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# Assessment of bacterial composition in the natural waters from Karaikal district, southeast coast of India in view of cholerae outbreak

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## ABSTRACT

***Karaikal, a rapidly developing port city at the southeast coast of India that is facing intensive human activities like rapid developments of harbour, urban and industries besides agricultural practices. Recent cholera outbreaks due to Vibrio cholerae contamination in the natural waters demanded this study to assess the natural water quality along with bacterial composition in and around Karaikal City. The essential water quality parameters had been tested to find out the variations in their levels which favour the bacterial contamination and hence, the essential parameters such as pH, temperature, salinity, dissolved oxygen, nitrite, nitrate, and ammonia were assessed and the values were recorded in the range of 6.9 - 7.4, 28 - 30°C, 1.0 - 20.5 psu, 1.4 - 8.6 mg/L, 0.09 - 0.157 µM/L, 0.012 - 0.174 µM/L, and 0.10 - 0.64 µM/L respectively. Most of the assessed parameters were found within the safety standard except salinity, dissolved oxygen and ammonia that were noticed with significant variations. The elevated levels of ammonia indicating potential contamination due to untreated urban sewage. Microbiological analysis confirmed the presence of pathogenic bacteria, including Staphylococcus sp., Escherichia coli and Bacillus species. Therefore, this study recommends a regular monitoring of the water quality parameters and microbial contamination in the natural potable waters to enforce effective control measures to avoid the recurrence of such diseases.***

***KEYWORDS: Karaikal, Cholera Outbreak, bacterial Contamination, Water Quality Parameters.***

## 1.INTRODUCTION

Aquatic and dependant ecosystems rely heavily on water quality management which supports life on the earth both plants and animals including human needs. Water quality is characterized by its standard physical, chemical and biological qualities. The natural water bodies such as ponds, lakes and rivers serve as vital sources of water for domestic usage (Chia et al., 2009). The increased demand for quality water for human need has led to the regular assessment of chemical, physical and biological properties of natural water resources (Sawant and Telave, 2009). Natural waters often contaminated with microbes particularly, by bacteria due to the discharges of sewage, industrial and agricultural wastes into the natural water bodies. Thus, contaminated water becomes the sources for waterborne disease like typhoid, cholerae, pneumonia, diarrhea etc. According to the World Health Organization, about 80% of illnesses and deaths among children globally are due to contaminated drinking water (WHO, 2003). Cholera epidemics, caused by *Vibrio cholerae*, have been reported in several countries, (Uzoigwe et al., 2012). In July 2022, Karaikal City experienced a cholera outbreak attributed to water contamination, resulting in a state of emergency. The outbreak affected approximately 2900 people and 750 with serious illness were reported (Fernando, 2022). Referring to this situation, an assessment of microbial quality and water quality parameters in the natural waters of Karaikal city was planned and executed to understand the extent of contamination and to make public consciousness and their responsibility in compliance with guidelines to ensure the potability of natural waters in and around Karaikal city and also ensure the effectiveness of the sewage treatment system etc.

## 2. MATERIALS AND METHODS

### 2. 1. Sampling sites

This study focused on five selected natural water bodies within the Karaikal District, each site faces unique challenges related to water quality. Karaikal medu (10°56'24.40"N, 79°50'45.26"E) has two rivers vulnerable to receive pollutants from the industrial discharges, agricultural runoff, urban sewage and fishing activities. T.R. Pattinam is a coastal town (10°50'13.47"N, 79°50'19.91"E) receives considerable amount of urban sewage and agricultural runoff that enough to cause severe impact in the water quality. The Karaikal fishing harbour (10°54'27.14"N, 79°50'8.04"E) is a commercial hub facing potential pollution from oil spillage and chemical waste affecting local ecosystems. Vanjur (10°52'27.02"N, 79°50'14.60"E) is being affected by the poor quality of water that is often polluted from industrial activities and improper waste disposal poses significant challenges. Thirunallar, a spiritual town (10°55'25.56"N, 79°48'50.59"E) confronts with water pollution from agricultural runoff, domestic sewage and tourism that impact nearby rivers and ponds (Fig: 1).

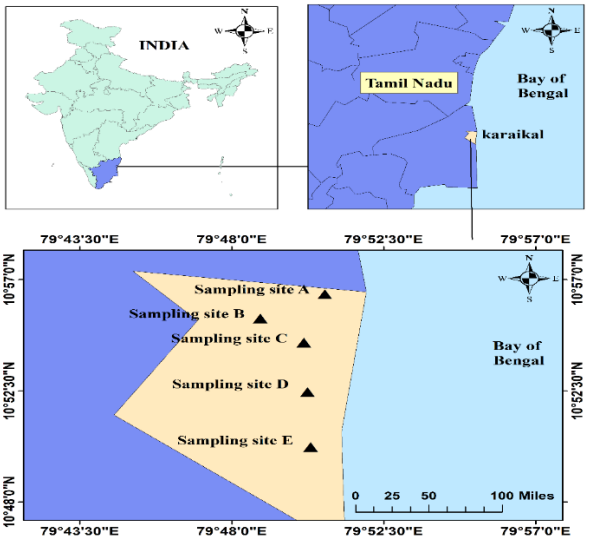


**Fig 1: Map showing the study area.**

### 2. 2. Sampling methods and analysis of water quality parameters

The water samples were collected in September 2022 (after two months of post cholerae outbreak) by using sterilised polyethylene bottles (1L) employing all standard sampling procedures. I*n situ* measurements of physical parameters such as temperature, pH, salinity was made. Remaining samples were preserved at 4°C and brought to the laboratory for the analysis of dissolved oxygen (DO) and nutrients (nitrate, nitrite and ammonia). The assessment was carried out by following the standard methods laid down in APHA (2011) and Trivedy & Goel (1987).

### 2. 3. Sampling methods and Microbiological Analysis

Water samples were collected aseptically from the pond at the specified sites. Using pre-sterilized (1L) bottles, triplicate samples were taken from depths of up to 20cm, at each location. All the samples were carefully preserved and transported to the laboratory for microbial analysis (Table 1).

**Table 1: Media used for isolation and enumeration of bacterial groups**

| SPECIFIC MEDIA | TYPES OF BACTERIA |
|---|---|
| Nutrient agar | Total Viable Counts (TVC) |
| Eosin Methylene Blue Agar | *Escherichia Coli* |
| Manitol Salt Agar | *Staphylococcus* species |
| MYP agar | *Bacillus* species |
| TCBS agar | *Vibrio* species |

All the media were prepared using double-distilled water and sterilized before use. Bacterial populations were estimated using the spread plate method, where 0.2 mL of appropriate dilutions was spread on nutrient agar and selective media. After inoculation, the plates were incubated and maintained at a temperature of 28 ± 2°C for 18–24 hours. Following incubation, the plates were examined, and the total Colony Forming Units (CFU) were estimated and expressed as CFU/mL. Total Viable Count (TVC) and pathogenic bacteria were assessed according to the APHA (2012) guidelines. All the experiments were conducted in triplicate. Representative colonies were subsequently sub-cultured for specific bacterial identification. Specific media plates were incubated at 37°C for 24–48 hours, and the final colony counts were noted. Colonies from the specific media were isolated and identified using biochemical and morphological tests, following the methods described by Bergey et al. (1994), along with gene sequencing

## 3. RESULTS AND DISCUSSION

### 3. 1. PHYSICO-CHEMICAL PARAMETERS

Water temperature was found ranged from 28°C to 30°C. The temperature did not show many variations, the slightly higher value was recorded in Vanjur (30°C) and the lower temperature in T. R. Pattinam (28°C) (Fig. 2a). Water temperature considered as a crucial factor that influences various biological processes within aquatic ecosystems, including metabolic rates, reproductive cycles and the distribution of aquatic organisms. At times at summer season, the temperature was reported to have highest levels as recorded by Elayaraj and Selvaraju, (2015) between 29°C and 41.1°C at Thachan Pond, Chidambaram. The consistency in temperature ranges across different studies underscores the influence of regional climatic conditions on aquatic ecosystems (Brown et al., 2013; Williams and Jha, 2015).

The minimum and maximum pH values in the water bodies of Karaikal District ranged from 6.9 (Vanjur) to 7.4 (Karaikal fishing harbor) (Fig. 2b). In general, the pH was found with optimal to the fresh water standard is concerned and usually it was reported that the urban and industrial wastes contain acid based contaminants that reduces the pH levels well below the optimal range (Mahananda et al., 2010). The recorded pH range in this study was comparable with previous findings of Ravisankar et al. (2008) in nearby Karaikal area, who had reported the pH values in the range of 6.5 to 7.07 in tsunami affected areas of Sirkazhi Taluk, Nagapattinam District, Tamil Nadu.

Salinity levels were found with wide variations, ranging from 1.0 to 20.5 (psu), with the highest salinity recorded at Karaikal Fishing Harbor (20.5 psu) and the lowest at T. R. Pattinam (1.0 psu) (Fig. 2c). These variations in salinity are influenced by several factors, including tidal effects, freshwater inflow, precipitation and several anthropogenic activities. Studies made by Venkatesan et al. (2013) and Kumar et al. (2015) had highlighted the impact of seasonal changes and influence of the human activities on salinity fluctuations in coastal and estuarine environments. This study indicates that the coastal ecosystems significantly affects the freshwater system through infiltration of saline water and alter the quality of natural waters which favours the microbial contamination.

Dissolved oxygen (DO) levels in the study area varied significantly, ranging from 1.4 mg/L (Karaikal Medu) to 8.6 mg/L (T. R. Pattinam) (Fig. 2d). DO is found to be a crucial parameter for assessing water quality, as it directly impacts the survival and health of aquatic organisms. The observed range of DO levels suggests that the natural waters in and around Karaikal city has lower to higher contamination of organic matter that requires significant amount of DO for effective decomposition. This variability highlights the influence of anthropogenic activities and natural processes on water quality. Similar studies made by Patil et al. (2012) and Nandan et al. (2017) reported comparable DO variations in other aquatic ecosystems emphasizing the importance of continuous monitoring and management to manage the impacts of contaminants and to avoid further unwanted consequences in future.

So far as the nutrient concentrations of nitrite, nitrate and ammonia are concerned, ammonia showed slightly

elevated levels which could be attributed to sewage contamination. The assessed nutrient values were found in the range between 0.012 to 0.157 µM/L, 0.012 to 0.174 µM/L, and 0.10 to 0.64 µM/L respectively. The lower and higher nitrite concentrations were recorded at T. R. Pattinam (0.012 µM/L) and Karaikal Medu (0.157 µM/L) respectively whereas the nitrate concentrations were found to have varied from 0.012 µM/L (Vanjur) to 0.174 µM/L (Karaikal Medu). Minimum level of ammonia was recorded from Karaikal Fishing Harbor (0.10 µM/L) and the maximum was recorded from T. R. Pattinam (0.64 µM/L) (Fig 2e, 2f and 2g). The values of the nutrients assessed in this study were found within the range values reported by Mishra et al. (2014), Elayaraj and Selvaraju (2015), Rajagopal et al. (2010), Kumar et al. (2018) and Patil et al. (2012). Elevated ammonia levels at certain sites indicate potential contamination from agricultural runoff and sewage, which requires further regular monitoring to prevent adverse environmental and health impacts.

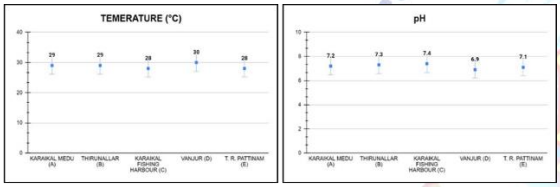


Fig: 2a. Temperature (°C)




Fig: 2b. Water pH

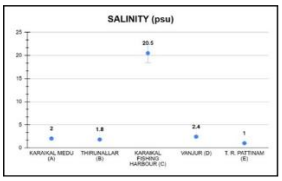


Fig: 2c. Salinity (psu)

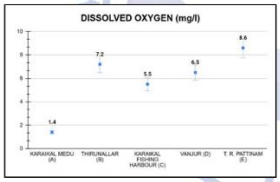


Fig: 2d. Dissolved Oxygen (mg/L)

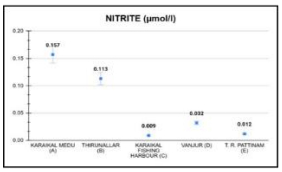


Fig: 2e. Nitrite (µM/L)

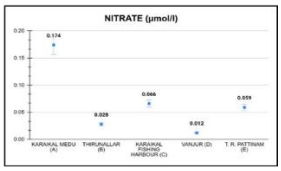


Fig: 2f. Nitrate (µM/L)

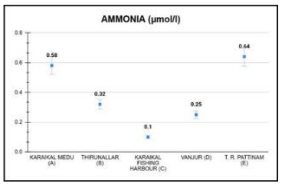


Fig: 2g. Ammonia (µM/L)

**Fig: 2. Shows the levels of water quality parameters in the above-mentioned sampling sites.**

### 3. 2. Bacterial Analysis

Bacteria were partially identified based on the morphological and biochemical analysis followed by Bergey's Manual of Determinative Bacteriology (Bergey's et al., 1994). The Total Viable Count (TVC) ranged from $3.24x10^6$ to $1.06x10^6$ CFU/mL/$10^3$. Pathogens such as *Staphylococcus* species (16 to 39 CFU/mL), *Bacillus* species (39 to 67 CFU/mL) and *Escherichia coli* (32 to 78 CFU/mL) were detected. The bacteriological quality of natural waters within the Karaikal district showed elevated levels of total viable count (TVC). The presence of pathogens such as *Escherichia coli*, *Staphylococcus* species and *Bacillus* species in the natural waters attributed to the water contamination whereas *Vibrio Cholerae* was not found detected in this study and this might be due to the effective treatment measure that was adopted after the outbreak of cholera. Previous studies carried out by Sankar Ganesh et al. (2015) reported the bacteriological composition in freshwaters in the Thirunallar region, Karaikal district who had reported elevated levels of faecal coliforms (75-400/mL). Kumarasamy et al. (2009) studied the seasonal variations of pathogens in the delta regions of the Cauvery River, South India. Their findings showed elevated levels of Total Viable Count ($6.2\text{-}26.0x10^4$ CFU/mL) during the monsoon season compared to the summer season ($5.2\text{-}20.0x10^4$ CFU/mL). They also reported enhanced levels of total coliforms ($4.1\text{-}21.0x10^3$ CFU/mL) and total streptococci ($2.3\text{-}120x10^3$ CFU/mL). These results exceed normal levels, indicating that natural waters in and around Karaikal district are being contaminated by human activities but there was no report available on the presence of *Vibrio Cholera*. However, Regulatory policy decisions are needed to ensure the potability of natural waters.

### 3. 3. Results of biochemical analysis

Bacteria screened using selective media from the collected water samples were subjected to biochemical tests for further confirmation. The tests included Gram staining, indole test, methyl red test, Voges-Proskauer test, nitrate reduction test, and others. The results confirmed the presence of *Staphylococcus* species, *Bacillus* species and *Escherichia coli*. Elevated levels of ammonia and the presence of pathogenic bacteria suggest that there is a need for improved waste water management practices, particularly in the areas affected by agricultural runoff and sewage discharge.

### 3. 4. Results of gene sequencing

The dominant species of *Staphylococcus caprae* and *Bacillus licheniformis* were confirmed through PCR identification using 16S rRNA F & R primers (standardized by CAGL). The analysis of DNA sequences using designated primers (Bact 16S 533 F and Bact 16S 1100R) from samples collected from a freshwater pond provided bacterial composition in these habitats. NCBI's BankIt protocol was followed for generating sequence information. All sequences were submitted with unique identification names and accession numbers (GenBank. S.No. OR731396 and GenBank S.No. OR759492).

***Staphylococcus caprae*:** The sequence derived from the sample (*Staphylococcus* species) displayed a 99% similarity with *Staphylococcus caprae*. It is a Gram-positive bacterium commonly found in various environmental niches, including freshwater habitats. (GenBank. S.No. OR731396).

***Bacillus licheniformis*:** The sequence derived from Sample (*Bacillus* species) shows a 99% similarity with *Bacillus licheniformis*. It is a Gram-positive bacterium known for its adaptability and potential applications in various fields. (GenBank S.No. OR759492).

Based on the nature, neither *Staphylococcus caprae* nor *Bacillus licheniformis* inherently pose significant harm to human health or the environment. However, it is important to note that they have ability to survive in the treated environment and it has to be considered that the potential for harm or disruption to the ecosystem can vary depending on the quantity or load of those species. Regular assessment of these bacteria along with *Vibrio cholera* within these natural waters becomes imperative to provide necessary procedures and measures.

### 3. 5. Phylogenetic analysis

The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Fig. 3). The analysis involved eight nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted using MEGA Version 10.1 (Kumar et al., 2018). The phylogenetic tree showed two major clades: one including *Bacillus licheniformis* (present study), *Bacillus* species (*in firmicutes*), *Bacillus haynesii* strain, and *Niallia circulans*, and the other including *Staphylococcus* species, *Staphylococcus caprae* and *Staphylococcus epidermidis*. The sample *Staphylococcus caprae* (OR731396) showed 33% similarity to *Bacillus* species (*in firmicutes*) (MH917294.1), while *Bacillus licheniformis* (OR759492) showed 65% similarity to *Staphylococcus* species (PP 434576.1).

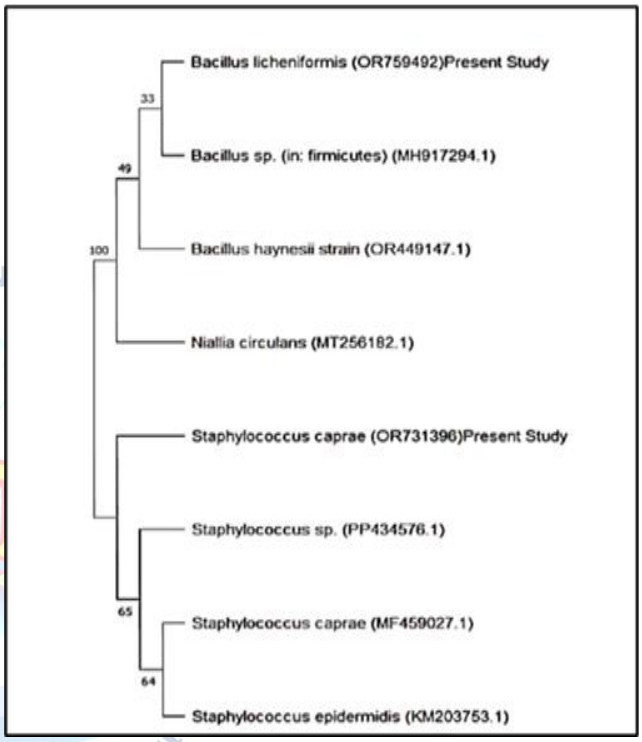


**Fig: 3. Shows the similarity index between the species.**

## 4. CONCLUSION

The assessed parameters were generally within acceptable ranges for human use. However, some locations were found significant variations of salinity, dissolved oxygen and ammonia which could be attributed that these variations would potentially alter the natural quality and harm aquatic life and affect human health. Therefore, this study proposes for regular assessment of these parameters in order to prevent contamination from the sewage and agricultural runoff. The microbial analysis confirmed the presence of *Staphylococcus caprae, Bacillus licheniformis* and *Escherichia coli* in the natural waters in and around Karaikal district but it was not confirmed for the presence of *Vibrio Cholera* due to stringent environmental management

system was under practice. However, Regulatory policy decisions re needed to ensure the potability of natural waters. In general, the present study confirms that the natural waters in and around Karaikal district are gradually being contaminated with sewage and agricultural wastes. The contamination by pathogens needs to be addressed seriously to prevent the recurrence of waterborne disease outbreaks.

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**Conflict of interest statement**

Authors declare that they do not have any conflict of interest.

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