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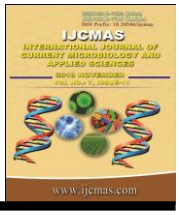

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Isolation and Identification of *Listeria monocytogenes* from Fish Intestines and Phylogenetic Analysis

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A B S T R A C T

Forty fish procured from retail shops located in and around from west Chennai were examined for the presence of *Listeria monocytogenes*. The cultural, biochemical, molecular and phylogenetic relationship characterization of isolates was performed to detect genetic variations *among L. monocytogenes* isolates. The selective enrichment procedure followed by selective isolation was used for *Listeria* isolation from fish intestines. Twenty (50%) *L. monocytogenes* were isolated and identified from fish intestines. Two different varieties were analyzed by the BLAST sequencing analysis of the isolates. The results indicated that the isolates might have originated from different sources, and fish contaminated with *L. monocytogenes* may cause serious health problems in humans.

Introduction

Listeriosis is one of the developing zoonotic foodborne illnesses commonly acquired through consumption of contaminated foods particularly foods of animal origin. *Listeria* species could spread the food supply chain over horizontal transmission and spoil the products like fish, chicken and meat products at any period of production, processing, distribution, marketing, handling and preparation of foods (Alsheikh *et al.*, 2012). Recently people are concerned to preserve the fish and meat products under cold temperature, in order to extend the shelf life or to make food at their suitable time. But, refrigeration condition and storage which inhibit the other contaminate organisms rather preserve and promote the survival of *Listeria* species in the fish, meat or meat products, thus endangering the health of consumers (Walker *et al.*, 1990; Farber and Peterin., 1991).

It also increases the growth environments conducive for psychotropic pathogens such as *Listeria*, letting them grow to unsafe levels in meat or other products (Francis and O"Beirne, 1998). These lead to the presence of *Listeria* species in different fish, meat and meat products in diverse geographical regions and promote causing in foodborne outbreaks (Borucki, 2003).

On the other side, the development of a rapid, precise and perceptive typing method is needed to observe Listeriosis outbreaks.

Of the various typing techniques established to differentiate *Listeria* at subspecies level, serotyping was the utmost usually and widely used (Seeliger *et al.,* 1979). Though, the limited value of serotyping in *Listeria* typing was stated in several studies, stemming from the fact that only a minority of serovars were detected from field isolates (Schuchat *et al.,* 1991). Similarly, various reports can be shown in the presence of *L. monocytogenes* in fish and fish products (Byun *et al.,* 2001 and Jrgensen *et al.,* 1998). Keeping in view the above facts in the present study an attempt was made to investigate the prevalence of *Listeria* spp. and its classification of phylogenetic relationship characterization from two different variety of fish collected from markets/ retail shops as well as roadside vendors located in and around West Chennai.

Materials and Methods

Sample collection

In the present study totally 40 fish samples were analyzed. The nature of the samples collected included the nature of retail outlets such as selling of fish of single species and/or multiple species, processed products and unbranded categories as relevant to the fish samples. The Study period was December 2017 to February 2018. In brief, a total of 100 samples comprising Catla fish (*Labeo catla*) (n=20) and Stingrays fish (*Dasyatis pastinaca*) (n=20), were randomly collected from the selected retail shop in Virugambakkam location in West Chennai city. The fish were placed into separate plastic bags in the icebox at 4°C and immediately transferred to the Microbiology Laboratory at MGR College, Hosur. Each fresh fish was opened with a sterile scalpel and about 1 g of intestine content was taken using a sterile swab for *Listeria* isolation.

Standard strains

The standard strain of *Listeria monoctogenes* (MTCC 1143), used in the study were procured from the microbial Type culture collection and gene bank, Institute of microbial technology, Chandigarh (IMTC), India.

Isolation and identification of *Listeria* sp.

ISO 11290 method was employed to isolate the organisms, whereby pre-enrichment of 25 g sample was done in 225 ml half strength Fraser broth containing selective supplements (Hi-Media) for 24 h at 30 °C, which was followed by second enrichment of 0.1 ml of pre-enriched Fraser broth content in 10 ml full strength Fraser containing selective supplements (Hi-Media) for 48 h at 37 °C incubation temperature. After the enrichment procedure, the inoculum was plated on PALCAM agar (Hi-Media) and incubated for 48 h at 37 °C.

The gray-green colonies surrounded by diffuse black zone on Polymyxin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol (PALCAM) agar was picked up and further purified on Tryptone Soya Yeast Extract Agar (TSYEA). Subsequently pinpoint colonies of TSYEA were subjected to identification procedures which included Gram' Staining followed by a microscopic examination, catalase test, and oxidase test. The characteristic Gram-positive, short rod-shaped organisms which were catalase positive and oxidase negative, were subcultured in TSYEA agar at 25 °C for 12 - 18 h. subsequently, culture showing typically tumbling motility were considered as "presumptive" *Listeria* isolates, which were in turn subjected to detailed biochemical tests viz.; methyl red,

Voges-Proskauer test, nitrate, and sugar fermentation tests with xylose, mannitol, glucose, galactose, maltose, lactose, fructose.

In-vitro pathogenicity tests

The isolates were tested for the type and the degree of hemolysis on 5 % sheep blood agar and the Christie Atkins Munch Peterson (CAMP test) was conducted using *Listeria* isolates, *S. aureus*, standard strains to observe for hemolysis between *Listeria* strain and the *S. aureus* owing to the synergistic effect of their hemolysins in case of a CAMP-positive reaction. In detail for Hemolysis test, inoculated heavily from TSYEA onto sheep blood agar by stabbing the plates by passing through the agar layer and incubated at 48 hours at 35 °C. The positive result for *Listeria monocytogenes* was produce slightly cleared zone around stab. On the other hand, for CAMP test streaked the hemolytic *Staphylococcus aureus* and were cultured in parallel and diametrically opposite to each other on a sheep blood agar plate, streak several test culture parallels to one another, but at right angles to and in between *Staphylococcus aureus* streaks. After incubation at 35°C for 24 - 48 hours the plates were examined for hemolysis. *Listeria monocytogenes* reaction is often optimal at 24 hours rather than 48 hours.

16S rDNA sequence determination

A colony PCR method was used for amplification of 16S rDNA. A single colony of the bacterial isolate was suspended into the 100µl sterile distilled water. Boiled for 25 minutes at 94˚C and centrifuged at maximum speed in a microcentrifuge for 15 minutes to pellet cell debris. 5 µl of the supernatant was used as DNA templates for PCR. Bacterial 16S rDNA was amplified from the extracted genomic DNA by using the universal bacterial 16S rDNA primers 27F forward primer (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R reverse primer (5' TACGGYTACCTTGTTACGACTT 3'). PCR was performed with a 25µl reaction mixture containing 5µl of DNA extract as the template, 1.5µL of Forward Primer and Reverse Primer, 5µL of deionized water, and 12µL of Taq Master Mix. Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean-up kit (Millipore).

PCR amplification

The PCR product was sequenced using the primers. Sequencing reactions were performed using an ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems) and consists of an initial 94°C denaturation for 3 min followed by 30 cycles of 94°C for 30sec, 60°C for 30sec, 72°C for 1 min, followed by a final extension at 72°C for 10 min. Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Nucleotide sequencing, alignment, and phylogeny

The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. Based on the scoring index, the program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar *et al.*, 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions

and divergent regions (Talavera *et al.*, 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering (Edwards *et al.*, 1989).

Results and Discussion

The study was undertaken to the occurrence of *Listeria* species in fish samples form *Labeo catla* and *Dasyatis pastinaca* (n=40) which were collected from different retail outlets located in and around from West Chennai (Figure 1). Samples were processed following the reference method ISO 11290:1996 with respect to isolation, identification and biochemical confirmation of *Listeria* species. Samples were broadly categorized based on the species slaughtered and type of processing at the retail outlets (Table 1).

The occurrence of *Listeria* species in fish

Colonies showing typical cultural and morphological characteristics such as grey-green shiny colonies having diffused black shadow were considered as characteristic of *Listeria* species on the selective PALCAM agar plates. Further, these colonies were streaked onto TSYEA slants and used for the biochemical analysis. The results of cultural (Figure 2 and 3) and biochemical reactions are shown in Table 2 and 3. Putative isolates showing typical biochemical reactions were confirmed. Of the total 20 Catla fish and 20 Stingrays fish samples analyzed for the presence of *Listeria* species using ISO11290:1996 method of isolation and identification and 20 samples showed the presence of *Listeria monocytogenes* and with an overall occurrence of 50 % of *Listeria monocytogenes* in fish samples.

16S rDNA sequence determination

Based on the morphological, physiological, biochemical characteristics (Table 2 and 3) showed that the ATCC 51779 strain is matched to the members of genus *Listeria monocytogenes;* accession number MH701890. The partially amplified for two different unknown samples correspondingly 978bp and 1045bp fragment of 16S rDNA sequence was submitted to NCBI database search using BLASTN to confirm the species of the bacterium. The highest sequence similarity revealed that sample from *Labeo catla* is closely related to *Listeria monocytogenes;* accession number MH701866, and similarly sample from *Dasyatis pastinaca* is closely related to *Listeria monocytogenes;* accession number MH701867.

Nucleotide sequencing, alignment, and phylogeny

The multiple sequence alignment and the phylogenetic relationship confirmed the highest sequence similarity with the ATCC 51779 strain *Listeria monocytogenes* (KF956740.1) with a distribution of the top 200 blast hits.

In contrast, the unknown samples were confirmed the highest sequence similarity with *Listeria monocytogenes* (CP011398.2) and (CP028411.1) with a distribution of the top 200 blast hits on 100 subject sequences for *Labeo catla* and *Dasyatis pastinaca* respectively.

The calculation and classification of a phylogenetic relationship was determined and PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data while being one order of magnitude faster (Figure 4). The program Tree Dyn 198.3 was used for tree rendering.

Fig.1 Appearance of raw material for the occurrence of *Listeria* species in fish samples (A) *Labeo catla*, (B) *Dasyatis pastinaca*

Fig.2 Identification of *Listeria* species in *Labeo catla*. A representative result of (A) The microbial strain grown on Oxford and PALCAM agar plate, (B) TSYEA plate, (C) Blood agar Christie Atkins Munch Peterson test, (D) Isolates from *Labeo catla* grown on blood agar

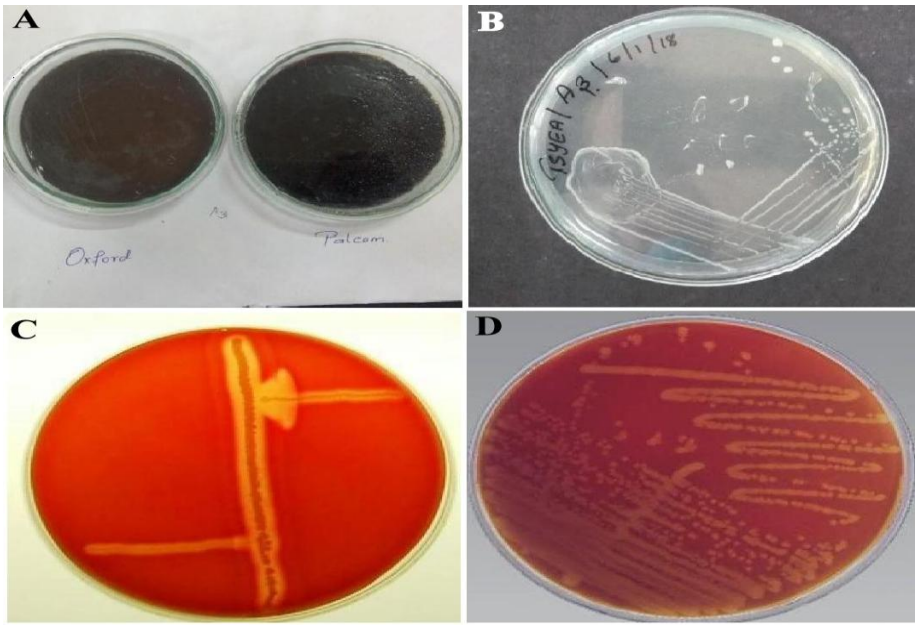

Fig.3 Identification of *Listeria* species in *Dasyatis pastinaca*. A representative result of (A) The microbial strain grown on Oxford and PALCAM agar plate, (B) TSYEA plate, (C) Blood agar Christie Atkins Munch Peterson test, (D) Isolates from *Dasyatis pastinaca* grown on blood agar

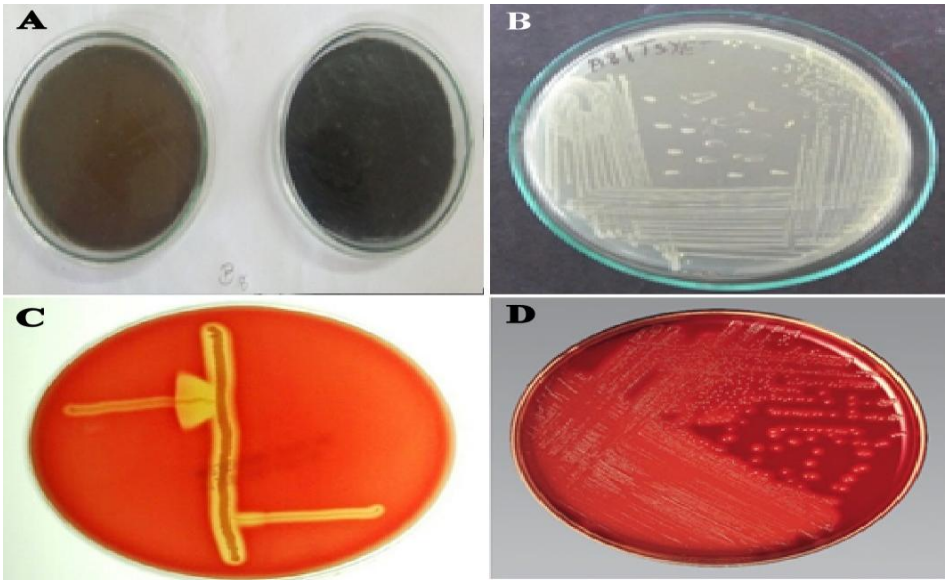

Fig.4 The phylogenetic relationship based on 16S rDNA sequence comparison showing the position of *Listeria monocytogenes* ATCC 51779 strain (KF956740.1), *Labeo catla* (CP011398.2), and *Dasyatis pastinaca* (CP028411.1). The GeneBank accession numbers for the 16S rDNA sequences are given after the strain in parenthesis. While scale bar corresponds to nucleotide sequence difference

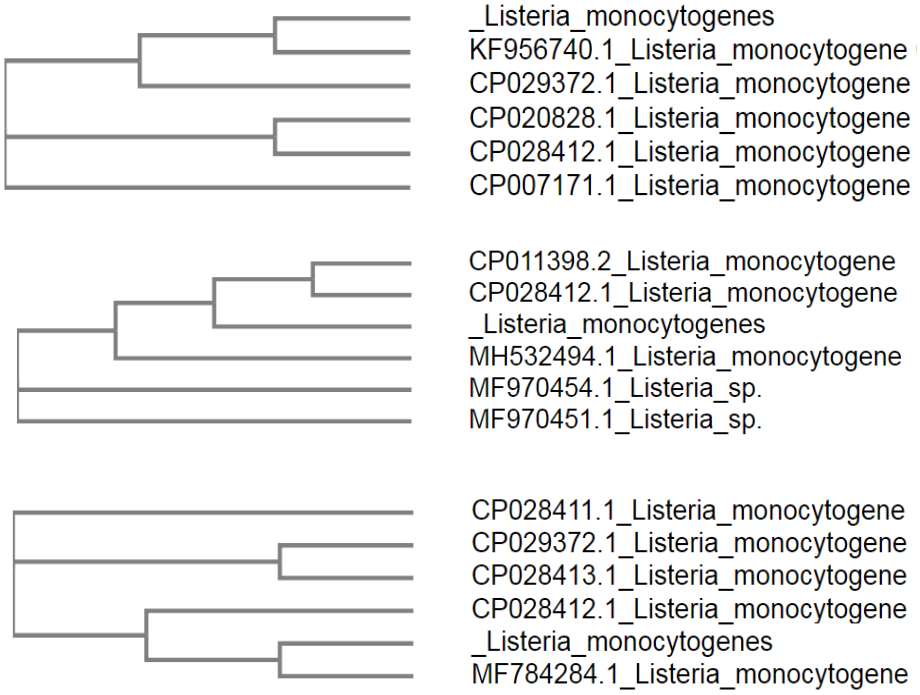

Table.1 Collection of samples for isolation of *Listeria* species in fish

Fish Type	Nature	Location	Collection (Nos)
Catla *(Labeo catla)*	Outlet selling only Fish (single species)	Chennai West	10
	Outlet selling Fish (multiple species)	Chennai West	10
Stingrays *(Dasyatis pastinaca)*	Outlet selling only Fish (single species)	Chennai West	10
	Outlet selling Fish (multiple species)	Chennai West	10

Table.2 Cultural and biochemical characteristics of *Listeria* species isolated from *Labeo catla*

***Labeo catla* Isolates**	**ATCC**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A 10**	**A 11**	**A 12**	**A 13**	**A 14**	**A 15**	**A 16**	**A 17**	**A 18**	**A 19**	**A 20**
Gram's staining	(P), rods	(P), rods	(P), coccus	(P), rods	(N), rods	(P), rods	(P), coccus	(P), rods	(P), coccus	(P), rods	(N). rods	(P), rods	(P), rods	(P), rods	(P), coccus	(P), rods	(N), rods	(P), rods	(P), coccus	(P), rods	(N), rods
Indole	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
MR	P	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
VP	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
Citrate	P	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
TSI	P	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
Nitrate	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Catalase test	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
Motility	P	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
Mannitol fermentation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Xylose fermentation	P	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
Glucose fermentation	P	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
Fructose fermentation	P	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
Galactose fermentation	P	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
Maltose fermentation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Lactose fermentation	P	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N

Abbreviation: P: Test Positive and, N: Test Negative, A1 to A20: 20 individual samples of *Labeo catla*.

Table.3 Cultural and biochemical characteristics of *Listeria* species isolated from *Dasyatis pastinaca*

Dasyatis pastinaca Isolates	ATCC 7644	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20
Gram's staining	(P), rods	(N), rod	(P), rods	(P), cocci	(P), rods	(N), rods	(P), rods	(N), rods	(P), rods	(P), rods	(P), rods	(N), rod	(P), rods	(P), cocci	(P), rods	(N), rods	(P), rods	(N), rods	(P), rods	(P), rods	(P), rods
Indole	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
MR	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
VP	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
Citrate	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
TSI	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
Nitrate	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Catalase	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
Motility	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
Mannitol fermentation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Xylose fermentation	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
Glucose fermentation	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
Fructose fermentation	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
Galactose fermentation	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
Maltose fermentation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Lactose fermentation	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P

Abbreviation: P: Test Positive and, N: Test Negative, B1 to B20: 20 individual samples of *Dasyatis pastinaca*.

Our results demonstrate that the two different type of fishes obtained from Chennai west retail shops contains *L. monocytogenes*, which is observed as an important human pathogen, in their intestines. Intestinal guts of fish generally infect the fish meat and this causes the contamination of foodborne pathogens such as *L. monocytogenes* during the factoring process. The occurrence of *L. monocytogenes* in freshwater fish was 6.6%. This rate is lower than that in a maximum of the published studies on fresh fish. In a study, 20 samples of *Labeo catla* and 20 samples of *Dasyatis pastinaca* were analyzed for the presence of *Listeria* spp. and *L. monocytogenes.*

The frequency of *Listeria* and *L. monocytogenes* in *Labeo catla* and *Dasyatis pastinaca* was 50% and 50%, respectively. Miettinen *et al.,* (2001) studied the external contamination and the occurrence of *L. monocytogenes* in fish dispensation factories. *Listeria* spp. was determined at a level of 45% and *L. monocytogenes* with at a level of 12%. In a different experiment, 56 fresh fish samples were analyzed for the occurrence of *Listeria* spp. and 15 of the samples were identified to be contaminated with *L. monocytogenes* and *L. innocua* were isolated from 3 and 12 samples, correspondingly (Vaz-Velho *et al.,* 2001). Fuchs and Surendran (1989), were unable to isolate *L. monocytogenes* in fresh fish from India, while 33% of the samples harbored *Listeria* spp. Conversely, Adesiyun (1993) documented a 2% occurrence of *L. monocytogenes* in fish and shellfish in India.

Comparatively, the outcomes of this experiment were higher than those of other, related experiments. All fish used in this experiment were acquired from the same area of the west Chennai retail shops and the sewage system of the area had been quitting into this region for several months. Hence, the isolation rate was anticipated to be high. Still, biological cleaning might not have been executed before discarding into the water bodies and this might have caused the isolation rate to be higher than that in other results.

The procedures used for the isolation of *L. monocytogenes* from foods materials are different. Many techniques involved in the enrichment and selective plating methods. The media used for enrichment and selective plating have also different methods (Karunasagar *et al.,* 2000). The procedure accepted by the standard ISO: 11290 (1996) method was used for the isolation of *L. myonocytogenes* in this study and followed by cultural, biochemical and molecular characterization and confirmation of isolates.

Listeria spp. was isolated from 50 % (20/40) of the sample of fish with the highest prevalence from fish samples. Intake of these fish foods, either raw or undercooked, may contribute to food-borne illness due to *L. monocytogenes*. *L. monocytogenes* in raw seafood may pose a health risk in pantries if contaminating ready to eat food. However, diseases could also get transmitted through the consumption of contaminated foods of animal origin and the extent of their occurrence has shown an increasing trend over the years.

In summary, our outcomes showed that the fish from Chennai west hypermarkets and street hawkers contain *L. monocytogenes* in their intestines and these fish may cause Listeriosis outbreaks as reported previously (Miettinen *et al.,* 1999 and Ericsson *et al.,* 1997). Humans consuming the contaminated fish and their foods are at risk of disease. The genotyping results indicated that the strains isolated have diverse genetic profiles. This may be the consequence of different sources of bacteria. More discriminative typing

methods could be used to disclose the relationship between human and fish isolates.

The high prevalence of *L. monocytogenes* in fish foods sold by hypermarkets and street hawkers pose a high risk to the consumer. Since fish foods are consumed directly after purchase with no further treatment applied to reduce *Listeria* in fish foods. Therefore, the materials have to maintain proper temperature before the sale and production of safe foods for the consumers. However, if one aims to detect *Listeria* spp. but not just *L. monocytogenes*, the combination use of two to three plating media are required to achieve higher proficiency of *Listeria* spp. detection.

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