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Prevalence of Antibiotic Resistant *Listeria monocytogenes* in Sea Foods of Tuticorin Coast, Southeastern India

K. Immaculate Jeyasanta and Jamila Patterson

Suganthi Devadason Marine Research Institute, Tuticorin, Tamil Nadu, India

Abstract: World Health Organization concludes that L. *monocytogenes* is an environmental contaminant mainly transmitted to humans through food. Although the incidence of listeriosis is at least 100 times lower than those of other food borne pathogens, such as *Campylobacter* and *Salmonella*, the seriousness of the disease it mainly affects vulnerable sections of the population, has focused a great deal of attention on *L. monocytogenes*. It has now become the target for much of the microbiological testing carried out by the food industry. Reports are very few regarding the pathogenesis of *L.monocytogenes* from seafood in Tuticorin region, Tamil Nadu, India. So the present study was proposed to evaluate the prevalence of antibiotic resistant *L. monocytogenes* from seafood in Tuticorin. In the present study 113 random samples of fresh seafood samples were purchased from landing site and fish markets were bacteriologically examined for detection of *L. monocytogenes*. Among that, 37 isolates were positive for *L.monocytogenes* and it was confirmed by biochemical test. The isolates were screened for antibiotic susceptibility test with 13 different antibiotics. *L. monocytogenes* isolates were highly resistant to Nalidixic acid (100%), gentamycin (75.67%) and streptomycin (78.37%). The isolates were highly sensitive to amoxicillin (100%). Antibiotic susceptibility studies revealed that sea foods from Tuticorin contained antibiotic resistant *L. monocytogenes* strains which may serve as a reservoir for antibiotic resistant genes in the seafood environment.

Key words: Seafoods · Biochemical Test · *L. monocytogenes* Species · Antibiotic Resistance

INTRODUCTION

Bacteria of the genus *Listeria* are Gram-positive, facultatively anaerobic, non-spore forming rods and are common in the natural environment. *Listeria* includes six different species which includes *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria innocua, Listeria welshimeri, Listeria seeligeri* and *Listeria grayi*. Both *Listeria monocytogenes* and *Listeria ivanovii* are pathogenic in mice, but only *Listeria monocytogenes* is consistently associated with human illness [1]. *L. monocytogenes* can cause severe, life threatening infections. Pregnant women, newborn babies, the elderly and the immuno-compromised are particularly at risk. The infective dose for vulnerable individuals is not known, but could be very low (<100 CFU/g) in some cases. The incubation period is typically 30 days, but can vary from 1-90 days. *Listeria monocytogenes* is unlike most other food-borne pathogens in that it only rarely causes the typical symptoms of gastroenteritis. Initial flu-like symptoms are often followed by vomiting and diarrhoea and in few cases potentially fatal meningitis and septicemia may develop. The route of transmission to humans occurs through consumption of contaminated food, responsible for 99% of human listeriosis [2]. However, rarely, persons without these risk factors can also be affected. According to CDC [3] there are about 1, 600 cases of listeriosis annually in the world and it is the third leading cause of death among major pathogens transmitted commonly through food. *L. monocytogenes* is ubiquitous in the environment and can be isolated from soil, plant material, animals and seafood. Fish and fish products are suitable vehicles for the transmission of the pathogens to human [4].

L. monocytogenes is known to persist and multiply in food and food-processing environments, being frequently isolated from fish, seafood and meat [5]. It has also been found in refrigerated foods because it is able to grow slowly at temperatures as low as 0°C and may therefore multiply to dangerous levels in refrigerated foods unless

Corresponding Author: K. Immaculate Jeyasanta, Suganthi Devadason Marine Research Institute, Tuticorin, Tamil Nadu, India. Tel: +91 461 2336488, Fax: +91 461 232569, E-mail: immaculatejayasantha@gmail.com.

controlled. *Listeria* species are also common colonizers of food utensils where they may form biofilms that are difficult to remove. These biofilms can act as reservoirs of persistent *L. monocytogenes* contamination for processed foods if not controlled. The presence of *Listeria* species in the utensils is often used as an indicator for *L. monocytogenes* contamination.

L. monocytogenes is psychrotrophic and halotolerant [6] and otherwise optimal conditions, grow in the range of 1 to 45°C. It can survive or even grow at pH values as low as 4.4 and at salt concentrations of up to 14%. As a consequence, it has been isolated from a variety of sources, including fish and fishery products [7-11]. Considerable contamination occurs during evisceration, fish handling and packing as a result of inadequate hygiene [12]. Frozen fish either the raw fish or the smoked product offers a good protection and that reduction of numbers of *L. monocytogenes* due to freezing is marginal. As a result, strict legislation governs both the detection limits and permissible levels of *L. monocytogenes* in sea foods. Many countries have introduced legislation to try to control the incidence of listeriosis and in many cases regulations require microbiological monitoring of *L. monocytogenes*. In the EU, the Microbiological Criteria Regulation requires regular testing of sea foods for *L. monocytogenes* and imposes limits of absent in 25g at the point of production. The U.S. Food and Drug Administration (FDA) maintains a zerotolerance policy [13, 14] while in Europe, legislation (No. 2073/2005) imposes a zero-tolerance policy in respect to certain foods destined for high-risk consumer groups and otherwise limits these bacteria to below 100 CFU/g [15].

In the past, those individuals who develop listeriosis have usually been treated with penicillin or ampicillin in conjunction with an amino glycoside [16] although tetracycline, erythromycin or chloramphenicol alone or in combination has also been used [17]. Current therapy of all forms of listeriosis is a combination of ampicillin and gentamycin [18]. Antibiotics once effective at controlling listeriosis infections are now ineffective due to bacterium's acquired resistance to these compounds. The resistant microbes may function as a potential source in the transportation of antimicrobial resistance to human beings. The use of antimicrobial drugs to control infectious diseases must be among the greatest achievements of medicine in the century. The disease threat from antibiotic resistant strains has increased in recent years. The occurrence of multiple antibiotic resistances among the enteric bacterial species could be a problem associated with transfer of resistance to human beings. Some reports have suggested the absence of *L. monocytogenes* in tropical fishes, but Ben Embarek [19] emphasized that more research is needed regarding the incidence of *L. monocytogenes* in tropical seafood. In India, there are very few reports on the incidence of *L. monocytogenes* in sea food samples [9, 20]. The prevalence of *L.monocytogenes* in tropical fresh shellfish and finfish ranges from 12.1 to 17.2% [20]. Since seafood may be a vehicle for *L. monocytogenes* and it is important to have information on the incidence of this pathogen. The widespread distribution of epidemiologically important *L. monocytogenes* and their resistance to commonly used antibiotics indicate a potential public health risk. The situation assumed a monitoring of antimicrobial resistance is extremely important to decide the proper treatment of listeriosis. Keeping in this view, the present study was carried out to isolate *Listeria monocytogenes* from marine food resources and evaluates the antibiotic susceptibility pattern.

MATERIALS AND METHODS

Sample Collection: The fresh seafood samples were purchased from the local fish venders and landing centers and transported to the laboratory in sterile bags with ice and processed within 2 hours of collection.

Isolation and Identification of *L. monocytogenes*: Samples were analysed for *Listeria* species using the enrichment selection and isolation protocol recommended by USFDA [21]. Five gram of each sample were blended with 45 ml *Listeria* enrichment broth and then incubated at 30°C for 48 hours. A loopful from *Listeria* enrichment broth was streaked onto a PALCAM agar plate media (Oxoid & UK) and then incubated at 37°C for 24 - 48 hours. Typical colonies (grey-green to black colonies with a black halo and a sunken center) were picked up and streaked onto a Trypticase soy agar, supplemented with 0.6 yeast extract (TSAYE) and incubated at 30°C for 24 hours till obtaining satisfactory pure separate colonies, then submitted to identification and confirmation.

Confirmation of *L. monocytogenes*: Up to 34 different individual colonies were subjected to confirmation analysis. Direct plating of suspected colonies on a blood agar plate. After incubation at 35 ± 2°C for 19 ± 3 h, the plates were examined for the presence of β-hemolytic colonies. If a colony was confirmed as *L. monocytogenes*, all the β- hemolytic colonies were considered. A clearly isolated β-hemolytic colony was subjected to further biochemical confirmation.

Microscopical Examination: Pure culture of the organism was stained with Gram's stain and examined microscopically for Gram staining and motility. *L. monocytogenes* showed Gram positive short rods, occurring in short chains with a typical diphtheroid arrangement.

Motility Test: Motility is one of the important determinants in making a final species identification [22]. *Listeria monocytogenes* move by means of flagella. Listeria motility medium have agar concentration of 0.4% used for detecting motility. The motility test is interpreted by making a macroscopic examination of medium for a diffused zone of growth flaring out from the line of inoculation. *Listeria monocytogenes* requires room temperature incubation before motility develops. Casein enzymic hydrolysate and peptic digest of animal tissue act as source of growth nutrients. The motility of *L. monocytogenes* is demonstrated by stab inoculating tubes of semisolid medium and incubating at room temperature (20 - 25°C). An umbrella-like zone of growth 2 to 5 mm below the surface of the medium is characteristic of *L. monocytogenes*. Motility at 35°C incubation is either absent or extremely sluggish.

Biochemical Identification: Biochemically identification of *Listeria monocytogenes* was carried out through catalase, oxidase, MR-VP, mannitol, Rhamnose and xylose fermentation test according to the outlines recommended by McClain and Lee [23].

Drug Sensitivity Test: Disc diffusion method [24] was used to examine bacterial susceptibility to antimicrobial agents. A total of 13 antibiotics discs (Hi media, Mumbai) of Ampicillin 10 mcg, Gentamycin 10 mcg, Chloramphenicol 30 mcg, Amikacin 30 mcg, Penicillin 10 U, Streptomycin 10 mcg, Tetracycline 30 mcg, Kanamycin 30 mcg, Vancomycin 30 mcg, Erythromycin 15 mcg, Ciprofloxacin 5 mcg, Amoxycillin 30 mcg and Nalidixic acid 30 mcg were used. The plates were examined and the diameter of the zones of complete inhibition to the nearest whole millimeter was measured. The zone diameter for individual antimicrobial agents was then translated into susceptible, intermediate and resistant categories according to the zone interpretation table of the Hi media, Mumbai.

RESULTS AND DISCUSSION

The prevalence of *Listeria monocytogenes* in sea foods collected from Tuticorin coast is presented in Table 1. From the 22 different seafood samples, 113 microbial cultures were made and among that 37 cultures were positive for *L.monocytogenes*. *Listeria* species were found in 32.74% of the samples. The highest positive results were obtained from crab (100%), followed by fishes (40- 62.5%), shrimps (25%), bivalves (60%) and cuttlefish (50%). No *Listeria* could be isolated from the Octopus, gastropods and dried fish samples. Reports from developed countries, like the United States of America, have implicated contaminated foods as a major vehicle of transmission for listeriosis [25, 26]. Fish and seafood harvested from natural environments have been identified as potential sources of *Listeria* in the human diet [27]. The organism has been isolated from fresh and seawater and from frozen or processed sea foods [28-30]. Adesiyun [31] noted a 2% incidence of *L.monocytogenes* in seafood samples from Trinidad. In India, the prevalence of *L.monocytogenes* in tropical fresh shellfish and finfish ranges from 12.1 to 17.2% [20]. In Latin America, the prevalence of *L.monocytogenes* in fish and fish products varies from 0 to 50% [32] and in India, 9% of fresh / raw seafood samples are contaminated with *L. monocytogenes* [33]. It can be concluded that Listeria can be important problem even for fisheries in tropical countries.

The fish and crab samples examined in this study had a higher incidence (40 -100%) of Listeria. This could be due to contamination of the seawater from which the fishes were caught. A number of earlier workers reported the absence of *Listeria* from tropical fish and attributed this to environmental factors [34 -36]. However, Jeyasekaram *et al.* [20] attributed it to inadequate isolation procedures. In a study conducted in Sokoto, Nigeria, Salihu *et al.* [37] reported an incidence of 25% *L.monocytogenes* in fish. *L.monocytogenes* pathogen gets transmitted through consumption of contaminated marine food resources. In the present study Listeria species were isolated from fresh fish, shrimp, crab, cephalopods, bivalves and dried fishes. In this out of 37 isolates 24 isolated from raw fish, 2 from shrimps, 2 from crab, 2 from squid, 1 from cuttlefish and 6 from bivalves were hemolytic on blood agar and identified as *Listeria monocytogenes*. Jeyasekaran *et al.* [20] reported higher occurrence of *Listeria monocytogenes* in fresh finfish from tropical seafood from India. Dhanashree *et al*. [38] isolated *Listeria monocytogenes* in 13% of fresh raw fish samples from Mangalore, India.

Yadollahi *et al.* [39] reported that from 300 seafood samples collected from retail shop in Iran, 6, 1, 0.33 and 0.66% of them were positive for *L. monocytogenes*, *L. ivanovii*, *L. innocua* and *L. seeligeri*, respectively but Rahimi *et al.* [40] previously reported from Iran on fresh

and frozen fish and shrimp showed that *L.monocytogenes* and *L. innocua* were detected in 1.9 and 5.7% of the samples analyzed respectively.

In the present study high prevalence of *L.monocytogenes* might be due to attack of Listeria from intestinal contents to other fish tissue. Ertas and Seker [41] reported that contact with intestinal contents is the risk factor for prevalence of *Listeria* spp. in seafood samples. In addition, cross contamination using contaminated equipment, fish manipulation and inappropriate transport, were introduced as risk factors [42]. An overall prevalence 32.74% of *L. monocytogenes* was observed in all the seafood of this current study could be due to contaminated coastal water, unhygienic processing and improper cleaning of processing utensils. But Miettinen and Wirtanen [43] reported prevalence of *L.monocytogenes* in pooled unprocessed fresh rainbow trout was on 35%. Study in Greece on fish and environment of fish markets showed that *Listeria* was more common species and the level of contamination of the environment of fish markets was higher than fish [44].

In Turkey, the incidence of *L.monocytogenes* from marine fish sample was 44.5% [45] which is higher than our results. In this study *L.monocytogenes* isolated from gastropods and bivalves, among these bivalves showed positive results out of 10 samples collected from landing site 6 were positive for this pathogen. Similarly Dhanashree *et al.* [38] reported that *L.monocytogenes* was isolated from 4.2% of raw clams from Mangalore, India. Abdellrazeq *et al.* [46] reported high prevalence 56.9% of *L. monocytogenes* in catfish. Chou *et al.* [47] showed that 25 to 47% of fresh channel catfish were contaminated with *L. monocytogenes*. Dhanashree *et al.*[38] reported smoked fish samples were free from Listeria spp.

In this study dried fishes werefree from this pathogen. Earlier reports suggested the absence of *L. monocytogenes*in tropical fish [34, 35, 37 and 48]. However, Jeyasekaran *et al.* [20] reported the incidence of *L.monocytogenes* in a variety of fish samples. In the present study, the incidence of *L. monocytogenes* in seafood was 32.74%. Baek *et al.* [49] reported the absence of *L. monocytogenes* in dried seafood in Korea. In the present study also *L.monocytogenes* was not isolated from the dried fish samples. Dhanashree *et al.* [38] suggested that the risk of acquiring listeriosis is higher through seafood in India. Samples that were positive for *L. monocytogenes* were raw seafood which could be cooked before consumption. Nevertheless, presence of this organism in raw seafood poses a health risk in kitchen where raw and cooked seafood may be stored and handled. Ben Embarek [19] reviewed the incidence of *Listeria* in seafood worldwide and found that the prevalence of *L. monocytogenes* varied from 14 to 22% in surveys from temperate areas. Farber and Peterking [27] reported the presence of *L. monocytogenes* in salmon from the United States, Chile, Norway and Canada. Other studies have found that the prevalence of *L. monocytogenes* in raw fish is quite low, ranging from 10 to 40% [50, 51]. Hartemink and Georgesson [52] stated that in Iceland 56% of fresh fish on sale were contaminated with *L. monocytogenes* and other *Listeria* species. An overall prevalence 30% of *L. monocytogenes* was observed in European fish [53].

In this study out of 113 isolates 37 isolates fulfilled the criteria of identification of *L. monocytogenes* (Table 2).

The widespread distribution of epidemiologically important serotypes of *L. monocytogenes* and their resistance to commonly used antibiotics indicate a potential public health risk. The situation assumed a monitoring of antimicrobial resistance (AMR) is extremely important to decide the proper treatment of listeriosis. The antimicrobial resistance of *L. monocytogenes* may be associated with the presence of a plasmid or may be determined by genes that are transferred by conjugation and mutational events in chromosomal genes [54].

Drug-sensitive strains of *L. monocytogenes* were isolated from clinical and food samples [38]. While, Sharma *et al.* [55] reported multi drug-resistance strains from milk samples. In the present study, antibiotic susceptibility pattern showed that all the isolates of *L. monocytogenes* from seafoods were susceptible to the 9 out of 13 antibiotics tested. However, few of the isolates were highly resistant to Nalidixic acid, Kanamycin, Streptomycin and Gentamycin (Table 2). Resistance of *L. monocytogenes* to Streptomycin and gentamycin, the drugs of choice for treatment of listeriosis was recorded [16]. Quendrea *et al.* [56] screened for the occurrence of *Listeria monocytogenes* in food and food-related settings and recorded that 35% of isolates were resistant to ampicillin (65%) and 24% of isolates were resistant to penicillin (82%) and our results agree to this. These results are especially relevant due to the importance of β-lactams in the treatment of human listeriosis.

Antibiotic susceptibility patterns in this study showed all the *L. Monocytogenes* isolates were resistant to Nalidixic acid (100%). Twenty eights isolates were resistant to gentamycin. However, they were susceptible to Amoxycillin and vancomycin (100%) and this coincides

Table 1: Prevalence of *Listeria monocytogenes* in sea foods collected from different sources

Sea food samples	Sources	No. of samples	No. of positive samples	Incidence (%)
Fin fishes				
Sardinellaalbella	Fish market	10	4	40
Stolephorusindicus	Fish market	10	6	60
Sauridatumbil	Fish market	10	4	40
Cephalopholisboenak	Fish market	5	2	40
Sillagosihama	Fish market	6	-	-
Gazzaminuta	Fish market	8	5	62.5
Parupeneusindicus	Fish market	5	3	60
Shrimp				
Penaeusindicus	Fish market	4	1	25
Penaeus japonicas	Fish market	4	1	25
Penaeussemisulcatus	Fish market	4	-	-
Crab				
Portunussanguinolentus	Fish market	2	2	100
Charybdis natator	Landing centre	2	-	-
Squid				
Loligoduvauceli	Fish market	2	1	50
Sepioteuthislessoniana	Fish market	2	1	50
Cuttle fish				
Sepia aculeate	Fish market	2	1	50
Sepia pharaonis	Landing centre	2	-	-
Octopus				
Octopus aegina	Landing centre	3	-	-
Gastropod				
Hemifuscuspugilinus	Inter tidal area	6	-	-
Turbinellapyrum	Inter tidal area	6	-	-
Bivalves				
Mimachlamyssanguinea	Estuary	10	6	60
Dried fish				
Caranx ignobilis	Dried fish market	5	-	-
Lutjanus rivulatus	Dried fish market	5	-	-
Total		113	37	32.74

Table 2: Biochemical identification of *L.monocytogenes*

Biochemical test	Results
Gram staining	Gram positive rods
Motility	Motile at 25°C
Catalase	Positive
Oxidase	Negative
Methyl red	Positive
Voges Proskaüer	Positive
Mannitol	Negative
L.Rhamnose	Positive
Xylose	Negative
β- haemolysis	Positive
Growth on blood agar after incubation at 35 – 37°C for 16-48 h	Colonies are 1.3 mm in diameter, smooth translucent and β- haemolysis extending 1-2mm from the edge of the colony

Table 3: Antibiotic susceptibility patterns of 37 selected strains of *Listeria monocytogenes* isolated from sea foods

	Resistant		Intermediate		Sensitive	
Antibiotics (dose/ disc)	% of positive strains	Inhibition Zone (mm)	% of positive strains	Inhibition Zone (mm)	% of positive strains	Inhibition Zone (mm)
Ampicillin 10 mcg	13.51 (5)	<13	29.72 (11)	15-16	56.75 (21)	>18
Gentamycin 10mcg	75.67 (28)	<8	-	13-14	24.32 (9)	>16
Chloramphenicol 30 mcg	5.40 (2)	<12	51.35 (19)	13-17	43.24 (16)	>22
Amikacin 30 mcg	16.21 (6)	<12	16.21 (6)	14-16	67.56 (25)	>16
Penicillin 10 U	37.83 (14)	<14	62.16 (23)	10-14	-	>15
Streptomycin 10 mcg	78.37 (29)	<11	21.62 (8)	14-18	-	>15
Tetracycline 30 mcg	32.43 (12)	<12	54.05 (20)	15-18	13.51 (5)	>20
Kanamycin 30 mcg	72.97 (27)	<11	27.02 (10)	14-16	-	>17
Vancomycin 30 mcg	10.81 (4)	<13	13.51 (5)	18-20	75.7 (28)	>15
Erythromycin 15 mcg	-	<10	-	13-16	16.21 (6)	>18
Ciprofloxacin 5 mcg	-	<13	-	14-18	21.62 (8)	>19
Amoxycillin 30 mcg	-	<14	-	13-16	100 (37)	>18
Nalidixic acid 30mcg	100 (37)	<12	-	14-18	-	>15

with Abdellrazeq *et al.* [46]. *L. monocytogenes* has been reported to be resistant to a number of these antibiotics, including ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, Sulphamethoxazole / trimethoprim and tetracycline (75%) [16, 57 & 58] and the present study ampicillin (35%), Chloromphenicol (32.43%), Gentamycin (75.67%), Nailidixic acid (100%) and Tetracyclin (32.43%) showed their resistant against the isolates.

This evolution of antibiotic resistance isolates in the seafood samples could be due to runoff from land to sea containing faeces contaminated discharges and other domestic pollution which carry antibiotic resistant microorganisms [59]. Ampicillin and tetracycline are often the first choice of drugs used in Egypt for the treatment of listeriosis [60], but in this study ampicillin showed 56.75% sensitivity, 13.51% resistant pattern, while tetracycline showed only 13.51% sensitivity and 32.43% resistant pattern against *L. monocytogenes* isolates. In this study all *L. monocytogenes* isolates were susceptible to erythromycin, ciprofloxacin and amoxicillin (100%). Ennaji *et al.* [57] reported that *L. monocytogenes* was resistant to cefotaxime (100%), nalidixic acid (100%), cephalothin (10%) and susceptible to ampicillin, chloramphenicol and gentamycin. Ruiz -Bolivar *et al.* [61] found the susceptibility of *L. monocytogenes* was 100% for ampicillin, amoxicillin, vancomycin and chloramphenicol, whereas for erythromycin, tetracycline and penicillin, isolates showed intermediate resistance. In this study, *L. monocytogenes* isolates showed intermediate resistance against ampicillin, chloramphenicol, amikacin, penicillin, streptomycin, tetracycline, kanamycin and vancomycin. In contrast, Soni *et al.* [62] reported that all *L. monocytogenes* isolates were resistant to ampicillin.

The study recommended that the seafood's are contaminated with the presence of this food borne pathogen so, attention also needs to be on seawater pollution monitoring, prevent highly polluted waste water discharges, improve lading site and marketing channel. Monitoring the antimicrobial resistance of *L. monocytogenes* in humans and animals to understand changes in the patterns of resistance to commonly used antimicrobials, to implement active measures to control the use of antimicrobial agents and to prevent the spread of multi-drug resistant strains, which can have many undesired consequences [63]. Okonkwo Lucy *et al.* [64] reported the way to avoid contamination in seafood by adequate measures which should be taken to improve the hygienic conditions of our markets, slaughtering and butchering facilities and our environments especially our homes. It is necessary to have sanitation controls measures that prevent contamination of product contact surfaces and eliminate niches where *L. monocytogenes* can establish itself, grow and persist.

CONCLUSIONS

The study showed that fresh seafood obtained from the market and landing site contains *L. monocytogenes*, which constitutes a public health hazard. An effective control measure for this pathogen has to target the processing utensils and the environments. Strict standard operating measures must be practiced during processing, handling and distribution. The coastal environment must be clean and unpolluted. Hazards from *L. monocytogenes* can be prevented by thoroughly cooking seafood and by preventing cross-contamination once the seafood is cooked.

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