Adversities of monsoon rain in Chennai (TN) and isolation of pathogenic leptospires from the Environment and Screen the effect of Sodium hypochlorite

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Abstract
The present study was carried out in order to find out environmental contamination of spirochetes and accounts for leptospirosis. We attempted for isolation of leptospires from 66 soil and 80 water samples collected from different zones of Chennai. Isolated strains allowed checking the efficacy of chlorine and it was correlated with meteorological data. Leptospires was found by dark-field microscopy in the cultures of 13 (16.25%) water samples and 11(19.5%) soil samples. Serovar which are isolated, found icterohaemorrhagiae, australis, hebdomodis, autumnalisand canicola. In the chlorine's efficacy test, autumnalis was completely inactivated in the study period of two hours.

The highest positive isolation frequency occurred during the months of August to December (18.7%) with maximum and the minimum temperature of 30°C and 13°C respectively. Samples with average 81 % of moisture content, 38 % organic content and pH of 7.2 had shown the highest positivity with different proportion isolation.

Keywords: Spirochetes, Leptospires, Serovars, Chlorine, Meteorological data.

Introduction
The Metropolitan city of Chennai is surrounded by river, rice field, farms and industries. Chennai has a tropical climate throughout the year with temperature ranging from 24-32°C and it receives around 1800 mm rainfall annually and highest rainfall is found in monsoon season. During this season, most of the water bodies are getting contaminated with leptospires from the urine of infected/carryer animals and sewage. Around 260 different species of the host which include rodents, wild and domestic animals adapted to carry and perpetuation of leptospiral serovars. Among them rats are the most common source and reservoir for human infections. Transmission of leptospires takes place through direct contact with urine of infected animal or ingestion of urine contaminated water or food indirectly.

People who engaged in outdoor activities where fresh water or damp soil is encountered may be at risk for leptospirosis.

It becomes a potential health problem of rural community’s mostly illiterate people, those who are working in poultry, cattle shed, sewage workers and farmers get the infection of leptospires due to barefoot walking. The scope of present study is detection of pathogenic leptospires in soil and water samples to provide an environmental contamination status and help to improve the sanitary condition for effective control and eradication of disease.

Material and Methods
Water and soil samples: A total of 80 freshwater samples were collected for isolation of Leptospira sp. They were collected from rivers, channels, rice fields and wet soils of different parts of Chennai (Plate 1a). Water samples were collected and transported in sterile 200-500-ml glass bottles. Soil from an area of 15 to 20 cm by 4 to 8 cm was taken after the removal of all loose surface materials (Plate 1b). The soil was immediately placed in a plastic bag which was then sealed. All 60 samples were protected against sudden temperature changes during transport to the laboratory and were processed within 12 hours.

Water and soil temperatures were taken in the field and pH was determined upon return to the laboratory. Water samples (100 ml) were passed through a sterile 0.22 µm pore size membrane filter and used for inoculation. Soil samples (100 g) were placed in a sterile 1-liter flask with 300 ml of leptospire-free distilled water and mixed by shaking. The suspension was allowed to settle for 5 to 7 min, and then 50 to 60 ml of suspension was allowed to centrifuge at 800 to 900 rpm in a clinical table-top centrifuge for 5 to 7 min. The supernatants were filtered using 0.22 µm membranes and used for inoculation.

Processing of Soil and Water Samples: Soil temperature has a significant role in helping to determine the rate of survival and growth of leptospires. The temperature of soil changes greatly with depth. The air temperature was measured by holding the thermometer at shoulder height still for about one minute and the temperature was recorded. Similarly, temperature of the surface of soil was measured by placing the thermometer flat on the ground and temperature was recorded after one minute. To determine the temperature below the ground surface, a glass rod marked at 1 inch, 2 inches and 6 inches was used.
A hole was made in the ground by pushing the glass rod into the ground till it reaches the desired depth, then the rod was removed and the thermometer was inserted for one minute, then the thermometer was removed and the temperature was recorded quickly. The water temperature was recorded by placing a thermometer in the water sample.

**Measurement of pH:** Soil pH determines the survival period and spread of the organism. Soil pH was normally measured by soil/water slurry. The presence of soluble salts in the soil sample will affect pH. So, Schofield et al.\(^{16}\) prescribed to use a mixture of soil and 0.01 M CaCl\(_2\). The excess salt in this solution masks the effect of differential soluble concentration in individual samples. To determine the soil pH, 5gms of soil sample was taken in the clean glass bowl and it was added to 10 ml of 0.01 M CaCl\(_2\) solution and mixed vigorously. Then the electrodes were placed in the slurry by swirl and recorded the readings like water testing.

**Measurement of moisture content:** The moisture content of the soil samples was determined by placing a known quantity of the sample (5 to 10 g) in a dry aluminum dish and then the sample was dried at 94 to 100°C until the weight remained constant. Then the percentage of moisture content was calculated.

**Determination of organic matter (OM):** Organic matter was determined by ashing 5 grams of moist free soil sample at 360°C for 2 hours in a Muffle furnace. The loss by weight of the sample during this ignition was calculated as the organic matter. Results were reported as percent organic matter by weight in the soil.\(^{13}\)

**Culturing and the determination of leptospires:** The medium used for the isolation of leptospires was the tween 80-albumin medium of Johnson and Harris.\(^8\) The medium was rendered semisolid by the addition of agar to a final concentration of 0.2%, and 5-Fluorouracil (5-FU) was incorporated at a concentration of 100 microgram per ml to minimize contamination. Then the medium was inoculated with 1 ml of sample and incubated aerobically at room temperature for 30 days and examined for the presence of leptospires by dark-field microscopy.

If leptospires were not detectable after 30 days of incubation, the sample was considered to be negative. When necessary, leptospires were separated from contaminants by placing a drop of the mixed culture in the center of Petri plates containing the Tween 80-albumin medium and 1% agar. The leptospires have the ability to migrate through the agar visualized as a veil of growth extending to the periphery of the plate.

**Characterization of isolates:** Characterization of isolates was carried out to find out the nature of organism (Parasitic or Biflexa). The cultures were maintained in EMJH medium containing 1 mg/ml of 5-Fluorouracil in a test tube at an incubation temperature of 30°C. Cells used in the temperature studies were 3 to 5 days old. By using an inoculation loop, one loop full of a cultured organism was transferred to 3ml of fresh EMJH medium containing 1 mg/ml of 5-Fluorouracil and incubated at 5 to 10°C for 7 days at a different interval and the growth was checked in DFM and number of the organism per field were noted.\(^{14}\)

In the low-temperature, parasitic group failed to grow. From these saprophytic and/or pathogenic leptospires were identified. Then, saprophytic leptospires were cultured selectively in medium containing 225 µg/ml 8-azaguanine. The same medium will differentiate saprophyte (which will grow in it) from pathogens (which will not). Later each one of them was sub cultured and stored for further study.

**Serovar identification of leptospires:** Leptospires isolated from the field were identified serologically based on microscopic agglutination test (MAT) as described earlier by using serovar specific monoclonal antibodies. MAT tested cultures were sub cultured in fresh medium and incubated for up to 10 days at 30°C and used for DNA extraction in PCR assay for further confirmation.

**Efficacy of sodium hypochlorite (Chlorine) on Leptospires:** To chlorinate the water, sodium hypochlorite was used. 1000 ml of well water was taken and filtered through Whatmann No. 1 filter paper and autoclaved, then the water was chlorinated in different concentration like 0.25 ppm, 0.5 ppm, 1 ppm, 2ppm, 3ppm, 4ppm by using 4% sodium hypochlorite. When chlorine is added to water, there is a formation of hydrochloric acid and hypochlorous acid. The hydrochloric acid is neutralized by the alkalinity of the water. The hypochlorous acid ionizes to form hydrogen ions and hypochlorite ions. Chlorine acts as the best disinfectant when pH is neutral because of the predominance of hypochlorous acid. When pH value exceeds 8, the hypochlorous acid gets ionized to hypochlorite ions.

\[
\begin{align*}
H_2O + Cl_2 & \rightarrow HCl + HOCI \\
HOCI & \rightarrow H+ OCl
\end{align*}
\]

**Estimation of free residual chlorine in chlorinated water:** Residual chlorine of water is estimated by orthotolidine method. For the test, 0.5ml of orthotolidine reagent was added with 5 ml of chlorinated water and it was mixed rapidly. The color produced was matched against suitable standard colors in the colorimeter. It is essential to take the readings in artificial light within 10 seconds after the addition of reagent to estimate free chlorine in the water.

**Inoculation of different Leptospiral strains in the chlorinated water:** 1 ml of chlorinated water samples in different concentration (0.25 ppm, 0.5 ppm, 1 ppm, 2ppm, 3ppm, 4ppm) were aseptically transferred into sterile vials and seven-day old leptospiral strains belong to following groups like *icterohaemorrhagiae, australis, hebdomodis, canicola* inoculated (0.36x10^7 organisms) as per the method described by Prabhu et al.\(^{13}\) For each strain, antigen control
was maintained without the addition of chlorinated water. Inoculated chlorinated water was examined at 30-minute intervals up to 2 hours using dark field illumination and the number of organisms per field was noted.

**Geographical Information System (GIS) Survey:** A survey was conducted during the seasonal period of heavy rainfall to geocodify the location which is more contaminated and prone to spread of Leptospirosis (Plate 2). Moreover, information was also collected by questionnaire and interview the local people and used for the interpretation of results.

**Meteorological Data:** Various meteorological factors influence health processes either directly or indirectly. Temperature, relative humidity, rainfall and Sunshine are perhaps the most important. Data were collected from government meteorological department, Kodampakkam, Chennai. This data was cross-referred with present research results and environmental monitoring data in geographical information system (GIS) for broad- scale analysis of associations between climate and disease.

**Statistical Analysis:** The sample size required was estimated from an expected prevalence of 50% with a 95% level of confidence and the desired accuracy of 5%. A chi square test was used to detect differences between proportions; a possibility of less than 0.05 was considered statistically significant.

**Results and Dissuasion**

**Soil and Water isolation:** Isolation of parasitic leptospires from environment samples provides an epidemiological status of illness and also helps measure prophylactic methods yet to adapt of regional and national level. In Chennai, no research has been carried out in leptospires isolation from contaminated environment till date. Hence an attempt was made in the present study to examine the distribution of leptospires in water and soil within a given area by an enrichment culture method using a standard volume of sample material. It is clear from the results that the density of leptospires from one body of water to the other is quite variable. The frequency of isolation was highest for the lake 7/16 (43.755%) followed by the pond (18.75%), the river 3/16 (12.5%) and 0% well water samples (Table 1).

The highest positive isolation frequency occurred during the months of August to December 7(53.8%)with maximum and the minimum temperature of 30°C and 13°C respectively. These results are more interconnected with serological report leptospirosis cases in Chennai in the same period.19 This peak period was followed by an interval, January through July in which the number of isolations decreased. Similar seasonal variations have been recorded for the total bacterial flora and the periphytic bacterial population of lakes. It has been reported that the minimal growth-supporting temperature for leptospires is 10 to 13°C and temperature changes within a lake correlated to variations in distribution, in numbers of specific organisms and to biochemical activity.23 There may be two possible explanations for this which may be interrelated. The lake might have reached a higher cell density during the monsoon as compared to the other source. It was also possible that the persistence of leptospires in the lake is correlated with the constant contact of the water with a reservoir of leptospires. This fact was coupled with organic content. A higher percentage of positive samples was achieved with medium or low organic content (45%) which would suggest increased contact with the soil. Since other factors remained nearly identical, in the population reached in the pond originating from the lake.

In this study, 60 soil samples were examined for the presence of parasitic leptospires and 11 (18.3%) were positive (Table 2). This is different from the isolation frequency for water samples taken during the same period and in the same and different areas. The percentage of positive soil samples was slight higher (2.05%) than water isolation. Such evidence indicated the survival of leptospires in the water and more/fewer soil samples. The above information coupled with two other reports of the isolation of leptospires from soils would indicate that the blanket term “water leptospires” may be valid.

Samples taken from the lake had a higher isolation percentage than those taken from other source. A high population density recorded in the immediate adjacent soil (86% of the soil samples taken within 5 meters of the source of the lake were positive) proves clearly that the interaction of the water and soil is responsible for the increase in the number of isolations observed in the lake. It had the highest consistent population of leptospires, and it would be feasible to conclude that this is, in part, due to its constant interaction with the soil and that it serves as the drainage route for the entire area.

In the laboratory as well as under natural conditions, the amount of soil moisture was shown to play a role in the survival of leptospires of the parasitic complex outside the host body. A fluctuation in the bacterial populations of soils has also been associated with variations in moisture and organic content. These two factors appear to play a role in the distribution of leptospires observed in the present study. The greatest number of positive soil samples occurred for those soils in which the moisture content was 65% or more. 83% were compared to 13% for those with less than 65% moisture. The largest numbers of positive soil samples (7/11) were obtained from marsh soils which are high in moisture content and organic matter and contributing 63.3% and it is followed by clay soil and sandy soil (18.1%). No samples were positive from soils strictly dry and gravel type in texture from all the areas. All the isolated soil samples consisted of medium to high organic content which is considered as a supporting factor for leptospires survival.
The present study has established that not all aquatic bodies of water are equally capable of supporting a leptospiral population, but a considerable variation may exist within a given area. The soil, particularly with the high organic matter, is a prime habitat not only for biflexa complex but also supporting to parasitic group. The importance is whether or not those habitats capable of maintaining high populations of free-living leptospires are also those in which members of the parasitic complex survive best outside the host and constitute a source of infection.

Table 1
Consolidated reports of leptospires isolated from water samples

<table>
<thead>
<tr>
<th>Water source</th>
<th>No. of +ve samples / Total No. of sample collected</th>
<th>% of +ve samples</th>
<th>Average temperature</th>
<th>Average pH</th>
<th>Serovar isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>River</td>
<td>2/16</td>
<td>12.50</td>
<td>28</td>
<td>7.5</td>
<td><em>Icterohaemorrhagiae</em> (2) Biflexa</td>
</tr>
<tr>
<td>Pond</td>
<td>3/16</td>
<td>18.75</td>
<td>25</td>
<td>7.2</td>
<td><em>Autumnalis</em> (2) Canicola</td>
</tr>
<tr>
<td>Lake</td>
<td>7/16</td>
<td>43.75</td>
<td>26</td>
<td>7.6</td>
<td><em>Australis</em> (3) <em>Icterohaemorrhagiae</em> (3) <em>Autumnalis</em> Biflexa</td>
</tr>
<tr>
<td>Well</td>
<td>0/16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pipe Line</td>
<td>1/16</td>
<td>6.25</td>
<td>26.3</td>
<td>7.4</td>
<td><em>Australis</em></td>
</tr>
<tr>
<td><strong>Grand Total</strong></td>
<td><strong>13/80</strong></td>
<td><strong>16.25</strong></td>
<td><strong>26.3</strong></td>
<td><strong>7.4</strong></td>
<td><em>Above</em></td>
</tr>
</tbody>
</table>

Table 2
Consolidated reports of leptospires isolated from soil samples

<table>
<thead>
<tr>
<th>Zone</th>
<th>Type of soil</th>
<th>No. of +ve samples/ Total No. of sample collected</th>
<th>Average temperature (°C)</th>
<th>Average pH</th>
<th>Average moisture content (%)</th>
<th>Average organic content (%)</th>
<th>Serovar isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Chennai</td>
<td>Clay</td>
<td>1/4</td>
<td>20</td>
<td>7.1</td>
<td>93.0</td>
<td>40</td>
<td><em>Icterohaemorrhagiae Biflexa</em></td>
</tr>
<tr>
<td></td>
<td>Sandy</td>
<td>1/2</td>
<td>21</td>
<td>7.2</td>
<td>70.0</td>
<td>28</td>
<td><em>Icterohaemorrhagiae</em></td>
</tr>
<tr>
<td></td>
<td>Marshy</td>
<td>2/5</td>
<td>18</td>
<td>7.0</td>
<td>84.0</td>
<td>40</td>
<td><em>Autumnalis</em> <em>Icterohaemorrhagiae Biflexa</em></td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gravel</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>East Chennai</td>
<td>Clay</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Sandy</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marshy</td>
<td>2/4</td>
<td>18</td>
<td>7.0</td>
<td>88.0</td>
<td>39</td>
<td><em>Icterohaemorrhagiae Autumnalis</em></td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>0/2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gravel</td>
<td>0/2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>South Chennai</td>
<td>Clay</td>
<td>0/2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Sandy</td>
<td>0/2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marshy</td>
<td>2/3</td>
<td>21</td>
<td>7.6</td>
<td>73.0</td>
<td>35</td>
<td><em>Hebdomodis Australis</em></td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>0/2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gravel</td>
<td>0/1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>West Chennai</td>
<td>Clay</td>
<td>1/3</td>
<td>19</td>
<td>7.3</td>
<td>92.0</td>
<td>30</td>
<td><em>Icterohaemorrhagiae Biflexa</em></td>
</tr>
<tr>
<td></td>
<td>Sandy</td>
<td>1/2</td>
<td>18</td>
<td>7.2</td>
<td>71.0</td>
<td>25</td>
<td><em>Icterohaemorrhagiae</em></td>
</tr>
<tr>
<td></td>
<td>Marshy</td>
<td>1/5</td>
<td>19</td>
<td>7.3</td>
<td>78.0</td>
<td>33</td>
<td><em>Autumnalis</em></td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gravel</td>
<td>0/4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total/Average</strong></td>
<td><strong>11/60</strong></td>
<td><strong>19.5</strong></td>
<td><strong>7.2</strong></td>
<td><strong>81.0</strong></td>
<td><strong>38</strong></td>
<td><em>Above</em></td>
<td></td>
</tr>
</tbody>
</table>

NC-North Chennai, EC-East Chennai, SC-South Chennai, WC-West Chennai
Plate 1a: Some of the soil and water sample collected area (GIS)

Plate 1b: Some of the soil and water sample collected area (GIS)
Efficacy of Sodium hypochloride: As the number of leptospiral cases increased during monsoon months in Chennai city, the present investigation was focused upon to unravel the efficiency of chlorine at different ppm on selected leptospiral strains. From that, autumnalis serogroups in Chennai city are found to be very sensitive to chlorine even at lower concentration and they are observed to be completely inactivated in the study period of two hours (Figure 1). Other predominant member of serogroups icterohaemorrhagiae, australis and hebdomidis were resistant at lower concentrations (up to 1.5ppm) (Table 3).

From this study it was found that the chlorine efficacy against leptospires was very minimal except for one or two serogroups, hence either we have to maintain desired concentration of chlorine or some other disinfective methods to be designed to prevent water borne leptospirosis through potable water supply in future.

Meteorological Analysis: Two significant troughs of low-pressure systems and TC Cliff were responsible for rains in all parts of Chennai forecasted by metrological department (Nungambakkam) which has also recorded a new high monthly rainfall during the month of October to December in last few years considered as major reason for spreading of pathogenic leptospires by water and soil contamination. Outbreaks of leptospirosis associated with watersports have demonstrated the ability of pathogenic leptospira species to survive in water for extended periods. Survival of pathogenic leptospires in the environment is dependent up on several factor, including pH, temperature, humidity and sunshine. In soil saturated with rainwater, leptospires have been found to survive for at least 3 weeks.

Extreme flooding or hurricanes during this time leads to the endemic outbreak of leptospirosis in and around Chennai. A case–control study showed that a 15-fold risk of disease was associated with walking through flooded waters. High-risk factors also included having rodents or dogs in the household, frequent contact with water or reservoir animals. More incidences of affected cases had shown two weeks after severe flooding in the area. Poor living conditions and sanitation were associated with increased risk.

According to the report of Smith and Self and Turner, the role of rain fall in leptospirosis outbreak was considered as follows: Leptospires were shed by reservoir hosts and get accumulated in moist soil during dry periods. The spirochete requires a warm, moist climate of 25°C, water, and soil with a pH level of 7.0–8.0 for optimal survival outside the host. When precipitation from a heavy rainstorm exceeds the capacity of the soil to absorb the moisture, leptospires are swept away from contaminated soil into water bodies and lead to increased outbreak.
Table 3
Preparation of different concentration of chlorine in water

<table>
<thead>
<tr>
<th>Water (ml)</th>
<th>Sodium hypochlorite (4%)</th>
<th>Chlorine concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0.00626 (or) 6.25 µl</td>
<td>0.25</td>
</tr>
<tr>
<td>1000</td>
<td>0.0125 (or) 12.5 µl</td>
<td>0.5</td>
</tr>
<tr>
<td>1000</td>
<td>0.025 ml (or) 25 µl</td>
<td>1</td>
</tr>
<tr>
<td>1000</td>
<td>0.05 ml (or) 50 µl</td>
<td>2</td>
</tr>
<tr>
<td>1000</td>
<td>0.075 ml (or) 75 µl</td>
<td>3</td>
</tr>
<tr>
<td>1000</td>
<td>0.1 ml (or) 100 µl</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 1: Effect of chlorine on leptospires serovars

Conclusion
By comparison of metrological data with our findings, it was concluded that heavy rainfall, higher humidity, and low temperature favor the survival of leptospires in the environment and spreading. Based on the isolation and observation, most the water/soil were contaminated with sewage, rodents, and animal excretions. The higher rate of soil and water isolation carried out in the north Chennai followed by the west Chennai indicates that these two regions are more prone to leptospirosis and need more attention.

For successful control, the passive surveillance system should be strengthened among the public, clinicians, physician, governmental and non-governmental organizations to reduce the incidence and fatal death cause to humankind and also minimize economic ablation of the individuals and the country.

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