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Virulence Factors Associated with *Staphylococcus Spp.* Isolated from Fish and Fishery Products

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Forty-eight isolates of *Staphylococcus* obtained from fish and fishery products [*Staphylococcus aureus* (25), *Staphylococcus hysicus* subsp. *chromogens* (7), *Staphylococcus simulans* (7), *Staphylococcus epidermidis* (7) and *Staphylococcus warneri* (2)] were used to study the virulence factors associated with these organisms. The virulence factors studied were lipase, phospolipase, phosphatase, thermo nuclease, enterotoxins and haemolysins. Lipase, phospholipase, and urease were produced by all the strains, where as thermo nuclease was produced by *S.aureus* strains only. In the case of other factors like haemolysins, proteases, phosphatases and enterotoxins, considerable strain-to-strain variation was observed. Most of the isolates produced more than one virulent factor. Enterotoxins were produced by 76% of *S.aureus* isolates. None of the coagulase negative staphylococci could produce enterotoxins. The study also shows that most of the isolates are capable of producing potential virulent enzymes and enterotoxins, which can cause infection and intoxication.

Key Words : *Staphylococcus spp*, Fishery products, virulence factors

The genus *Staphylococcus* placed in the family of *Micrococcaceae* consists of 33 known species. They are facultative anaerobic or aerobic, Gram-positive bacteria that are generally catalase positive and oxidase negative. Among the Staphylococci, *Staphylococcus aureus* is the most significant human pathogen. Other species are considered opportunistic. The clinical significance of *Staphylococcus* is largely due to the ubiquity of the species. *Staphylococcus aureus* is found in wide range of habitats including human skin. Many strains are commensals that may be clinically significant or contaminants of food Sandel & Mc Killip, (2004).

Staphylococcus aureus is an important food borne pathogen because of its ability to produce a wide range of extracellular protein toxins and virulence factors that contribute to the pathogenicity of the organism (Boerema *et al.*, 2006). *Staphylococcus aureus* produces a wide variety of infection from simple abscess to fatal sepsis and toxinoses such as food poisoning and toxic shock syndrome toxin (Fueyo *et al.*, 2005). The ability of some *Staphylococcus aureus* strains to produce heat stable enterotoxin is of relevence to the food processing industry since staphylococcal food poisoning ranks as one of the most prevalent cause of gastroenteritis worldwide (Dingis *et al.*, 2000). Evans & Niven, (1950) reported that most enterotoxigenic Staphylococci were members of the coagulase positive group.

Staphylococci especially *S.aureus* is frequently isolated from fish and fishery products that are subjected to extensive human handling. The incidence of *S.aureus* in fresh (Nambiar & Iyer, 1990), dried (Sanjeev *et al.*, 1985; Sanjeev & Surendran, 1994), frozen (Sanjeev *et al.*, 1986; Nambiar & Iyer, 1990) and smoked (Basti *et al.*, 2006) fish and shellfish have been reported by various authors. So far no attempt has been made to study the virulence factors associated with *Staphylococcus* isolated from fish and fishery products. The present study denotes the virulence traits and associated virulence factors in *Staphylococcus* isolated from different fish and fishery products from the retail markets and processing factories of Cochin area.

Materials and Methods

A total of 118 fish and shellfish samples consisting of fresh fish (66 samples), frozen fishery products like shrimps (both IQF and

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block frozen) and finfishes (29 samples), dried fish/shellfish (16 samples) and battered and breaded products (7 numbers) were collected from retail markets, processing plants and cold storages of Cochin area.

A 25g 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 Baird-Parker agar (Oxoid) plates and incubated at 36 ± 1°C for 36- 48 hours. All the gray-black colonies with an entire whitish margin accompanied by a clearance around and an adjacent opaque opalescent area were picked and streaked on nutrient agar slants.

The isolates were initially confirmed as *Staphylococcus* based on Gram staining, Catalase test, Oxidase test, O/F (glucose) test and urease test (Surendran *et al.*,2003). The isolates were further classified to species level as per the scheme of Harvey & Gilmour, (1985).

Coagulase test was carried out using rabbit plasma with EDTA (Difco) as per manufacturer's instruction. Briefly, 0.5ml aliquotes of reconstituted plasma was mixed with two drops of a heavy suspension of the *Staphylococcs* culture in BHI broth (24hrs) in sterile coagulase test tubes (100mmx10mm dia) and incubated at 37°C in a serological water bath and examined for clotting of the contents every 30 minutes up to 4 hours.

The proteolytic activity was evaluated by plating *Staphylococcus* isolates on nutrient agar plates supplemented with 5% skimmed milk. Isolates were inoculated in the form of streaks on the surface of pre–dried plates and incubated at 36±1°C for 24-48 hours. The zone of clearance formed around the bacterial colony due to proteolysis was examined after 48 hours.

The lypolytic activity was assayed using tributyrin agar. The tributyrin agar was prepared by supplementing nutrient agar with 2% tributryin (Sigma). The isolates were spotted on pre-set plates of tributyrin agar and incubated at 36±1°C for 24-48 hours. The isolates were examined for lipolytic activity. Colonies with a zone of clearance around was considered as positive for lipolytic activity.

Phospholipase activity was evaluated by plating *Staphylococcus* isolates on nutrient agar supplemented with 5% egg yolk emulsion. The isolates were inoculated in the form of spot on the surface of pre-dried plates and incubated at 36±1°C for 24-48 hours. The plates were examined for lecithinase activity after incubation. A distinct gray to white precipitate around the colony indicated free fatty acid liberated by lecithinase action on phospholipid.

Haemolytic activity was evaluated using blood agar. nutrient agar supplemented with human blood (5%) for blood agar plates. The isolates were both stab inoculated and spotted on the surface of pre-dried plates of blood agar plates were incubated at 36±1°C for 24-48 hours. Haemolysis was indicated by the clearance (partial or total) around the colony. Based on the reactions in the plate the haemolysins was classified as α (partial hydrolysis), β (complete hydrolysis), γ (no clearence). Haemolysis was indicated by the clearance (partial or total) around the colony.

Christensen's urea agar was used for urease test. The cultures were streaked on urea agar slops and incubated at 36 ±1°C for 24-48 hours. After incubation the cultures were observed for urease production. A pink colour indicated a positive reaction and the negative cultures retained the media colour.

Phosphatase activity was tested on Nutrient agar with 0.01% Phenolphathelene phosphate. The pre-dried plates were spot inoculated with cultures and incubated at 36±1°C for 18-24 hours. After incubation the plates were exposed to ammonia vapour. Pink or red colonies indicated a positive

reaction and colourless colonies a negative reaction.

Nutrient agar with 1% DNA was used for testing thermo nuclease activity. Pre-dried plates were spot inoculated with the cultures and incubated at 36±1°Cfor 18-24 hours. After incubation the plates were flooded with 1N HCl. A clearance around the colony indicated nuclease activity.

The cultures were inoculated in BHI broth and incubated at 37°Cfor 18-24 hours. After incubation the cultures were centrifuged at 900g for 20 minutes at 4°C.The pellets were discarded and the supernatant was taken for assay. The assay for enterotoxins was carried out using SET-RPLA test kit (Oxoid). The assay was carried out as per manufacturer's instructions.

Results and Discussion

All the colonies with a smooth convex, glistening and jet-black colour with a white margin and zone of clearance were taken as *Staphylococcus*. The average size of the colony varied between 1.7- 2 mm with an opaque halo, surrounded by a 2-4 mm zone of clearance. All the cultures were Gram-positive spheres appearing singly or in pairs and in clusters. All the isolates gave a positive catalase reaction with 30% hydrogen peroxide and all were oxidase negative.

The coagulase test differentiated the cultures into coagulase positive and coagulase negative. The coagulase test showed that out of the total 75 isolates only 25 were coagulase positive. The rest were coagulase negative even though they showed very typical colony morphology similar to that of coagulase positive isolates. Table-1 gives the incidence of *S.aureus* and coagulase negative staphylococci in different fish and fishery products. It can be seen that maximum incidence of *S.aureus* was noticed in battered and breaded products and the maximum incidence of coagulase negative staphylococci was detected in dried fish and shellfish.

Further studies were carried out with 25 *S.aureus* cultures and 23 coagulase negative staphylococci. The coagulase negative staphylococci isolates were classified up to species level as per the scheme of Harvey and Gilmour (1985) and they were identified as *S.simulans, S.warneri, S.epidermidis* and *S.hysicus* subsp. *chromogens*. The distribution of virulent factors of coagulase positive and negative Staphylococci are given in Table 2.

The exotoxins produced by *Staphylococcus* are considered to be an expression of pathogenicity and most of these characteristics are strain specific(Bhat *et al.*,1990). The enzymes like lipases, proteases, phosphatases, phospholipases help these bacteria in the invasion of the host tissue. The *in-vitro* studies showed a strain-to-strain variation in these enzymatic activities. Some enzymes like lipases, phospho lipases and urease were produced by almost all the isolates, while some other enzymes like gelatinase, haemolysins etc were produced by only a certain percentage of the isolates.

Table 1. Incidence of Staphylococci in fish and fishery products

Samples	Total No	No. of samples containing coagulase +ve *Staphylococcus*	% Occurrence	No. of samples containing Coagulase –ve *Staphylococcus*	% Occurrence
Fresh fish &Shellfish	66	15	22.72%	30	45.45%
Battered and breaded products	7	4	57.14%	3	42.85%
Dried fish	16	4	25%	12	75%
Frozen fish & Shellfish	29	9	31.03%	12	41.38%
Total	118	32	27.1%	57	48.31%

Table 2. Distribution of virulence factors in *Staphylococcus* species isolated from fish and fishery products (%)

Test	*S.hysicus* subsp.*chromogens* N=7	*S.simulans* N=7	*S.warneri* N=2	*S.aureus* N=25	*S.epidermidis* N=7
Thermo nuclease	Nil	Nil	Nil	96%	Nil
Phosphatase	100%	Nil	100%	100%	85.71%
Lipase	100%	100%	100%	100%	100%
Phospholipase	100%	100%	100%	100%	100%
Urease activity	100%	100%	100%	100%	100%
Gelatinase	28.57%	28.57%	Nil	20%	14.28%
Caseinase	100%	100%	100%	100%	100%
α-hemolysins	Nil	Nil	Nil	8%	28.57%
β-hemolysins	Nil	71.42%	Nil	60%	57.1%
Non-haemolytic	100%	28.58%	100%	36%	42.8%

All the coagulase positive and negative *Staphylococcus* isolates in the present study showed urease activity. Although similar result was reported by Bhat *et al.* (1990), they found lower percentage (15%) of coagulase negative *Staphylococcus* from clinical isolates as urease producers. Langlois *et al,* (1990) reported a low incidence of urease positive *S.aureus* from milk (6%) and food handlers (14%), but they obtained a 100% urease activity by *S.chromogens, S.epidermidis* and *S.simulans* isolates of bovine origin.

There was a good correlation between thermo nuclease activity and coagualase activity. 96% of the *S.aureus* isolates were thermo nuclease positive. None of the coagulase negative isolates showed positive reaction for thermo nuclease. Similar results were obtained by Da Silva *et al,* (2000) and Bennet & Lancette (1995). These authors reported that 97.8% and 93% of *S.aureus* isolated from milk and environmental samples, and food ingredients respectively as thermo nuclease positive. Bhat *et al,* (1990) also reported that all coagulase positive clinical *S.aureus* isolates produced thermo nuclease and this test could be used in the identification of *S.aureus* in addition to coagulase test. They also reported the non-production of thermo nuclease by coagulase negative Staphylococci.

Phosphatase activity was shown by all isolates of *S.aureus*. Among the coagulase negative *Staphylococcus,* all strains of *S.simulans* and 14.29% strains of *S.epidermidis* were phosphatase negative. All other coagulase negative strains were found to possess phosphatase activity. Phosphatase activity is considered another important virulence factor. Bhat *et al,* (1990) reported phosphatase activity by 100% of *S.aureus* and 30% of coagulase negative *Staphylococcus* from clinical isolates but Langlois *et al,* (1990) showed a different picture of phosphatase activity by coagulase negative Staphylococci of human and bovine origin. They reported phosphatase activity by 70.8% of *S.epidermidis* of human origin and 91.5% of *S.chromogens* and 82.6% of *S.epidermidis* of bovine origin. About 8% of *S.simulans* showed a weak positive activity and none of the *S.warneri* isolate could produce phosphatase. However in the present study both the *S.warneri* isolates produced phosphatase and none of the *S.simulans* isolate could produce this enzyme.

Assay for enterotoxin production showed that 76% of the *S.aureus* isolates were toxigenic. They produced either single or multiple toxins, i.e staphylococcal enterotoxin A,B,C (SEA,SEB,SEC) and none of the isolates produced the toxin SED. Enterotoxin B and C was produced by 40% of the isolates

and SEA was produced by 20% of the isolates. Three isolates produced SEA and SEB and one isolate produced SEA and SEC. None of the coagulase negative isolate produced enterotoxin. Enterotoxin B and C were the dominant toxin types produced by *S.aureus* in the present study. Rosec *et al.* (1997) reported that 74.4% of the isolates of human origin produced enterotoxin C (SEC). Rajalakshmi & Rajyalakshmi, (1982) reported that majority of *S.aureus* isolates recovered from bacterial food poisoning in India were found to produce enterotoxin C. But contrary to this result Sanjeev *et al.*(1985;1986) and Sanjeev & Surendran, (1994) reported the predominance of SEA producing isolates from dried fishery products and frozen fishery products respectively.

Several workers have reported the presence of enterotoxigenic coagulase negative *Staphylococcus* in products like goats milk and cheese Vernozy- Rozand *et al.*(1996) and from restaurant workers Udo *et al.* (1999). However, in the present study none of the coagulase negative *Staphylococcus* strain was found to be enterotoxigenic

Lipase activity was displayed by all the isolates of both coagulase positive and negative Staphylococci. All the isolates could hydrolyze lecithin and tributyrine. Nearly 29% of *S.hysicus* subsp. *chromogens* and *S.simulans*, 20% of *S.aureus* and 14.28% of *S.epidermidis* showed gelatinase activity. None of the *S.warneri* strains could hydrolyze gelatin. However all the *Staphylococcus* isolates could hydrolyze casein. The presence of lipase in *Staphylococcus* has been considered as an important factor in the initiation of boils and carbuncles in humans and majority of the *S.aureus* isolates from human infection posses this enzyme Milgrom & Flanagan, (1982). In the present study also, all the isolates of both coagulase positive and negative staphylococci possessed a strong lipolytic acivity on tributyrin agar. Bhat *et al*,(1990) reported lipolytic activity by 83.7% of *S.aureus* and 17.5% of coagulase negative Staphylococci.

Staphylococcal haemolysins are identified as important virulence factors that contribute to bacterial invasion and escape from the host immune response (Salyers & Whitt, 1994). Jarvis & Lawrence (1971) reported the production of haemolysins by both enterotoxgenic and non- enterotoxigenic strains of *S.aureus*. The β-haemolysin production is indicative of animal strains (Meyer, 1967). In the present study 60% of the *S.aureus* isolates showed β-hemolytic activity, 8% were α hemolytic and rest were non-hemolytic. Among the coagulase negative *Staphylococcus*, 8% of the isolates were α haemolytic, 40% were β-haemolytic and rest of the isolates were non-haemolytic.

It can be concluded from the study that *Stapylococcus* species are major contaminants in fish and fishery products. Most of these organisms are major inhabitants of man and other lower warm-blooded animals and their presence in fish and fishery products indicates unhygienic handling. The study also shows that most of the isolates are capable of producing potential virulent enzymes and enterotoxins, which can cause infection and intoxication.

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