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Seroepidemiological Review of Leptospirosis and its Co- Infection Between 2007 to 2009 in Chennai, Tamil Nadu - A Doctoral Thesis Report

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The intention of this study was to detect leptospirosis in suspected pyrexia cases with unidentified sanitary status in and around Chennai. A total of 458 serum samples from human were screened for presence of *Leptospira* antibodies. Of these, 176 serum samples were found positive for *Leptospira* antibodies and yielding an overall seropositivity of 38.40% with anicteric leptospirosis (87.4%.). Higher rate of seropositivity has been reported during the period of October 2008 to February 2009 and male comprise 62.00%. The occurrence of *autumnalis* was found to be 23.2%, *icterohaemorrhagiae* 21%, *hebdomadis* 12.6, *australis* 10.8%, *grippotyphosa* 9.7%, *pomona* 9%, *copenhageni* 6.9%, *bataviae* 4.6% and mixed 2.2% also found, though at lower frequencies. All the patients had fever and myalgia, head ache, vomiting, body pain with lower frequencies. Association of leptospirosis and urinary tract co-infection infection was found in 7.40% of cases, causative organisms isolated from the patients were *pseudomonas aeruginosa, Escherichia coli,* and *Enterococcus faecalis.*

Key words: Leptospira, Leptospirosis, Serovars, UTI, Co-infection.

India with an 8,129 km long coastline with of plenty of natural resources has one of the major important coastal areas of Chennai. Attribute to the rapid ecological changes in the region during the past decade many new zoonotic diseases have emerged and resulted in epidemics leading to significant morbidity and mortality in human. Leptospirosis is one among them and it is caused by spirochetes belonging to *Leptospira interrogans* groups and is characterized by wide spectrum of clinical manifestations in human being and leads to multiple organ involvement with fatal complications [1]. Chennai receives around 1800 mm rainfall annually and high rainfall during monsoon season particularly in the month of October, November and December. This monsoon floods cause the rodent infested sewer systems to run over the streets and contaminate almost all the water bodies and produce human infection. Leptospirosis become a potential health problem of in Chennai city [2]. No major investigation on either the epidemiology or clinical picture was carried out on human infection in Chennai. A better understanding of clinical and epidemiological status of leptospirosis is necessary for HS control and prevention. The present investigation was done to define major clinical presentation and epidemiology by serologically suspected cases of leptospirosis admitted to major hospitals and samples received in clinical laboratories of Chennai, Tamil Nadu, and India.

MATERIALS AND METHODS

Collection of Samples

This study included patients admitted to government and private hospitals of Chennai that

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draws most of the patients from the Chennai town and surrounding villages. The infection criteria consisted of the presence of fever with headache or body aches associated with jaundice, decreased urine output, or bleeding tendencies including subconjunctival hemorrhage, vomiting, diarrhea etc (Table 4). A total of 458 patients were selected and blood samples were collected from them. Urine samples were collected specifically from 27 patients with severe clinical manifestation of leptospirosis and UTI on the day of admission for direct microscopic observation and suspicious for UTI co-infection. Attempts were made to collect a second sample, 7 - 10 days after collection of the first sample from doubtful cases. Serum was separated from all the blood samples and stored at 4°C until use. Ten blood samples were also collected from healthy individuals for comparison and cross check.

Bacterial Strains

The leptospiral serovars used in the present study includes the following serovars in the species, a) *Leptospira interrogans – Pomona* (strain *Pomona*), *Icterohemorrhagiae* (strain *Lai*), *Australis* (strain *Ballico*), *Autumnalis* (strain *Rachmat*), *Hebdomadis* (strain *Hebdomadis*), *Canicola* (strain *Canicola*), *Louisiana* (strain *Lanka*), b) *Leptospira kirschneri* - serovar *Grippotyphosa* (strain *Moskva* V) c) *Leptospira borgpetersenii- Javanica* (strain *Poi*), d)*Leptospira biflexa* - serovar *Patoc* (strain *Patoc* I). All strains were received from the Regional Medical Research Center, Port Blair, Andaman and Nicobar Islands. These serovars were maintained in semisolid (1% agar) EMJH supplemented with 10% enrichment (Hi-media, India) at 30°C in screw capped test tubes.

Dark Field Microscopic examination (DFM)

Thin smear of serum and urine was prepared in a microscopic slide after centrifugation. Using the high power objectives of a Dark field microscope, one edge of the film was focused to observe the *Leptospira*. The presence of *Leptospira* was observed and count was made. If no *Leptospira* was seen in HPFs it is believed to be negative [3].

Culture and Isolation of *Leptospira*

Out of 458 patient patients, blood from 65 cases, were subjected to culture. Few drops of freshly collected blood was immediately added to 3 ml of Ellinghausen-Mccullough-Johnson-Harris medium (EMJH) and enriched with 5-fluorouracil (100 mM) (Hi-media Laboratories, Bombay). Growth of leptospires were checked Dark field microscope for a period of seven weeks.

Macroscopic Slide Agglutination Test (MSAT)

MSAT is a simple and quick screening test to detect leptospira genus – specific antibodies in human sera. This is a slide agglutination test using formalized and heat killed *patoc-1* antigen pooled with leptospires belonging to different serovar commonly prevalent in and around Chennai [4]. 7 µl of phosphate buffered saline, 6µl of patient's sera and 13 µl of MSAT antigen were placed on a cavity slide. The antigen drops were carefully mixed with an applicator stick and rotated for 4 minute at 100 rpm. Agglutination was observed and positive results are graded from1+ to 4+ depending on the intensity of agglutination described by Brandao, 1998[5]and Sumathi *et al.*, 1995[4].

Microscopic Agglutination Test (MAT)

Microscopic agglutination test (MAT) is performed with a battery of 10 live cultures (*Pomona*, *Icterohemorrhagiae*, *Australis*, *Autumnalis*, *Hebdomadis*, *Canicola*, *Louisiana*, *Grippotyphosa*, *Javanica* and *Patoc)*, which were grown in EMJH medium. Approximately, an inoculum size of $1\text{-}2\times10^8$ CFU/ml was used as antigen. The patient's sera were diluted (1:10) in sterile saline in separate tubes. The test was done in micro titre plates. To each well 20 ml of sterile PBS, was added by using micropipette to make the serum dilution as 1:20. Then double fold serial dilution was made like1:20, 1:40, 1:80, 1: 160 and so on by the micropipette. To all the diluted wells, 20 ml of live antigen was added after testing the concentration and allowed for agglutination. This procedure was performed with all serovars independently. Agglutination and lyses were observed after 2- 4 hours incubation at normal room temperature.

IgM ELISA - Enzyme Linked Immunosorbent Assay

ELISA is a sensitive serological screening test used to detect IgM to distinguish specific current infection. IgM ELISA was carried out by using commercially available kit (SERION Pvt. Ltd, Germany) obtained from Lister Laboratory. The method was followed as per the manufacturer's

protocol and absorbance of microtitre plate done at 450 nm in spectrophotometer.

Detection of leptospires from infected blood and urine sample

Blood films were prepared and allowed to stain by modified Fontana method described by Gandhadhar & Rayasekjar (1998). The spirochetes were stained brownish-black on a brownish-yellow background and it is observed by DFM.

Random Amplified Polymorphic DNA (RAPD)

The RAPD fingerprinting method was carried out with DNA sample by methods described by Balakrishnan *et al*., 2009[7], Sharma *et al.*, 2006[8] and Ramadas *et al*., 1997[9] was used with a few modifications. Briefly, each PCR was carried out in a mixture containing about 50ng of leptospiral DNA isolates, 10 mM Tris-HCl, 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM of deoxynucleoside triphosphates, forward (GGGAAAAATAAGCAGCGATGTG) and reverse (ATTCCACTCCATGTCAAGCC) primers at concentration of 300 pM each (Medox Pvt. limited, Chennai) and 0.5 U of *Taq* DNA polymerase. The whole mixture was overlaid with 50 µl of mineral oil and amplification was carried out in a thermal cycler. The first two cycles consisted of denaturation at 95°C for five minutes, annealing of primers for 5 minutes at 40°C and extension for 5 minutes at 72°C. The successive 40 cycles consisted of denaturation for 1 minute at 95°C, annealing of primers for 1 min at 60°C, and extension for 3 min at 72°C, with a final extension step for 10 minutes at the last cycle. The amplified product and DNA molecular weight marker were electrophoresed in 1.5% agarose gel with DNA molecular weight marker and observed under UV light as a single band in gel.

Diagnosis of Concomitant Infection in Leptospirosis Cases

In this study, we included 27 patients who are serologically confirmed for leptospirosis with UTI symptoms which include a frequent urge to urinate, a painful and burning feeling in the area of the bladder and urethra during urination. This study was done with people living in and around Chennai between July 2008 and September 2011. Leptospirosis was diagnosed in patients who had at least a fourfold increase in antibody titer against serotype of leptospirae in paired serum samples by a microscopic agglutination test (MAT). UTI was diagnosed urine cultures with Nutrient agar, Blood agar plates and McConkey agar using a calibrated loop (0.01ml). A colony count of 103 or more CFU / ml of urine were considered significant. Then it was sub cultured in selective mediums (EMB agar and Manitol salt agar) based on growth characteristics and morphology appearance in Gram's staining. After the growth achievement in selective medium, biochemical tests were carried out for further conformation in species level (Table 5).

Meterological Data

Meterological Data: Temperature, relative humidity, rainfall and Sunshine were collected from Government meterological department, Kodampakkam, Chennai. This data were cross referred to present research results and environmental monitoring data in a geographical information system (GIS) for broad scale analysis of associations between climate and disease (Tables 24-28).

Statistical Analysis

Obtained data were stored in Microsoft Office -Excel sheet and allowed to calculate absolute frequencies, percentages and comparison test for proportions were calculated using EPIDAT 3.1 statistical software.

RESULTS AND DISCUSSION

Serological and cultural tests

During the period of June 2007 to November 2009, 458 suspected cases having leptospirosis was selected for the present investigation and diagnosis was made based on modified Faine's criteria described by Sivakumar [10]. Score was given for positive samples, a score of 25 and more believe it to be positive either by combination or single part (Table-1) and based on the above criteria, leptospirosis cases were recorded. Current leptospirosis was confirmed in 176 cases out of the 458 patients, whose sera were examined by the MSAT, MAT, ELISA, Dark field Microscopic observation (DFM) and by Culture method (Table -2 and Plate-1 & 2). Seroprevalance of leptospirosis was determined to be 38.4%. 144 patients provided single serum samples, and 32 provided repeat samples.

Dark field microscopic observation of Blood, Urine and Blood culture

Among the 76 persons blood tested 9 of

them (11.8%) were positive by DFM. Blood culture was given positive report in 15 (19.7%) cases. Growth was confirmed by intermittent observation of EMJH medium by Dark field microscopy and Dinger's ring formation just 1-2 cm below the surface of medium. As of 27 urine samples 5 (17.8%) given over all positivity. This lower level positive rate of leptospires by microscopy and culture methods were probably because the samples were taken during the first 10 days of illness [10, 11, 12].

Polymerase Chain Reaction

PCR method targeting 16S rRNA gene on DNA extracted from culture resulted in a high specific analytic method ; pathogenic Leptospira spp. were detected while other bacteria species were negative. The primers that amplify 571bp

Table 1. Modified Faine's criteria (Shivakumar, 2008)

S. No.	Clinical features (Part - A)	Score	Laboratory tests (Part-B)	Score	Epidemiological factors (Part-C)	Score
1	Fever	2	ELISA (IgM)	15	Rainfall	5
2	Headache	2	MSAT	15	Contaminated environment	4
3	Temperature > 39 deg.C	2	MAT- single positive high	15	Animal contact	1
4	Myalgia	4	MAT- paired sera.	25		
5	Conjunctival suffusion	4				
6	Meningism	4				
7	Jaundice	1				
8	Albuminuria / elevated BUN	2				
	Total		Total		Total	
Cumulative score						

Note: A score of 25 &more considered as positive either by combination or single part

Table 2. Role of various tests in diagnosis of Leptospirosis in our study

Tests	No. of sample positive / Total no. of samples tested	Percentage(%)
I. SEROLOGY		
1. MSAT	171/458	37.30
2. MAT	176 /458	38.40
3. ELISA (IgM)	144/176	81.80
4. Microscopy (DFM)	9/76	11.80
II. CULTURE		
EMJH medium	7/76	9.20
III. MOLECULAR		
PCR	5/5	100
IV. CO-INFECTION	5/27	5.40
UTI		

Table 3. Incidence of leptospirosis based on age group

S.No.	Age group	Total number of samples tested	Positive samples	Percentage of positivity
1	1-20	132	54	40.90
2	21-40	168	79	47.00
3	41-60	96	27	28.10
4	Above 60	62	16	25.80
Total	458	176	38.40	

fragment of 16S rRNA gene of pathogenic leptospira were used for carrying out PCR[13]. The sequence of the forward primer was 5'–GGGAAAAATA AGCAGCGATGTG–3' and the reverse primer was 5'–ATTCCACTCCAT GTCAAGCC–3'. The analysis of PCR product was carried out in 1.3% agarose gel stained with ethidium bromide (0.5 mg/ml). 100 bp DNA ladder (Medox) and appropriate controls were incorporated to rule out false positive or negative results. The gel was observed under UV transilluminator. The presence of 571 bp amplicon was detected in the present study that confirms the amplification of 16S rRNA gene of pathogenic Leptospires [7,14].

Table 4. Clinical Features found in Leptospirosis cases

S. No	Symptoms	No. of Patients	n=176%
1	Fever	149	84.6
2	Myalgia	132	75.0
3	Head Ache	113	64.2
4	Vomiting	57	32.3
5	Body Pain	54	30.6
6	Abdominal Pain	42	23.8
7	Joint Pain	41	23.2
8	Diarrhoea	33	18.7
9	Anaemia	28	15.9
10	Muscle Tenderness	26	14.7
11	Jaundice	24	13.6
12	Meningitis	19	10.7
13	Renal failure	16	09.0
14	Breathlessness	14	07.9
15	Conjunctival suffusion	14	07.9
16	Dimness of vision	12	06.8

Concomitant Urinary Tract Infection in leptospirosis cases

Out of twenty seven suspected cases on urine analysis, seven patients had UTI co-infection. *Enterococcus faecalis, Pseudomonas aeruginosa,* and *Escherichia coli* are the causative agents of UTI infection. Among them *Pseudomonas aeruginosa* was found in two cases and *Enterococcus faecalis* caused infection in two patients. *Escherichia coli* was also found in one of the cases. Table 5 reveals the cultural and biochemical results of the above identified organisms. *L.interogans* showed high positive result against serovar *autumnalis* and *icterohaemorrhagiae* ranging from 1:160 to 1:320 (Plate 3). The percentage of UTI co-infection in leptospirosis cases was found to be 7.40 % with predominant symptoms of fever, chill, headache, myalgia, pain in urinary bladder & urethra and urine itself found cloudy in appearance. Out of five cases, three were female and two were male. Among that a male had undergone minor appendicitis operations recently with diabetic complications. All five co-infection cases were with a median age group of 33 years (Table 3). Overall, observations revealed a more severe clinical presentation associated with co-infection than with UTI or

Table 5. Cultural and biochemical characteristics of UTI co-infecting organisms

S.No	Test	*Pseudomonas aeruginosa*	*Enterococcus faecalis*	*Escherichia coli*
1	Nutrient Agar	+	+	+
2	MacConkey agar	Pink color	White	Pink color
3	Blood agar	Beta hemolytic	-	-
4	EMB agar	-	-	Metalic sheen
5	Manital salt agar.	-	Yellow	-
6	Gram's staining	Gram-negative rods	Gram-positive cocci	Gram-negative rods
7	IMViC	- + - +	-	+ + - -
8	Bile Esculin test	-	+	-
9	Catalase test	+	-	-
10	Coagulase Test	-	-	-
11	Urease test	-	-	-
12	TSI	Acid- slant/butt, No gas, No H_2S	Acid-slant/butt, Gas, No H_2S	Acid- slant/butt, Gas, No H_2S

EMB- Eosin-methylene blue agar, IMViC- Indole, Methyl red, Voges-Proskauer and Citrate test, TSI- *Triple sugar iron* agar.

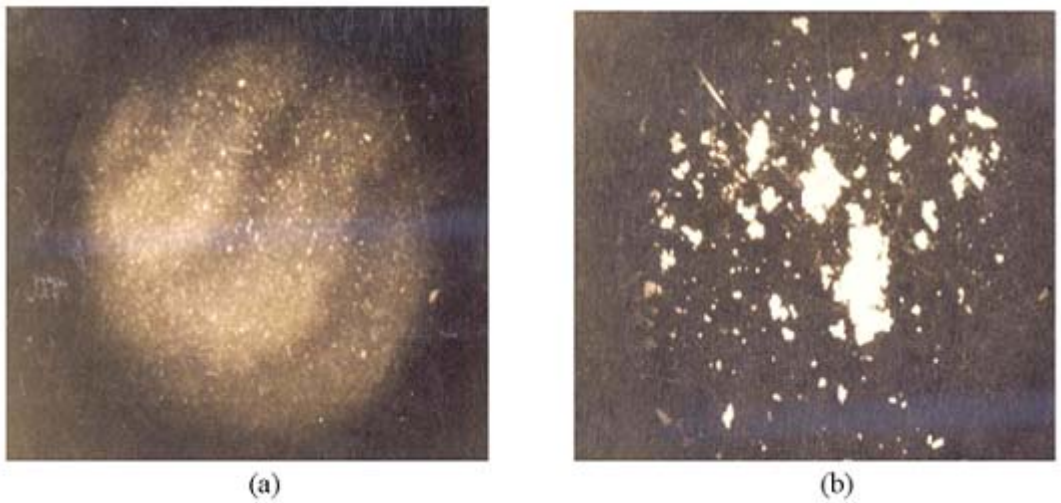

Plate 1 : MSAT (a) Clear suspension - No clumps and (b) Test positive Agglutination of Serum (4+)

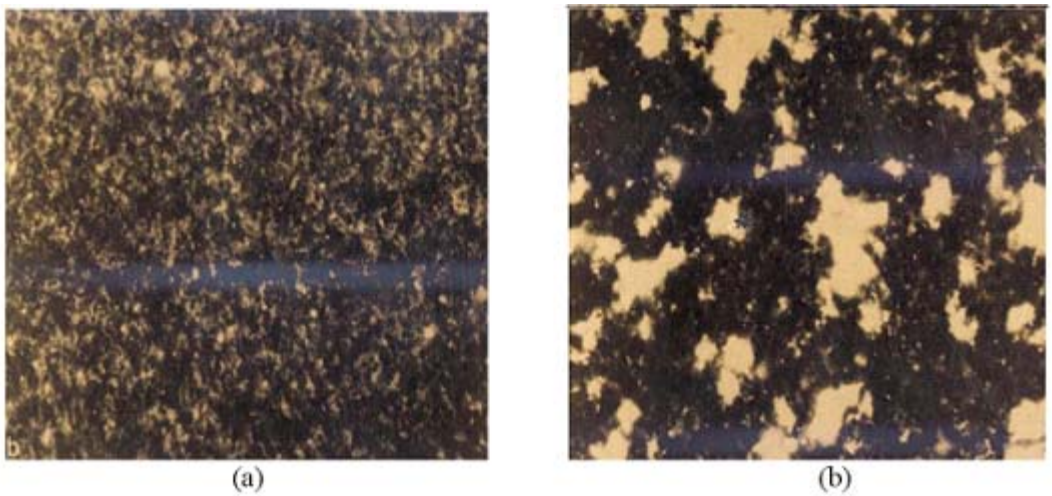

Plate 2 : MAT (a) Test Negative : Free Leptospires - No clumps and (b) Test Positive : Agglutinated leptospires (Clumps)

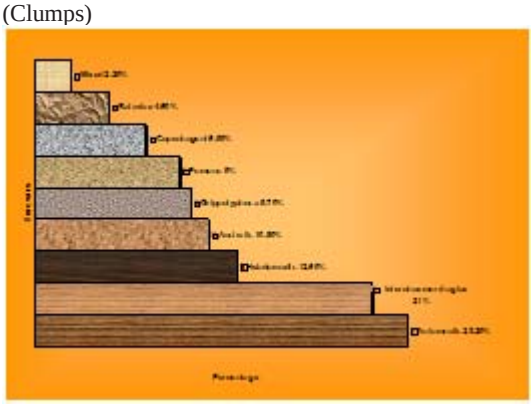

Plate 3: Frequency of *Leptospira* serotype among patients

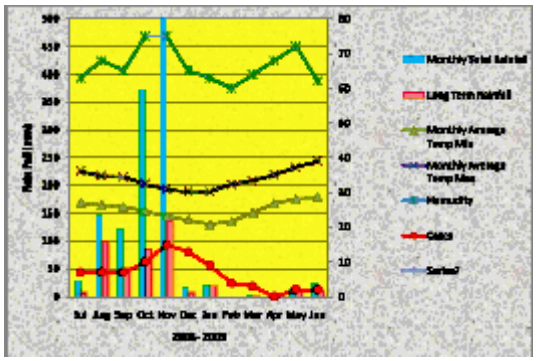

Plate 4: Month wise report of Rain fall, Temperature, Humidity and Number of cases from July 2008 – October 2009

leptospirosis mono-infection.

Meteorological Report

Two significant troughs of low pressure systems and TC Cliff were responsible for heavy rain in various parts of Chennai. Nungambakkam Metrological Department, recorded a new high monthly rainfall record of 556.6 mm during the month of Nov. 2008. The remaining parts of the country received average to below average rainfall. The seasonal rainfall outlook for the October and December period in Chennai in 2007 and 2008 received heavy rain especially in November 2008 owing to Nisha cyclone (Plate 3). Rainfall was recorded almost all places of Chennai, moderate to heavy rainfall resulting in localized flooding. Maximum air temperatures were mainly average to above average during the month of May to July 2008. The highest positive anomalies were recorded at Nungambakkam (39.5°C) and minimum air temperatures were also average to below average in Chennai. The minimum anomaly of 21.7°C was recorded in the month of December - January 2008 and 2009. Relative Humidity at 09.00 hrs was above average at Chennai. The greatest positive anomalies were recorded at Nungambakkam was 75% during the period of December 2007 (IMD, 2007 - 2009).

The present study has been conducted based on modified Faine's criteria for diagnosis of leptospirosis and its correlation with other bacterial urinary tract infection. This criteria is a simple but comprehensive guideline for physicians and microbiologists in both urban and rural areas. The outbreak in Chennai typically belongs to tropical, anicteric and urban kind according to Faine [15,16] epidemiological patterns and scores found to be 25 to 35. Out of 176 leptospirosis positive cases, males were 109, females were 67 in number and their mean age was calculated to be 39.6 years (Table 3). Fever, headache, myalgia was the common clinical manifestation observed in the present work (Table 4). Jaundice occurred in 13.6% but no renal failure cases had been reported. *Leptospira* positive cases were reported throughout the year, but 60.2% cases were recorded during rainfall and it been proved by correlating with metrological data of rain and humidity (Plate 4). After Nisha cyclone in Nov- Dec 2008, the report of leptospirosis cases has been drastically increased due to environment contamination (86%) and animal contact (93%). Outdoor manual workers were found to be important occupational risk factors. Contaminated environment includes inefficient garbage disposal attracting rodents and stray dogs, cattle, inadequate drainage facilities leading to stagnant water, barefoot walking, and rearing of cattle and other domestic animals.

According to the report of Koteswaran [17] revealed that the seroprevalance of leptospirosis in human samples was found to be 57.5% during 1998-99 and it declined to 32.8% (2002-2003) and 10% (2005-06) in Tamil Nadu, whereas Sumathi *et al*. 2008[18] reported 18.6% in 2004 and with an increased record of 28.5% in 2006, which is in conformity with the present investigation (38.4%). So present results clearly indicates that the incidence of leptospirosis is once again re-emerging in Chennai, which may be due to over and floating population, unplanned inhabitations and more over negligence among public.

CONCLUSION

Finally, it was concluded that, a significant rise in the incidence of leptospirosis was the documented in and around. The increased awareness among physician about different clinical symptoms of leptospirosis and early laboratory diagnosis shall help to condense morbidity associated with *leptospira* and its co-infection disease. For successful control, the active and standard surveillance scheme in animals and humans should be strengthened and implement in all major hospitals and primary health centers. This surveillance should include improved simple techniques for rapid and cost-effective analysis, identification of risk factors, identification of reservoir animals, successful methods of prevention and control can be designed. In addition, agencies such as International Leptospirosis Society, Indian Council of Medical Research (ICMR) give sustained support to monitor the disease particularly in rainy season.

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