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Isolation and Identification of Pathogenic Bacteria in Edible Fish: A Case Study of Mogral River, Kasargod, Kerala, India

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ABSTRACT

Water is one of the most valued natural resource and hence the management of its quality is of special importance. In this study, an attempt was made to compare the aquatic ecosystem pollution with particular reference to the upstream and downstream quality of river water. Water samples were collected from Mogral River and analysed for physico-chemical and bacteriological parameters. Healthy fish samples from the river basin were subjected to bacteriological studies. The direct bacterial examination of the histological sections of the fish organ samples were also carried out. Further, the bacterial isolates were taxonomically identified with the aid of MALDI-TOF MS. The physico-chemical parameters monitored exceeded the recommended level for surface water quality in the downstream segment. Results of bacteriological analysis revealed high level of faecal pollution of the river. The isolation of enteric bacteria in fish species in the river also served as an indication of faecal contamination of the water body. Comparatively, higher bacterial density was found in the liver samples of the fish collected from the downstream, than in other organs of the fish collected from the upstream segment. Taxonomical identification revealed the presence of eight pathogenic bacterial strains from the fish samples, all of which represents a potential hazard to humans. The mean bacterial load of the isolates was found to be markedly higher than the recommended public health standard value adopted by the standard prescribed by World Health Organisation (WHO).

Introduction

Rivers are the most important freshwater resource for man. Unfortunately, river waters are being polluted by indiscriminate disposal of sewage, industrial waste and plethora of human activities, which affects their physico-chemical characteristics and microbiological quality [1]. Pollution of the aquatic environment due to the heavy release of industrial, agricultural and commercial chemical discharges have led to various deleterious effects on aquatic organisms. Aquatic organisms, including fish, accumulate pollutants directly from contaminated water and indirectly *via* the food chain [2]. Disposal of sewage wastes into a large volume of water could increase the biological oxygen demands to such a high level that all the available oxygen may be removed, consequently causing the death of all aerobic species, e.g., fish [3].

It is apparent that fish are continuously exposed to the microorganisms present in water and in sediment including the contaminants in sewage/faeces [4]. Pathogenic microbes cause many diseases in both wild and cultured fish. They may vary from a primary pathogen to that of an opportunist invader of a host rendered moribund by some disease process [5]. Fish may harbour pathogens on or inside its body after exposure to contaminated water or food. It is recognised that, extraneous bacteria are capable of surviving in fish. The faecal indicator organism *Escherichia coli*, was found to survive and even multiply in the digestive tract of rainbow trout (*Oncorhynchus mykiss*) after ingestion via contaminated food [6].

The infections of various pathogens can significantly affect the overall behaviour, metabolism, body condition, fecundity and survival of fish and is a critical concern in areas that lead to human contact or food consumption.

Most bacterial species cause different diseases in fish and some cause diseases in humans. Human diseases that can be caused by bacteria in fish include: - food poisoning and gastroenteritis, diarrhoea [7], superficial wound infections and ulcers [8], bacillary dysentery (Shigellosis), clonorchiasis, dracunculiasis and paragonimiasis due to larvae and metacercariae ingested in fish and crustaceans [9], Cholera [10], typhoid and paratyphoid [11] etc. The microbial association with fish compromises safety and the quality for human consumption is particularly critical, when the micro-organisms are opportunistic and / or pathogenic in nature. Therefore, fishery products which are of great importance for human nutrition and health worldwide can act as a source of food borne pathogens and may be a potential source of diseases [12].

There have been great economic losses reported due to food borne illness such as dysentery and diarrhoea resulting from consumption of contaminated fish. These circumstances prompted the present study, to investigate the occurrence of any human bacterial pathogens in the fish that was being caught from the upstream and downstream portion of Mogral River basin.

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Materials and Methods

Study Area

The study was conducted in Mogral River, which is a west-flowing river in the Kasaragod district in the Indian State of Kerala. The river is 34 km long and has its entire course within Kerala. The geographical map of the course of Mogral River showing the study area and sampling points is shown in (fig 1). Two sampling stations were fixed for the study Muliyar (MG 1) which is the upstream portion of the river and Mogral Puthur (MG 2), the downstream portion.

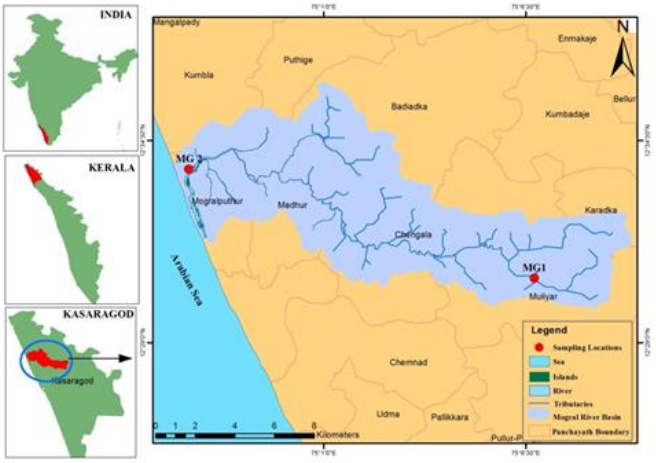

Fig 1. Map of Mogral River segment showing study area and sampling points.

Water and Fish sampling

Water samples were collected from upstream and downstream portion of Mogral river with replicates for physico-chemical and bacteriological analysis. The samples for physico- chemical and bacteriological analysis were collected in 1litre plastic cans and 100 ml sterilized bottles [13]. The samples were collected following the standard sampling guidelines and methods. Further the samples were transported immediately to the laboratory in ice cold condition and subjected to physico-chemical and bacteriological analysis.

Fish samples were collected from Mogral River during pre monsoon season (April, 2016). *Etroplus suratensis*, commonly known as Green chromide which are the common inhabitants of brackish and freshwaters sources of Kerala was selected for the study. It is also, one of the edible and economically important fin fishes of Mogral River. Four samples of *Etroplus* were collected aseptically and immediately transported in a thermal bag to the laboratory and the samples were kept in the refrigerator ($4^oC – 8^oC$).

2.3 Physico-chemical analysis of water samples

The collected River water samples were analyzed in the laboratory for pH, temperature, electrical conductivity (EC) and total dissolved solids (TDS) using EUTECH multi-parameter tester digital-electrode pH meter. The biological oxygen demand (BOD) of the samples was determined by Wrinkler's titration method [13].

2.4 Bacteriological analysis of water samples

Quantitative bacteriological analysis of the water samples were carried out by using standard plate count (SPC) to identify the general bacterial load of the sampling points. Total heterotrophic bacterial population was quantified using R-2-A agar medium. Total coliforms and total thermotolerant coliforms were detected and quantified with the use of Eosin methylene blue (EMB) agar. Their counts were expressed in cfu/100ml of the water.

Fish sample preparation

Sample preparation was made using the method described in the literature [14]. Approximately, 10 g of the fish sample was cut from the head, middle and tail regions with a sterile knife. The cut samples were crushed into small pieces in a sterile mortar with about 10 ml sterile water. From the crushed sample, 1 ml aliquot volume was measured out and homogenized in a clean, dry sterile beaker containing 9 ml of distilled water giving a 1:10 dilution.

Enumeration of bacteria from skin, liver, intestine and tissues

The bacterial counts on the external surfaces, liver, intestines and tissue were estimated as follows:

Skin Surfaces

Sample from different locations of the skin of the raw fish was taken by rubbing the sterilized cotton swab over the skin and then inoculated into 100 ml of peptone water and further kept for incubation at 37^0C. Ten fold serial dilution of the bacterial suspension already inoculated in peptone water was prepared in duplicate. Bacteria were enumerated using spread plate method with Nutrient agar, MacConkey agar, King's agar and TCBS agar. The plates were incubated at $35±2^oC$ for 24h. The observed colony growth were counted using Coulter™ Colony counter according to plate count method.

Intestines, Liver and Tissues

One gram of the fish sample was dissected out, blended and mixed properly in a mortar. It was aseptically transferred to a sample bottle containing 9 ml of 0.1% sterile peptone water. The bottle was closed and shaken thoroughly for 10 minutes and allowed to stand for 20 minutes, after which a 10 fold serial dilution was carried out in duplicates and viable aerobic bacterial counts were enumerated in standard plate count agar after incubation at 37^oC for 48 hrs as described by [15]. The observed colony growth were counted using Coulter™ Colony counter according to plate count method.

Estimate of mean colony forming unit per gram (CFU g^{-1})

The mean colony forming unit per gram (CFU g^{-1}) denoted by (x) was calculated as $\Sigma f\chi/\Sigma f$, where Σfx is the sum of the products of number of colonies and the colony forming unit per gram; while Σf is the summation of the number of colonies.

Examination of Bacterial Density in Tissues

Demonstration of bacteria in the fish tissues was done using special staining by gram's staining modifications [16, 17]. Thin sections of the body tissue, liver and the intestine of the fishes from both the upstream and downstream were stained by special gram's staining method for bacterial examination.

Bacterial isolation and presumptive identification

The experiments were carried out immediately after collection .Bacteria isolated from the sampling sites (described above), were grown on nutrient agar at 37°C for 18 to 24 hours. Each colony was the subject of the following tests: morphological, physiological and biochemical tests. Then our results were compared to the known characteristics of bacteria in Bergey's Manual of Systematic Bacteriology [18].

Whole cell MALDI-TOF mass spectrometry analysis

The MALDI -TOF mass spectrometry protein analysis was carried out following [19, 20]. The identification of the isolates by MALDI-TOF MS was performed on a Microflex LT instrument with FlexControl (version 3.0)

software (Bruker Daltonics) for the automatic acquisition of mass spectra in the linear positive mode within a range of 2 to 20 kDa, according to the instructions of the manufacturer. Automated analysis of the raw spectral data was performed by the MALDI BioTyper automation (version 2.0) software (Bruker Daltonics); it makes use of a large database containing reference spectra for more than 3200 reference strains [21].

The score value is defined by three components, the matches of the unknown spectrum against the main spectrum, the matches of the main spectrum peaks against the unknown spectrum, and the correlation of intensities of the matched peaks. This leads to a first score, from 0 (no match) to 1,000 (perfect identity), which is converted into a log score from 0 to 3. When the score is greater than 2.0, it is considered to indicate good species-level identification and scores above 2.3 correspond to excellent species-level identification. Values between 1.7 and 2.0 correspond to reliable genus-level identification and values below 1.7 indicate no identification (no significant similarity) [22, 23].

Results and Discussion

The results of the physicochemical parameters of the water samples, from the two sampling points on the river segment are presented in Table 1. The temperature of the water at the two sampling points were 30.8°C and 31.4°C which is slightly higher than the normal temperature range supportive of good surface water quality which is 0°C to 30°C [24]. The pH was found to be almost normal ranging having values of 7.02 and 7.64. Those values fall within the accepted range of 6.0–8.5 indicative of good water quality. The values of the total dissolved solids (TDS) were reported to be 40.00 µs/cm in the upstream and 2582.00 µs/cm in the downstream portion of the river. The highest value for TDS was recorded at the sampling point MG2, which is the downstream segment of the river. The significantly high TDS of the water are implicative of a high level of pollution of the sampling point when compared to the prescribed standards set under Environment Protection Rules [24].

The BOD at the upstream point was 2.85 mg/l and at the downstream station was found to be 7.33 mg/l. There was a consistent increase in the BOD values along the sampling points from upstream to downstream. The BOD value at downstream exceeded the primary quality criteria set by Environment Protection Rules [25]. Unpolluted waters typically have BOD values of 2 mg/L or less, whereas those receiving wastewaters may have values up to 10 mg/L or more. The BOD value was high, probably due to the discharge of domestic wastes especially defecation activities and poorly executed agricultural activities near the river banks which was observed during survey of the area.

The mean total viable count of heterotrophic bacteria (TH), total coliforms (TC) and total thermotolerant (fecal) coliforms (TTC) of the water samples collected from the upstream and downstream points are presented in table 2. In all the cases the highest count of bacteria was reported at the downstream stations.

Table 1. Physico-chemical characteristics of water samples collected from Mogral River segment.

Parameters measured	Sampling points	
	MG1 (point 1)	MG2 (point 2)
pH	7.02 ± 0.01	7.64 ± 0.02
Temperature (^oC)	30.8 ± 0.15	31.4 ± 0.35
Electrical Conductivity (µs/cm)	58.00 ± 1.53	3590.00 ± 1.00
Total Dissolved Solids (µs/cm)	40.00 ± 0.40	2582.00 ± 2.08
Biological Oxygen Demand (mg/l)	2.85 ± 0.04	7.33 ± 0.02

An alarming high mean TCC was obtained at Mogral Puthur sampling point. The mean total viable count of heterotrophic bacteria (TH), total coliforms (TC) and total thermotolerant (fecal) coliforms (TTC) of the water samples collected from the upstream and downstream points are presented in Table 2. In all the cases, the highest count of bacteria was reported at the downstream stations. The TTC values were all relatively higher than the recommended limit for river water quality. The significant decrease in the TDS content of water samples collected at point MG1 could be linked to the observed correspondingly lowest count of the total heterotrophic bacteria enumerated at the same point. This observed decrease could be linked to the effect of self-purification process at the flowing stream which could reduce the microbial population.

A faecal coliform index obtained by a ratio of faecal coliforms to total coliforms, has been proposed and recommended by [24] instead of the formally used total coliform index for evaluating the microbiological suitability of freshwaters for recreational uses. The proposed maximum acceptable limit was 200 faecal coliforms per 100 ml and 126 *E. coli* per 100 ml [24]. This preference was selected because faecal coliforms were more faecal-specific and less subject to variation than, total coliforms which were greatly influenced by storm water run-off. It has also been found that, usually, more than 95 percent of thermotolerant coliforms isolated from water are the gut organism *Escherichia coli*, the presence of which is definitive proof of faecal contamination [26]. The alarming high number of total coliforms and thermotolerant (faecal) colifoms per 100 ml obtained from the water samples, which exceeded at least ten times the recommended limit, indicates high level of faecal pollution of the river water which potentially poses a high health risk for recreational purposes, let alone for drinking purpose. This clearly implies that the organic pollution at the downstream segment of the river is more of faecal origin. The total number of heterotrophic bacteria reflects the contamination extent by the easily decomposable organic matters, while the faecal coliform bacteria number gives an idea of the contamination size by faecal substance [27].

The standard plate count of the fish samples, in different growth media ranged between 0.36 x 10^4 and 22.53 x 10^4 cfu/g as shown in table 3. Out of the two fish samples analysed, the skin had the lowest isolation with 1.03 x 10^4 to 3.53 x 10^4

Table 2. Mean Total Heterotrophic, Total Coliform and Total Thermotolerant Coliform counts of the water samples from two sampling points on Mogral river Segment.

Sampling points	Total Heterotropic count (TH)	Total Coliform count (TC)	Total Thermotolerant Coliform count (TTC)	Freshwater quality standard limit (Fecal coliforms/100ml)*
MG1 (point 1)	12.3x10^4 ± 0.23	9.3x10^4 ±1.41	2.0 x10^4 ±0.70	200
MG2 (point 2)	22.4 x10^4 ±2.64	31.4 x10^4 ± 3.60	3.29 x10^4 ±4.00	200

*Source: [24]

cfu/g in upstream and in downstream 1.63 x 10^4 to x 9.40 10^4 cfu/g respectively. The coliform count which was evident from the growth in Macconkey plates was highest in the fish collected from downstream portion (MG2) as compared to the other fish. The presence of faecal coliforms in fish indicates the level of pollution of their environment because coliforms are not the normal flora of bacteria in fish. The intestine, liver and tissues were also heavily populated by bacteria with the maximum load reported in the tissue of *Etroplus suratensis* (22.53 x 10^4 cfu/g), followed by the intestine (22.23 x 10^4 cfu/g). Likewise, the liver of *Etroplus suratensis* exhibited the highest colonization rate of 21.40 x 10^4 cfu/g and 20.70 10^4 in Macconkey and Nutrient agar respectively. In TCBS plates the skin of the fish from upstream exhibited no bacterial growth. Also, TCBS plates showed a low isolation rate in all samples analysed as generally compared with other growth media. The diversity of potential bacteria from the samples of fish is of concern particularly at a time when many in our communities are immunologically compromised as a result of various illnesses.

Examination of the direct bacterial load in various parts of the fish samples were carried out using special gram staining technique. The photomicrographs of the differentiated bacterial cells present in the tissue, liver and intestine tissues of *Etroplus suratensis* from the upstream and downstream points of Mogral river segment is shown in figure 2(A,B), 3(A,B) and 4(A,B) respectively. As the isolated bacteria belonged to the gram negative group, shades of red colour in the figures denoted both the bacteria and hepatocytes. Figure 2(B) shows numerous gram-negative cells which are concentrated and dispersed in some areas within the tissue section. The gram-negative cells are more densely populated in the liver than the other tissue sections, indicating more red shades on the plate.

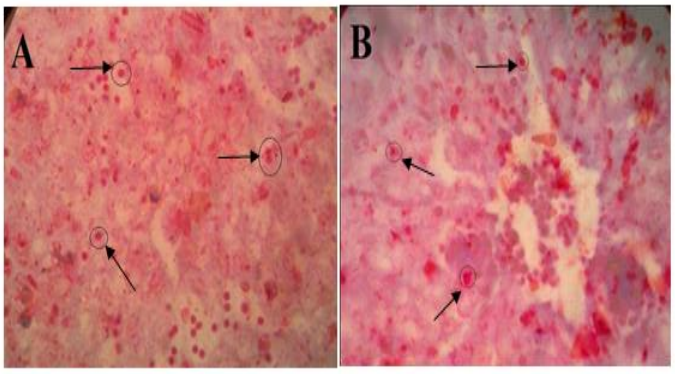

Fig 2 A. Examination of bacteria in liver of *Etroplus suratensis* obtained from upstream segment; (B) Examination of Bacteria in liver of *Etroplus suratensis* obtained from downstream segment

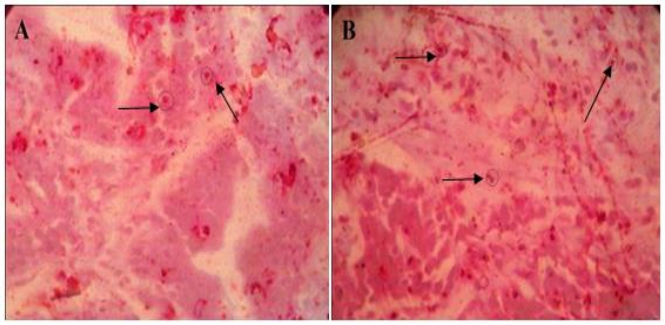

Figure 3 A. Examination of bacteria in intestineof *Etroplus suratensis* obtained from upstream segment; (B) Examination of Bacteria in intestine of *Etroplus suratensis* obtained from downstream segment.

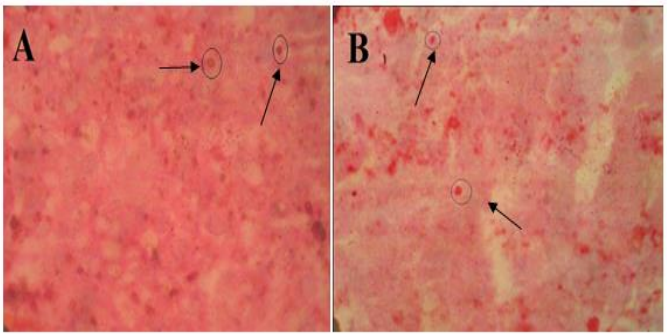

Figure 4 A. Examination of bacteria in tissues of *Etroplus suratensis* obtained from upstream segment; (B) Examination of Bacteria in tissues of *Etroplus suratensis* obtained from downstream segment.

There was a small distinction with a little increase in the bacterial population in the intestine of *Etroplus suratensis* from the upstream point over the bacterial population in downstream point of the river segment. It is also evident from Figure 4(A) and 4(B) that the concentration of gram-negative bacterial population was scanty in the tissue sections when compared to the bacterial concentration in liver and intestinal sections.

Nevertheless, the high microbial load observed on the gram-stained histological slides of the liver, intestine and tissue sections of the fish (figures 2, 3 and 4) is in agreement with the results of the physicochemical and bacteriological examination of Mogral river pollution. There are comparative distinctions. The bacterial population observed in the fish intestine of both upstream and the downstream could be due to the enteric region of the fish. The increase in microbial load observed in the fish liver from MG1 when compared to MG2 reveals pollution effect in the fishes of the river. This work was in accordance with the work of [28] where he reported that the invasion of fish flesh by pathogenic bacteria is very likely if the fish are reared in water containing over 10^4 of faecal coliforms (*Escherichia coli*) and, that high concentrations of pathogenic microorganisms might occur in the digestive tract and intraperitoneal fluid of the fish even at low numbers of indicatory bacteria.

Table 3. Mean count of the bacteria present at different parts of examined sampled fishes.

Sampling point	Parts of fish	Nutrient agar (cfu/g)	MacConkey agar (cfu/g)	King's agar (cfu/g)	TCBS agar (cfu/g)
MG1 (point 1)		10^4	10^4	10^4	10^4
	Skin	2.56 ± 2.08	3.53 ± 1.52	1.03 ± 1.52	-----
	Intestine	3.40 ± 2.64	4.26 ± 2.08	1.43 ± 2.08	0.36 ± 2.51
	Liver	2.26 ± 3.05	4.00 ± 1.00	1.00 ± 1.00	0.36 ± 1.50
	Tissue	1.63 ± 1.52	2.50 ± 3.00	1.00 ± 2.00	0.43 ± 1.52
MG2 (point 2)	Skin	8.36 ± 2.08	9.40 ± 2.64	4.23 ± 2.51	1.63 ± 1.52
	Intestine	19.36 ± 3.21	22.23 ± 3.21	5.53 ± 3.51	2.30 ± 2.64
	Liver	20.70 ± 2.64	21.40 ± 2.64	6.26 ± 2.08	1.86 ± 1.52
	Tissue	22.53 ± 0.57	20.20 ± 2.60	5.90 ± 1.00	2.20 ± 1.00

The findings, of the present study confirm that, the microbiological quality of the fishes from river is poor and unsafe for consumption.

The eight bacterial isolates from the fish samples were designated as B1-B8 and were further subjected to strain identification by whole-cell MALDI-TOF MS analysis. This method discriminates bacteria on the basis of screening of characteristic peaks observed as biomarkers for bacterial identification. Large numbers of peaks are retained for each reference strain, constituting a spectrum typical of the species concerned. This strategy is improved by the use of several reference strains for each species, which must be included in the database. In our experiment, we used the software MALDI Biotyper (Bruker Daltonics) to compare the collected spectra of our eight bacterial cultures with the reference database, to generate a numerical value (score) based on the similarities between the observed and stored datasets. As shown in table 4, all the eight spectra aligned with the MALDI Biotyper database were correctly identified to the species level (scores ≥ 2).

The identification studies had clearly indicated that, the bacteria isolated from the fish samples were present in all sorts of environment of human involvement, majority of them are human as well as animal pathogen.

Table 4. Identification of bacteria based on the score value of MALDI-TOF MS.

Bacteria	Identification according to MALDI Biotyper	Scores
B1	*Rahnella aquatilis*	2.211
B2	*Enterobacter clocae*	1.746
B3	*Escherichia coli*	2.413
B4	*Enterobacter absuriae*	2.005
B5	*Aeromonas hydrophila*	1.958
B6	*Enterobacter kobei*	2.297
B7	*Enterobacter ludwigii*	2.146
B8	*Pseudomonas aeruginosa*	1.964

Among the eight bacterial strains isolated from the fish samples, four samples belong to the *Enterobacter* species. The presence of enteric bacteria can be attributed to fecal contamination due to improper sewage disposal and/or water pollution. The presence of *E.coli* was attributed to the contamination of the fish samples by raw sewage that is discharged directly into the tributaries feeding the river. The fish in this study also harboured human disease causing organism like *Pseudomonas* which is most likely to cause food-borne diseases. Another bacterium named *Rahnella aquatilis* (Enterobacteriaceae) was detected from the samples, which is highly capable of causing food borne illnesses with severe antibiotic resistance [26]. *Aeromonas hydrophilia* which is disease causing bacteria in fish was also isolated from this study. These organisms can cause "hemorrhagic septicaemia" which includes lesions of septicaemia when the bacteria or bacterial toxins are present within the organs or skin of the fish. The results from this study and according to the published microbiological guidelines cited by [27] suggest that the microbiological quality of the fish examined is unacceptable and pose a potential risk to public health.

In this investigation, more than one species of bacteria were isolated from the fish from downstream, which may be associated with the nature of poor water quality observed in the segment. Bacterial species like *Rahnella aquatilis*, *E.coli*, *Aeromonas hydrophilia*, *Enterobacter absuriae* and *Enterobacter kobei* were specifically isolated from the fish samples collected from the downstream segment.

All these bacterial species are known to be present in all sorts of environment of human involvement mainly on sewage, intestinal tracts of humans and animals etc. Also, bacterial species like *Enterobacter cloacae* and *Pseudomonas aeruginosa*, which mostly occurs as commensals in water and soil were abundantly found in the fish samples collected from the upstream segment. In this study the extent of pollution was found to be higher in the downstream section of Mogral River. The higher incidence of diverse enteric bacteria in fish indicates a major health concern, by representing a potential hazard to human health. It is therefore recommended to coordinate different efforts at the level of the community dwellers and the government to rescue the downstream segment of Mogral River and its aquatic life from the current hazard-posing environmental problems.

Conclusion

The study based on the water quality analysis and identification of pathogenic bacteria in the fish indicated that, the Mogral River especially in the downstream is polluted. The study has been able to track the type of pollution to be more of fecal contamination by the examination of the bacteriological quality of the river water samples. Eight human bacterial pathogens were isolated and identified from the fish samples, and the highest load was reported to be from the downstream point. However, further investigation of the bacteria present in the fish organs portrayed a very high bacterial density in the liver compared to the other parts. The implications of these findings pointed to the fact that, people dependant on Mogral river water for the uses like fishing and farming are exposed to public health risks. The fish collected from the river act as a reservoir of highly pathogenic agents and can cause disease to susceptible individuals especially the immune-compromised consumers. Moreover the recoveries of various organisms, which are potentially pathogenic to humans, in the fish suggest that if they are improperly handled, undercooked or consumed in raw, may contribute to the intake of pathogens. Further examination, for the presence of pathogens during handling, storage and up to the very point of consumption is needed for the protection and maintenance of public health.

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