Original Research

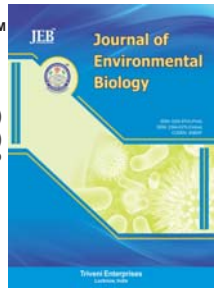
Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

JEB™

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

DOI: https://doi.org/10.22438/jeb/38/3/MS-256



Study on retail fish markets: Possible occurance and transmission of emergeing pathogen from faecal indicators

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Key words

E. coli,
Faecal indicators,
Faecal streptococci,
Sulphite reducing clostridia

Publication Info

Paper received : 09.02.2016
Revised received : 23.10.2016
Re-revised received : 27.10.2016
Accepted : 09.11.2016

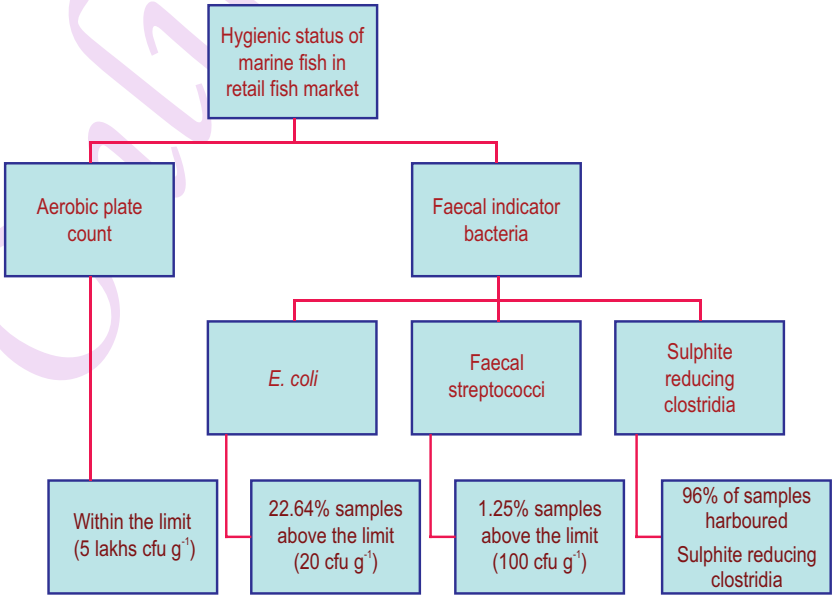
Abstract

Aim: Most of the Indian consumers *hitherto invouge* prefer to procure the fish in retail markets despite of its poor unhygienic condition. Hence, multiyear environmental study was carried out to scrutinize the hygienic status by faecal indicator estimation in retail markets of Navi Mumbai region.

Methodology: In total, 159 marine fish were collected and analysed for estimation of aerobic plate count and faecal indicator bacteria *viz*., *E. coli*, faecal streptococci and sulphite reducing clostridia.

Results: Aerobic plate counts of all fish were within the limit; but the level of *E. coli* was higher in retail fish *i.e.,* 22.4%. The average level of *E. coli*, faecal streptococci and sulphite reducing clostridia were 14, 31, 15 cfu g^{-1}, respectively. Higher ratio was observed between the faecal streptococci and *E. coli i.e*., 1: 0.45.

Interpretation: Fish in retail markets harbour noteworthy number of faecal indicator bacteria which indicates considerable number of faecal contamination and poor hygienic status of the retail market. Repeated use of same water for washing fish may be reason for the elevated level of contamination. So, cleaning of fish with potable water may reduce contamination. In addition, these retail markets need to be monitored by the controlling authority at regular intervals with stringent control policy in order to provide safe seafood.



Introduction

Faecal indicator bacteria are index organisms that generally exist as commensal in the intestine of warm blooded animal; but, not in fish (Huss, 1994). Hence, the presence of these indicators in fish show definite faecal contamination (Kulshrestha and Sharma, 2006; Sharma and Chaturvedi, 2007). *E. coli,* faecal streptococci and Sulphite reducing clostridia are considered as important indicators for assessment of faecal contamination (Tyagi *et al.,* 2006). Presence of mere faecal indicator bacteria in fish is not directly connected with pathogenic bacteria; but, there is high chance of occurrence of pathogenic bacteria in water bodies. Hence, existence of these faecal indicator bacteria in fish needs to be considered very critically to control the waterborne pathogenic bacteria/virus/parasitic oocyst contamination in fish (Ashbolt *et al*., 2001).

E. coli is a primary indicator for faecal contamination; abundant in warm blood animal faeces. So, *E. coli* is considered as a sole indicator for faecal contamination (Tallon *et al*., 2005). But, it exists in environment for short period and rapidly destroyed while freezing the food materials (Huss, 1994). Another group of faecal indicator bacteria *viz.* faecal streptococci is being used to monitor the potable water resources (EC, 1998; Krishnan *et al*. 2007). It has better survivability in environment and in freezing temperature thus make the bacteria can stay longer period in environment than *E. coli* (Huss, 1994). Around 84% of sewage contaminated polluted water sources contain faecal streptococci; hence, it is considered as true faecal indicators. The term faecal streptococci and Enterococci are used alternatively because Enterococci are considered as sub set of faecal streptococci. Recent report says that these faecal streptococci are not only an indicator bacteria but also a pathogenic bacteria *i.e., Enterococcus faecalis* and *Enterococcus faecium* are responsible for endocarditis, intra-abdominal infection, surgical wound infection and urinary tract infections in human (Ross, 1998; Barrell *et al*., 2000). It has also been recorded that *E. faecalis* and *E. faecium* are regularly isolated from cheese, fish, sausages, minced beef and pork (Foulquie *et al.,* 2006; Klein, 2003). *E. faecalis* and *E. faecium* are the most prevalent species isolated from humans clinical cases. *E. faecium* is responsible for most Vancomycin-Resistant Enterococci infections.

Sulphite reducing clostridia are group of bacteria that reduce sulphate to sulphite; presence of these sulphite reducing clostridia in water resources indicate the remote faecal contamination. Since sulphite reducing clostridia spores are highly resistance in nature and withstand sanitation, it is used to evaluate the virus, cyst inactivation and to assess the effect of sanitation in drinking water disinfection processes (WHO, 1996; Borrego *et al*, 2002).

In the past year, faecal streptococci are considered as an indicator for faecal contamination; but, recent research report from hospitals are frequently associated with the faecal streptococci as a prime pathogenic responsible of urinary infection and other critical diseases. Since, fish are handled by various persons, it has been found that a considerable number of faecal streptococci in fish and elevated level of faecal streptococci in some of the fish and shell fish. But, permissible limits/standards have not yet been defined by Food Control Authority in India, as well as international for faecal streptococci. In case of other faecal indicators such as *E. coli* and *C. perfringens*, permissible limit has been defined by Indian Food Control Authority *i.e*., 20 cfu g^{-1} and absence in 25 g^{-1}respectively (FSSAI 2012). Considering the emergence of pathogenic Faecal streptococci, in the present study Faecal streptococci and other Faecal Indicator Bacteria were investigated in marine fish and shell fish, available in retail markets with reference to different species, and discussing about the possible source of contamination and prevention. Since, most of the Indian consumers prefer local retail outlet for the procurement of fish, the present study mainly focused on the local retail markets.

Materials and Methods

Fish sample from retail fishery market : In total, 159 marine water fish were collected randomly, based on the availability of the fish in different fish markets (Table 1) in a sterile polythene bags with ice and brought to laboratory within four hours. Name of the collected fish and shell fish are as follows *Chirocentrus dorab, Johnius dussumieri, Epinephelus sp., Congresox talabonoides, Tylosurus crocodilus, Parastromateus niger, Lates calcarifer, Rastrelliger kanagurta, Mugil cephalus, Sillago sihama*, *Nemipterus japonicus, Eleutheronema tetradactylum, Dasyatis sp., Sardinella longiceps, Scomberomorus sp., Penaeus sp., Pampus argenteus, Cynoglossus sp., Loligo duvauceli.*

Aerobic plate count : Twenty five grams of fish sample was aseptically taken from upper part of the fish and transferred to 225 ml of 0.1% peptone saline; the fish meat was then blended in a stomacher (Seaward, UK) for 2 min. The blended material was serially diluted upto 10^{-6} dilution; then, 0.5 ml of sample was transferred to Tryptone Glucose Beef Extract agar (Hi Media, #M791) plates and spread over the surface and incubated at 35° C for 2 days for aerobic plate count enumeration (APHA, 2015).

***Escherichia coli* :** Enumeration of *E. coli* was carried out by ISO, 9308-1 (ISO, 1990) with slight modification *i.e*., 0.5 ml of serially diluted fish sample was transferred to Modified Tergitol-7 (Hi Media, #M6161) plates supplemented with 0.25 ml of 1% Triphenyl Tetrazolium Chloride (Himedia, FD057) and spread over the surface; then, all the plates were incubated at 35° C for 24 hrs. Flat dry yellow colonies with red tinge were further streaked over eosin methelene blue agar plates; after incubation, colonies showing greenish metallic sheen were further subjected to IMViC for further confirmation (ISO, 1990).

Faecal streptococci : The faecal streptococci were enumerated, based on the pour plated method. One ml of dilute sample from each test tube was transferred to sterile empty petri plates. Then Kenner faecal (KF) streptococcal agar base (Hi Media, #M248) was boiled and cooled to 48°C then supplemented with 1ml of 1% of 2,3,5-triphenyl-2H-tetrazolium chloride (FD057). Finally, agar medium was poured in petri plate containing 1 ml of diluted fish meat and rotated firmly for uniform mixing. After the plates were dried, 5ml of KF agar medium was overlaid on the surface of each plate and allowed to dry in room temperature. All the plates were then incubated at 35°C for 48 hrs. Brown colonies surrounded by a halo zone subjected to biochemical test for further confirmation (APHA, 2015; Sugumar *et al*., 2008).

Sulphite reducing clostridia : Sulphite reducing clostridia numbers were determined by a three tube MPN technique using Differential Reinforced Clostridial Broth (DRCB) (M549, Himedia). All black colour tubes were confirmed by streaking on to Tryptose Sulfite Cycloserine (TSC) agar and the characteristic colonies were again confirmed by biochemical reactions as described by Collee *et al.* (1996).

Results and Discussion

Aerobic plate count for the collected samples were below 5,00,000 cfu g^{-1} and ranged between 2,000 – 4,00,000 cfu g^{-1}. But, 22.64% of fish samples harboured higher level of *E. coli i.e.*, more than the recommended level (20 cfu g^{-1}); an average of 14.62 cfu g^{-1} of *E. coli* was noticed in the fish of retail fish market. Higher level of *E. coli* (56.67 cfu g^{-1}) and sulphite reducing clostridia (51.67 cfu g^{-1}) was noticed in Shrimp species (*Penaeus sp.*). Higher level of faecal streptococci (60 cfu g^{-1}) was noticed in seer fish. Average of 31.93 and 15.80 cfu g^{-1} was observed for the faecal streptococci and sulphite reducing clostridia respectively (Table 1).

Correlation coefficient was used in the study to assess the impact of faecal indicator on aerobic plate count using Excel, Window 7 and it was found that there is no significant correlation was observed between and aerobic plate count and faecal indicator bacteria (Table 2). The ratio between the faecal streptococci and *E. coli* was 1: 0.45.

Aerobic plate count is a prime indicator of food spoilage; even though aerobic plate counts are not hazardous to human, it indicates the level of spoilage. As per Indian and international standard, the limitation for aerobic plate count in fish was 5,00,000 cfu g^{-1} (FSSAI, 2012; IS, 1978; ICMSF, 1986). In the present study, the aerobic plate count level of all the collected samples were within 5,00,000 cfu g^{-1} the reason may be that fishes are procured on the basis of freshness index such as color, appearance and firmness.

Eventhough, aerobic plate count was within the limit, there is a huge possibility of presence of hazardous microorganism in the fish. Because, most of the fish were exposed to contamination while handling/processing. Indian Food Control Authority has given clear guidelines such as the fish sample should not have *E. coli* higher than 20 cfu g^{-1}. In the present investigation among 159 samples, 36 samples consisted of higher *E. coli i.e.,* 22.64% of the samples had higher *E. coli* count than the recommended level. Among the collected seafood, shrimp species were highly contaminated (Table 1), which might be due to the use of contaminated water in the fish market, and led to the accumulation of *E. coli* between the shrimp shell and the body, thus increase the contamination. On an average around 14 cfu g^{-1} of *E. coli* were present in the fish of retail market, which indicates a considerable number of faecal contaminations were observed in the retail markets.

Even though, faecal streptococci are the important indicator, there is no international limit in fish/ meat. But, European council recommended level of for *Enterococcus sp*. (sub set of faecal streptococci) in potable water is 0/250 ml (EC, 1998); but not for food or fish. In India, Bureau of Indian Standard (BIS) recommends that level of faecal streptococci should not exceed 100 cfu g^{-1} of fish (IS: 4780 – 1978). So, as per Indian standard IS-4780- 1978 (IS, 1978) in the present investigation two samples were exceeding the recommended limit of faecal streptococci *i.e.*, 100cfu g^{-1} (IS: 4780 – 1978). In India, implementing and monitoring authority *viz.,* Food safety standards authority of India (FSSAI) has not mentioned the faecal streptococci level in fish. 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, endocarditis and meningitis in humans (Ross, 1998; Barrell *et al.*, 2000). *E. faecalis* recorded as a main cause of life-threatening infections in humans, especially in the nosocomial (hospital) environment with high levels of antibiotic resistance (Ryan and Ray, 2004). Around 30% to 90% of root canal-treated teeth are infected with *E. faecalis*. About nine times increase in recurrence of *E. faecalis* as a secondary infection in root canal infection than primary infections (Molander *et al*., 1998; Rocas *et al*., 2004). Vancomycin resistant Enterococci *i.e., E. faecalis* is an emerging problem in worldwide in the hospital, it is resistant to most commonly used antibiotics (Amyes *et al*., 2007).

Presence of *E. faecalis* in fish can be transferred to the handlers during handling process. In addition, these faecal streptococci are heat resistant and it can withstand pasteurization, temperature, it may survives in partial cooking and it will not be affected by ingestion (Sorensen *et al*., 2001); thus makes the bacteria to spread easily to handlers and consumers. So, proper and hygienic handling is needed for fish handlers and consumers; because, the present study confirms the presence of noteworthy numbers of faecal streptococci in retail fish markets *i.e*., average of 31 cfu g^{-1} of faecal streptococci was observed in

Table 1 : Microbiological parameters of different fish and its average and range

Scientific name	Common name	Marathi name	No. of samples	APC/g		*E. coli*/g		FS/g		SRC/g	
				Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range
Arius arius	Catfish / Thread fin sea catfish	Shingala	9	124522.22	25000 - 44000	4.44	0 - 40	33.11	0 - 100	39.60	1.4 - 140
Chirocentrus dorab	Silver bar/ Dorab wolf - herring	Karli/ Datali	19	84765.79	47000 - 218750	13.00	0 - 80	27.71	0-60	18.45	0.3 - 110
Johnius dussumieri	Croaker	Dhoma/Dhomi	4	48250.00	6000 - 73000	10.00	0 - 20	18.00	10 - 40	1.20	0.4 - 2
Epinephelus sp.	Grouper	Hekru/Gobra	2	119000.00	78000 - 160000	20.00	0-40	40.00	20 - 60	20.00	10 - 30
Congresox talabonoides	Conger eel	Wam	2	131000.00	92000 - 170000	40.00	40	40.40	14 - 60	1.80	1.1- 2.5
Tylosurus crocodilus	Hound needle fish	Tolki	5	64660.00	52900 - 81000	9.60	0 - 20	13.43	0-20	28.08	0.3-110
Parastromateus niger	Black pomfret	Halwa	7	63457.14	28000 - 95000	7.14	0 - 20	10.00	0-20	19.60	0 - 110
Lates calcarifer	Asian seabass	Jitada	2	65100.00	37200 - 93000	25.00	0 - 50	30.00	0-50	15.30	0.6 - 30
Rastrelliger kanagurta	Indian mackerel	Bangada	6	98000.00	4000 - 217000	10.00	0 - 20	33.64	0-80	8.58	0.9 - 45
Mugil cephalus	Grey mullet	Boita	11	43940.91	2700 - 7100	20.91	0 - 60	4.33	0- 10	15.98	0.4 - 45
Sillago sihama	Indian whiting	Murdus / Renvi	3	106900.00	85000 - 125700	10.00	0 - 30	24.06	0-60	4.37	1.1 - 95
Nemipterus japonicus	Pink perch	Rani	17	82029.41	37000 - 200000	16.59	0 - 40	45.83	0- 110	7.76	0 - 45
Eleutheronema tetradactylum	Indian Salmon	Rawas	12	135437.50	56000 - 37000	17.42	0 - 40	35.86	0- 100	24.78	0 - 110
Dasyatis sp.	Marine sting ray	Pakat	14	74892.86	50000 - 145000	11.93	0 - 100	48.86	10 - 90	9.98	0.7 - 45
Sardinella longiceps	Indian oil sardine	Tarli	7	133083.33	56000 -380000	6.67	0 - 40	10.00	0-20	21.80	0 - 110
Scomberomorus sp.	Seer fish	Surmai/ Towar	6	30933.33	2000 - 63000	0.00	0	60.00	40-80	6.83	0 - 30
Penaeus sp.	Shrimp	Kolbi / zinga	3	97000.00	39000 - 140000	56.67	30 - 100	0.00	0	51.67	20- 110
Pampus argenteus	Silver pomfret	Saranga/Paplet	2	175250.00	88000 - 262500	5.00	0 - 10	53.33	0-140	10.20	0.4 - 20
Cynoglossus sp.	Sole fish	Lep /Shivra	12	140600.00	53000 - 324000	20.83	0 - 20.33	31.29	0-90	11.92	1.5 - 25
Loligo duvauceli	Squid	Nal Mhakul	16	95944.12	94000 - 401000	16.00	0 - 50	33.11	0 - 100	8.72	0.7 - 45
				Mean ± SE		**14.62± 1.43**		**31.93±2.22**		**15.80±2.29**	

Table 2 : Correlation between APC, *S. aureus* and Faecal indicators

APC and Faecal indicator bacteria	*APC*	*E. coli*	*FS*	*SRC*
APC	1			
E. coli	0.065914	1		
FS	0.212045	0.228605	1	
SRC	0.253649	0.124246	0.076177	1

the retail fish markets. Since, *E. faecalis* is major concern in the Enterococcus genus; enumeration of *F. faecalis* will be sufficient instead the enumeration of total Enterococcus/faecal streptococci in fish.

Sulphite reducing clostridia are the group of Clostridia includes mainly *Clostridium perfringens, C. bifermentans, C. difficile, C. sporogenes, C. botulinum and C. septicum* (Kouassi *et al*., 2011). Among the group, *C. perfringens* is a key species and commonly found in human and animal intestine. Since, *C. perfringens* is specific and more pathogenic, recent EC directive has amended the regulation *i.e*., *C. perfringens* has to be analysed in place of sulphite reducing clostridia. Previous EC directive recommended that the level of sulphite reducing clostridia should be absent in 20 ml of potable water; but the recent 98/83/EC Directives recommend absence of *C. perfringens* in 100 ml of potable water (Barrell *et al*., 2000). In the present study shows that among the 159 sample 154 samples contained sulphite reducing clostridia (Table 1); hence, there is huge possibility of *C. perfringens* in most of the samples; thus attributes that most of the fishes are contaminated with faecal materials. Since, sulphite reducing clostridia can withstand the cooking temperature and other food processing techniques, most of the fish still carry the sulphite reducing clostridia even after cooking/processing or any other preservation method. Hence, the hygienic handling practices have to be followed to avoid the sulphite reducing clostridia/C. *perfringens* in seafood.

In the present investigation, twenty different types of fish and shell fish were evaluated for presence of faecal indicator bacteria (Table 1). The outcome, shrimps samples were harbouring elevated level of *E. coli* and sulphite reducing clostridia. The reason for the elevated level of *E. coli* and sulphite reducing clostridia in shrimp may due to either contamination in pond (brackish water) or repeated use of contaminated water for cleaning of shrimps in the retail market. The unique feature of shrimp exoskeleton; especially the below the exoskeleton may entrap most of the contaminant, thus favours the high level of *E. coli* or sulphite reducing clostridia in shrimp. Eventough fishes encounter the same contaminated water; it may not accumulate as like shrimp. So, results suggest that most of the shrimp samples are highly prone for faecal contamination. In case of seer fish higher number of faecal streptococci with completely devoid of *E. coli*. Perhaps, seer fishes are more in demand and fetch higher cost in the retail markets; transported/retailer might have stored in the fish below the freeing point to retain the better quality of fish. The below freezing temperature might have destroyed the *E. coli*. Similarly, the costly/high demand fishes *viz.* black pomfret and silver pomfret had lower level of *E. coli*.

Butniaux and Mossel (1961) reported that per gram of faecal matter contains 40×10^6 coliform, 4×10^8 of *E. coli* and 4×10^6 Enterococcus sp. Hence, 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. But, in the present study faecal streptococci with *E. coli* ratio was 1: 0.45 *i.e.,* very high level of *E. coli* in fish samples, which is possible due to multiplication of *E. coli* in fish samples. So, the present study clearly indicates that the marine fish samples favour the multiplication of the *E. coli.*

Since marine environment is free of faecal indicator bacteria, most of the contamination take place in post-harvest handling (Visnuvinayagam *et al*., 2015a; 2015b; Sivaraman *et al*., 2016a; 2016b; Murugadas *et al.,* 2016), these contaminations starts from the un-cleaned fish storage tank, contaminated water used for cleaning of the fish in harbours and retail market. Repeated use of same water for cleaning of the fish in retail market would play a major factor responsible of the contamination of fish (Visnuvinayagam *et al*., 2015b). Correlation co-efficient was carried out between aerobic plate count and within faecal indicator bacteria (Table 2); the study found that there is very poor correlation was observed between the aerobic plate count and faecal indicator bacteria i.e., 0.07 to 0.25. Similarly poor correlation was observed between the faecal indicator bacteria *i.e.,* 0.07 to 022, which may due to that the distribution of the faecal indicator bacteria in the fish flesh/surface may not be uniform and multiplication of faecal indicator in fish. High level of *E. coli* and sulphite reducing clostridia in the shrimp species, high number of faecal streptococci with absence of *E. coli* seer in the present investigation is need to be confirmed by analysing more number of samples.

It is concluded that 22.4% of the fish available in the fish market are unfit for human contamination due to excess level of *E. coli* in the fishes. Contamination can be reduced by providing a potable water to clean the fish to remove the surface adhered bacteria and retail markets need to be monitored by the controlling authority at regular interval with stringent control policy to sell safe seafood. So, fish handlers should aware of good hygienic handling practices of seafood.

Acknowledgments

The microbiological work carried out by Thriveni G. Adiga, Assistant Chief Technical officer of Mumbai Research Centre of CIFT is thankfully acknowledged. The author also thank the Director, ICAR-CIFT, Cochin for funding the project.

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