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Occurrence and Characterization of *Staphylococcus aureus* (Rosenbach, 1884) in Seafood from Landing Centres and Retail Markets of Thoothukudi, South-east coast of India

A. Mercy Amala, G. Sugumar* and B. Chrisolite
Fisheries College and Research Institute, Tamil Nadu Fisheries University, Thoothukudi - 628 008, India

Abstract

The prevalence of *Staphylococcus aureus* (Rosenbach, 1884) in 88 samples of seafood from fish landing centres and retail markets of Thoothukudi were analysed. Two hundred and twenty nine isolates were confirmed as *Staphylococcus* by biochemical tests and these isolates were tested for the production of extracellular enzymes, antibiotic resistance profile and enterotoxin gene profile. There was not much difference in aerobic plate count from freshly landed fish ($5.80 \times 10^5 - 5.20 \times 10^6$ cfu g^{-1}) and fish from market ($7.80 \times 10^5 - 1.10 \times 10^7$ cfu g^{-1}). Coagulase positive and negative staphylococci were confirmed in 9.09 and 31.82% of the freshly landed seafoods and in 11.36 and 54.55% of the market samples, respectively. Haemolysin, lipase, lecithinase, gelatinase and thermostable deoxyribonuclease enzyme were produced by 73.36, 66.81, 77.29, 79.22 and 46.28% of the isolates, respectively. Five groups of staphylococci classified based on their ability to produce coagulase and other extracellular enzymes were tested for antibiotic susceptibility. Results of antibiotic sensitivity test showed that most isolates were resistant to Penicillin-G and Nalidixic acid, while none was resistant to Gentamicin. Three genes, *sea, sec* and *seg* were detected in *S. aureus* by PCR. The presence of enterotoxigenic *S. aureus* in fishes raises concern and calls for proper implementation of better hygienic and sanitary practices both in landing centre and market.

Keywords: *Staphylococcus aureus*, seafood, enterotoxin

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* *E-mail: jsotnfu@gmail.com*

Introduction

Staphylococcus aureus are ubiquitous in nature and are commonly present in human beings and animals. Human being serve as the major reservoir of these organisms and these are lodged on the skin and nasal cavity (Jay, 1996). About 40 to 44% of healthy people carry this organism in their throat and nose. Since they are normally present in human and transmitted to food materials, they are referred to as indicators of personal hygiene (Huss, 1995).

Occurrence of *S. aureus* has been documented in marine products and environment. Sanjeev et al. (1985) studied the occurrence of enterotoxigenic *Staphylococcus* in cured fishery products collected from Cochin and found that 20.5% of cured fishery products contained enterotoxigenic *Staphylococcus* which produced enterotoxins A,B,C,D and E either singly or in combination. Nambiar & Iyer (1990) reported *S. aureus* in 8.5% of the frozen fish from the retail markets of Cochin, however, Sanjeev & Surendran (1994) reported a higher level of incidence of *S. aureus* in frozen fish samples from the same area. *S aureus* was detected in 17% of fishery products and 54% of fish processing factory workers of Cochin (Simon & Sanjeev, 2007). Vazquez-Sanchez et al. (2012) reported the incidence of this organism in 25% of fishery products marketed in Galicia, Northwest Spain.

Occurrence of coagulase positive staphylococci was found to be comparatively higher in cooked fishery products, evidently due to the involvement of human handling after cooking (Iyer, 2002). The presence of coagulase positive staphylococci was confirmed in 22.7% of fresh fish samples and coagulase negative staphylococci in 44.45% of the samples (Sindhu & Surendran, 2006).

Another characteristic of *S. aureus* is the ability to produce various extracellular enzymes such as haemolysins, thermostable deoxyribonuclease, lipase and lecithinase, many of which are toxins to humans and some animals. Thermostable nuclease is a characteristic enzyme of *S. aureus.* Park et al. (1978) suggested the evaluation of staphylococcal thermostable deoxyribonuclease assay as a means of screening foods for growth of staphylococci and possible enterotoxin production. Production of coagulase is a characteristic used to distinguish pathogenic from non-pathogenic strains of *S. aureus*, but it cannot be used as the sole determinant of pathogenicity. Staphylococcal food poisonings result from the ingestion of food containing staphylococcal enterotoxins (SEs) performed by enterotoxigenic strains. Growth of enterotoxigenic strains of *S. aureus* to a population of 10^6 or more cells g^{-1} of food has been reported to be necessary for production of enterotoxin. Staphylococcal enterotoxins (SEs) are a group of single chain, low molecular weight (26,900 to 29,600) proteins produced by some species of staphylococci, primarily *S. aureus*. They are similar in composition and biological activity, but they are identified as separate proteins due to their difference in antigenicity. Of the twenty types of Staphylococcal enterotoxins known to date, SEA, SEB, SEC, SED and SEE are associated with food poisoning (Aydin et al., 2011).

Several staphylococcal species other than *S. aureus* reportedly produce SEs (Jay, 1996). Among the coagulase negative species, *S. cohnii, S. epidermidis, S. xylosus* and *S. haemolyticus* isolated from ewe's milk were found to produce one or several SEs (Baustica et al*.,* 1988). Enterotoxin A was reported to be the most potent toxin in causing food poisoning followed by SED, SEC and SEB. Not all staplylococci are pathogenic in nature and therefore there is a need for clear understanding on the different types of staphylococci, especially the coagulase positive and coagulase negative saphylococci in seafood. The paper deals with the occurrence of coagulase positive and coagulase negative Staphylococci in fish and shellfish samples from landing centres and retail markets of Thoothukudi and their enterotoxin and virulence potential.

Materials and Methods

Samples of sardine (*Sardinella* sp.), emperor fish (*Lethrinus* sp.), shrimp (*Penaeus* sp.) and blue crab (*Portunus pelagicus)* were procured from Thirespuram landing centre and V.O.C retail market at Thoothukudi. In each variety, eleven samples were collected at the landing centre and at the retail market separately at different months which accounts for a total of 88 samples. Samples were collected in sterile containers and brought to the laboratory within 15 min for analysis. Each sample represents a pool of six fish.

25 g of meat sample was taken aseptically and homogenized with 225 ml of physiological saline in sterile bags using stomacher. The homogenized sample was serially diluted and inoculum was taken for enumerating total plate count and presumptive staphylococcal count. Isolated colonies from the selective medium, Baird Parker Agar (BPA) were chosen based on morphology, purified on trypticase soy agar (TSA) medium and further characterised based on Gram reaction, catalase, anaerobic utilization of glucose and mannitol and stock cultures were maintained.

Extracellular enzyme activity of isolates of *S. aureus* was tested by growing the culture on media with appropriate substrates. The isolates were screened for the production of coagulase (AOAC, 1995), thermostable deoxyribonuclease (Lachica et al., 1971), gelatinase, lecithinase, lipase (West & Colwell, 1984) and haemolysin (Surendren et al., 2003). The isolates were also tested for their antibiotic sensitivity by disc diffusion method (Bauer et al., 1966) using different antibiotic discs *viz.,* Bacitracin (10 units), Chloramphenicol (30 mcg), Erythromycin (15 mcg), Gentamicin (10 mcg), Nalidixic acid (30 mcg), Penicillin-G (10 units), Sulphafurazole (300 mcg), Trimethoprim (5 mcg) and Vancomycin (30 mcg).

Bacterial DNA was extracted and genes were amplified using the following primers (McLauchlin et al., 2000) (Table 1) and the PCR products were visualized using gel documentation system (Biorad, USA).

Results and Discussion

In the present study, aerobic plate count (APC) of freshly landed seafood samples was found to range from $5.8 \times 10^5 - 5.2 \times 10^6$ cfu g^{-1} in landing centre samples and $7.8 \times 10^5 - 1.1 \times 10^7$ cfu g^{-1} in market samples (Table 2). Such high APC in seafoods landed in Thoothukudi fishing harbour has already been reported (Anand et al*.,* 2002; Chrisolite et al., 2006). Generally, bacterial loads in freshly landed

Table 1. Description of staphylococcal enterotoxins primers

Primer designation	Nucleotide sequence 5′ to 3′	Enterotoxin	Fragment size (bp)
A1	5′ TTG GAA ACG GTT AAA ACG AA3′	A (*sea*)	120
A2	5′ GAA CCT TCC CAT CAA AAA CA 3′		
B1	5′ TCG CAT CAA ACT GAC AAA CG 3′	B (*seb)*	478
B2	5′ GCA GGT ACT CTA TAA CTG CC 3′		
C1	5′ GAC ATA AAA GCT AGG AAT TT 3′	C (*sec*)	257
C2	5′ AAA TCG GAT TAA CAT TAT CC 3′		
D1	5′ CTA GTT TGG TAA TAT CTC CT 3′	D (*sed*)	317
D2	5′ TAA TGC TAT ATC TTA TAG GG 3′		
E1	5′ TAG ATA AAG TTA AAA CAA GC 3′	E (*see*)	170
E2	5′ TAA CTT ACC GTC GAC CCT TC 3′		
G1	5′ TCG TAT CGA CAC ACT ACA ACC 3′	G (*seg*)	704
G2	5′ CCA GAT TCA AAT GCA GAA CC 3′		
H1	5′ CGA AAG CAG AAG ATT TAC ACG 3′	H (*seh)*	495
H2	5′ GAC CTT TAC TTA TTT CGC TGT C3′		
I1	5′ GAC AAC AAA ACT GTC GAA ACT G 3′	I (*sei*)	630
I2	5′ CCA TAT TCT TTG CCT TTA CCA G 3′		
T1	5′ ATG GCA GCA TCA GCT TGA TA 3′	TSST (*tsst*)	350
T2	5′ TTT CCA ATA ACC ACC CGT TT 3′		

Table 2. Bacteriological status of fish from landing centres and retail markets of Thoothukudi

S. No	Details of sample	Source	No. of samples analysed	APC (cfu/g) (Mean ± SD)	PSC (cfu/g) (Mean ± SD)
1	Sardines (*Sardinella* spp.)	Landing centre	11	$1.7 \pm 3.8 \times 10^6$	$6.6 \pm 9.3 \times 10^4$
2	Emperor fish (*Lethrinus* sp.)	Landing centre	11	$5.8 \pm 6.6 \times 10^5$	$3.8 \pm 5.1 \times 10^4$
3	Shrimp (*Penaeus* spp.)	Landing centre	11	$5.2 \pm 9.1 \times 10^6$	$1.1 \pm 2.4 \times 10^5$
4	Blue crab (*Portunus pelagicus*)	Landing centre	11	$2.1 \pm 4.5 \times 10^6$	$2.0 \pm 2.5 \times 10^4$
5	Sardines (*Sardinella* spp.)	Retail market	11	$7.8 \pm 7.7 \times 10^5$	$7.6 \pm 7.0 \times 10^4$
6	Emperor fish (*Lethrinus* sp.)	Retail market	11	$3.7 \pm 6.9 \times 10^6$	$5.1 \pm 4.9 \times 10^4$
7	Shrimp (*Penaeus* spp.)	Retail market	11	$1.1 \pm 1.5 \times 10^7$	$4.3 \pm 7.4 \times 10^5$
8	Blue crab (*Portunus pelagicus*)	Retail market	11	$4.7 \pm 8.8 \times 10^6$	$3.5 \pm 6.4 \times 10^5$

tropical seafoods were reported to be high in the range of 10^3 to 10^7 cfu g^{-1} (Karunasagar et al., 1992). This might be due to the fact that the quality of raw materials harvested from different fishing areas depend upon several factors including sanitary status of the landing centres. The slightly higher counts in market samples might be due to possible contamination during handling and transport, the

longer distance between the landing centre and retail market which increases the storage time and hence the microbial load in the fish. The results are comparable with those on fresh sardines in retail markets of Cochin (Nambiar & Iyer, 1990) and fishes in Bombay (Iyer et al., 1986). However, the results of the present study were higher than the APC recommended by ICMSF (1986).

The high presumptive staphylococcal count of freshly landed seafood samples (up to 10^5 cfu g^{-1}) and fishes sold in market (up to 10^6 cfu g^{-1}) (Table 2) reflected poor personal hygiene of fish handlers and poor handling practices. *Staphylococcus,* although commonly present in seafood, is not a normal inhabitant of the fish and therefore, its presence in seafood at high level is considered to be due to direct human contamination or water pollution. Presumptive streptococcal counts were higher in crustacean samples both from landing centre and market. Although the results were comparable with the studies of Anand et al. (2002), they were higher (Antony et al., 2002; Sindhu & Surendran, 2006) and lower (Ellender et al., 1995) than earlier reports.

Coagulase positive and negative staphylococci were confirmed in 9.09 and 31.82% of the freshly landed seafoods and in 11.36 and 54.55% of the market samples respectively. Among the freshly landed samples, crab registered a higher incidence of both coagulase positive and negative staphylococci followed by shrimp and emperor fish samples (Table 3). Among retail market samples, sardines registered higher incidence of both coagulase positive and negative staphylococci. This might be due to excessive human handling, as it is one of the much preferred fish in local market involving repeated sorting and handling in retail trade. The results were comparable with those on fresh and frozen fishes in retail market of Cochin (Nambiar & Iyer, 1990; Sindhu & Surendran, 2006). However, presence of *S. aureus* in 100% of the frozen crab meat and frozen shrimp samples was also reported (Sanjeev et al., 1985). *S. aureus* was detected in 33.3% and 48.70% of fresh and frozen samples from Tehran seafood market, Iran (Sharifi-Yazdi et al., 2016), 32-62.70% of 330 imported fresh fish samples from three countries Egypt, India and Yenam (Obaidat et al., 2015), in 19.60% of retail fish samples in Japan (Saito et al., 2011) and 9% in fish and fish products in Korea (Oh et al., 2007). The occurrence of *S. aureus* in 9.09 and 11.36% of landing centre and retail market sample in the present study is lesser than most of the earlier studies, however higher aerobic plate count in all samples could be due to the unhygienic practices of fish handlers, delay in transport, storage conditions, contact with contaminated work surface etc.

Enzymes produced by microorganisms interfere with mammalian functions and some of these enzymes contribute to the virulence of microbial pathogens by enabling the microorganisms to invade body tissues (Atlas, 1989). The production of coagulase is an important characteristic of pathogenic *S. aureus.* It has been reported that 24% of

Table 3. Occurrence of staphylococci in seafood samples from landing centre and retail market of Thoothukudi

Sl. No.	Sample	Source	No. of samples analysed	Samples positive for coagulase positive staphylococci		Samples positive for coagulase negative staphylococci	
				No.	%	No.	%
1	Sardine	Landing centre	11	0	0	3	27.27
2	Emperor fish	Landing centre	11	1	9.09	2	18.18
3	Shrimp	Landing centre	11	1	9.09	2	18.18
4	Blue crab	Landing centre	11	2	18.18	7	63.64
5	Sardine	Retail market	11	3	27.27	9	81.82
6	Emperor fish	Retail market	11	0	0	3	27.27
7	Shrimp	Retail market	11	1	9.09	4	36.36
8	Blue crab	Retail market	11	1	9.09	8	72.72

environmental staphylococcal isolates and 78.6% of clinical isolates were coagulase positive (Bhat et al., 1990). Compared to these data, the incidence of coagulase positive staphylococci was very low (5.70%) in the present study (Table 3). Among the enzymatic characters, the ability to produce TDNase and coagulase correlated well as already reported (Bhat et al., 1990). However, a weak TDNase reaction was reported in a few coagulase negative staphylococci (Lachica et al., 1969; Stickler & Freestone, 1971) as also observed in the present study, where several of the coagulase negative staphylococci (46.30%) produced TDNase on prolonged incubation.

Mannitol fermentation has been considered as an important indicator of enteropathogenicity in staphylococci. About 90% of the staphylococci fermented mannitol both aerobically and anaerobically, however, reaction on salt mannitol agar did not serve as a substitute for tube coagulase test as only 5.7% of the total isolates were coagulase positive. Haemolysin, lipase, lecithinase, gelatinase and thermostable deoxyribonuclease enzyme were produced by 73.36, 66.81, 77.29, 76.86 and 46.29% of the isolates respectively (Table 4). Lipase has been considered as an important agent in the initiation of boils and carbuncle in humans and majority of *S. aureus* strains isolated from human infection possess this enzyme (Bhat et al., 1990). In the present study, 82.35% of coagulase positive strains and 56.47% of coagulase negative staphylococci produced lipase. Lecithinase hydrolyses lecithin, a lipid component of cell membranes. Lecithinase also acts as a haemolysin, causing the lysis of red blood cells in addition to destroying various other tissues (Atlas, 1989). Although all the β-haemolytic strains (92%) of the 229 isolates in this study produced lecithinase, not all lecithinase positive strains (82%) produced β-haemolysis. The ability of *S. aureus* to produce haemolysin is more associated with virulence.

The 5 groups of staphylococci classified based on their ability to produce coagulase and other extracellular enzymes did not show greater variation in their antibiogram. Most isolates were resistant to Penicillin-G and Nalidixic acid, while none was resistant to Gentamicin (Table 5). The percentage of cultures showing resistance to Vancomycin, Bacitracin, Trimethoprim and Erythromycin varied between these groups, however, no relationship could be drawn between antibiogram and enzyme potential of these isolates. Resistance to Penicillin was found higher in the isolates than those already reported (Sanjeev et al., 1985; Sanjeev & Iyer,

Table 4. Toxin potential (extracellular enzymes) of *S. aureus* isolates from seafoods

Sl. No.	Sample	Source	No. of samples analysed	No. of isolates	Coagulase		TDNase		Haemolysin	Lipase	Lecithinase	Gelatinase
					on MSA	in rabbit plasma	6 h	48 h				
1.	Sardine	Landing centre	11	9	8(88.88)	0	0	6 (66.67)	2(22.22)	0	3(33.33)	3(33.33)
2.	Emperor fish	Landing centre	11	7	7(100)	3(42.86)	3(42.86)	3(42.86)	7(100)	4(57.14)	7(100)	3(42.85)
3.	Shrimp	Landing centre	11	5	5(100)	1(20)	1(20)	4(80)	1(20)	0	1(20)	5(100)
4.	Blue crab	Landing centre	11	81	71(88.65)	2(2.47)	2(2.47)	36(44.44)	70(86.42)	70(86.42)	70(86.42)	73(90.12)
5.	Sardine	Retail market	11	30	29(96.67)	4(13.33)	4(13.33)	11(36.67)	25(83.33)	19(63.33)	30(100)	22(73.33)
6.	Emperor fish	Retail market	11	14	12(85.71)	0	0	10(71.43)	7(50)	4(28.57)	7(50.00)	7(50)
7.	Shrimp	Retail market	11	6	5(83.33)	1(16.67)	1(16.67)	3(50)	2(33.33)	3(50)	5(83.33)	2(33.33)
8.	Blue crab	Retail market	11	77	65(84.42)	2(2.60)	2(2.60)	33(42.86)	54(70.13)	53(68.83)	54(70.13)	61(79.22)

Figures in parenthesis indicate percentage.

1988). Due to indiscriminate and uncontrolled use of antibiotics, *S. aureus* strains might have lost their sensitivity against the common antibiotics such as Penicillin. A higher resistance to Penicillin was exhibited by *S. aureus* from Egypt, India and Yenem (Obaidat et al., 2015), China (Lv et al., 2014) and France (Pereira et al., 2009). The results of antibiotic sensitivity showed that Chloramphemicol, Sulphafurozole, Vancomycin and Bacitracin were effective against the strains of staphylococci. Resistance to chloramphenicol was not observed in Portugal by Pereira et al. (2009) however, Vazquez

Table 5. Antibiotic resistance pattern (percentage) of *S. aureus* isolates

Sl.No	Isolate code	B	Cl	E	G	Na	P	Sf	Tr	Va
1	Coagulase positive *S. aureus* isolates	25	8.3	0	0	91.6	100	0	33.3	8.3
2	Non-haemolytic coagulase negative staphylococci with lipase, lecithinase and gelatinase potential	8.3	0	16.6	0	100	75	0	0	8.3
3	Coagulase negative staphylococci with delayed thermonuclease reaction	0	0	25	0	91.6	75	8.3	25	0
4	Coagulase negative staphylococci with gelatinase, lecithinase and harmolysin potential	0	0	50	0	100	91.6	0	0	8.3
5	Coagulase negative staphylococci with least toxin potential	0	0	8.3	0	100	75	0	8.3	0

B - Bacitracin; Cl - Chloramphenicol; E - Erythromycin; G - Gentamicin; Na - Nalidixic acid; P - Penicillin-G; Sf - Sulphafurazole; Tr - Trimethoprim; Va - Vancomycin

Tabl 6. Enterotoxin gene profile of selected isolates of coagulase positive staphylococci as tested by PCR

Strain No.	Enterotoxin genes								
	sea	*seb*	*sec*	*sed*	*see*	*seg*	*seh*	*sei*	*tsst*
MS_1	-	-	-	-	-	-	-	-	-
MS_3	-	-	-	-	-	-	-	-	-
CN_4	-	-	-	-	-	-	-	-	-
LC_5	-	-	-	-	-	-	-	-	-
LC_6	-	-	+	-	-	+	-	-	-
LL_7	-	-	-	-	-	-	-	-	-
MS_8	-	-	-	-	-	-	-	-	-
LP_{10}	-	-	-	-	-	-	-	-	-
MC_{11}	+	-	+	-	-	-	-	-	-
CN_{14}	-	-	-	-	-	-	-	-	-
CS_{15}	-	-	+	-	-	-	-	-	-
LL_{16}	-	-	-	-	-	-	-	-	-
LL_{17}	-	-	-	-	-	-	-	-	-

se - Staphylococcal enterotoxin; *tsst* - Toxic shock syndrome toxin

–Sanchez et al. (2012) found 100% resistance to Chloramphenicol from isolates in fish from Spain. It is difficult to compare the resistance profile of *S. aureus* isolates among studies in different parts of the world as different antibiotics have been used in each study. However, majority of the studies have shown resistance of *S. aureus* to β lactam antibiotics such as Penicillin-G, Ampicillin (Obaidat et al., 2015; Arfatahery et al., 2016) Information concerning the drug resistance pattern of the prevailing pathogenic bacteria and the appearance of new resistant characteristics is of utmost value for proper selection of an antimicrobial agent for therapeutic purposes, and that bacteria of environmental origin are more susceptible to antibiotics than that from hospital or clinical species which possess plasmid mediated resistance.

Of the nine SET gene studied in the present study *sea, sec* and *seg* were detected in *S. aureus*. Studies by Simon & Sanjeev (2007) reported that 41% of *S. aureus* form fishery products and 28% from processing workers were enterotoxigenic. They also reported that *sec* (57%) was the predominant gene followed by *sea* (43%). *Sea* was the predominant gene in *S. aureus* from fish samples from Iran (Arfatahery et al., 2016). Although clinical isolates of coagulase positive *S. aureus* were found to be highly positive (74.4%) for either one or more of the enterotoxin genes (Rosec et al., 1997), the occurrence of enterotoxin genes in the field isolates was reported to be low, as recorded in the present study. Zschock et al. (2000) registered enterotoxin genes only in 36% of *S. aureus* from sub clinical cases of bovine mastitis. Three of the 13 isolates which possessed *set* genes carried gene coding for SEC (Table 6), confirming the observation made by Sneha (2004) who reported that 50% of the isolates from fish and fish products produced *sec* followed by *sea* and *seb*. *sea* detected in one of the isolates has been described as the most potent toxin due to its high resistance to proteolytic enzyme (LeLoir et al., 2003). Further, it is stated that detection of *set* genes need not necessarily indicate the ability of the organism to produce intact and biologically active toxin or to produce sufficient toxin to induce disease (McLauchlin et al., 2000). However, detection of enterotoxin gene in environmental *S. aureus* indicates the potential of the organism to cause disease and therefore needs caution. Seafood is one of the commonly traded international commodities in fresh and processed form and therefore, care has to be exercised to check the bacterial level within the permissible limits to avoid trade / health issues. The fish handlers need to be educated on the hygienic methods of handling fish, to check contamination and build-up of toxigenic microorganisms to ensure safety.

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