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PREVALENCE OF *VIBRIO SPP*. AND THEIR VIRULENCE GENES IN VEMBANAD LAKE AND ASSOCIATED CANALS AND COASTAL WATERS DURING THE PRE-MONSOON SEASON

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ABSTRACT

Vibrio cholerae, the pathogen responsible for cholera, flourishes in environments characterized by inadequate sanitation and contaminated water sources. This study aimed to assess the prevalence of *Vibrio cholerae* and its associated virulence genes in coastal waters and selected locations within Vembanad Lake through a culture-independent methodology. Metagenomic DNA extracted from water samples was subjected to screening utilizing PCR primers targeting the outer membrane protein (*omp*), cholera toxin gene (*ctx*), and toxin-regulating protein (*toxR*). The findings revealed the presence of *Vibrio cholerae* at all 14 monitoring stations within the connecting canals, whereas 58% of the coastal water samples tested positive for the *omp* gene. Notably, none of the samples obtained from Vembanad Lake indicated the presence of *Vibrio cholerae*. Among the 21 positive samples analyzed, 8 exhibited the *toxR* gene, while none contained the *ctx* gene, thus excluding the presence of toxigenic *Vibrio cholerae*. Nevertheless, the detection of the *toxR* gene suggests a potential for pathogenicity through horizontal gene transfer under favourable conditions. This research underscores the ecological dynamics of *Vibrio cholerae* within the Vembanad Lake ecosystem and highlights the imperative for continuous monitoring to avert potential outbreaks.

Keywords: Cholera, *Vibrio cholerae*, Vembanad Lake, *omp* gene, *toxR* gene, *ctx* gene

INTRODUCTION

Microbial pollution in drinking water sources and aquatic ecosystems is a major global concern. It is estimated to cause approximately half a million diarrheal deaths each year due to water-associated diseases. The death rate of diarrhoea in India is 45 per 100,000 population (Behera and Mishera,2022) Therefore, it is crucial to prevent human interaction with contaminated water bodies.

Cholera, caused by *Vibrio cholerae,* is a significant waterborne disease. (Ojeda Rodriguez *et al.,*2024). Cholera outbreaks are common in areas with sewage contamination of drinking water, faecal contamination of water sources (Muzembo *et al.,*2022).

Many of the coastal water's fauna and flora, including phytoplankton and zooplankton, act as reservoirs and vectors of *Vibrio cholerae* (Racault *et al.,*2019). These reservoirs and vectors may help *Vibrio cholerae* and other *Vibrio* species to survive in unfavourable conditions or aid in their regional transportation (Stewart *et al.,*2008). In the current study, molecular tools are employed to monitor the distribution of *Vibrio* species and *Vibrio cholerae* in selected locations along the coastal regions near Vembanad lake in Kerala.

Prevalence of *Vibrio cholerae* were confirmed by the presence of *omp* gene and the pathogenic potential of *Vibrio cholerae* were assessed by with the presence of *toxR* gene and *ctx* gene. The toxin regulatory gene(*toxR*) control the expression of *ctx* gene and the cholera toxin gene (*ctx*) encodes cholera toxin, which causes diarrhoea.

MATERIALS AND METHODS

Sample Collection

39 water samples were collected from selected locations of Vembanad Lake, associated canals and coastal waters during pre-monsoon season (12 seawater samples, 13 water samples from Vembanad lake and 14 water samples from associated canals of Vembanad lake). (Table 1, Fig 1). The water samples were collected in sterile polypropylene bottles of carrying capacity 1 litre and the sampling bottles were labeled properly.

Table 1: Table Showing the List of Water Samples Collected for Study

SL NO	Sample Code	Sampling Stations
1	WID1A	MUNAMBAM SHORE
2	WID1B	MUNAMBAM 1/2KM
3	WID2A	CHERAYI SHORE
4	WID2B	CHERAYI 1/2KM
5	WID3A	KUZHUPILLY SHORE
6	WID3B	KUZHUPILLY 1/2KM
7	WID4A	PUTHUVYPIN SHORE
8	WID4B	PUTHUVYPIN 1/2KM
9	WID5	KANNAMALLY
10	WID6A	CHELLANM SHORE
11	WID6B	CHELLANAM 1/2 KM
12	WID7	ANDHAKARNAZHI
13	WOS1	MUNAMABAM DIPNET
14	WOS3	KUZHUPILLY CANAL
15	WOS4	PUTHUVYPIN
16	WOS5	GCDA
17	WOS6	MATTANCHERY
18	WOS7	MARINE DRIVE
19	WOS8	NETTOOR CAGE
20	WOS9	KANNAMALY CANAL
21	WOS10	AROORKUTTY
22	WOS11	ANDHAKARNAZHI CANAL
23	WOS KBM	COCHIN BARMOUTH
24	WOS CC	CHELLANAM CANAL
25	WOS KCK	KUZHUPILLY CHEMEENKETTU
26	WOS MC	MUNAMBAM CAGE
27	VLS1	BARMOUTH
28	VLS2	VALLARPADAM
29	VLS3	WELLINGTON ISLAND
30	VLS4	AROOR
31	VLS5	VADUTHALA
32	VLS6	PERUMBALAM
33	VLS7	MURIMJAPUZHA
34	VLS8	THAVANAKKADAV
35	VLS9	THANNERMUKKAM
36	VLS10	KANNANKARA
37	VLS11	PATHIRAMANAL ISLAND
38	VLS12	MUHAMMA
39	VLS13	NORTH KAINAGIRI

- 1/2 KM means 1/2KM away from shore
- WID- code for sea water samples
- WOS -code for samples collected from associated canals of Vembanad lake
- VLS-code for water samples collected from different sample stations of Vembanad lake

Fig 1: Sampling Site Map

Sample Processing

The total microbial load of the 1L water sample was concentrated by filtration onto membrane discs (pore size: 0.2 µm and diameter 47mm) made of mixed cellulose esters (Millipore; GTTP2500). The membranes after filtration were stored under sterile condition in fresh zip lock covers at

-20ºC freezer until DNA extraction. All the samples were labelled properly.

DNA Extraction from Filter paper

DNA was extracted from the sample using NucleoSpin Soil kit (Macherey-Nagel, Germany) with minor modifications (Vautier *et al.,*2020). The membrane samples were cut into small pieces aseptically with the aid of sterile scissors and forceps and transferred into a microcentrifuge tube of carrying capacity 2 ml. 525µl of lysis buffer were added to the microcentrifuge tube containing chopped filter paper. It is vortexed in the presence of 2-3 sterile glass beads diameter 3mm for 1 minute. Then 5 µl lysozyme (50 mg/ml) was added and the tube was incubated at 37^{0}C for 1 hour. After incubation to the same tube 60 µl 10% SDS and 3 µl Proteinase K (20 mg/ml) were added and mixed properly. The tube was again incubated; at 55^o C for overnight in a dry bath. The next day, one volume of Chloroform–Isoamyl Alcohol (24:1) was added to the sample; mixed properly and then spun at 10,000xg for 10min at RT. Then the aqueous phase from the sample was transferred in to a fresh labelled tube. This step was repeated once again and then to the aqueous phase collected, 150 µl of Buffer SL3 was added and vortexed for 5 seconds and then the tube was incubated at 0-4°C for 4 min. The tube was spun at 11,000xg for 2 min and the clear supernatant was passed through spin column for Inhibitor removal. The collected flow through was treated with 250µl of Buffer SB; mixed by vortexing for 5 seconds and then transferred to spin column assembly for DNA capture. The membrane with DNA was washed four times; 1st time with Buffer SB (500µl) and then with Buffer SW1 (500µl) and 3rd and 4th wash with buffer SW2(650ìl). A Final spin at 11,000xg for 2 minutes was given to dry the spin column; then the collection tube was replaced with a fresh sterile Microcentrifuge tube. The DNA on the membrane was eluted with 60µl of Buffer SE. The quality of DNA was checked by agarose gel electrophoresis.

PCR Based Screening of Samples for *Vibrio cholerae*

The presence of *Vibrio cholerae* in water samples were confirmed by PCR amplification with *Vibrio cholerae* specific primer pair. The PCR was set in a 20µl reaction volume containing 1 µl DNA (10–50 ng), 1µl each of Forward and Reverse primers (10 picomoles ìl-1) and 10µl Emerald Amp GT PCR master mix (Takara). The cycling conditions used are as follows: initial denaturation at 95 °C for 2 min, followed by cycle denaturation at 95 °C for 40 s, annealing at 58-60 °C for 40 seconds, extension at 72 °C for 45 seconds for a total of 30 cycles and a final extension for 7 minutes at 72 °C The success of PCR reaction was confirmed by running 5µl PCR product on 1% agarose gel (impregnated with ethidium bromide) at 120V; for ~45 minutes in 1X TAE Buffer. The image of the gel was recorded with the UV gel documentation system for future reference. 100bp DNA ladder from Thermo was loaded parallel to PCR products as a size marker. Three pairs of primers were used in this work. 1st set pair is specific for *Vibrio cholerae omp*(F5'CACCAAGAAGGTGAC TTTATTGTG 3'; R- 5' GAACTTATAACCAC CCGCG 3'). The 2nd and 3rd set primers are used for checking if the Cholerae positive samples contain any virulence genes in them; or they are simple environmental *Vibrio cholerae* which are non-pathogenic in nature. *toxR* (F- 5' CCTTCGATCCCCTAAGCAATAC 3'; R-5' AGGTTAGCAACGATGCGTAAG 3') and *ctx*(F-5' AACTCAGACGGGATTTGTTAGGC 3'; R-5' TCTCTGTAGCCCCTATTACGATGT 3') respectively(Krishna *et al.,* 2020).

RESULTS

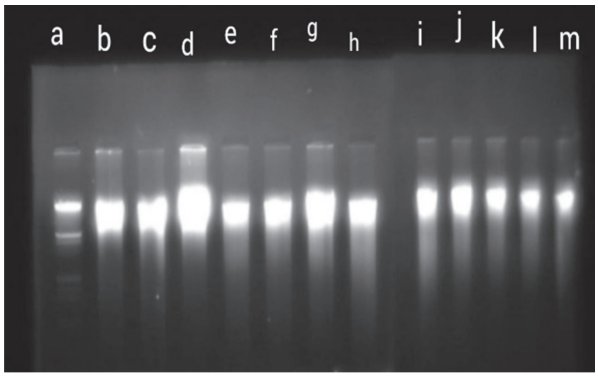

Fig 2: Representative images of genomic DNA extracted from filter paper

Table 2 SAMPLE STATION DETAILS

COASTAL STATIONS		ASSOCIATED CANALS OF VEMBANAD LAKE STATIONS		VEMBANAD LAKE SATIONS	
WID	RESULT	WOS	RESULT	VLS	RESULT
WID 1 A	Positive	WOS 1	Positive	VLS 1	Negative
WID 1B	Negative	WOS 3	Positive	VLS 2	Negative
WID 2A	Negative	WOS 4	Positive	VLS 3	Negative
WID 2B	Positive	WOS 5	Positive	VLS 4	Negative
WID 3A	Positive	WOS 6	Positive	VLS 5	Negative
WID 3B	Negative	WOS 7	Positive	VLS 6	Negative
WID 4A	Negative	WOS 8	Positive	VLS 7	Negative
WID 4B	Negative	WOS 9	Positive	VLS 8	Negative
WID 5	Positive	WOS 10	Positive	VLS 9	Negative
WID 6A	Positive	WOS 11	Positive	VLS 10	Negative
WID 6B	Positive	WOS KBM	Positive	VLS 11	Negative
WID 7	Positive	WOS CC	Positive	VLS 12	Negative
		WOS KCK	Positive	VLS 13	Negative
		WOS MC	Positive		

DNA isolation was followed by PCR amplification to determine the prevalence of *Vibrio cholerae*. As shown in Table 2, out of 12 coastal water samples (WID), 7 tested positive for *Vibrio cholerae*. All the 14 water samples collected from the associated canals of Vembanad Lake (WOS) were positive for *Vibrio cholerae*, whereas none of the 13 water samples collected from Vembanad Lake (VLS) showed any presence of *Vibrio cholerae*.

The presence of *Vibrio cholerae* was confirmed by PCR amplification targeting a fragment of the outer membrane protein (*omp*) gene, which is specific to *Vibrio cholerae* (Fig 3).

In total, 39 water samples examined,21 tested positive for the *omp* gene, confirming the presence of *Vibrio cholerae*. A summary comparison of sample station results is provided in Table 3.

Table 3: Table showing the *comp*arison between different sample stations for the prevalence of *Vibrio cholerae*

Sampling Area	No: of samples examined	No: of samples showing positive results for the presence of *Vibrio cholerae*
Coastal waters	12	7
Associated canals	14	14
Vembanad lake	13	0

PCR Based Screening to Check the Virulence of *Vibrio cholerae*

PCR amplification was repeated for the samples which shown the presence of *Vibrio cholerae*. The cholera positive samples were re-examined for the presence of *toxR* gene (Figure 4) and *ctx* gene (Fig 5). *toxR* genes are toxin regulator genes, which refers to the transcriptional regulatory genes that control the expression of cholera toxin genes (*ctx*).

As the table 4 indicates 1 out of 7 WID and 7 out of 14 WOS water samples showed the presence of *toxR* gene. None of the VLS samples were examined for the presence of *tox R* gene because of the complete absence of *Vibrio cholerae* in these water samples. And none of the water samples tested positive for the presence of *ctx* gene.

Table 4: Table showing the c*omp*arison between different sampling areas for the presence of *ToxR* Gene

Sampling Area	No: of Samples Analyzed	No: of samples in which tox R gene is present
Coastal waters	7	1
Associated canals	14	7
Vembanad lake	0	0

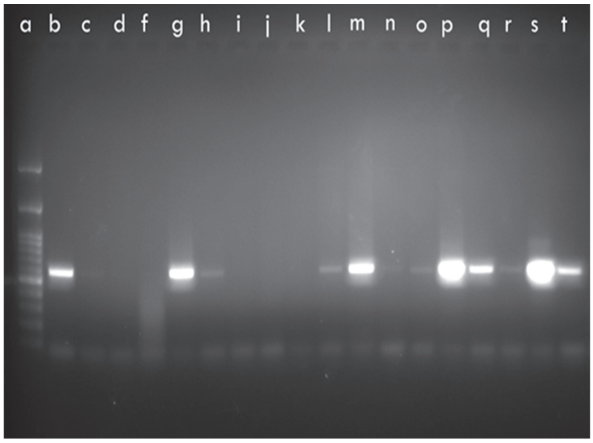

Fig 3: Representative image obtained after screening for *Vibrio cholerae*

Well (a). 100bp ladder; Well (b). Positive control; Well (c)-(t). test samples

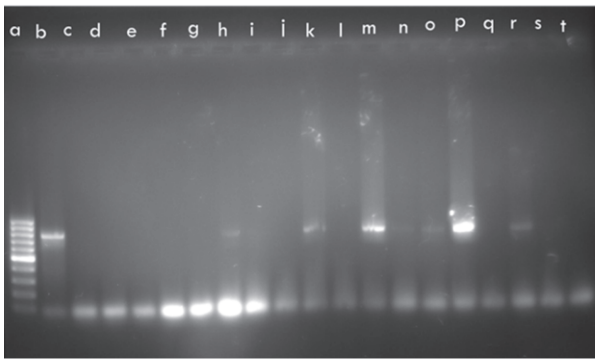

Fig 4: Representative image obtained after screening for *toxR* Gene

Well (a). 100bp ladder; Well (b). Positive control; Well (c)-(t). test sample.

Table 5: Table showing the results obtained after PCR amplification for the prevalence of *omp* gene, tox R gene and *ctx* gene

SL NO	Sample code	V.cholerae *omp* gene Result	V.cholerae *toxR* gene Result	V.cholerae *Ctx* gene Result
1	WID1A	+	_	_
2	WID1B	_	_	_
3	WID2A	_	_	_
4	WID2B	+	_	_
5	WID3A	+	_	_
6	WID3B	_	_	_
7	WID4A	_	_	_
8	WID4B	_	_	_
9	WID5	+	_	_
10	WID6A	+	_	_
11	WID6B	+	+	_
12	WID7	+	_	_
13	WOS1	+	+	_
14	WOS3	+	_	_
15	WOS4	+	_	_
16	WOS5	+	+	_
17	WOS6	+	_	_
18	WOS7	+	+	_
19	WOS8	+	+	_
20	WOS9	+	_	_
21	WOS10	+	+	_
22	WOS11	+	+	_
23	WOS KBM	+	_	_
24	WOS CC	+	+	_
25	WOS KCK	+	_	_
26	WOS MC	+	_	_
27	VLS1	_	_	_
28	VLS2	_	_	_
29	VLS3	_	_	_
30	VLS4	_	_	_
31	VLS5	_	_	_
32	VLS6	_	_	_
33	VLS7	_	_	_
34	VLS8	_	_	_
35	VLS9	_	_	_
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37	VLS11	_	_	_
38	VLS12	_	_	_
39	VLS13	_	_	_

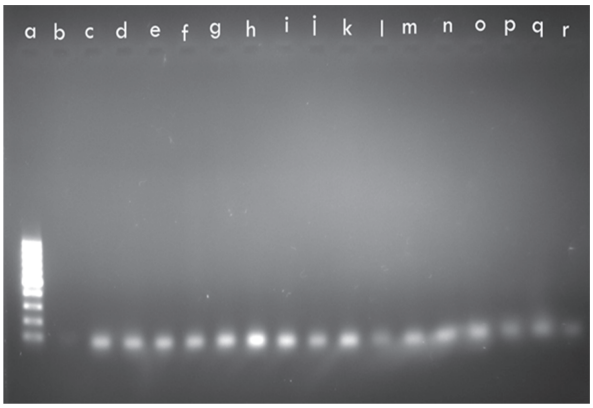

Fig 5: Representative image obtained after screening for *ctx* Gene

Well (a). 100bp ladder; Well (b)-(r) test samples

DISCUSSION

Cholera is specifically caused by the bacterium *Vibrio cholerae*, particularly the toxigenic strains of the O1 and O139 serogroups. These strains produce cholera toxin (*ctx*), which is responsible for the severe watery diarrhoea characteristic of the disease. Other non-O1 and non-O139 strains of *Vibrio cholerae* exist, but they typically did not cause epidemic cholera. However, they can sometimes cause milder gastrointestinal infections or localized outbreaks (Van Kessel& Camilli,2024). The prevalence of *Vibrio cholerae* was confirmed by the presence of *omp* gene, which acts as a reliable molecular signature for detecting the same, especially when combined with other virulence makers for c*omp*rehensive analysis. Among the 39 water samples, 21 water samples showed positive result for the presence of *omp* genes. 7 out of 12 coastal water samples, all the 14 water samples collected from associated canals of Vembanad lake and none of the water samples collected from different sampling stations of Vembanad lake shown the presence of *Vibrio cholerae.*

Environmental strains of the bacterium may inhabit in aquatic ecosystems and generally do not pose direct health risks unless they acquire virulence genes. Hence, the second part of the study were extended to check the pathogenic potential of *Vibrio cholerae* isolates. The presence of *toxR* indicates that the isolates can potentially became pathogenic upon acquisition of temperate phage *ctx*; while a positive result in *ctx* PCR indicates that the organism already contains the toxic gene and so the particular water sample contains a pathogenic *Vibrio cholerae* in it. But out of the 21 samples only 8 were tested positive for the presence of *toxR* gene. And *ctx* were absent among all samples. Hence, it was concluded that the isolates were simple environmental *Vibrio cholerae* and non-pathogenic in nature. Environmental strains are predominantly non-toxigenic and exhibit significant genetic variability, which supports their long-term persistence (Lutz *et al.,*2013).

The study was conducted during the pre-monsoon season, which creates favorable ecological conditions such as warmer temperature, moderate salinity and high nutrient loads that support the increased prevalence of *Vibrio cholerae*. Shackleton *et al.,* (2023) studied about the seasonality of cholera in Kolkata and the influence of climate and found out that, a bi-annual pattern of cholera cases with two peaks coinciding with the increase in temperature in summer and the onset of monsoon rains. Vembanad Lake's distinctive blend of ecological diversity, intense human activity, and complex environmental dynamics creates an ideal setting for studying *Vibrio cholerae*. Investigating its behavior within this ecosystem can provide insights for developing targeted public health strategies and enhancing sustainable water management practices. In the context of Vembanad lake, the largest lake in Kerala, Anas *et al.,* (2021) investigated the environmental reservoirs of *Vibrio cholerae* and highlighted the significant influence of variability on the dynamics of these reservoirs. Their findings emphasize that fluctuations in climatic factors can directly impact the quality of natural water resources, potentially affecting their safety for human consumption.

So, the present study offers valuable insights into environmental drivers of cholera outbreaks, supports risk mitigation efforts, and enhances public health preparedness. Future studies may face challenges due to climate change, induced alterations in rainfall patterns, temperature fluctuations, and salinity levels, which could impact *Vibrio cholerae* distribution and complicate long-term predictions. Additionally, increasing anthropogenic activities and land use changes around Vembanad lake may introduce new environmental variables, necessitating more complex and adaptive monitoring approaches.

CONCLUSION

This study highlights the prevalence of *Vibrio cholerae* in coastal waters and associated canals of Vembanad Lake, with 21 out of 39 water samples testing positive for the *omp* gene, confirming the presence of the bacterium. Notably, all canal samples and 7 coastal samples showed contamination, while Vembanad Lake itself remained unaffected. Further analysis revealed the *toxR* gene in 8 samples, indicating potential pathogenicity, though the absence of the *ctx* gene suggests these strains are currently non-toxigenic. The findings underscore the importance of continuous monitoring of *Vibrio cholerae* in aquatic ecosystems, especially in regions reliant on these waters for daily use. Understanding its distribution and potential virulence is crucial for predicting outbreaks, improving water management, and implementing preventive strategies. This study also highlights the broader implications of environmental changes on microbial dynamics, offering valuable insights for public health and ecosystem conservation. So, ensuring clean water sources is vital for avoiding the spread of cholera, making this study relevant.

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