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Levels of Faecal Indicator Bacteria and Biofilm-Producing *Escherichia Coli* in Vembanad Lake, Kerala, India

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Abstract

Most of earlier investigations on pollution monitoring of bacteria have relied on the levels of *E. coli* in the aquatic environment with limited investigation on their biofilm producing ability. The capacity of a bacteria to produce biofilm is closely related to its pathogenicity. Vembanad Lake in Kerala is the longest lake in India, with 1.6 million people living on its shores and relying on it for their livelihood either directly or indirectly. The study was conducted to assess the level of biofilm-producing *E. coli* in Vembanad Lake. A Niskin water sampler was used to collect samples after mapping 34 locations and were used as sampling points. These location points covered the entire stretch of Vembanad Lake; Ernakulam, Kottayam, and Alappuzha regions. Faecal coliform (FC), *E. coli* and Faecal Streptococci (FS) in the water samples were enumerated using the 5-tube MPN method. The samples yielded 102 *E. coli* isolates, which were confirmed by polymerase chain reaction. All the isolates were checked for their biofilm formation ability. The study revealed that the Ernakulam region had the highest number of FC, FS and *E. coli* among the three regions. Among the *E. coli* isolates, 68, 14, 12 and 3 were weak, moderate, strong and very strong biofilm producers, respectively. It can be inferred that high prevalence of biofilm producing *E. coli* in Vembanad Lake indicate a potential hazard. It is recommended that the biofilm-forming capacity of *E. coli* be assessed along with routine monitoring since this ability of bacteria is associated with bacterial survival and invasiveness.

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Introduction

Vembanad Lake is the longest lake in India (Alex, 2017) and the largest lake in the state of Kerala with an area of 2033.02 km² (Gopakumar & Takara, 2009) and an associated mangrove area of 2114 Km². It is the second-largest Ramsar site in India (Ramsar Convention, 2004) that lies 0.6–2.2 m below mean sea level (MSL) along the south-west coast of India and has a permanent connection with the Arabian Sea at bar-mouth (Chandran et al., 2008). Over 1.6 million people live on the banks of the Vembanad lake and are directly or indirectly dependent on it for their livelihood (https://www.vembanad.org). House boats in Vembanad Lake are at the heart of Kerala Backwaters tourism with hundreds of houseboats plying on it and numerous resorts established on its banks. The lake is bordered by three districts of Kerala State *viz.,* Alappuzha, Kottayam, and Ernakulam (Alex, 2017).

Bacterial populations that are enclosed in extracellular polymeric materials are called biofilms. A critical component of bacterial pathogenicity and essential for microbial survival is the capacity to produce biofilms (Donlan & Costerton, 2002). Both biomaterial-related ailments and many chronic diseases that do not respond to conventional antibiotic treatment are primarily brought on by biofilms. It also determines pathogenicity of bacteria and plays a critical role in the infection process. The development of biofilms on many medical devices impedes healing and poses a serious health concern (Francolini et al., 2014). *E. coli* biofilm development is linked to invasiveness of bacteria, which leads to increased pathogenicity (Martinez-Medina et al., 2009).

Materials and Methods

Using a Niskin water sampler, 34 water samples were collected from three distinct sections of Vembanad Lake; Alappuzha, Kottayam, and Ernakulam. All the samples were taken at a depth of one meter, transferred to sterile plastic containers, and transported in chilled condition (4 ºC) to the laboratory. Details of the sampling points were based on Vaiyapuri et al. (2021).

Enumeration of *E. coli* and Faecal Streptococci of the Vembanad Lake water was carried out using 5-tube-MPN (Multiple Tube Fermentation Technique) as described by American Public Health Association (APHA) (Baird et al., 2017). A loopful of culture from each positive tube of *E. coli* broth (EC broth) were streaked on Eosin methylene blue (EMB) agar and the colonies that had metallic sheen were subjected to the IMViC test i.e., Indole, Methyl red, Voges–Proskauer and Citrate Utilization for bio-chemical confirmation (Visnuvinayagam et al., 2023).

The biochemically confirmed isolates were subjected to Polymerase chain reaction (PCR) for further confirmation as described by Vaiyapuri et al. (2021). The confirmation of *E. coli* was based on the amplification of *uidA* gene primers (Visnuvinayagam et al., 2023). PCR reaction was performed in a thermocycler (Veriti, Applied BioSystem) using *uidA* primers (Godambe et al., 2017). PCR products were analyzed in 2 % agarose gel with 1× TAE buffer at 80 V for 1 h in a horizontal gel electrophoresis system and visualized by a gel documentation system (Bio-Rad, USA) (Fig. 1).

The isolated organisms were tested for their ability to form biofilm using a microtiter plate as described by Christensen et al. (1982) with slight modification. 180 µl of BHI broth with 2 % glucose was added to all the wells of the microtiter plates and 20 µl of overnight grown bacterial cultures were inoculated in all the wells and incubated for 24 h. The microwells were washed 3 times with normal saline and dried at 55 ºC. After the wells were completely dried, 200 µl of methanol was added to each well and kept undisturbed. Methanol was drained off and the plate was air-dried. Finally, the biofilms were stained by adding 200 µl of 0.1 % crystal violet in each well of the microtiter plate. Plates were then kept at room temperature in undisturbed condition for 30 minutes. The plates were then rinsed 3 times with normal saline and vigorously tapped over the tissue paper to remove the unbound dye. The microwells were air dried for about 1 h and the biofilm-bound dye was collected by adding 200 µl of 33 % acetic acid to each microwell and kept undisturbed condition at room temperature for 15 min and checked for absorbance in a microtiter plate reader (Biotek) at 585 nm.

Results and Discussion

The uniqueness of the Vembanad Lake is due to the presence of brackish water in the north (Ernakulam area) and fresh water in the south (Kottayam and Alappuzha region). The separation was made possible by the installation of salt water regulator (Fig. 1) (Chandran et al., 2008). All three regions of Vembanad Lake were included in the study. Many activities such as harvesting of clams (*Villorita cyprinoides*), paddy cultivation, boating, and other recreational activies are in operation at different portions of the Vembanad Lake (FEJI, 2017). Total of 34 points were identified, and samples were obtained from those locations. The average level of faecal coliform (FC) was very high in the Ernakulam region (Table 1). Alappuzha region had lesser level of FC compared to Kottayam and Ernakulam regions. The reason for the high level of contamination in the Ernakulam region may be due to higher population density in the region. Similar to the FC, the Faecal Streptococcal (FS) count was highest in the Ernakulam region (Table 2). Alappuzha and Kottayam regions had lesser levels of FS.

Faecal coliforms are employed as the indicator bacteria for assessing the water quality, according to the Centre for Central Pollution Control Board (CPCB). As per CPCB, marine water is classified into five categories i.e., SW-I (For salt pans, mari-culture and ecologically sensitive Zone), SW-II (For bathing, contact water sports and commercial fishing), SW-III (For industrial cooling, recreation non-contact) and aesthetics, SW-IV (For Harbour Waste) and SW-V (navigation and controlled waste disposal) (CPCB, 1993). In SW-II, the limit for faecal coliform is 100 MPN/100 ml. In the case of SW-III, the limit for the faecal coliform is 500/100 ml (CPCB, 1993). Among the three regions, presence of FC was highest in the Ernakulam region. The other two regions were less contaminated. Among the 34 points, only three points contained a higher level of FC than the recommended limit i.e., 500/100 ml. Out of them, two points were located in the Ernakulam region while one point was located in Kottayam region.

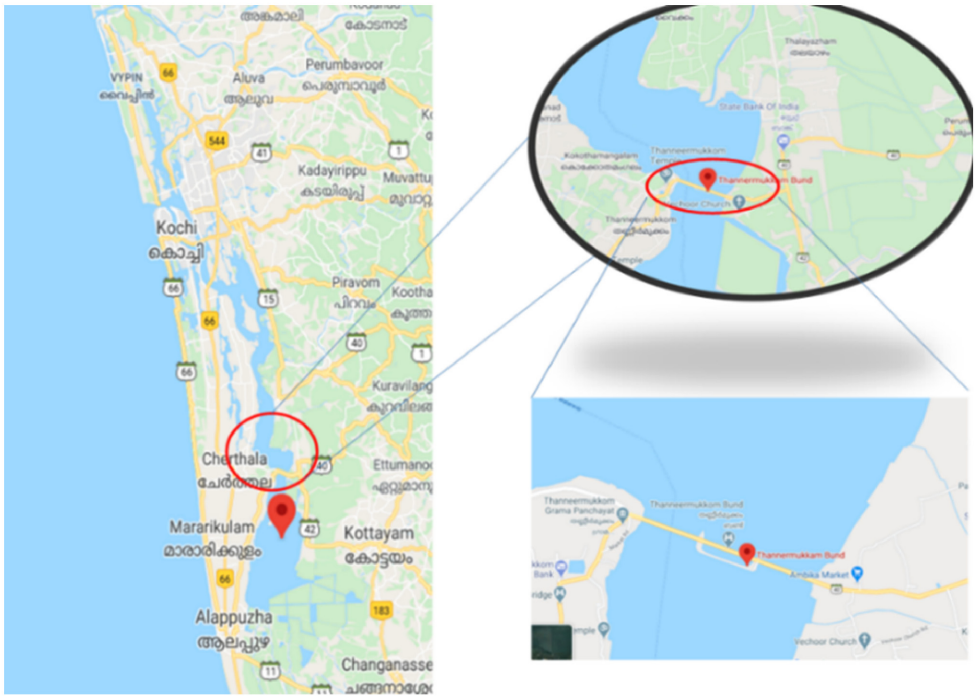

Fig. 1. Vembanad lake map
(Separation of salt and fresh water by salt water regulator)

The cutoff values of faecal streptococci for categorisation of water as A, B, C and D are <40, 41-200, 201- 500 and >500 per 100 mL respectively (WHO, 2021). In the present study, 4 locations in Ernakulam and 2 locations in Kottayam were found to be above the 200 MPN/100 mL (Table 2). Based on average value; Alappuzha and Kottayam are under Category B. However, due to higher value of FS in the samples, the Ernakulam region falls into Category 'C.' As a result of the contact water activities in Ernakulam, gastroenteritis (GI) and acute febrile respiratory illness (AFRI) may occur (WHO, 2021). FC is an index organism for determining pollution levels in marine water according to Indian standards (CPCB, 1993); conversely, WHO recommends Enterococci (a subset of FS) as an index bacterium for determining pollution levels in marine water. Slanetz & Bartley (1965) compared the viability of coliforms, faecal coliforms, and faecal streptococci in dialysis bags suspended in seawater and sewage effluent water and observed that coliform and faecal coliform numbers in sewage increased 3-10 times in the first 1-2 days and stayed at these levels for about 7 days. However, there was no rise in the number of faecal streptococci in the bags, and within 4 - 6 days, there was a sudden drop in their numbers (Sinton et al., 1993). Based on these results the authors concluded that faecal Streptococci would be the most consistent indicator of feacal contamination in saltwater through runoff.

A total of 105 suspected *E. coli* cultures were streaked on EMB agar (Eosin Methylene blue agar) and the characteristic dry colonies with metallic

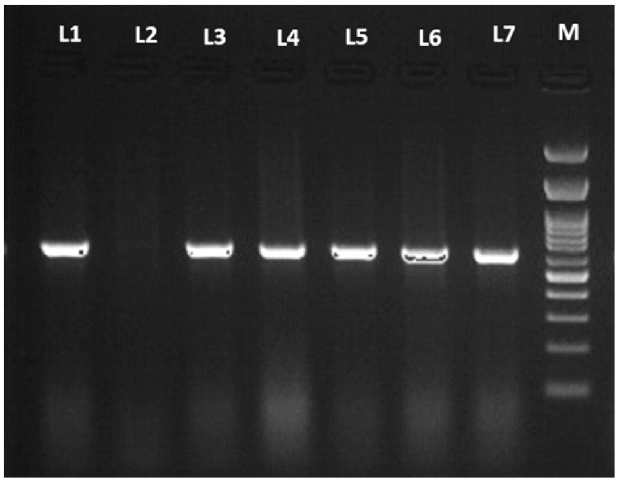

Fig. 2. Confirmation of *E. coli* by amplification of *uidA* gene (603 bp) by agarose gel electrophoresis

Lane 1: Positive control; Lane 2: Negative control; Lanes 3-7: *E. coli* isolates of Vembanad lake Lane M: Marker

sheen were selected for further analysis. Around 3 colonies were taken from each sample and confirmed by IMViC Test. These colonies were further confirmed by polymerase chain reaction (PCR) specific for detection of *E. coli* (Fig. 2).

E. coli (n=102) were screened for biofilm-forming ability using the crystal violet method (Fig. 3). The level of biofilm production was calculated as described by Shakerimoghaddam et al. (2017). The level of biofilm production by *E. coli* was categorized based on the OD values; weak biofilm producers with OD between 0.1 - 0.2, moderate biofilm producers with OD between 0.2 - 0.3, and strong biofilm producers with OD between 0.3 to 1.0 OD. In the present study, OD > 1.0 were considered as very strong biofilm producers (Table 3). Among the 102 isolates, 5 were non-biofilm producers; 68 were weak biofilm producers, 14 were moderate biofilm producers, 12 were strong biofilm producers and 3 were very strong biofilm producers (Fig. 3; Table 3).

Table 1. Levels of faecal coliform in water samples by 5 tube MPN method

Region	Sample code	Faecal coliform	Mean ± SE and Range
Alappuzha	A	110	105.59 ± 16.65; 9.3 - 210
	B	84	
	C	94	
	D	170	
	E	15	
	F	9.3	
	G	58	
	H	48	
	I	70	
	J	170	
	K	120	
	L	150	
	M	210	
	N	170	
Ernakulam	O	280	425.6 ± 155.05; 32 - 1600
	P	32	
	Q	70	
	R	1600	
	S	540	
	T	920	
	U	84	
	V	280	
	W	280	
	X	170	
Kottayam	Y	110	150.66 ± 54.67; <1.8 - 540
	Z	350	
	AA	220	
	AB	540	
	AC	84	
	AD	<1.8	
	AE	<1.8	
	AF	47	
	AG	58	
	AH	94	
Vembanad Lake		Total	
		Mean ± SE	212.96 ± 52.86
		Range	<1.8 - 1600

Table 2. Faecal Streptococci in water samples by 5 tube MPN method

Region	Sample code	Faecal streptococci	Mean ± SE and Range
Alappuzha	A	48	60.25 ± 11.85; 1.8 - 140
	B	32	
	C	31	
	D	110	
	E	<1.8	
	F	<1.8	
	G	32	
	H	33	
	I	47	
	J	110	
	K	58	
	L	79	
	M	140	
	N	120	
Ernakulam	O	170	239.28 ± 89.76; 6.8 - 920
	P	6.8	
	Q	40	
	R	920	
	S	220	
	T	540	
	U	32	
	V	150	
	W	220	
	X	94	
Kottayam	Y	70	87.22 ± 37.77; 1.8 - 350
	Z	240	
	AA	130	
	AB	350	
	AC	3.6	
	AD	<1.8	
	AE	<1.8	
	AF	12	
	AG	17	
	AH	46	
		Mean ± SE	120.84 ± 31.05
		Range	<1.8 - 920

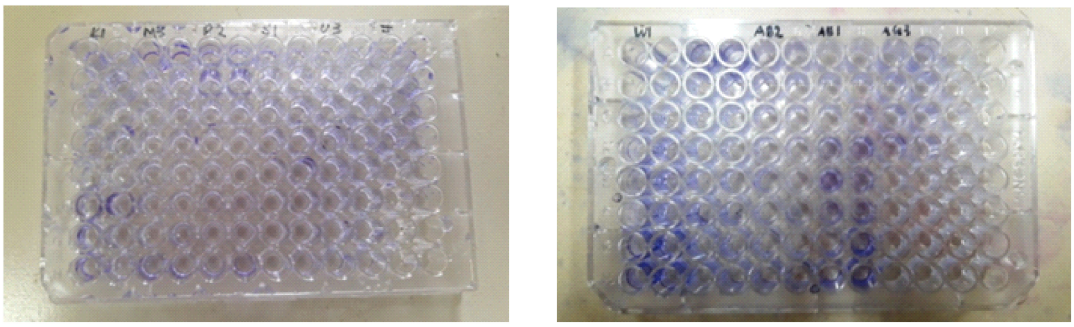

Fig. 3. Biofilm producing ability of *E. coli* determined by crystal violet method

In order to take into account the variation in the sampling size and also various degrees of bio-film production, normalization was carried out (Table 4). Weak, moderate, strong and very strong biofilm producers were multiplied by 1, 2, 3 and 4 respectively. To assess the region-wise biofilm production of *E. coli*, the normalized value was divided by the number of isolates. Based on the normalization, a higher value was observed in the Ernakulam region, followed by Kottayam and Alappuzha region. Ernakulam region had higher levels of *E. coli*, faecal coliform and biofilm-producing *E. coli*.

Most retail fish vendors in India typically source their fish from landing centres. It is common practice for them to clean the fish using nearshore seawater (Visnuvinayagam et al., 2015; 2017; 2019), which often contains elevated levels of *E. coli*/FC, thereby leading to potential contamination (Whitman et al., 2003). According to Noble et al. (2003), the typical survival rate of *E. coli* in marine water is limited to 19 hours. However, Gerba et al. (1976) have found that the presence of high levels of organic matter or debris and low salinity can enhance the survival rate of *E. coli*. Water from the nearshore was found to have higher bacterial load than farshore areas (Whitman et al., 2003). Therefore, there is an increased possibility that *E. coli* that produces biofilm might enter the food chain through fish.

Table 3. Level of biofilm producing *E. coli* from Vembanad Lake

	Non biofilm producers	Weak biofilm producers	Moderate biofilm producers	Strong biofilm producers	Very strong biofilm producers	Total biofilm producers
Alappuzha (42 isolates)	4(3.92 %)	30(29.41 %)	2(1.96 %)	3(2.94 %)	3(2.94 %)	38
Kottayam (30 isolates)	0(0.00 %)	22(21.57 %)	6(5.88 %)	2(1.96 %)	0(0.00 %)	30
Ernakulam (30 isolates)	1(0.98 %)	16(15.69 %)	6(5.88 %)	7(6.86 %)	0(0.00 %)	29
All Regions	5(4.9 %)	68(67.67 %)	14(13.73 %)	12(11.76 %)	3(2.94 %)	97

Table 4. Biofilm levels in three regions of Vembanad Lake after normalization

Regions of Vembanad Lake	Weak Biofilm producer	Moderate Biofilm producer	Strong Biofilm producer	Very Strong Biofilm producer	Normalized value	Normalized value/ Number of Isolates
Alappuzha	30	4	9	12	55	55/42 = 1.309
Kottayam	22	12	6	0	40	40/30 = 1.333
Ernakulam	16	12	21	0	49	49/30 = 1.633

It can be concluded that the average levels of faecal coliforms, feacal Streptococci as well as biofilm-producing *E. coli* were highest in the Ernakulam followed by the Kottayam and Alappuzha region. The results indicate that in future, in addition to enumerating the *E. coli* count, assessing the biofilm production ability of the strains must be included to determine the true level of environmental risk. Consistent training, frequent monitoring and stringent regulations, are needed to restrict the spread of pathogenic biofilm-forming bacteria.

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