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Hygienic Status in Domestic Fish Trade of Mumbai, India with Special Reference to Salmonella Contamination

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An attempt has been made to study the prevailing hygienic status in domestic fish trade with special emphasis on pathogenic *Salmonella* contamination. In this study samples of fish, prawn and fish handling environments obtained from retail fish markets, fish landing centres and processing plants of Mumbai, India were analysed for total bacterial load and also for incidence of salmonella. The Total Plate Count (TPC) of different samples varied between 3.28 log cfu/g and 7.04 log cfu/ g. The TPC level in fish and prawn samples from fish landing centres and fish markets were always on higher side and varied between 5.87 and 7.04 log cfu/g. More than 18 % of the samples from the fish markets were contaminated with *Salmonella*, while none of the fish or shrimp samples from landing centres or the processing plants was found to be contaminated. A total of 26 *Salmonella* strains, belonging to 11 serovars, were isolated, of which as high as 9 serovars were isolated from different samples of fish markets. Most of the serovars isolated in this study were pathogenic in nature and reported to be responsible for food-borne gastroenteritis in human. Polymerase Chain Reaction (PCR) analysis to test the pathogenicity of the isolated strains confirmed their invasive nature. Looking at the extent of *Salmonella* contamination with variety of serovars and probable contamination with other pathogens in the samples of fish markets, the domestic retail outlets, a great deal of care is suggested while preparation of such contaminated fish.

Keywords : Bacteriological quality, fish & prawn, retail outlet, *Salmonella*, PCR

Microbiological quality and safety of foods has gained great attention among present day consumers, food processors and regulatory agencies and this continues to increase day by day. In fish, this is more pronounced because of the apparent distinction from other foods in terms of extreme perishable nature and shortened shelf life (FAO, 1973). It has been reported that quality of fish sold in domestic market in India is poor compared to that of export trade and were mostly contaminated with pathogenic microorganisms (Nambiar & Iyer, 1990). The bacteriological quality of freshly landed as well as retail market fish and other seafood in different parts of the country have been studied by many workers (Lakshmanan *et al.*, 1984; Iyer *et al.*, 1986; Varma *et al.*, 1988; Nambiar & Iyer, 1990). Most of these studies have established that a sizeable portion of the fish available in the market for consumption is not meeting the quality criteria prescribed by Indian Standards.

Human infections due to many pathogenic bacteria are reported to have transmitted through fish, shellfish and other seafood products (Okuda *et al.*, 1997). USFDA has recognised 14 pathogenic bacteria as significant in terms of their capability of causing food borne diseases (FDA, 2001). Among them, Salmonellae are recognised as one of the leading cause of food borne disease outbreaks and infections in many countries (Tirado & Schimidt, 2001). It is the leading cause of foodborne gastroenteritis in the United States (Olsen *et al.*, 2000) and is one of the most commonly reported causes of food borne disease outbreaks in the western world. Non-typhoid salmonellosis accounts for 1.3 billion cases of acute gastroenteritis/

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diarrhoea with three million deaths annually (Pang *et al.*, 1995). Typhoid fever caused by *S. typhi* remains an important public health problem in India and other developing nations of the world with 16 million cases and six lakh deaths annually (Nasser *et al.*, 2002). Besides, there are several reports on estimates of devastation due to *Salmonella* spp. infection worldwide (Todd, 1989; Schmidt, 1995 and FAO, 1998). It is estimated that 95% of salmonellosis cases involved foodborne transmission (Tauxe, 1991). The genus *Salmonella* comprises 2541 serovars (serotypes) as on 2002 and new serovars are being listed in annual updates of Kauffmann-White scheme (Euzeby & Berrett, 2007) and most, if not all, of these serovers are considered pathogenic to humans and domestic animals (Jay, 1986). Being a major source of food borne diseases, *Salmonella* has been recognised to serve as an indicator of the safety of the food and water supply of a country (Anon., 1993).

The present study was aimed to analyse whether the increasing consumer awareness on food quality of recent years has improved the microbiological quality of fish and fish handling environments at domestic retail outlets of Mumbai, one of the advanced metro-cities of India. It was also aimed to assess the possibility of pathogenic contamination taking *Salmonella* spp. as the reference pathogen. Pathogenicity of the isolated *Salmonella* strains was also ascertained by PCR test targeting one of the most commonly reported pathogenicity related genes. Diverse sampling sites were selected in this study to assess the influence of post-harvest handling on the microbiological quality of fish meant for domestic trade.

Materials and Methods

Three different types of sampling Sites, viz., Fish Landing Centres (2 Nos.), Fish Markets (3 Nos.) and Fish Processing Plants (2 Nos.) of Mumbai were selected for the study. Fish (both freshwater and marine), shrimp, water and swab samples from fish contact surfaces were collected from these sampling sites at bi-monthly interval for a period of six months. A total of 183 samples were analysed from 8 different sampling sites. Fish and shrimp samples were collected randomly in triplicate from the designated site in sterile polythene bags. Samples of water used for washing and cleaning at the sampling sites were collected randomly in duplicate sterile polypropylene bottles. Similarly, swab samples from sorting or dressing surfaces/containers were collected in triplicate using sterile cotton swab (HiMedia Laboratories, India). Swabbing on an area of 25 sq. cm. and put in to a separate sterile polythene bag formed one sampling unit. All the samples were brought to the laboratory in insulated thermocole box under ice and processed as such immediately.

Total Plate Count was estimated by pour plating method following USFDA guidelines (FDA, 2001). Briefly, 25 g each of the fish and prawn samples was aseptically weighed and homogenized in 225 ml sterile physiological saline, while 25 ml of water sample was mixed with 225 ml saline solution. Similarly, swab samples drawn on 25 sq. cm. surface area was aseptically dispensed into 10 ml saline solution and serial decimal dilutions were prepared using same diluent. One ml each of the appropriate dilutions was pour plated in duplicate on Plate Count Agar (PCA) (Himedia Laboratories, Mumbai) and incubated at 37°C. TPC was then enumerated following the standard methods and represented as cfu/g for fish and shrimp samples, cfu/ml for water samples and cfu / sq.cm for contact surfaces.

Salmonella spp. was isolated from the samples following the USFDA protocol (FDA, 2001) with slight modification following the method of International Organization for Standardisation (ISO, 1991). The media and reagents used in this experiment were prepared following the specifications of AOAC international (AOAC, 2000). In brief, 25 g or ml of sample was homogenized and pre-enriched in 225 ml Buffered Peptone Water (BPW) at 37°C for 24 h. Selective enrichment of *Salmonella* was carried out in Tetrathionate (TT) broth and Rappaport-Vassiliadis (RV) medium in thermostatically controlled water bath. Each of these enriched cultures was streaked separately on to four selective plates viz., Bismuth Sulfite Agar

(BSA), Brilliant Green Agar (BGA), Xylose Lysine Desoxycholate Agar (XLDA) and Hektoen Enteric Agar (HEA). Presumptive positive colonies, based on the typical and atypical colony characteristics on different selective plates were transferred to PCA slants. The colonies sere subjected to biochemical and serological tests following USFDA protocol (FDA, 2001). *Salmonella typhi* (MTCC 734) and *Escherichia coli* (MTCC 1687) were used as standard cultures. The positive strains were then serotyped at National *Salmonella* Centre (Vet.), Indian Veterinary Research Institute (I.C.A.R.), Izatnagar, Bareilly, Uttar Pradesh (India). While, the common chemicals and media like NaCl, BPW, PCA etc. were from Himedia Laboratories, India; all the selective medias and antiserums were procured from Difco, Detroit Michigan, USA.

The isolated strains were tested for their pathogenicity by PCR using primers targeting *inv*A gene. Template DNA was prepared by rapid-boiling lysis method described by Malorny *et al.* (2003), with little modification. Briefly, single isolated colony of the bacteria of interest was picked from PCA plates and inoculated in 5 ml Luria Bertani broth (Himedia Laboratories, India) and incubated at 37°C with rigorous shaking for overnight. 1.5 ml of the culture was pelleted down by centrifuging at 8,000 x g for 5 min at 4°C. The supernatant was discarded; pellet was washed and then resuspended in 100 il TE buffer [10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0)]. The suspension was then incubated for 10 min at 100°C and immediately chilled on ice. After centrifugation at 12,000 x g at 4°C for 5 min, the supernatant containing DNA was transferred to a fresh tube. A 5 µl aliquot was used as the template DNA for PCR reaction.

The PCR reaction was performed following methods described by Malorny *et al.* (2003) in a Thermocycler (Mastercycler®, Effendorf, Germany). After the reaction, an aliquot of 10 µl each of the PCR products were electrophorosed in 1.8% Agarose (Sigma-Aldrich, USA) incorporated with ethidium bromide. The DNA bands were viewed under UV light and documented using gel documentation system (Syngene Inc., Cambridge, UK). While the primers were synthesized by Operon Inc., USA, the chemicals for PCR reaction including PCR buffer, dNTPs, Taq polymerase, DNA molecular weight marker, gel loading dye etc. were procured from MBI Fermentas, Germany.

Results and Discussion

Results of the bacteriological analysis pertaining to microbiological quality have been presented in Table 1. Taking together all the samples, the average Total Plate Count (TPC) varied between 3.28 log cfu/g and 7.04 log cfu/g. The TPC level in the processed shrimp samples from the processing plants were always the lowest with the average value of 3.28 log cfu/g. On the other hand, the average TPC level of the samples from fish landing centres and that of the fish markets always remained towards higher side and the average values fell in a range of 4.72 to 7.04 log cfu/g. Among these two categories of sampling sites, the average TPC level of water used for washing the fish and the swab samples from containers/dressing surfaces were usually lower than that of the fish and shrimps (i.e., between 5.87 and 7.04 log cfu/g).

It was noted that average TPC for samples of marine fish and shrimp from fish landing centres were 5.99 and 6.45 log cfu/g, respectively and that of fish markets in the range of 5.87 and 6.11 log cfu/g, respectively. This may be attributed to the handling practices followed in each station. It was observed that un-hygienic handling practices was being followed in fish landing centres of Mumbai, where the fish or shrimp catch were heaped for auction on the same floor that was being used by fishermen and traders to move around. There was neither any elevated platform nor the practice of using containers that would have reduced the bacterial load. On the other hand, the same fish / shrimp when taken to fish market and being retail sold, the trader takes some simple steps, may be to make the fish and shrimp attractive, like multiple washing and displaying on a proper platform. These simple steps would have reduced the bacterial load, particularly the one clinging

to the external body surfaces to a great extent. This is also evident from the bacterial load on swab samples obtained from sorting or dressing surfaces / containers at both the sampling sites (Table 1).

The average TPC of fish and shrimp samples in the present study were in tune with many of the previous studies involving samples from Bombay (Iyer *et al.*, 1986) and Mangalore (Jadhav & Magar, 1970). Similarly, Nambiar & Iyer (1990) and Lakshmanan *et al.* (1984) have reported TPC level of marine fish on a wider range of 3 to 7 log cfu/g. The average TPC of all the processed shrimp samples fell within the allowable limit of 5.699 log cfu/g, while that of fish and shrimp samples meant for domestic consumption at the retail fish markets and also of the fish landing centres were always higher than the allowable limit of 5×10^5 cfu/g prescribed by EIC of India for fresh and frozen finfish and shellfish meant for export.

The fish landing centres of the present study were among the sources of raw material for the processing plant. In spite of that, the TPC levels at the originating stations were on a higher side than that at the processing plants. This reduction in bacterial load in the processed samples may be attributed to the effective treatments in the processing plants during washing, dressing and also during other methods of processing. Besides, the overall sanitation and hygienic practices in the processing plants and the extra care taken to process bacteriologically sound shrimps might have contributed towards such reduction in TPC level (Iyer & Shrivastava, 1989a). On the other hand, such care is totally lacking for the fish and shrimp meant for domestic consumption at the retail fish markets, which might be attributed to the absence of stringent regulation in this regard.

Out of 183 samplings made in the present study, 26 samples were found to be contaminated with *Salmonella* (Table 2). It is evident from the table that more than 18 % of the samples from the fish markets were contaminated. Among the different samples of the fish markets, six freshwater fish samples out of 9 (66.67%), 5 marine fish samples out of 18 (27.77 %) and 2 shrimp samples out of 27 (7.41%) were found to be contaminated with *Salmonella*. Similarly, water and swab samples from both fish markets and fish landing centres were contaminated. On the other hand, it is interesting to note that none of the fish or shrimp samples of the landing centres and the processing plants was contaminated with *Salmonella*.

Contamination of fish and fishery products with *Salmonella* has been reported

Table 1. Total Plate Counts of samples from various sampling sites

Type of sample	Fish Landing Centres			Fish Markets			Fish Processing Plants		
	No. of samples	TPC Range	Average TPC	No. of samples	TPC Range	Average TPC	No. of samples	TPC Range	Average TPC
		cfu g^{-1}			cfu g^{-1}			cfu g^{-1}	
Freshwater Fish	ND*	-	-	9	9.5×10^6- 1.2×10^7	1.1×10^7 (7.04)	ND	-	-
Marine Fish	18	2.1×10^5 - 4.3×10^6	9.7×10^5 (5.99)#	18	6.3×10^5 - 8.7×10^5	7.5×10^5 (5.87)	ND	-	-
Shrimp	18	3.7×10^4 - 1.7×10^7	2.8×10^6 (6.45)	27	1.1×10^6 - 1.4×10^6	1.3×10^6 (6.11)	18	6.2×10^2- 3.1×10^3	1.9×10^3 (3.28)
Water	12	1.3×10^4 - 6.9×10^5	1.3×10^5 (5.11)	18	8.2×10^5 - 1.1×10^6	9.4×10^5 (5.97)	ND	-	-
Swab	18	4.8×10^3 - 2.0×10^6	4.0×10^5 (5.60)	27	5.1×10^4 - 5.4×10^4	5.2×10^4 (4.72)	ND	-	-
Total	66			99			18		

* - Not Done

Value in the parenthesis indicate log cfu g^{-1}

by many workers in their studies over different parts of India (Iyer & Shrivastava, 1989a; Nambiar & Iyer, 1991; Bandekar *et.al.*, 1995; Panda, 2002 and Kumar *et al.*, 2003). Incidence of *Salmonella* in the water and swab samples of landing centres was in tune with the findings of Gore *at al.* (1980). Absence of *Salmonella* in the fish and shrimp samples of the landing centres may be attributed to the fact that fishes caught from open sea are free from *Salmonella* (Shewan, 1962) and salmonellae from the contaminated coastal environment had not been transferred to the freshly landed fish and shrimp by the time of sampling. At the same time, incidence of the pathogen in the samples of fish markets may be attributed to external contamination (Iyer & Shrivastava, 1989b) and poor handling at ambient temperatures (Al Jedah *et al.*, 1999). Besides, incidence of *Salmonella* in the freshwater fish samples may also be attributed to initial contamination from polluted culture tanks. In general, incidence of *Salmonella* was found to be higher in fish samples compared to that in shrimps, which may be attributed to the difference in the methods of handling fish and shrimps (Iyer & Shrivastava, 1989a).

Source wise distribution of *Salmonella* serovars has been given in Table 3. All the 26 *Salmonella* strains isolated in the present study were classified in to 11 serovars. It is also evident from the table that, maximum variety of serovars totaling as high as 9 were found to harbour in different samples obtained from the fish markets. Among the different samples of fish markets, 3 serovars each was isolated from the samples of freshwater fish, marine fish and swab samples. Similarly, 2 serovars each was isolated from shrimp and water samples. At the same time, all the 8 strains of *Salmonella* isolated from water and swab samples of fish landing centres belong to only 2 serovars namely, S. Typhimurium and S. Ohio. On thorough observation on the source of isolation of each of the serovars, no particular trend however could be established indicating the probable source of initial contamination of the pathogen and its route of transmission. However, one thing seems very much pertinent here that the method of post-harvest handling plays some crucial role influencing the contamination status of the fish and shrimp.

Saxena *et al.* (1983) have reported that *S.* Typhimurium and *S.* Saintpaul are the most common serotype isolated from human stool in India. Besides, *S.* Saintpaul and *S.* Typhimurium are the dominant serotypes in animals and sewage, respectively. Thus isolation of *S.* Typhimurium from coastal waters can be attributed to fecal contamination. Similarly, isolation of *S.* Saintpaul from freshwater fish samples may be attributed to its contamination at source or during handling and transportation. *S.* Stanley and *S.* Heidelberg are rarely isolated in India and most of these isolations were from human and frog legs (Saxena *et al.*, 1983). However, Iyer & Srivastava (1989b) have reported isolation of *S.* Stanley and *S.* Ohio from fresh fish of Mumbai and *S.* Senftenberg from frozen lobsters off Cochin. Further, the pattern of

Table 2. Level of contamination of the samples with *Salmonella* strains

Sample Type	Fish Landing Centres		Fish Markets		Fish Processing Plant	
	No. of samples tested	No. of samples positive (%)	No. of samples tested	No. of samples positive (%)	No. of samples tested	No. of samples positive (%)
Freshwater Fish	ND	-	9	6 (66.67)	ND	-
Marine Fish	18	Nil	18	5 (27.77)		
Shrimp	18	Nil	27	2 (7.41)	18	Nil
Water	12	6 (50.0)#	18	2 (11.11)	ND	-
Swab	18	2 (11.11)	27	3 (11.11)	ND	-
Total	66	8 (12.12)	99	18 (18.18)	18	Nil

- figures in parenthesis indicate percentage

Table 3. Source-wise distribution of *Salmonella* serovars in the fish markets and landing centres

Salmonella serover	Antigenic formula	Fish Landing Centres				Fish Markets				Processing Plant		
		Fish	Shrimp	Swab	Water	Fresh water fish	Marine Fish	Shrimp	Swab	Water	Shrimp	TOTAL Nos isolated
S. Senftenberg	1,3,19:g,s,t:-	-	-	-	-	2	-	-	-	-	-	2
S. Saintpaul	4,5,12:e,h:1,2	-	-	-	-	2	-	-	1	1	-	4
S. Reading	4,5,12:e,h:1,5	-	-	-	-	2	1	-	-	-	-	3
S. Stanley	4,5,12:d:1,2	-	-	-	-	-	3	1	-	-	-	4
S. Sarajane	4,5,12:e,n,x	-	-	-	-	-	1	-	-	-	-	1
S. Sandiego	4,5,12:e,h(r):e,n,z_{15}	-	-	-	-	-	-	-	1	-	-	1
S. Heidelberg	4,5,12:r:1,2	-	-	-	-	-	-	1	-	-	-	1
S. Reinickendorf	4,12:1,z28:e,n,x	-	-	-	-	-	-	-	-	1	-	1
S. Bradford	4,5,12:r:1,5	-	-	-	-	-	-	-	1	-	-	1
S. Typhimurium	4,5,12:i:1,2	-	-	-	1	-	-	-	-	-	-	1
S. Ohio	6,7:b:1,w	-	-	2	5	-	-	-	-	-	-	7
TOTAL	11 Serovers	-	-	2	6	6	5	2	3	2	-	26

Salmonella serotypes isolated from India has been reported by National Salmonella and Escherichia Centre, Central Research Institute, Kasauli (Anon. 1984, 1985 and 1986), Nambiar & Iyer (1991) and Bandekar *et al.* (1995). However, serotypes isolated in the present study like *S.* Reading, *S.* Sarajane, *S.* Sandiego, *S.* Reinickenfort and *S.* Bradford are rarely reported from fish and fishery products in India.

The *Salmonellae* may be divided into three groups based on their host predictions (COS, 1969). None of the serovars isolated in the present study belong to typhoid or paratyphoid group that has human as the specific host and causing systemic typhoid fever. Only one serovar, *S.* Typhimurium causes diseases in human and many other animals (Baumler *et al.*, 1998). Except this, all other serovars isolated in the present study, particularly the serovars isolated from fish markets do not have well known host specificity. These un-adapted serovars of *Salmonella* are responsible for food-borne gastroenteritis in human (Jay, 1986). The PCR analysis (Fig. 1) showed that *inv*A gene, a gene involved in invasion of host cells during *Salmonella* infection, was present in all the strains of *Salmonella* and thus all of them are invasive in nature. Therefore, frequent isolation of these serovars from fish markets is of great significance from the point of human health hazard. Since *Salmonella* was taken in the present study as the representative of the pathogenic contamination, the possibility of contamination by other dreaded pathogens cannot be ruled out. Looking at the extent of *Salmonella* contamination with variety of serovars and probable contamination with other pathogens in the samples of fish markets, the domestic retail outlets that cater fresh fish to the local inhabitants, a great deal of care is suggested while preparation of such contaminated fish.

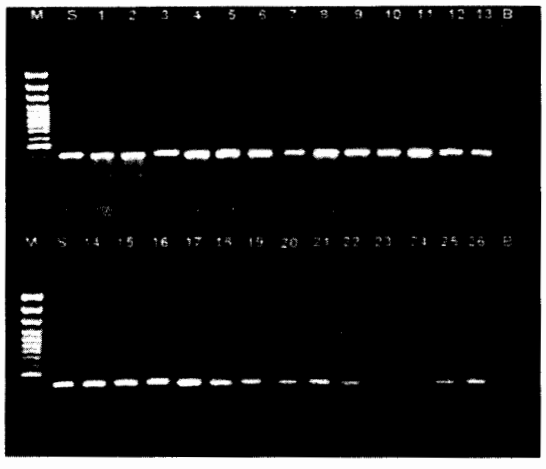

Fig. 1. Test for pathogenecity of the isolated *Salmonella* strains (Sl. No. 1 to 26) by PCR targeting *inv* a gene

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