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RESEARCH ARTICLE

Detection of Multi Drug Resistant Bacteria in Retail Fish Market Water Samples of Vashi, Navi Mumbai

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Abstract Occurrence of faecal indicator bacteria (FIB) is being reported regularly by various researchers. But there is no information about the microbiological quality of the water used in the retail fish markets. Hence to understand the hygienic status of the water used in retail outlets, about 51 water samples were collected from the retail outlets of Navi Mumbai region and microbiological parameters such as aerobic plate count (APC), faecal indicator bacteria viz., *Escherichia coli*, faecal streptococci (FS) and sulphite reducing clostridia (SRC) were enumerated. Antibiogram was also carried out for 57 isolates against 20 antibiotics. Results indicated that all water samples harboured higher levels of APC, *E. coli*, FS and SRC with an average value of 558, 41, 51 cfu/mL and 2.42/20 mL respectively. Ratio between the FS and *E. coli* i.e., 1:1.25 indicates the multiplication of *E. coli* in the water used in the retail market. Higher level of resistance was observed for Augmentin and Colistin. Four multi drug resistant (MDR) *E. coli* were observed in the water samples. Checking of water in the retail fish market is the most neglected area where high level of contamination and MDR bacteria have been detected. So, it was inferred that the repeated use of same water without replacement is a major cause of higher levels of FIB and MRD bacteria in the retail fish market water samples that further leads to their transfer from water to fish. Hence, running water facility should be used to clean the fish.

Keywords Faecal indicator bacteria · *Escherichia coli* · Faecal streptococci · SRC · Potable water · Retail fish market

Significance statement Most of the researches explore the microbial quality of the fish in the retail market; but not on the water used in fish market. It was found to have presence of around 4000 higher level of multidrug resistant *E. coli* in the water used in the fish market.

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Introduction

Quality of the water used for fish processing should be of good quality and potable in nature in order to avoid the transfer of pathogens from water to fish. Since monitoring of all pathogens is a cumbersome procedure, European Council directives have recommended testing the faecal indicator bacteria instead of all pathogens. Faecal indicator bacteria (FIB) generally exist as commensal in intestine of warm blooded animals [1]. Hence, the presence of these indicator organisms in fishes shows definite faecal contamination [2]. Even though, existence of mere FIB in fish is not directly linked with the pathogenic bacteria; there is high possibility of the presence of pathogenic bacteria as well. *Escherichia coli* faecal streptococci (FS) and sulphite reducing clostridia (SRC) are considered as important indicators for the assessment of faecal contamination [3]. Hence, the presence of these bacteria in potable water used for cleaning of the fish is to be considered seriously to overcome their transfer from the water to fish [4].

Escherichia coli is abundant in warm blood animal faeces; more over 90% of the coliform isolated from the faecal materials are confirmed as *E. coli* [5]. Hence, it is

considered as the best and primary indicator for the faecal contamination [6]. Moreover, it is an index organism for *Salmonella*, coliphages and human enteric viruses [7]. But it is highly labile and gets rapidly destroyed on freezing the food materials [1]. Faecal streptococci (FS) are a group of bacteria having better survivability in environment as well as freezing temperature than *E. coli* [1] which is used to monitor the quality of potable water resources [8]. Majority of FS isolated from the polluted water are of true faecal origin [9]. Similarly sulphite reducing clostridia (SRC) are group of bacteria that reduce sulphate to sulphite. Owing to their wide distribution and their ability to form spores that withstand very harsh environmental conditions, the presence of these SRC in water resources indicates remote faecal contamination. Since the SRC spores are highly resistant in nature and withstand the sanitation, it is being used to evaluate the virus, cyst inactivation and to assess the effect of sanitation in the drinking water disinfection processes [7]. Higher incidences of SRC in the freshwater and marine fishes were also reported in Cochin, Kerala, India [10].

Most of the researches in India are based on the quality checking of either inland or pond water [11, 12]. Clear standards were described by the Central Pollution Control Board (CPCB) to analyze the water quality for irrigation or drinking purpose [13]. But, clear guidelines regarding water used for fish processing were not described by CPCB. But, Bureau of Indian standard (BIS) has given clear guidelines regarding the parameter to be analyzed for ice/water used in fish processing industry i.e., IS-14517:1998 [14]. Recent emerging multi drug resistant bacteria viz., Methicillin resistant *Staphylococcus aureus* (MRSA) also reported in Indian retail fish market [15, 16]. High level of multi drug resistant *E. coli* was also reported in many Indian retail fish market [17]. High level of FIB bacteria such as *E. coli,* FS and SRC were found in the fresh water fishes of Indian retail market of Navi Mumbai region [18]. Similarly, enormous literatures were reported about the level of contamination on marine water [19–21]. But no detailed research has been carried out on the quality of the water used in the retail markets. Only one report was available regarding the water used in the fish retail market. Kumar et al. [22] collected 12 water samples of fish retail outlets along with fish sample's microbiological quality checking and found that 8 samples i.e., 66% of the samples harbour *E. coli*. In India, most of the consumers prefer to procure fishes from the retail fish markets; but the quality of the water used in the fish retail marker is the most neglected area in fish market. Hence, the present study was given importance to understand the quality of water used directly or indirectly in the retail market. Hence, a study was carried out to understand the quality of water used in the retail fish markets of Vashi, Navi Mumbai.

Material and Methods

Collection of Water Samples

Total 51 water samples were collected from retail fishery outlets of Navi Mumbai region using sterile conical flasks and analysed for aerobic plate count (APC), faecal indicator bacteria such as *E. coli,* faecal streptococci (FS) and sulphite reducing clostridia (SRC).

Aerobic Plate Count (APC)

One mL of water sample was serially diluted and poured over the Tryptone Glucose Beef Extract (TGBE) agar (Hi Media, #M791) plates and spread over the surface and incubated at 35 °C for 2 days for APC enumeration and the colonies between the 30–300 were counted for enumeration [23].

Escherichia coli

Most probable number (MPN) method of analysis is suitable method for coliform/faecal coliform/*E. coli* analyses in water sample (MPN/100 mL). But, in the present study, one mL contains average of around 40 *E. coli*, therefore, all test tubes were positive for *E. coli* (> 1400) for 100 mL MPN. Hence, in the present study, for enumeration of *E. coli,* ISO 9308-1 protocol was followed with slight modification. i.e., one mL of water was spread over the Tergitol 7 agar (Hi Media, #M6161) plates supplemented with 0.25 mL of 1% Triphenyl Tetrazolium Chloride (TTC) (Himedia, FD057) and the plates were incubated at 37 °C for 24 h [29]. Flat dry yellow colonies with or without red tinge were further checked on Eosin methelene blue (EMB) agar plates for characteristic dark purple colony with greenish metallic sheen. These green metallic sheen colonies were further subjected to IMViC tests for further confirmation [24].

Faecal Streptococci (FS)

The faecal streptococci were enumerated based on the pour plated method. The diluted sample (1 mL) from each test tube was transferred in the sterile empty petri plates. Beforehand, Kenner faecal (KF) streptococcal agar base (Hi Media, #M248) was boiled and cooled to 48 °C and supplemented with 1 mL of 1% TTC (FD057). The agar medium was poured into the petri plates containing 1 mL of diluted water sample and rotated firmly for uniform mixing. After the plates were dried, 5 mL of the KF agar medium was overlaid on the surface of each plate and allowed to dry in the room temperature. All the plates were

incubated at 35 °C for 48 h. Brown colonies surrounded by a halo zone were subjected to biochemical test for further confirmation [23].

Sulphite Reducing Clostridia (SRC)

SRC numbers were determined by a three tube most probable number (MPN) technique using Differential Reinforced Clostridial Broth (DRCB) (M549, Himedia). All black colour tubes were confirmed by streaking over Tryptose Sulfite Cycloserine (TSC) agar and the characteristic colonies were again confirmed by biochemical reactions [25].

Antibiogram

Antibiogram was carried out for all 57 confirmed *E. coli* isolates. The isolated strains were inoculated in Brain Heart Infusion (BHI) broth. After reaching the 0.5 Mc Farland level turbidity, the culture was spread plated uniformly onto pre set Mueller–Hinton agar (HiMedia # M173,) plates (pH 7.2–7.4) by cotton swab. Then commercially available antibiotics impregnated disc for 20 antibiotics disc (Himedia, # IC002) were placed over the plates. The zone diameter was measured after 24 h using the scale and the resistance level was interpreted based on the breakpoint using the standard chart [26].

Results and Discussion

Of 51 water samples collected from the retail fish markets of Vashi, Navi Mumbai, APC values of all the samples were more than the recommended limit. As per the EC (1998), the APC should be 20 cfu/mL at 37 °C [8]. But, in the present study, the average APC was 558.92 cfu/mL of water sample (Table 1) i.e., around 27 times higher than the European commission recommendation. Similarly, Bureau of Indian Standard (BIS) has published certain microbial limitation in 1998 for the APC related to the water used for fish processing i.e., IS-14517:1998 [14] the limit for the APC is 100 cfu/mL. Hence, as compared to BIS, the APC average was 5.5 times higher than the recommended limit.

As per IS-14517 recommendation, *E. coil* should be absent in 100 mL [28]; but in the present study, *E. coli* counts in all 51 samples were more than the recommended level, with the average count of 41.37 cfu/mL. As per BIS, water used in the fish processing Industry is contaminated 4137 times higher than the recommended limit. However, it is difficult to compare the coliform level with the CPCB standard of *E. coli* level; because, the limit for the coliform count is 50 MPN/100 mL. In general, higher level of *E. coli* was observed in the water.

For quality standards, the recent EC directive 98/83 recommends the level of Enterococci (sub set of faecal streptococci) absent in 250 ml [8]. FS in the water samples was 51.92 cfu/mL on an average. Recent dreadful report suggested that, the Enterococcus species viz. *E. faecalis* and *E. faecium* are responsible for endocarditis, intra-abdominal infection, surgical wound infection, and urinary tract infections in humans [27, 28]. Moreover, these FS are heat resistant; even withstanding pasteurization temperature. In addition, they are not affected by the ingestion [29]; thus enabling the bacteria to spread easily by handling.

As per the previous EC directive [28] recommendation, the level of SRC was absent in 20 ml. Since, *C. perfringens* is a more suitable indicator in SRC, recent 98/83/EC Directives amended as absence of *C. perfringens* in 100 ml of water [8]. Among the 51 samples tested, SRC counts of 8 samples were nearly zero (0.6–0.8 cfu/mL). The average levels of SRC were 2.42/20 mL. Since SRC can withstand the cooking temperature and other food processing techniques, most of the fishes carry the SRC even after cooking/processing or any other preservation method. Hence, hygienic handling practices have to be followed to avoid the SRC/*C. perfringens* in seafood.

The presence of the faecal indicators in the samples indicated that the water samples were highly contaminated with the faecal materials. Butniaux and Mossel [30] reported that polluted pond water contains 40×10^6 coliform, 4×10^6 of *E. coli* and 4×10^6 *Enterococcus sp*. per gram of faecal matter. The presence of 10 number of *E. coli* in the food is equal to contamination by 2.5 g faecal materials in fish. They also observed that ratio between the *Enterococcus* with *E. coli* is 1:0.1 respectively. But, in the present study, FS with *E. coli* ratio was 1: 1.25. i.e., very high level of *E. coli* in the water sample, which is possible due to multiplication of *E. coli* in water samples. In addition, correlation coefficient determination was carried out between the APC and Faecal Indicator bacteria and within Faecal Indicator bacteria and found that better correlation was found between the *E. coli* and FS i.e., 0.45; which indicates that a multiplication of the FS also takes place along with the *E. coli*. It was the reason for the higher level of the FS in the water samples. But, in case of the SRC, no multiplication happened, because of its anaerobic nature. So, the present study clearly indicates that the water samples used in the fish retail outlet act as a reservoir for *E. coli* multiplication.

Of 57 *E. coli* strains isolated from the samples, antibiogram was carried out with 20 antibiotics (Fig. 1; Table 1). Most of the isolates were resistant to augmentin (73%) and colistin (26%) (Table 1). Augmentin is a combination of amoxicillin trihydrate and potassium clavulanate (a beta-

Table 1 Resistant level of the *E. coli* isolates against the different antibiotics

Name and dose of antibiotics	No. of isolates	Resistant	Intermediate resistant	Percentage of resistant
Augmentin (AMC) 30 μg	57	42		73.68
Colistin (CL) 10 μg	57	15	30	26.32
Cefpodoxime (CPD) 10 μg	57	13	17	22.81
Nitrofurantoin (NIT) 300 μg	57	6	8	10.53
Co-Trimoxazole (COT) 25 μg	57	4	-	7.02
Nalidixic acid (NA) 30 μg	57	3	-	5.26
Imipenem (IPM) 10 μg	57	2	6	3.51
Levofloxacin (LE) 5 μg	57	1	-	1.75
Cefoxitin (CX) 30 μg	57	1	2	1.75
Gentamicin (GEN) 10 μg	57	1	2	1.75
Ciprofloxacin (CIP) 5 μg	57	-	-	0.00
Tobramycin (TOB) 10 μg	57	-	4	0.00
Moxifloxacin (MO) 5 μg	57	-	-	0.00
Ofloxacin (OF) 5 μg	57	-	-	0.00
Ceftazidime (CAZ) 30 μg	57	-	-	0.00
Norfloxacin (NX) 10 μg	57	-	1	0.00
Gatifloxacin (GAT) 5 μg	57	-	-	0.00
Amikacin (AK) 30 μg	57	-	-	0.00
Aztreonam (AT) 30 μg	57	-	-	0.00
Ceftriaxone (CTR) 30 μg	57	-	-	0.00

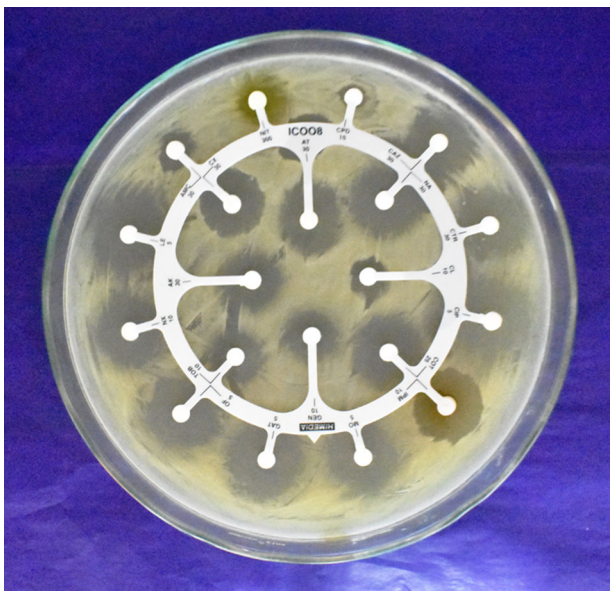

Fig. 1 Antibiogram for the *E. coli* isolates with 20 antibiotics. Decreased zone of Inhibition was observed for augmentin (AMC) and cefpodoxime (CPD)

lactamase inhibitor); combination results in antibiotic with an increased spectrum against beta-lactamase. Augmentin is prescribed as a last choice of penicillin class resistant bacteria. Bacteria resistance to Augmentin indicates their MDR nature. It is a drug of choice for extended spectrum of beta lactamase (ESBL) producing bacteria. Hence, the resistant to Augmentin indicates higher resistant bacteria present in the water samples. The resistant bacteria are generally mutant; which multiply more in number due to clonal selection and also potential agent for the transfer of the resistant gene in the water samples. Generally, Augmentin is not used in the pond water treatment and hence is an indication of the handler's contamination.

Colistin (polymyxin E) is a polymyxin antibiotic recommended for the last-resort antibiotics for multidrug-resistant bacteria. It is commonly administered as injection. Generally the injectable resistant antibiotics are rarely encountered in the environmental samples. But, in the present study, higher level of Colistin is viewed seriously because of the potential involvement of the handlers, not by the environmental contamination. The remaining isolates were resistant to cefpodoxime (22%), nitrofurantoin (10%), and co-Trimoxazole (7%). Antibiotics such as nalidixic acid, imipenem, levofloxacin, cefoxitin and gentamicin were observed to have 1–5% resistance. No resistance was observed for ciprofloxacin, tobramycin, moxifloxin, ofoxacin, ceftazidime, norfloxacin, gatifloxacin, amikacin, aztreonam and ceftriaxone. Even though, these isolates were resistant to augmentin and cepodoxime, they were susceptible to aztreonam and

ceftriaxone and hence these isolates were non-extended-spectrum beta-lactamase (ESBLs) producer. About four isolates were found to be resistant to ≥ 3 antibiotic classes and hence categorized as multi drug resistant (MDR) bacteria.

Conclusion

The present study was concerned about the most neglected area in fish market i.e., water used in the fish market for cleaning of fishes. The study inferred that retail markets are potential sources of FIB and MDR bacteria. Reason for the occurrence is due to repeated use of contaminated water for cleaning of fish in retail fish markets i.e. dipping of the fish for cleaning in the same water without replacement. Hence, the retailers need to follow stringent hygienic practices by use of clean potable free flowing water for washing of fish. Meanwhile, researchers need to examine the water quality of the retail fish market while collecting the fish samples to assess the transfer of MDR pathogens from water to fish in retail fish outlets. The current data provides an insight of the prevailing unhygienic condition of retail fish markets thus providing information to the consumers, producers and regulators to establish appropriate public policies. Similar studies are mandatory for creating general awareness among consumers regarding the quality of water and ultimately the fish in retail market thus instigating the consumer to demand/insist for the supply of safe and quality fish.

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Compliance with Ethical Standards

Conflict of interest It is declared that there is no conflict interest among the authors to publish this manuscript.

References

1. Huss HH (1994) Traditional microbiological quality control. In: Assurance of seafood quality, FAO Fisheries Technical Paper, p 334. http://www.fao.org/docrep/003/t1768e/t1768e04.htm
2. Kulshrestha H, Sharma S (2006) Impact of mass bathing during Ardhkumbh on water quality status of river Ganga. J Environ Biol 37:437–440
3. Tyagi VK, Chopra AK, Kazmi AA, Kumar Arvind (2006) Alternative microbial indicators of faecal pollution: current perspective. Iran J Environ Health Sci Eng 3:205–216
4. Ashbolt NJ, Grabow WO, Snozzi M (2001) Indicators of microbial water quality. http://www.who.int/water_sanitation_health/dwq/iwachap13.pdf
5. Hurst CJ, Crawford RL, Knudsen GR, McInerney MJ, Stetzenbach LD (2002) Manual of environmental microbiology, 2nd edn. ASM Press, Washington
6. Tallon P, Magajna B, Lofranco C, Leung K (2005) Microbial indicators of fecal contamination in water: a current perspective. Water Air Soil Pollut 166:139–166
7. WHO (2002) World Health Organization, guidelines for drinking water quality. Health criteria and other supporting information, vol 2, 2nd edn. World Health Organization, Geneva
8. EC (1998) Council Directive 98/83/EC of 3 November 1998, on the quality of water intended for human consumption. L 330/32 EN Official Journal of the European Communities5.12.98. http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:1998:330:0032:0054:EN:PDF
9. Pinto B, Pierotti R, Canale G, Reali D (1999) Characterization of fecal streptococci as indicators of faecal pollution and distribution. Environ Appl Microbiol 29:258–263
10. Visnuvinayagam S, Viji P, Binsi PK, Chakrabarti R, Thriveni A, Joshy CG, Lalitha KV (2015) Effect of monsoon on sulphite reducing clostridia levels in fish from retail outlets in Maharashtra. Fish Technol 52:198–201
11. Dalvie VV, Amte GK, Naber Bela (2013) Analysis of microbial load in Kalkhadi lake, Kharbav, (dist: Thane), India. Pollut Res 32:765–767
12. Purnima G, Dhruba S (2013) Microbial contamination of community pond water in Dibrugarh district of Assam. Curr World Environ 8:85–89
13. CPCB (1995) Central Pollution Control Board, classification of inland surface waters (CPCB Standards). Water Qual Parivesh 1(4):6
14. IS (1998) IS:14517:1998, fish processing industry—water and ice-technical requirements. Bureau of Indian, New Delhi
15. Visnuvinayagam S, Toms CJ, Murgadas V, Chakrabarti R, Lalitha KV (2015) Status on methicillin resistant and multiple drug resistant staphylococcus aureus in fisheries of Cochin and Mumbai Cost, India. J Environ Biol 36:571–575
16. Visnuvinayagam S, Murthy LN, Viji P, Sivaraman GK (2017) Study on retail fish markets: possible occurrence and transmission of emerging pathogen from faecal indicators. J Environ Biol 38:465–470
17. Murugadas V, Joseph TC, Lalitha KV (2016) Distribution of pathotypes of *Escherichia coli* in seafood from retail markets of Kerala, India. Indian J Fish 63:152–155
18. Visnuvinayagam S, Viji P, Murthy LN, Jeyakumari A, Sivaraman GK (2016) Occurrence of faecal indicators in freshwater fishes of Navi Mumbai in retail outlets. Fish Technol 53:334–338
19. Sivaraman GK, Vanik D, Prasad MM, Visnuvinayagam S, Basha KA, Nadella RK, Jha AK, Vaja C (2017) Detection of chromosomal AmpC, bla CTX-M in extended spectrum beta lactamase producing *Escherichia coli* in seafood processing effluent. Proc Natl Acad Sci India Sect B Biol Sci. https://doi.org/10.1007/s40011-017-0868-6
20. Shibata T, Solo-Gabriele HM, Fleming LE, Elmir S (2004) Monitoring marine recreational water quality using multiple microbial indicators in an urban tropical environment. Water Res 38:3119–3131
21. Sivaraman GK, Visnuvinayagam S, Jha AK, Renuka V, Remya S, Vanik D (2016) Assessment of microbial quality of fish processing industrial effluent in bar-mouth at Bhidia landing site, Veraval, Gujarat, India. J Environ Biol/Acad Environ Biol India 37:537–541

22. Kumar HS, Parvathi A, Karunasagar I (2005) Prevalence and antibiotic resistance of *Escherichia coli* in tropical seafood. World J Microbiol Biotechnol 21(5):619–623
23. APHA (2001) American Public Health Association, Downes FP, Ito K, compendium of methods for the microbiological examination of foods, 4th edn. APHA, Washington
24. ISO (1990) ISO: 9308-1. International Organization for Standardization (ISO), 1990, Draft ISO/DIS 9308-1
25. Collee JG, Fraser AG, Marmion BP, Simmons A (1996) Mackie and McCartney, practical medical microbiology, 14th edn. Churchill Livingstone, London
26. CLSI (2002) Clinical and Laboratory Standards Institute. Performance Standards for antimicrobial disk susceptibility tests for bacteria isolated from animals. Approved Standard M32-A2. 2nd ed. CLSI, Wayne, PA
27. Ross PW (1998) Streptococcus and enterococcus. In: Greenwood D, Slack R, Peutherer J (eds) Microbiology, a guide to microbial infections: pathogenesis, immunity, laboratory diagnosis and control, 15th edn. Produced by Addison-Wesley Longman. China Limited, Hong Kong, pp 9–25
28. Barrell RA, Hunter PR, Nichols GC (2000) Microbiological standards for water and their relationship to health risk. Commun Dis Public Health 3:8–13
29. Sorensen TL, Blom M, Monnet DL, Frimodt-Møller N, Poulsen RL, Espersen F (2001) Transient intestinal carriage after ingestion of antibiotic-resistant *Enterococcus faecium* from chicken and pork. N Engl J Med 345:1161–1166
30. Butniaux R, Mossel DAA (1961) The significance of various organisms of faecal origin in foods and drinking water. J Appl Bacteriol 24:353–364