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Enterotoxigenicity of Coagulase Positive and Negative *Staphylococcus* Species Isolated from Fish and Fishery Products

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Seventy-five strains of coagulase positive and negative *Staphylococcus* species were isolated from different fish and shellfish products. These strains were investigated for enterotoxin production using Reverse Passive Latex Agglutination (RPLA) method. The different samples analysed include fresh, frozen and dried fish and shellfish, battered and breaded fish/shellfish products and fish kheema. Either one or a combination of three enterotoxins, viz., Staphylococcal Enterotoxin A, B, C (SEA, SEB, SEC) was produced by most of the strains but none were found to produce the enterotoxin SED. Out of the 75 isolates tested, 25 isolates were coagulase positive and all these strains were positive for thermonuclease activity. 76 % of the coagulase positive strains were toxigenic Among these, enterotoxins SEA,SEB and SEC were produced by 26.31 %, 52.63 %, and 47.36 % of the isolates respectively.None of the isolate produced SED. Multiple enterotoxin production was also observed. While 5.26 % of the strains produced SEA & SEC ,15.78 % of strains SEA & SEB and 5.26% of the strains produced SEB & SEC. None of the coagulase negative isolates produced enterotoxin.

Key words : Enterotoxin, *Staphylococcus aureus*, fishery products, Reverse Passive Latex Agglutination

Seafood is high quality protein supplement available at low cost. There is an increasing demand for fish and fish products around the world due to its health benefit roles. (Feldhusen, 2000). However there is substantial evidence that fish and fishery products are high on the list of foods associated with outbreaks of food-borne diseases (Huss, 1997). Staphylococcal food poisoning resulting from the growth of enterotoxigenic staphylococcus is one of the important food safety problem (Lopes *et. al.*, 1993). Fish and fish products are good proteinaceous substrate and support the growth of Staphylococci. Usually staphylococcal conta mination occurs during handling and processing. Staphylococci are poor competitors with other bacteria and in the absence of competitive growth by other saprophytes, they can cause food poisoning hazard under improper storage conditions. Occurrence of

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enterotoxigenic staphylococci in fish and fish products has been reported previuosly (Sanjeev *et. al.* 1986; Sanjeev & Surendran 1994; Sanjeev *et, al.*, 1985;).

Staphylococcal enterotoxins belong to a group of pyrogenic exotoxins sharing common phylogenic relationships, structure, function and sequence homology. Staphylococcal food poisoning is caused by the ingestion of food containing heat stable enterotoxins produced by certain species of staphylococci (Su,Y & Lee Wong, 1997). Heat stability is one of the most important properties of Staphylococcal enterotoxins in terms of food safety. The biological activity of toxins remains unchanged even after thermal processing of food. They are similar in composition and biological activity but are identified as separate proteins due to their difference in antigenicity (Casman *et al.* 1963) and twelve serologically distinct staphylococcal enterotoxins are currently known. They are designated as SEA, SEB, SEC1, SEC2, SEC3, SED, SEE, SEG, SEH, SEI, SEJ, and SEK based on their serological behavior (Balaban & Rasooly, 2000).

The aim of the present investigation was to study the incidence of coagulase positive and coagulase negative staphylococci from various fish/shellfish and their products and also to investigate the enterotoxin production potential of these isolates.

Materials and Methods

A total number of 118 fish/shellfish and fishery products were collected from various sources viz. retail markets, processing plants and cold storages situated in and around Cochin. The different fresh fish samples included in this study were Indian oil sardine (*Sardinella longiceps*), Indian mackerel (*Rastrilleger kanagurta*), tilapia (*Orechromis mossambicus*), reef cod (*Epinephelus spp*), seer fish (*Scomberomorous spp*), Chinese pomfret (*Pampus chinensis*), rohu (*Labeo rohitha*) etc. Shellfishes like tiger prawn (*Penaeus monodon*), white prawn (*Penaeus indicus*), black clam (*Villoritta cyprinoides*), Oyster (*Crassostrea madrasensis*), green mussel (*Perna viridis*), frozen products , shrimp (IQF and block frozen), crab meat, seer fish and tuna and dried fishes, anchovy, Otolithus, shark, mullan secutor and shrimp were also studied. Other fishery products studied were battered and breaded products like fish fingers, fish balls, and fish kheema etc.

Quantitative estimation of *Staphylococcus aureus* was carried using Baird-Parker agar (Oxoid) for all the samples. 25 g of the sample was homogenized with 225 ml of sterile normal saline (0.85 % NaCl w/v) in a stomacher blender (Seward Medicals, London, U.K) at 230 rpm for one minute. Serial dilutions were prepared and 0.5 ml of the appropriately diluted homogenate was spread plated on pre-set surface dried Baird-Parker agar (Oxoid) plates. Plates were incubated at 36 ± 1°c for 36- 48 hours. 2-4 gray-black colonies with an entire whitish margin accompanied by a clearance around and an adjacent opaque opalescent area from each plate were picked and streaked on nutrient agar slants for further studies. Morphological characteristics of the isolates were determined. The characteristic isolates were identified using the tests, Gram staining, Catalase (Surendran *et al.*, 2003), Coagulase and thermo nuclease.

The tube method of coagulase test was carried out using lyophilized rabbit plasma with EDTA as per the manufacturers instructions (OXOID). Based on the results the isolates were classified as coagulase positive and coagulase negative. All the isolates were studied for thermo nuclease activity by employing the method described by Barry *et al.* (1973). They were spot inoculated on pre-set surface dried plates of DNase test agar and the plates were incubated at 36±1°c for 18-24 hours. After incubation the plates were flooded with 1N

Hydrochloric acid. The precipitation pattern was observed and the results are recorded as positive or negative.

All the isolates were tested for enterotoxin production potential. Toxin typing was carried out according to the procedures outlined by the manufactures, using the SET-RPLA Kit (Staphylococcus Enterotoxin-Reverse Passive Latex Agglutination, TD 900-Oxoid). Based on the agglutination pattern with latex sensitised with antienterotoxin A,B,C & D, isolates were noted as enterotoxignic A,B,C or D.

Results and Discussion

The quantitative study of *S. aureus* showed that the count ranged between 10^2-10^4cfu/g for all the samples analysed. Characteristic colony from different samples were isolated and further confirmed as *S. aureus* based on colony morphology and biochmical features before carrying out coagulase test. All the staphylococcus strains presented a strong coagulase reaction (4 +). The incidence of coagulase positive and negative staphylococci in different fresh fish and shellfish and their products is given in Table 1.

The presence coagulase positive *S. aureus* was confirmed in 22.72 % of the fresh samples and coagulase negative staphylococci in 44.45% of the samples. The staphylococcal count of samples were in the range $2x10^2$- $4x10^4$ cfu/g. The presence coagulase positive *S. aureus* was maximum in value added fishery products like kheema followed by frozen and dried products. The incidence rate was lowest in fresh samples. This can be attributed to the presence of competing micro flora which out grows staphylococcus. Out of the 7 battered and breaded products, four samples were found to harbour coagulase positive *S. aureus*. The high incidence of *S.aureus* in these products can also be attributed to high human handling of these products thereby causing a high initial load .

In the case of dried fish and shellfish samples *S. aureus* was detected in 25% of the samples analysed. All the coagulase positive isolates were toxigenic. Staphylococcal counts in these samples were in the range of $3x10^3$-$4x10^3$. The very high incidence of staphylococci in dried fish and shellfish can be attributed to cross contamination and unhygienic handling during drying operations, since most of the staphylococci are inhabitants of man and other warm blooded animals. *Staphylococcus aureus* is an osmotolerant organism that may flourish in salt brines or other low water activity conditions

Table 1. Incidence of Staphylococci in Fish and Fish Products

Samples	Total No. of samples	No. of samples containing coagulase+ve *S.aureus*	% Occurr ence	No. of samples containing coagulase - ve *S.aureus*	% Occurr ence
Fresh fish & Shellfish Fish fingerFish keema	66	15	22.72	30	45.45
Fish ball	7	4	57.14	3	42.85
Dry fish	16	4	25	12	75
Frozen fish& Shellfish	29	9	31.03	12	41.38

Table 2. Distribution of Enterotoxin among Enterotoxigenic staphylococcal isolates

Enterotoxin type	No. of isolates	% Isolates
A	5	26.31 %
B	10	52.63 %
C	9	47.36 %
D	-	-
AB	3	15.7 %
AC	1	5.26 %
BC	1	5.26 %

(Troller & Stinson, 1978). Staphylococcus was isolated from heavy-salted cold smoked fish (Basti *et. al.*, 2004; Viswanath *et al.* 1998). They also reported that Staphylococci could grow best in salty and low water activity foods in which the competing microorganisms are in low numbers. *Staphylococcus aureus* has been isolated from 23.2% of the dry fish samples (Sanjeev *et. al.*, 1985) from Cochin.

In the present study out of the 29 frozen samples analysed 9 (31.03%) showed the

presence of *S. aureus* and 41.38% coagulase negative species. The total plate count was in the range of 100-1000cfu/g. Sanjeev & Surendran (1994) reported 68.52% incidence of *Staphylococcus aureus* in frozen fish samples. Comparing with this data, the incidence rate in present study is low. The high incidence of staphylococcus in frozen products might be due to the fact that frozen products are relatively free of most of the competing bacteria, which suppress the growth of staphylococci or due to poor survival at low temperature While Sanjeev *et. al.* (1986) reported the presence of *S. aureus* in 100% of the frozen crabmeat and 52% of frozen shrimps, Nambiar & Iyer (1990) reported their incidence in 8.7% of the frozen fish from the retail markets of Cochin. Iyer & Shrivastava (1988) reported the incidence of coagulase positive Staphylococci in various fishery products. The variation in occurrence of staphylococci in these samples may be due to factors like the difference in native flora, their number and variety of environmental factors to which these foods are subjected.

Different methods have been used for checking the thermo nuclease activity like simplified thermo nuclease test (Lachica, 1976), accelerated procedure for thermo nuclease activity (Lachica 1980), Barry *et al.*(1973) method, etc. All the three methods were tried in this study and it was found that the method by Barry *et al.*(1973) was the best simple and accurate method. All the coagulase positive *S. aureus* isolates were positive for thermo nuclease activity, but none of the coagulase negative isolates showed thermo nuclease activity. Comparison of the thermo nuclease activity of 24 and 48 hr old cultures were showed that consistent results could be obtained in the case of 24 hours at 36±1°C. This can be due to the reason that nuclease production reached a maximum at 16-24 hours but had decreased by 30 % after 48 hours (Jarwis & Lawrence, 1971).

Out of the 25 *Staphylococcus aureus* tested, 19 were found to be toxigenic. All the isolates obtained from dried fishes and value added seafood products were toxigenic. In the case of frozen samples 77.77 % of the isolates were toxin producers while only 50% of the isolates from the fresh fish were toxigenic. Sneha (2004) reported that 41.1% of the isolates from fish and fish products produced enterotoxin. The author also reported that 71.4% of the isolates from frozen PD prawns and 40% isolates from fish cutlets as toxin producers.

Enterotoxin A was reported to be the most potent toxin in causing food poisoning followed by SED, SEC and SEB (Casman & Bennet, 1965). The distribution of enterotoxins among enterotoxigenic staphylococcal isolates is given in Table 2. It was found that the distribution pattern of the types of staphylococcal enterotoxin is quite different from those reported earlier. In the present study, it was observed that enterotoxin B was the dominant toxin (52.63%) followed by SEC (47.36%), SEA (26.31%). SED was not detected from any of the samples. This observation differed from Sanjeev *et.al.*, (1985, 1986) where the authors recorded the predominance of SEA followed by SED from frozen and dried fish products. Sneha (2004) reported that 50% of the isolates from fish and fish products produced SEC followed by SEA (33%) and SEB (16.7%). The author also could not detect any SED producing staphylococci in their study. This may be probably due to the fact that SED is the toxin most likely to go undetected in culture of enterotoxigenic staphylococci. Rajalakshmi and Rajyalakshmi, (1982) reported that majority of the *Staphylococcus aureus* isolates recovered from cases of bacterial food poisoning in India were found to produce enterotoxin C. In this study also Enterotoxin C producers formed a percentage of 47.36 %. It was reported that in the case of human origin isolates 74.4 % were

enterotoxin C producers (Rosec *et al.*, 1997). Lopes *et al.*, (1993) reported the enterotoxin production in foods by *S. aureus* strains that produce more than one enterotoxin. They also reported that enterotoxin B was produced in large amounts in culture media. In the present study most of the isolates were single enterotoxigenic. However multiple enterotoxigenic strains were also detected in small numbers. Among the isolates 15.7 % produced both SEA and SEB, 5.26 % produced both SEA & SEC and 5.26 % produced both SEB and SEC.

Coagulase negative *Staphylococcus* species were found as a major contaminant of both fresh fish and fishery products. Their presence in dried products and frozen products indicates their ability to survive at different conditions viz. high salt content, low temperature etc. All the coagulase negative staphylococci isolated were found negative for toxin production. But there are reported cases of toxin production by coagulase negative staphylococci in different products like meat, poultry, milk and cheese (Vernozy-Rozand *et al* 1996).

The presence of enterotoxigenic Staphylococci in fish and fish products in Cochin points to the food poisoning risk associated with these products. Since unhygienic handling and abuse of temperature during storage leads to proliferation of *S.aureus*, care should be taken to prevent any contamination at any point from catch to consumption.

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