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## Coagulase gene polymorphism of *Staphylococcus aureus* in fish and its multiple drug resistant pattern

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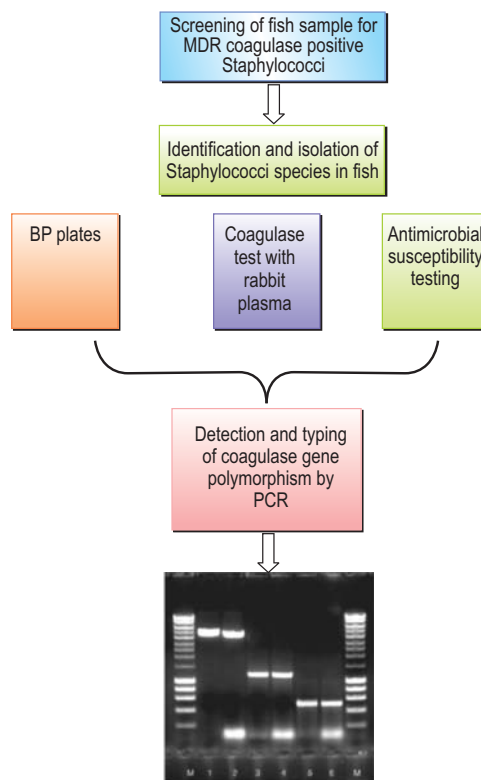
### Abstract

**Aim:** *Staphylococcus aureus* is a common inhabitant of human skin and nose of healthy people and animals. But this well-known opportunistic pathogen that can cause a broad range of infections including mild skin infections, invasive diseases, toxin mediated diseases and food poisoning. *S. aureus* is considered as one of the most frequently occurring food-borne pathogen worldwide and cause food poisoning. So the main objective of the study was to monitor the presence of multi drug resistant coagulase positive staphylococci and its coagulase gene polymorphism in fish species of retail outlets in Gujarat, India.

**Methodology:** *S. aureus* was identified using FDA, (2015) standard procedures on Baird Parker (BP) agar plates with supplements of egg yolk emulsion and potassium tellurite solution. The characteristic Staphylococci colony was purified on Tryptic Soy Agar plate and tested for coagulase with rabbit plasma. The antimicrobial sensitivity test was carried out by disc diffusion method on Mueller Hinton agar with 4% NaCl and the multi drug resistant staphylococci was interpreted as per CLSI, 2014 guidelines. Polymerase Chain Reaction was carried for the detection and typing of coagulase gene polymorphism.

**Results:** The present investigation revealed the incidence of 11.52% coagulase positive staphylococci in fish species of retail outlets in Gujarat with Staphylococcal count of  $2.0 \times 10^1$  to  $7.8 \times 10^2$  cfu.g<sup>-1</sup>. Almost all the CPS isolates were resistant to at least one antibiotic and many are resistant to multiple antimicrobials. The *coa* gene coding for coagulase protein showed multiple bands and were used for diagnosis of *S. aureus*.

**Intpretation:** Seafood may be contaminated with multidrug resistant coagulase positive *Staphylococci* due to poor hygienic profile of the handlers, processing and unhygienic environment.



## Introduction

*Staphylococcus aureus* is a Gram positive bacteria and commonly found on the skin surfaces and noses of 25% of healthy people and animals. It may not causes disease in healthy individuals and will cause food poisoning when eat the contaminated food products. *S. aureus* considered to be one of the most frequently occurring food-borne pathogen worldwide and cause food poisoning (CDC, 2010; Popovic *et al.*, 2010). The number of outbreak and number of staphylococcal gastroenteritis is much higher than other microbial food-borne outbreak (Jay, 2000). Several studies clearly suggest the possibility of potential public health threat resulting from contamination of seafood mainly due to unhygienic handling, processing, storage and unhygienic environment. In India, the rate of staphylococcal infection is still higher because of warm and humid climate (Bhatia and Zahoor, 2007). Coagulase production by *S. aureus* is considered to be an important criterion for the safety of seafood as well as for clinical microbiologists. Though the quality and safety of food is followed mostly but food- borne outbreaks from microbial contamination, chemicals and toxins are most common in many countries today (WHO, 2007). The widespread use of antibiotics will lead to an exponential increase of antibiotic resistance in the recent years worldwide. The contamination of antibiotic resistant bacteria in food materials is the great concern to public health, as this resistance can be transferred to human (Chiu *et al.*, 2002, Diana Gutiérrez *et al.*, 2012). Thus, multidrug-resistant *S. aureus* strains are most common in hospitalised patient and farm animals but recently reported in animal food products such as milk, meat and fishery products (Le-Loir, 2003). In view of the above, the present study was carried out to monitor the presence of multi drug resistant coagulase positive staphylococci in fish species of retail outlets in Gujarat, India.

## Materials and Methods

**Fish samples:** A total of 408 fish samples such as Horse Mackerel, Indian Mackerel, Tuna, Ribbon fish, Seer fish, Croaker, Ghol, Dhoma, Sardine, Prawns, Shark, Ray fish, Dried fishes, Cephalopods and Surmi, etc., were collected in and around Veraval region, Gujarat during Sep, 2011 to Mar, 2015. The fish samples were collected in pre-sterilized containers, kept at  $-4^{\circ}\text{C}$  in ice storage box and immediately transported to the laboratory within an hour.

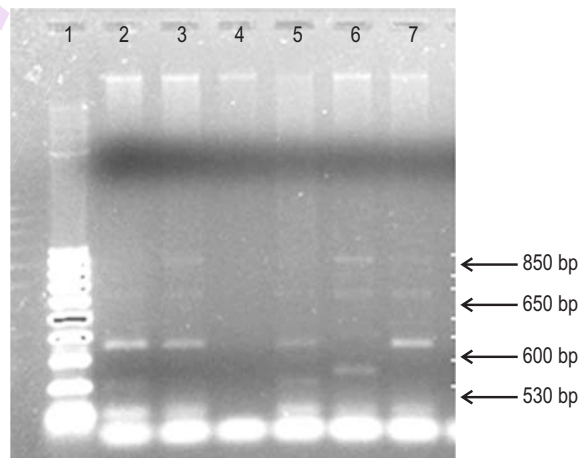
**Isolation and identification:** *S. aureus* was identified using FDA, (2015) standard procedures. In brief, 10g of fish samples was added to 90 ml of sterile normal saline solution and blended in stomacher for 90 sec @ 250 rpm. A serial dilution of  $10^1$  to  $10^5$  was made and aseptically transferred 1 ml sample suspension of 0.3, 0.3 and 0.4ml from each dilution to triplicate plates of Baird Parker agar (Oxoid, UK) containing supplements of sterile 5 ml of 50% egg yolk emulsion and sterile 1 ml of 1% potassium tellurite solution and incubated at  $35^{\circ}\text{C}$  for 48 hrs. Staphylococci showed

typical colony with a circular, smooth, convex, moist, 2-3 mm diameter, grey to jet black colony with white line of margin, opaque outer zone around it (Fig. 1). From these plates, 5-6 typical colonies were streaked on to Tryptic Soy Agar plate for isolation and purification and then confirmed by Gram staining, motility, oxidase, catalase and mannitol utilization.

**Coagulase test:** 5-6 typical colonies of staphylococci isolated from each fish samples from Baird parker plates were selected and streaked on to TSA plates and then a well isolated colony was inoculated in Brain Heart Infusion broth (Oxoid, UK) and incubated at  $25^{\circ}\text{C}$  for 18- 24 hrs. Coagulase tube test were carried out with 0.5 ml of rabbit plasma with EDTA (Difco, USA) with 2 drops of 18- 24hrs grown BHI broth culture, incubated at  $37^{\circ}\text{C}$  in serological water bath and observed every 30 min up to 4 hrs for coagulation (Fig 1. B) and further confirmed with anaerobic glucose fermentation and mannitol utilization (Fig 1. C).

**Antibiotic sensitivity test (ABST):** The antimicrobial sensitivity test to 24 antibiotics (Dodeca Staphylococci-1 and 2, HiMedia, Mumbai) were carried out by disc diffusion method (Bauers *et al.*, 1966) on Mueller Hinton agar with 4% NaCl. The concentrations and resistance patterns were measured as specified by the CLSI, 2014. The list of antibiotics used with their required concentrations is listed in Table 1. Multi drug resistant (MDR) *staphylococci* were considered as showing resistance to penicillin along with at least three classes of antibiotics (Shittu *et al.*, 2006). *S. aureus* ATCC 25923 was used as a standard positive control strain for ABST.

**PCR method for detection of coa gene:** DNA was extracted from putative *S. aureus* isolates by using the Bacterial Genomic DNA Kit (Sigma- Aldrich, Spain) according to the manufacturer's



Lane 1: 100 bp DNA ladder; Lane 2-7: Coagulase positive staphylococci isolates from fish samples and amplified with different size DNA fragments

**Fig. 1 :** Characterization of coagulase positive Staphylococci in fish samples using PCR with Coa gene

recommendations. The *coa* gene was amplified by PCR with 5'-ATAGAGATGCTGGTACAGG-3' and 5'-GCTTCCGATTGTTCCGATGC-3' primers (Hookey *et al.*, 1998). The PCR reaction was carried out with 75 pM  $\mu\text{l}^{-1}$  of each primer, 2.5  $\mu\text{l}$  of 10X buffer, 2.5 mM of  $\text{MgCl}_2$ , 1.5 unit of Taq polymerase, 10 mM of dNTP mix and 50 ng  $\mu\text{l}^{-1}$  of DNA to 50  $\mu\text{l}$  reaction volume with 92°C for 5 minutes followed by 92°C for 45 sec, 55°C for 30 sec and 72°C for 45 sec of 35 cycles and 72°C for 5 minutes (Sure Cycler, Agilent, USA). The PCR products (Fig. 1) were electrophoresed on submarine electrophoresis and stained with ethidium bromide and visualized under UV light with a Gel Doc (BioRad, USA).

### Results and Discussion

The present investigation revealed the incidence of coagulase positive staphylococci in fish species of retail outlets in Gujarat. A total of 235 isolates of coagulase positive staphylococci (CPS) were recovered from 408 seafood samples showing the overall incidence of 11.52%. Staphylococcal count ranged between  $2.0 \times 10^1$  to  $7.8 \times 10^2$  cfu  $\text{g}^{-1}$  and exceeded the recommended limit of 100 cfu  $\text{g}^{-1}$  (ISO, 2015) in seafood. Further, 12.77% of the isolates were coagulase positive with rabbit plasma antisera. Similar to the present study, Bujamma and Padmavathi (2015) found that an overall of 24.47% of fish samples showed the presence of *S. aureus* in the range of 4.16% to 50.0% in the fish market of Guntur, Andhra Pradesh, India Vázquez-Sánchez *et al.* (2012) reported that 25% fishery products such as fresh, frozen, salted, smoked, ready to cook, surmi, roe and ready to eat fishery products was

contaminated with *S. aureus* in Galicia (NW Spain) retail market. Whereas Sergelidis *et al.* (2014) reported 7.0% contaminate of the ready to eat fish products with *S. aureus* in Greece and Tulayion Elal Mus *et al.* (2014) also observed that 6% of the retailed fresh fish, mussel and shrimp samples were contaminated with *S. aureus* in Turkey. Moreover, much higher rate incidence of *S. aureus* contamination were reported for fresh (43%) and frozen (30%) fishery products in Spain by Vazquez-Sanchez *et al.* (2012) also, Ezzeldeen *et al.* (2011) reported *S. aureus* contamination low salt sardine (86.7%), salted *Mugil cephalus* (93.3%) and salted *Hydrocynus froskallii* (90%) fish samples in Egypt. The difference in the prevalence rate of *S. aureus* between seafood and its products might be associated with pre-harvest and post-harvest method, storage and handling conditions (Simon and Sanjeev, 2007; Bujamma and Padmavathi, 2015).

These coagulase positive staphylococci isolates were also assayed for susceptibility to 24 numbers of recommended antibiotics for Gram positive Staphylococci (Dodecca staphylococci 1 and 2, HiMedia, Mumbai) and showed a variable range resistant pattern to these antimicrobials tested (Table 1). Almost all of these Coagulase Positive Staphylococci isolates were resistant to penicillin with at least 3 groups of antibiotics revealing the presence of multidrug resistant (MDR) staphylococci from these seafood samples. The antibiotic resistant pattern clearly indicated that the overall high percentage of Coagulase Positive Staphylococci isolates were 97.67 %

**Table 1 :** Antimicrobial patterns of coagulase positive beta lactam producing Staphylococci from fish species of retail outlet in Gujarat

Name of antibiotic discs	Number of isolates resistant	% of isolates resistant	ATCC 25923
Penicillin (100U)	29	96.67	Susceptible
Azithromycin (15 $\mu\text{g}$ )	29	96.67	Susceptible
Erythromycin (15 $\mu\text{g}$ )	26	86.67	Susceptible
Clarithromycin (15 $\mu\text{g}$ )	26	86.67	Susceptible
Linezolid (30 $\mu\text{g}$ )	2	6.67	Susceptible
Co-Trimoxazole (25 $\mu\text{g}$ )	2	6.67	Susceptible
Vancomycin (30 $\mu\text{g}$ )	0	0	Susceptible
Cefoxitin (30 $\mu\text{g}$ )	0	0	Susceptible
Ciprofloxacin (5 $\mu\text{g}$ )	29	96.67	Susceptible
Gatifloxacin (5 $\mu\text{g}$ )	29	96.67	Susceptible
Ofloxacin (5 $\mu\text{g}$ )	26	86.67	Susceptible
Clindamycin (2 $\mu\text{g}$ )	2	6.67	Susceptible
Tigecycline (15 $\mu\text{g}$ )	0	0	Susceptible
Moxifloxacin (5 $\mu\text{g}$ )	26	86.67	Susceptible
Gentamicin (10 $\mu\text{g}$ )	2	6.67	Susceptible
Rifampicin (5 $\mu\text{g}$ )	0	0	Susceptible
Lomefloxacin (10 $\mu\text{g}$ )	28	93.33	Susceptible
Norfloxacin (10 $\mu\text{g}$ )	25	83.33	Susceptible
Novobiocin (30 $\mu\text{g}$ )	0	0	Susceptible
Teicoplanin (15 $\mu\text{g}$ ),	4	13.33	Susceptible
Nitrofurantoin (300 $\mu\text{g}$ )	23	76.67	Susceptible
Pristinomyacin (15 $\mu\text{g}$ )	26	86.67	Susceptible
Ampicillin- Sulbactam (10 10 $\mu\text{g}^{-1}$ )	14	46.67	Susceptible
Piperacillin- Tazobactam (100 10 $\mu\text{g}^{-1}$ )	0	0	Susceptible

resistant to Penicillin, Azithromycin, Ciprofloxacin and Gatifloxacin, 93.33% resistant to Lomefloxacin, 86.76% to Erythromycin, 76.67% to Nitrofurantoin Clarithromycin, Ofloxacin, Moxifloxacin, and Pristinomycin, 83.33% to Norfloxacin, 46.67% to Ampicillin- Sulbactam, 13.33% to Teicoplanin and 6.67% to Linezolid, Co-Trimoxazole, Clindamycin and Gentamicin, respectively. Almost all the CPS isolates were resistant to at least one antibiotic and many were resistant to multiple antimicrobials (93.33%) except few isolates. *S. aureus* has developed multidrug resistance worldwide and indicates that as potential hazards to food supply and to human health (Gutiérrez, 2012). These results are in agreement with the previous report wherein the incidence of antibiotic resistance among *S. aureus* strains isolated from different foods showed relatively high incidence rates of resistance to different antibiotics (Vázquez-Sánchez, 2012), whereas Marino *et al.* (2011) found that the incidence of antibiotic resistance among *S. aureus* strains isolated from food was not remarkably high, excluding Penicillin.

The *coa* gene, being highly polymorphic because of variable sequences at its 3' coding region and produces different sizes of amplicon. Similar to the present investigation, Salasaia *et al.*, 2004, Guler *et al.*, 2005 also reported different size of the *coa* gene from staphylococcal isolates in bovine milk and could be used as specific/host specific diagnosis of *S. aureus* infection.

The present study revealed that fish species procured from retail outlets in Gujarat was frequently contaminated with multidrug resistant coagulase positive *S. aureus* which could possibly be due to poor hygienic profile of the handlers, processing and unhygienic environment of the fish source. Strict hygienic measures are required to reduce the staphylococcal contamination, thereby to provide the wholesomeness of fish.

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