6 cattles sample were intermediately pathogenic or saprophytic). Similarly, 93 urine samples (63 from cattle, 10 from dogs and 20 from goats) were collected from the rural/tribal areas out of which 12 (19.0%) cattle, 1 (10.0%) dog and 1 (5.0%) goat samples were found positive for the presence of *Leptospira* (Table 2).

The seasons trend in *Leptospira* positivity in various ecological niches indicates that the highest positivity of *Leptospira* was found during monsoon followed by post-monsoon and pre- monsoon season (Table 3).

Discussion

The literature indicates that the environmental and occupational factors are very important predictors of the seasonal development of leptospirosis [2] . The role of water is well established for the transmission of pathogenic *Leptospira* [23]. The wet surroundings, streams nearby dirty surroundings, contact with animals, field work and rat infestation has been noted as high risk environmental factor for the transmission of the *Leptospira* in the UT of Dadra and Nagar Haveli. [24]. A wide difference in the positivity (3% to 83%) of *Leptospira* in water samples has been reported from various environmental entities [9]. The positivity of *Leptospira* from water samples obtained from the South Andaman Island, India, has been reported 53.98 % from urban and 50.33 % from rural [13]. The current study conducted in the UT of Dadra Nagar Haveli, shows that the water samples were found less positive (37.62 % and 36.52 %) from urban and rural/tribal areas respectively, when compared to the high positivity rates of *Leptospira* identified from the water samples of the South Andaman Islands, India. The recovery of leptospiral DNA from the soil samples, from urban and rural areas were less in comparison of the soil samples obtained from the Peninsular Malaysia [7].

In soil samples obtained from near stable and rice field of the rural /tribal areas of the UT of Dadra and Nagar Haveli were found highest positivity for pathogenic leptospiral DNA. Especially, where the animals are present, hence the risk for causing infection among farmers and animal husbandry workers are higher.

It is well explained in the literature that the mammalian species excretes pathogenic leptospires through their urine, mixing of these with rain water results in contamination of sewage, household drainage, ponds and river, making these reservoirs to become the sources of transmission of leptospirosis [16].

The literature indicates that the prevalence of other leptospires (intermediately pathogenic or saprophytic) were more than the pathogenic *Leptospira* [13]. The

Table 3. Seasonal variation in the presence of *Letospira* in the UT of Dadra & Nagar Haveli.

Area	Type of sample	Pre-monsoon N (%)		Monsoon N (%)		Post-monsoon N (%)	
		Pathogenic	Non-pathogenic	Pathogenic	Non-pathogenic	Pathogenic	Non-pathogenic
Urban	Water	1 (3.0)	8 (24.2)	4 (11.8)	14 (41.2)	2 (5.9)	9 (26.5)
	Urine	0 (0.0)	1 (3.1)	1 (2.9)	2 (5.9)	2 (6.3)	3 (9.4)
	Soil	0 (0.0)	0 (0.0)	1 (11.1)	0 (0.0)	0 (0.0)	2 (25.0)
Rural	Water	2 (8.7)	6 (26.1)	5 (10.9)	12 (26.1)	4 (8.7)	13 (28.3)
	Urine	0 (0.0)	2 (6.9)	0 (0.0)	6 (17.6)	1 (3.3)	5 (16.7)
	Soil	0 (0.0)	0 (0.0)	2 (15.4)	1 (7.7)	1 (9.1)	1 (9.1)

high prevalence of intermediately pathogenic or saprophytic *Leptospira* in comparison of pathogenic *Leptospira* in the present study confirms the result of the previous study. The behaviour or reservoirs of nonpathogenic (saprophytic) *Leptospira* in the environment has to be studied yet. However, the literature indicates that leptospires are most frequently associated with the soils of high moisture, and the reservoir of the saprophytic *Leptospira* could be in the soil [8]. It has also been suggested that saprophytic *Leptospira* survives in wet soil during dry days and appears on the surface area of water during rainy days [10]. Hence the depth of sampling point is also a factor that can affect the ratio of various leptospires (pathogenic. intermediately pathogenic or saprophytic) detected in the environment.

The appearance of *Leptospira* positive clinical cases are frequently associated, with exposure to environmental risk factors, such as rainfall and flooding [2]. In this context, the highest positivity of *Leptospira* in the environment is identified during the rainy season. These findings reinforce the concept that, rainfall affects the prevalence of adapted and incidental *Leptospira* in the environment. That may lead to be a contributing factor for the development of *Leptospira* infection.

Conclusions

The results of PCR reveals that the pathogenic leptospires were identified from the samples of water, soil and animal urine collected from the urban and rural/tribal area of the UT of Dadra and Nagar Haveli. The highest positivity of *Leptospira* was reported from household drainage in the urban areas and in the water from the rice fields in the rural areas. The data obtained from the present study may help and guide in developing preventive measures for leptospirosis in the UT of Dadra and Nagar Haveli.

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