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Isolation and molecular characterization of microbial population from the Fish 'Tilapia' collected from Vembanad Lake, Kerala, India

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Abstract

Water bodies provide a large amount of easily accessible fresh water which stabilizes any population. Kuttanchaal is a beautiful island surrounded by Vembanad Lake, in Thycattusserry, Cherthala, Alappuzha, Kerala, India. The major livelihood of the people living in this island is fishing and coir industry. For the study of the fish, swabs from the face, gut and gills were taken directly and analysed for microbial content. The water sample was also subjected to physicochemical analysis. The temperature, salinity and specific gravity of water sample was 32.7℃, 4-7 ppt and 1.004-1.007 kg/m^3 respectively. Bacteriological analysis of water and fish samples were done using Thiosulphate Citrate Bile salt Sucrose (TCBS), Eosin Methylene Blue (EMB) and Bismuth Sulphite (BS) agar and the colonies were studied and antibiotic sensitivity assay was also done. *Vibrio cholerae* and *Ferrimonas balearica* showed more susceptibility towards Norfloxacin and least towards Furazolidone. *Salmonella typhi* and *E. coli* showed more susceptibility towards Cephalexin and Furazolidone whereas least towards Norfloxacin and Cephalexin respectively. The bacterial species *Ferrimonas balearica* was found in the gut of the fish sample. The sequence was submitted to NCBI (Accession number MT322629.1).

1. Introduction

Water is a requisite for life on earth and the pollution of water bodies has severe ecological and public health issues. People, especially in developing nations live without access to safe water and is perhaps the most widely denied right in the world. Globally, due to poor sanitation and lack of safe drinking water, humans are being infected severely with microbes. Microbial contaminants in water pose severe public health problems [1].

Kuttanchaal is a beautiful island surrounded by Vembanad Lake, in Thycattusserry, Cherthala, Alappuzha, Kerala state, India. Many microorganisms are found naturally in fresh and saltwater. These include bacteria, cyanobacteria, protozoa, algae, and tiny animals such as rotifers [2]. These can be important in the food chain that forms the basis of life in the water. For example, the microbes called cyanobacteria can convert the energy of the sun into the energy it needs to live. The plentiful numbers of these organisms in turn are used as food for other life. The algae that thrive in water are also an important food source for other forms of life.

Like humans and other animals, fish also suffer from various diseases. Fish defences against these diseases by specific and non-specific methods. Non-specific defences include skin and scales, as well as the mucus layer secreted by the epidermis that traps microorganisms and inhibits their growth. If pathogens breach these defences, fish can develop inflammatory responses that increase the flow of

blood to infected areas and deliver white blood cells that attempt to destroy the pathogens. All fish carry some type of pathogens. Disease is a prime agent affecting fish mortality, especially at their younger stages. Fish can limit these pathogenic impacts with their behavioural or biochemical means, and such fish have reproductive advantages. Low grade infection becomes fatal as a result of interacting factors. In particular, things that cause stress, such as natural droughts or pollution or predators, can precipitate outbreak of disease [3].

Human infections caused by pathogens transmitted from fish or the aquatic environment are quite common and depend on the season, patients' contact with fish and related environment, dietary habits and the immune system status of the exposed individual. They are often bacterial species facultatively pathogenic for both fish and human beings and may be isolated from fish without apparent symptoms of the disease. The infection source may be fish kept for both for food and as a hobby [4]. Mycobacteriosis is particularly significant among infections transmissible from fish to human beings. Many mycobacterial species, both rapid and slow growing, have been isolated from affected fish tissues [5]. Primary septicaemia or the gastrointestinal illness in human beings may follow after ingesting raw seafood, particularly of ocean filter feeder (e.g. oysters and clams) and fish infected by *V. vulnificus* [6, 7].

Human infections and intoxications with the following bacteria have been recorded: *Mycobacterium* spp., *Streptococcus iniae, Photobacterium damselae, Vibrio alginolyticus, Vibrio vulnificus, Vibrio parahaemolyticus, V. cholerae, Erysipelothrix rhusiopathiae, Escherichia coli, Aeromonas* spp., *Salmonella* spp., *Staphylococcus aureus, Listeria monocytogenes, Clostridium botulinum, C. perfringens, Campylobacter jejuni, Delftia acidovorans, Edwardsiella tarda, Legionella pneumophila,* and *Plesiomonas shigelloides* [8]. Fish tissue histamine intoxications of people have frequently been described. The purpose of the present paper was to elaborate an overview of significant bacterial causative agents of human diseases transmitted from fish used as food or by handling them.

2. Methodology

2.1 *Sample collection*

Water samples were collected aseptically using 100ml screw cap sterilized bottles, from locality in Kuttanchaal, Thycattusserry, Thuravoor, Cherthala, Kerala state, India, at a distance of 1 meter apart from the lake shore, during the first and last week of April, 2019. Meanwhile fish sample was collected from the same location and kept in a 1000 ml sterilized containers. All samples were labelled properly and transferred to the laboratory by using icebox and processed within 3 hours of arrival.

2.2 *Physical Parameters*

The pH of all water samples were checked using pH meter (Systronics 361, India). The temperature was measured using standard mercury filled centigrade thermometer. The electrical conductivity and Total Dissolved Solids (TDS) were measured using pre calibrated conductivity TDS meter (Systronics 308, India). Salinity and specific gravity of the water samples were estimated using a hand held refractometer (Erma, ERS10, Tokyo Japan) [9].

2.3 *Heterophilic plate count of water sample*

Heterophillic plate count (HPC) provides an indication of general microbial population in the sample. Water sample to be analyzed for quantitative bacterial analysis, were plated on Glucose Tryptone Agar (GTA) [10,11] fungal analysis was done on plating the samples in Potato Dextrose Agar (PDA) plates and the actinomycetes count was taken using Starch Casein Agar plates (HiMedia Laboratories,

Mumbai, India) [12, 13]. The bacterial plates were incubated at 37°C in an incubator and the number of colonies was counted after 24 hours. The fungal and actinomycetes plates were kept for incubation at 28°C and the number of colonies was counted after 48 hours of incubation.

2.4 *Isolation of microorganisms*

The bacterium was isolated from water and fish samples using standard dilution plate techniques [14]. For the isolation of *Salmonella* sp., 10 ml of water sample was inoculated into l00 ml of selenite enrichment broth and incubated at 37°C for 12-18 hours and swabs from the selenite broth were then streaked on BS agar (HiMedia Laboratories, Mumbai, India). To detect the presence of *Vibrio sp*., water sample was enriched by adding 50 ml water sample in 100 ml of double strength alkaline peptone water (pH 8.6) and incubated at 37°C for 24 hours and swabs from the alkaline peptone water were then streaked on TCBS agar (HiMedia Laboratories, Mumbai, India) and further incubated at 37°C for 24 to 48 hours [15, 16]. Similarly, for the isolation of bacterial population from fish sample, sections from the face, gut and gills of fish were taken. Swabs from these parts were taken directly and enriched as per the procedure mentioned above. In addition to this, for the isolation of *E. coli*, the swab containing organisms was inoculated into l00 ml of buffered peptone water and incubated at 37°C for 12-18 hours and swabs from the buffered peptone water were then streaked on EMB agar.

2.5 *Antibiotic sensitivity assay*

The antibacterial disk diffusion assay was carried out on Mueller Hinton agar plates following the method described by Gupta et al., [17]. The bacterial suspension was prepared from overnight-grown cultures (24 h) in nutrient broth containing tween-80 (0.1 % v/v; Merck), and the turbidity was adjusted equivalent to 0.5McFarland standard (approximately 1.2×10^8 CFU/ml). Aliquots (100 µl) of inoculum were spread over the surface of agar plates with a sterile glass spreader. Antibiotic discs such as Norfloxacin (10 µg), Oxytetracycline (30 µg), Cefotaxime (30 µg), Cephalexin (30 µg), Co-Trimoxazol (25 µg) Chloramphenicol (30 µg), Nalidixic acid (30 µg) and Furazolidone (50 µg) were placed on the agar plates [18]. The plates were then incubated at 37 °C for 48 h, and the zone of bacterial growth inhibition around the disk was measured. The assay was repeated twice, and mean of the three experiments was recorded. The antibiotic discs were procured from HiMedia Laboratories, Mumbai, India.

2.6 *Molecular identification of strain*

Isolation of genomic DNA

1.5 ml of the fresh culture was centrifuged at 10,000 rpm for 5 min to obtain the pellet and dissolved in 0.5 ml of lysis buffer (100 mMTris pH 8.0, 50 mM EDTA, 10% SDS). 0.5 ml of saturated phenol and 0.2 ml chloroform were added and incubated at 55°C for 10 min. Then it was centrifuged at 10,000rpm for 10 min. To the supernatant equal volume of Chloroform: isoamyl alcohol (24.:1) and 1/20th volume of 3M sodium acetate were added. Centrifuged at 10,000rpm for 10 min and to the supernatant 3 volumes of chilled absolute alcohol was added. Precipitated DNA was separated by centrifugation. Pellet was washed with 70% alcohol, dried and dissolved in TE buffer (10 mMTris pH 8.0, 1 mM EDTA) and store at -20°C [19].

Separation of DNA by AGE

The agarose gel electrophoresis (AGE) was carried to observe the isolated DNA. Total DNA was mixed with 6X loading buffer and loaded onto 0.8% agarose gel along with 1kb molecular weight marker for electrophoresis at 80V for 30 to 45 minutes. Gel documentation system (Biorad) was used

to analyse the DNA and was then photographed. The quality of the DNA was examined by using spectrophotometer.

Analysis of 16S rRNA sequence

The 16S rRNA genes were amplified using primers forward primer 27F5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 1392R5'-GGTTACCTTGTTACGACTT-3'. The PCR mixture contained DNA, 2µl of 16s forward primer and 16s reverse primer, 20µlPCR master mix (Takara), and made to 50µl by adding molecular biology grade water and amplified in an automated PCR thermal cycler (BioRad). The following conditions were used for the PCR amplification. Initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 2 minutes. The PCR products were electrophoresed on 1% agarose gel with 500 bp DNA ladder. PCR products were purified using GeneiPure TM Quick PCR Purification Kit and was sequenced [20, 21].

DNA sequencing

DNA sequencing of purified PCR product was carried out at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India. The sequencing was done with both forward and reverse primers used in PCR. The obtained partial sequence was searched for homology against National Center for Biotechnology Information (NCBI) 16S database using Basic Local Alignment Search Tool (BLAST) [22]. We have retrieved the FASTA Sequence of blast hits and closest organisms from NCBI and executed Multiple Sequence Alignment (MSA) using Clustral W algorithm and a phylogenetic tree was made using MEGA6 software.

3. Results and Discussion

3.1 Physicochemical characteristics of water

Water samples collected from Kuttanchaal were subjected to physicochemical analysis and the results were noted in Table 1. The pH for the first week and last week of April was 6.6 and 6.5 respectively. It shows slightly a difference of 0.1. The temperature for the first and last week were 32.7°C and 32.6°C respectively.

Table 1. Physicochemical characteristics of water

Physical Parameters	Values in the month of April	
	First week	Last week
pH	6.6	6.5
Temperature	32.7°C	32.6°C
Total dissolved solids (TDS)	14.07 ppm	13.57 ppm
Specific gravity	1.004 kg/m^3	1.007 kg/m^3
Salinity	4 ppt	7 ppt
Electronic conductivity (EC)	28.01 mS	26.86 mS

The Total dissolved solids (TDS) were 14.07 ppm and 13.57 ppm respectively for the first and last week. The specific gravity was 1.004 and 1.007 kg/m^{3}for the first and last week respectively. The salinity showed a difference in the value from 4 to 7 ppt in the first and last week respectively. The electric conductivity also showed difference in their value from 28.01 mS to 26.86 mS in the first and last week respectively.

3.2 *Microbiological analysis of water*

Heterophilic plate count

More number of bacteria and less number of fungi were seen in the water samples collected from Kuttanchaal and the results were noted in Table 2. Bacteria and actinomycetes were high compared to that of fungi. Actinomycetes (58×10^3 cfu/ml) were more and fungi (2×10^3 cfu/ml) were less in number.

Table 2: HPC of lake water sample

Bacteria (cfu/ml)	Fungi (cfu/ml)	Actinomycetes (cfu/ml)
53×10^3	2×10^3	58×10^3

3.3 *Isolation of microorganisms*

Water samples enriched in alkaline peptone water and selenite broth for the detection of *Vibrio* sp. and *Salmonella* sp. respectively were prepared for the microbiological analysis of water. These were streaked in TCBS and BS agar respectively. Yellow coloured colonies were obtained in the TCBS agar which confirmed the presence of *Vibrio cholerae* as shown in Figure 1 and black-brown coloured colonies were obtained in the BS agar which confirmed the presence of *Salmonella typhi* as shown in Figure 2.

Figure 1:*V. cholerae* in TCBS agar

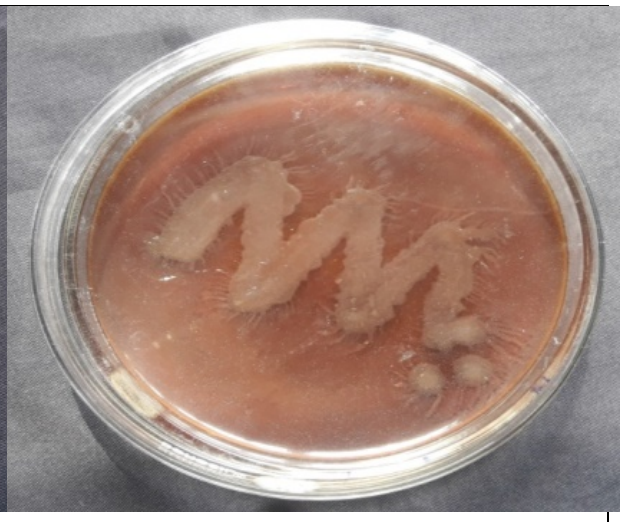

Figure 2: *S. typhi* in BS agar

3.4 *Bacteriological analysis of fish*

The samples from face, gills and gut of the fish were streaked and each were cultured on TCBS, BS and EMB agar media for bacteriological analysis and the results were shown in Table 3. In TCBS agar, yellow coloured

colonies of *Vibrio cholerae* from the face and gut, dark green coloured colonies of *Ferrimonas balearica* (Figure 3) and green coloured colonies of *Vibrio parahaemolyticus* from the gut were obtained. In EMB agar, pink coloured colonies of *Enterobacter aerogens* and purple coloured colonies of *E. coli* were obtained from face, gut and gills. In BS agar, brown coloured colonies of *Salmonella typhi* were obtained from gut and gills. Highest number of *Vibrio cholera* was obtained from the face, *Enterobacter aerogens* and *E. coli* from the gut and *Salmonella typhi* from the gut.

Table 3: Bacteriological analysis of fish

Sl. No.	Medium	Area swabbed	No. of colonies	Colour of colony	Organism
1.	TCBS agar	Face	134	Yellow	*Vibrio cholerae*
		Gut	25	Yellow	*Vibrio cholerae*
			2	Dark green	*Ferrimonasbalearica*
			26	Green	*Vibrio parahaemolyticus*
		Gills	Nil		
2.	EMB agar	Face	4	Pink	*Enterobacter aerogens*
			2	Purple	*E. coli*
		Gut	26	Pink	*Enterobacter aerogens*
			28	Purple	*E. coli*
		Gills	23	Pink	*Enterobacter aerogens*
			4	Purple	*E. coli*
3.	BS agar	Face	Nil		
		Gut	153	Brown	*Salmonella typhi*
		Gills	10	Brown	*Salmonella typhi*

Figure 3: *Ferrimonas balearica* in TCBS agar

3.5 Antibiotic sensitivity assay

Antibiotic sensitivity of the microbes from gut flora of fish sample collected from Kuttanchaal was done and results were noted and given in Table 4. *Vibrio cholerae, Ferrimonas balearica, Salmonella typhi* and *E. coli* were the organisms isolated from the gut. When the antibiotic sensitive pattern were assessed for *V. cholerae*, it was resistant for Co-Trimoxazol, Chloramphenicol, Nalidixic acid and Oxytetracycline and susceptible for Norfloxacin (25mm), Furazolidone (7mm), Cefotaxime (15mm)

and Cephalexin (10mm). For *Ferrimonas balearica*, it was susceptible for all the eight antibiotics used, i.e., Norfloxacin(30mm), Oxytetracycline(25mm), Cefotaxime (20mm), Cephalexin (12mm), Co-Trimoxazol (11mm), Chloramphenicol (24mm), Nalidixic acid (25mm) and Furazolidone(10mm).

Table 4: Antibiotic sensitivity pattern

Sl No.	Media	Antibiotic sensitivity		Zone of inhibition (mm)
		Resistant	Sensitive	
1	*Vibrio cholerae*	Co-Trimoxazol	Norfloxacin	25
		Chloramphenicol	Furazolidone	07
		Nalidixic acid	Cefotaxime	15
		Oxytetracycline	Cephalexin	10
2	*Ferrimonasbalearica*	Nil	Norfloxacin	30
			Oxytetracycline	25
			Cefotaxime	20
			Cephalexin	12
			Co-Trimoxazol	11
			Chloramphenicol	24
			Nalidixic acid	25
			Furazolidone	10
3	*Salmonella typhi*	Nalidixic acid	Norfloxacin	17
			Oxytetracycline	19
			Cefotaxime	25
			Cephalexin	29
			Co-Trimoxazol	24
			Chloramphenicol	19
			Furazolidone	18
4	*E. coli*	Nil	Norfloxacin	28
			Oxytetracycline	18
			Cefotaxime	29
			Cephalexin	17
			Co-Trimoxazol	23
			Chloramphenicol	23
			Nalidixic acid	25
			Furazolidone	30

For *Salmonella typhi*, it was resistant to Nalidixic acid and susceptible to Norfloxacin (17mm), Oxytetracycline (19mm), Cefotaxime (25mm), Cephalexin (29mm), Co-Trimoxazol (24mm), Chloramphenicol (19mm) and Furazolidone (18mm). For *E. coli,* it was susceptible towards all the eight antibiotics used, i.e., Norfloxacin (28mm), Oxytetracycline (18mm), Cefotaxime (29mm), Cephalexin (17mm), Co-Trimoxazol (23mm), Chloramphenicol (23mm), Nalidixic acid (25mm) and Furazolidone (30mm). *Vibrio cholera* and *Ferrimonas balearica* showed more susceptibility towards Norfloxacin and least towards Furazolidone. *Salmonella typhi* and *E. coli* showed more susceptibility towards Cephalexin and Furazolidone whereas least towards Norfloxacin and Cephalexin respectively.

3.6 Molecular identification of strain

Bacterial genomic DNA was isolated under the standard protocol (Hoffman and Winston, 1987). The presence of isolated bacterial genome was confirmed by stained on 0.8% agarose gel with ethidium bromide. An intense band was seen together with the DNA marker. To amplify the 16srRNA gene as a template, the extracted DNA was used. The 16S rRNA universal primers, 27F 5'(AGAGTTTGATCCTGGCTCAG) 3' and 1492R 5'(TACGGYTACCTTG TTACGACTT)3', were used to amplify the genomic DNA of the bacterial isolates. It has been found that the optimum temperature for annealing was 58^{o}C. The PCR products were electrophorized by 1% agarose gel, stained by ethidium bromide, and visualized by a UV transilluminator. According to the results, PCR products of approximately 1,500 bp were visualized (Figure 4). The PCR products were purified with a PCR purification kit (Qiagen). The PCR product was then subjected to Sanger sequencing.

The sequence results of the PCR product, on the basis of the BLAST public database of homologous genes on the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/blast/), showed that the bacterial isolate was identified as *Ferrimonas balearica* strain OMFB19 16S ribosomal RNA gene, partial sequence, exhibiting 98.9% sequence similarity. The sequence was submitted to NCBI (Accession number MT322629.1). Figure 5 showed the phylogenetic relationship of 16S ribosomal RNA gene, partial sequence of the isolated strain and other strains and species related to *Ferrimonas balearica* in the Gen-Bank database.

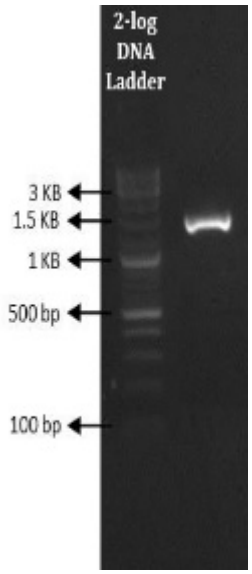

Figure 4: Agarose gel electrophoresis

The increased prevalence of indicator and pathogenic bacteria in the enclosed southern part of Vembanad Lake may be resulting from the altered flow patterns due to the salt water regulator [23]. In addition to this, resistance of microorganisms to antibiotics of clinical interest has previously been reported in the area.An investigation was carried out on Vembanad Lake in the Eramalloor region of Ernakulum district, Alappuzha, Kerala. The study was carried out for a period of 11 months from September 2015 to July 2016. During their study, the average temperature was 29.0^{o}C, average salinity was 19.02% and pH was about 8.1 [24]. But from the present study which we conducted in the month of April, the temperature was 32.6^{o}C, salinity ranged from 4 to 7 in the first week to last week of the month and pH was noted as 6.60.

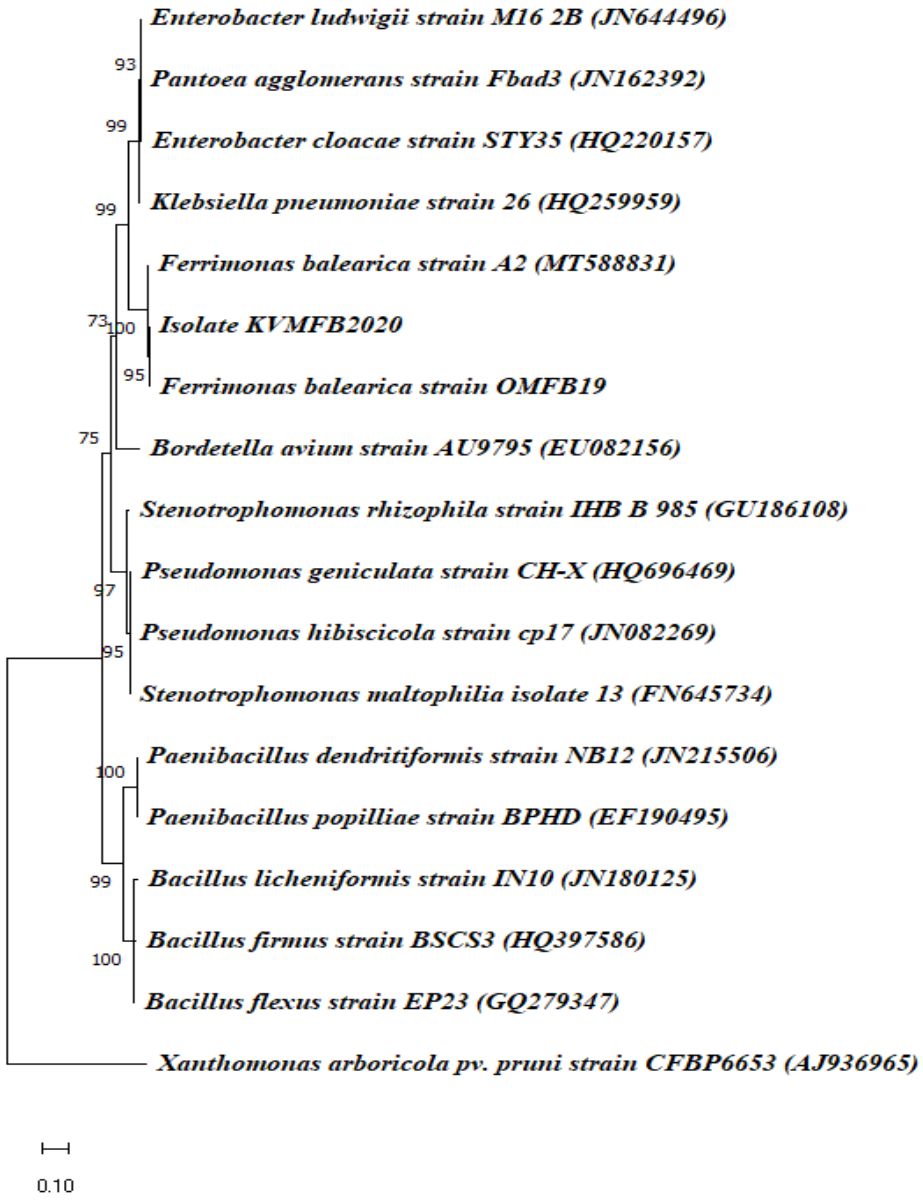

Figure 5: Phylogenetic tree

The results came out with interesting facts that we could find *Ferrimonas* sp. in the gut of Tilapia fish from that area. One of the plates with the gut culture swabbed produced certain black colour deposits near the dark green colour colonies. Colonies of *Ferrimonas balearica* produce a black iron precipitate when the cells are grown on TSI agar [25]. We were successful in finding a new species which was not found in Kerala. But we were unable to find the disease causing organism which killed the fishes in that area. The presence of *Salmonella* and *Vibrio* species were detected in the water sample collected from the same area. A change in salinity and specific gravity were observed while testing the water sample in the first and last week of April. To find an affected fish was really a tough thing compared to the lab procedures. The results can be exploited in many ways. The *Ferrimonas* sp. found in that area can be further used to study about their pathogenic properties and their abundance in the gut of the fish and the sediments in the lake. The people living there were affected with certain skin diseases. From the survey conducted there, we could assume that it was due to their work (like fishing) and by the contact with the sediments in the river. Certain lesions or swellings can be found in their skins.

Conclusion

An evaluation of the bacteriological quality of surface water in the present study confirmed the presence of various bacterial species including opportunistic pathogens such as *Vibrio* and *Salmonella* sp. These organisms were resistant to several classes of antibiotics. Undesirable properties of water quality caused by the presence of drug-resistant bacteria can pose a negative impact on human health. The species *Ferrimonas balearica* identified along the study is the first species of this genus to be reported from Kerala. This species was first reported in the Spain [25]. *Ferrimonas balearica* is the type species of the genus Ferrimonas, which belongs to the family Ferrimonadaceae within the Gammaproteobacteria. The species is a Gram negative, motile, facultatively anaerobic, non spore forming bacterium. Future studies are required on this organism for identifying its pathogenicity and how far it affects the fish and also the humans.

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Disclosure statement: *Conflict of Interest:* The authors declare that there are no conflicts of interest. *Compliance with Ethical Standards:* This article does not contain any studies involving human or animal subjects.

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